THE CHELIDONURA TSURUGENSIS–SANDRANA (GASTROPODA: CEPHALASPIDEA) SPECIES COMPLEX: DO REPRODUCTIVE DECISIONS MAINTAIN COLOUR POLYMORPHISM?

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ABSTRACT

Cephalaspid gastropod molluscs are increasingly being utilized in studies of tradeoffs associated with conflicts in simultaneous hermaphroditic reproduction. Chelidonura sandrana exhibits a wide range of colour forms, from black to yellow-spotted or marked with white. One of these colour morphs was described as Chelidonura tsurugensis Baba & Abe, 1959, while others have been accepted as natural variation within C. sandrana Rudman, 1973. Previous work has mostly assumed (1) that colour forms represent the same species and (2) that reproductive behaviour does not differ among colour forms. Mating behaviour between three co-occurring colour forms (simplified to ‘black’, ‘yellow’ and ‘white’) of the Chelidonura tsurugensis–sandrana species complex was investigated to test the above assumptions. Individuals of each colour form were crossed with individuals of the same form, and then with each of the two other colour forms, and with additional species as controls. Mating frequency, duration and an individual’s role of sperm donor or receiver were recorded. The results show that there was no evidence for positive assortative mating with respect to colour form. Instead, at least one colour form was shown to mate significantly more often, and for longer, with individuals of colour forms other than its own. There was no evidence for trading of intromissions or intromission duration among colour forms. Although all three colour forms readily mate and behave like a single biological species, the questionable identity of C. tsurugensis prevents synonymization of C. sandrana with this species. Conservatively, we suggest that until a systematic revision of Chelidonura is carried out, the application of the name C. tsurugensis should be restricted to Japan, and elsewhere the yellow-spotted colour form should be referred to as C. sandrana.

INTRODUCTION

Chelidonura is a genus of heterobranch gastropod molluscs belonging to the clade Cephalaspidea sensu stricto. Species are elongated, cylindrical, often brightly coloured and have a small internal shell in the centre of the body (Fischer, 1887). They also have a series of sensory bristles at the front of the head and two parapodia which fold over the centre of the body and extend posteriorly as a pair of uneven ‘tails’ (for a recent morphological review, see Mikkelsen, 1996). Chelidonura species are typically found on sand on shallow tropical reefs and are reported to feed on turbellarians (Rudman, 1973). Chelidonurids are simultaneous hermaphrodites, but are unable to self-fertilize (Baur, 1998). Mating can be unidirectional or simultaneously reciprocal, and these alternatives can be distinguished by the position of the mating pair (Anthes & Michiels, 2005).

Some confusion exists regarding the geographic range and validity of taxa contained in the Chelidonura tsurugensis–sandrana species complex. Originally described from Japan, C. tsurugensis (Baba & Abe, 1959) was described as black with varying numbers of yellow or golden-brown spots, with opaque white markings on the anterior edge of the head shield. Rudman (1973) described C. sandrana from Zanzibar in the western Indian Ocean as black with yellow spots that were surrounded by white markings. The holotype was an orange and white individual, but he considered a single black specimen to be conspecific (and noted it mating with the typically coloured specimens). Another likely member of this species complex is C. babai Gosliner (1988) from Aldabra Atoll. Gosliner (1988)
noted that both yellow-spotted and entirely black specimens were present and would copulate, and that some spotted individuals also had white pigmentation. In one colour form or another, *C. sandrana* has been recorded from the Red Sea (Yonow, 1994a), the Maldives (Yonow, 1994b), Chagos (Yonow, Anderson & Buttress, 2002), Australia (e.g., Rudman, 1998), Malaysia (Elliot, 2003), the Philippines (Köhler, 2002) and Indonesia (Warren, 2000).

An additional complication is that sympatric black colour forms of multiple *Chelidonura* species appear to exist. For example, at Hoga Island, Indonesia, there are two black forms that differ in the amount of white pigmentation across the anterior portion of the head shield. One form has a much broader band, but the differences are only qualitative. However, Anthes (2006) distinguished the two by body shape and referred to the more elongate form with the broader white anterior band as ‘C. cf. sandrana black’. This form appears to be differentiated by COI sequence data (Anthes, 2006; not shown by Anthes, Schulenburg & Michiels, 2008), but not in the more conservative 16S + 28S analyses (Anthes et al., 2008).

In recent years, *C. sandrana* has been used as a model organism in studies of hermaphroditic reproductive behaviour (Dall & Wedell, 2005). Studies of this species by Anthes, Michiels and colleagues have described sperm trading (Anthes & Michiels, 2005), body size effects (Sprenger et al., 2009), multiple mating effects (Sprenger, Anthes & Michiels, 2008a), parental effects (Sprenger et al., 2010) and sex-role preferences (Anthes, Putz & Michiels, 2006; Sprenger, Lange & Anthes, 2011). Although Anthes & Michiels (2007a) mentioned that individuals of *C. sandrana* with differences in body shape and/or colouration can display different reproductive behaviours between colour forms have not been investigated in detail. If *C. sandrana* is to continue to be used as a model organism it is critical that any differences in reproductive behaviours between colour forms be fully investigated and characterized, and any resulting taxonomic issues clarified.

All three putative colour forms of *C. sandrana* occur sympatrically on the island of Hoga, southeastern Sulawesi, Indonesia. Previous anecdotal observations described mating between all three colour forms here (Warren, 2001). The aims of this study were to describe mating behaviours (i.e., mating frequency, intromission duration, sex role and its alternation) within the *C. tsurugensis–sandrana* species complex, and to determine whether any assortative mating occurs among different colour forms, whether by individual sex-role mating decision or differential sperm donation.

**MATERIAL AND METHODS**

The study animals were found in abundance on the sand flats between the reef crest and the beach around Hoga Island (Home Reef, 5°28’59”S, 123°45’14”E). We refer to the three colour forms occurring on Hoga as ‘black’, ‘yellow’ and ‘white’ (Fig. 1). The yellow form roughly corresponds with the *Chelidonura tsurugensis* morphotype, and the white form to *C. sandrana*. Individuals of *C. hirundinina* also co-occurred with the three colour forms and were collected by snorkel to act as a sympatric control. Individuals of *C. varians* were not found in the same area but were collected subtidally using SCUBA (Blue Bowl, 5°26’60”S, 123°45’44”E, approximate depth 14 m) to act as an allopatric mating control. Once in the laboratory the length of each specimen was measured, to the nearest millimetre, when the animal was in a standard crawling posture, i.e. not contracted or excessively stretched out (Pennings, 1991). Forty specimens each of yellow and white were collected with a length of 5.46 ± 0.22 and 5.68 ± 0.18 mm, long respectively. Twenty specimens of black were collected measuring 5.70 ± 0.24 mm long. Twenty specimens of *C. hirundinina* and eight of *C. varians* were collected measuring 5.80 ± 0.27 mm and 3.88 ± 0.13 mm long, respectively. All individuals were maintained in separate Petri dishes for at least 24 h to acclimatize before any experiments commenced. Sea water was changed daily.

During mating observations, pairs were placed in shallow trays (18 × 10 × 2 cm) with an approximate volume of 360 ml and filled with fresh sea water. Pairs were allowed 5 min to acclimatize prior to the experiment. Pairs were then continuously observed for 2 h during which time penis intromission duration (see Anthes & Michiels, 2003, for definition), mating direction and intromission frequency were recorded. Unidirectional sperm donation was identified by the sperm donor occupying the posterior position of the mating pair. Simultaneous reciprocal mating did not occur in any trials, although it has been observed in other chelidonurids, i.e., *C. flavolobata* and *C. inornata* (Anthes & Michiels, 2007a).

Five replicates were carried out for each cross between the three colour forms or control species. Within each replicate, four individuals were paired with all other individuals. For example the yellow and white cross consisted of four individuals of each of the two colour forms (in total 20 individuals of yellow and white individuals of white were used). This provided 16 pairing combinations for this cross (Table 1). Following
completion of these 16 pairing combinations, the yellow individuals were then paired with all the other yellow individuals and the white individuals were paired with all the other white individuals to give yellow × yellow and white × white crosses (a further 12 pairing combinations, of six yellow and six white). This was repeated five times. Observation time for this cross was therefore 28 × 2 h × 5 replicates = 280 observational hours. A further 20 individuals of yellow and white as well as 20 individuals of black were collected to examine mating behaviour between yellow and black, and white and black colour forms. In each of these (five) replicates there were four individuals of each of the three colour forms, yellow, white and black. As before, the yellow and black cross therefore totalled 16 pairing combinations, as did the white and black cross. The black × black cross totalled six pairing combinations. Experimental design was randomized, i.e. not all mixed colour-form crosses took place prior to the single (i.e. black × black) colour-form crosses. Experiments were carried out during daylight hours over a 23-d period, with one researcher monitoring up to four pairings at any one time. A replicate (16 pairing combinations) normally took 2 d of observational time. Individuals were rested for at least 5 min between mating observations. Control crosses involved a randomized sample of individuals of yellow, white and black being paired with individuals of C. hirundinina (20 individuals) and C. varians. It proved more challenging to collect suitably sized C. varians for use in this study, so only eight individuals were utilized in a randomized design for crosses involving C. varians.

The statistical analyses are based on the outcomes of single mating trials and each mating observation entered the analysis as a single independent data point. All statistical analyses were carried out using MINITAB 15. The normal distribution of data was verified using the Anderson–Darling test prior to all statistical tests. Where data were not normally distributed, they were transformed (as noted in figure legends). Parametric tests such as one-way analysis of variance (ANOVA) were carried out on data (or transformed data) that exhibited normal distributions. Post hoc testing was carried out using Tukey’s tests when significant differences were indicated by ANOVA. If transformation of the data did not produce normally distributed datasets, nonparametric Kruskal–Wallis or Scheirer–Ray–Hare (nonparametric version of two-way ANOVA) were used. Mann–Whitney tests (with Bonferroni correction for multiple testing) were used to investigate differences highlighted by the results of the Kruskal–Wallis or Scheirer–Ray–Hare tests.

RESULTS

Intromission behaviour among colour forms

The different colour forms of the Chelidonura tsurugensis–sandrena species complex mated with each other but not with either of the control species (Table 2). Significantly fewer penis intromissions were recorded for the yellow × yellow cross compared to the mixed colour-form crosses (Fig. 2A). However, for white and black there was no significant difference in the number of intromissions between the same colour crosses and the mixed crosses (P = 0.099 and 0.198 for white and black, respectively) (Fig. 2B, C). Comparing the whole dataset in a single Kruskal–Wallis analysis followed by Mann–Whitney tests (with Bonferroni correction) to look at differences gave the same results (results not shown).

When individuals of yellow were given the opportunity to mate with individuals of white there was no significant difference in the frequency of decision to be either solely sperm donor or sperm receiver, or to alternate between the two roles, or to refrain from mating (P = 0.226) (Fig. 3A). However, when individuals of yellow were given the opportunity to mate with individuals of black, unilateral mating was more frequent than reciprocal mating (P = 0.009 with yellow as sperm donor; P = 0.01 with yellow as sperm receiver) with no difference between which colour form was acting as sperm donor in unilateral matings (P = 0.270) (Fig. 3B). Similarly, when individuals of white were given the opportunity to mate with individuals of black, unilateral mating was more frequent than reciprocal mating (P = 0.015 with white as sperm donor; P = 0.011 with white as sperm receiver) with no difference between which colour form was acting as sperm donor in unilateral matings (P = 0.997) (Fig. 3C).

The majority of sex role alternations (for figure, see Supplementary material) involved just one alternation of sex role. Of the eight mating trials where more than one sex-role alteration took place in the yellow and white, and yellow and black pairings, the yellow colour form took the role of sperm donor on average for 68% of each mating trial. In the white and black pairings where more than one sex role alternation took place (five mating trials), on average equal numbers of individuals of black (47%) and white (53%) took the role of sperm donor.

Intromission duration among colour forms

Penis intromission duration was significantly shorter for the yellow × yellow cross compared to the yellow × white.
Similarly, intromission duration was significantly shorter for the white/white cross compared to the white/black (\(P = 0.016\)) cross (Fig. 4B). In contrast, there was no significant difference between intromission duration for the black/black cross compared to the black/yellow or black/white (\(P = 0.556\)) crosses (Fig. 4C). Comparing the whole dataset in a single Kruskal–Wallis analysis followed by Mann–Whitney tests (with a Bonferroni correction factor) to look at differences gave the same results (results not shown).

There was a significant effect of both the number of sex-role alternations (Scheirer–Ray–Hare \(\chi^2 = 0.986, df = 2, P = 0.014\)) and colour-form cross (Scheirer–Ray–Hare \(\chi^2 = 0.956, df = 5, P = 0.044\)) on intromission duration; however, the number of sex-role alternations was not dependent on colour-form cross (Scheirer–Ray–Hare \(\chi^2 = 0.619, df = 10, P = 0.381\)) (Fig. 5). Thus, overall and regardless of colour form cross, in those instances where there was more than one sex-role alternation, intromission duration was significantly longer for the first intromission compared to the second intromission (\(P = 0.03\)) or the third intromission (\(P = 0.013\)). There was no significant difference in intromission duration between the second and third intromissions (\(P = 0.213\)). However, the results seemed to be heavily skewed by the results for the yellow/black cross, which showed a marked difference between first and second intromission duration (Fig. 5). If these yellow/black data were excluded, there was no significant effect of number of sex-role alternations (\(P = 0.143\)) or colour-form cross (\(P = 0.283\)) on intromission duration (results not shown).
This study is the first to examine detailed reproductive behaviour specific to colour forms in a cephalaspis species. Individuals of the *Chelidonura tsurugensis–sandrana* species complex from our sampling site all had the same external morphology, with the only differences between individuals being in body colouration. Our results indicate significant variability in mating decisions and behaviour among the three colour forms that likely contribute towards maintaining colour polymorphism. No significant differences supported assortative mating relative to colour form.

Overall, the results show that colour forms can have a greater tendency to mate with a colour form different from themselves, more often and/or for a longer time (Figs 2–5). For instance individuals of yellow mated for significantly less time, and took part in significantly fewer intromissions with other individuals of yellow, than with individuals of white or black. Furthermore, when individuals of yellow were given the opportunity to mate with those of black, or *vice versa*, there was a significant tendency for these individuals to undertake the role of sperm donor rather than to show sex-role alternation (Fig. 3). This is in contrast with results presented by Anthes & Michiels (2005, 2007a), Anthes et al. (2006) and Sprenger et al. (2009), which suggested that a single stereotypic sex-role alternation was the norm for *C. sandrana*. These authors all sourced *C. sandrana* from Lizard Island, Australia, where it appears the majority of the population comprises the white colour form (Anthes & Michiels, 2007a). Our data show no significant difference between sperm donation and alternating roles for white individuals, consistent with the data of Anthes and colleagues, but highlight the behavioural biases introduced by colour form. Although we did not specifically test for the trading of intromissions, the fact that individuals of different colour form did not reciprocate mating equally (either in duration or number of intromissions) shows there was no evidence for trading of intromissions or intromission durations (Fig. 3).

Furthermore, we also demonstrate the effect that colour form can have on intromission duration. Contrary to Anthes & Michiels (2005), we found that there was a significant difference between the length of the first and second intromission when sex role alternation had taken place. But this result was clearly being driven by the large differences observed in the yellow × black cross (Fig. 5) and, when these data were excluded, intromission duration was not significantly different for the first and subsequent intromissions. The number of multiple matings has been shown to be a key driver in the regulation of offspring size in *C. sandrana* (Sprenger et al., 2008a, b), with those ‘females’ mated four times with the same ‘male’ showing reduced offspring size. In comparison, ‘females’ mated multiple times with different ‘males’ produced larger egg capsules and veligers. Therefore it appears that sperm-donor diversity (rather than amount of sperm) can lead to fitness benefits, and this may explain why intromission duration decreases with multiple intromissions (Sprenger et al., 2008a, b).

Cephalaspidids (*Anthes, Putz & Michiels, 2005; Anthes & Michiels, 2007b*), especially *C. sandrana* (*Anthes & Michiels, 2005; Anthes et al., 2006*), are increasingly being utilized as the system of choice for studies of reproduction in simultaneous hermaphrodites, especially aspects of their reproductive behaviour (*Anthes & Michiels, 2007a; Sprenger et al., 2011*). Recent investigations using *C. sandrana* have included work examining sperm trading and sex-role alternation (Anthes et al., 2006), the effect of body size on reproductive behaviour (Sprenger et al., 2009) and the effect of multiple matings on offspring size (Sprenger & Michiels, 2006a, b). In nearly all of these studies, the colour form used in experiments was not explicitly stated (but see Anthes & Michiels, 2005). The kind of significant among-morph variation we have demonstrated can clearly skew the outcome of such studies. It is likely that the frequency of colour morphs varies among populations and that variation attributed to geographic partitioning may, by proxy, actually be partly caused by colour-morph variation. For example, the alternation of sexual roles was more frequent in the Red Sea population (1.01 alterations on average, white form) compared to both Lizard Island populations (black form with an average of 0.84 alterations and white form with an average of 0.57 alterations; Anthes & Michiels, 2007a; colour pictures in *Anthes, 2006*). However, it also appears that the black form of *Anthes* (2006) included some yellow and white forms (as defined by our study) and may represent a mixed population relative to our results for the black form.

In the absence of any evidence for assortative mating by colour form there is no support for the classification of the different colour forms as separate species or subspecies. However, in view of the colour and morphological variation recorded in this study and by others it is clear that further work is needed to investigate the systematics of the *C. tsurugensis–sandrana* species complex. Colour polymorphism has been previously recorded in another cephalaspis species complex, *Melanochlamys cylindrica*. 

**DISCUSSION**

**Figure 4.** Time per intromission (min) comparing same-colour crosses and mixed colour crosses in the *C. tsurugensis–sandrana* species complex. **A.** Yellow (y) (Kruskal–Wallis $H = 25.16, df = 2, P < 0.001$). **B.** White (w) (Kruskal–Wallis $H = 12.17, df = 2, P = 0.002$). **C.** Black (b) (Kruskal–Wallis $H = 1.17, df = 2, P = 0.556$). Kruskal–Wallis tests were used for statistical analysis. In A and B Mann–Whitney tests (with a Bonferroni correction for multiple testing) were used to investigate differences highlighted by the results of the Kruskal–Wallis test. Values are means ± SE. Bars sharing the same letters are not significantly different from each other. Note that crosses such as y–w and w–y are repeated for clarity ($n = 30–80$ pairings).
forms represent distinct species (Krug morphological analyses, was used to confirm that these colour Journal of Molluscan Supplementary material is available at species complex at Hoga Island could be C. tsurugensis–sandrana. In that case molecular work, coupled with traditional investigations. Regardless of the underlying mechanism that maintains these colour polymorphisms, our results challenge previous assumptions that the colour forms do not differ from density dependence. Additionally, the possible influence of pre-reproductive behaviour to mate with colour morphs other than their own may not be linked to colour itself, but rather additional mate choice factors such as pheromones. Future investigations should try to link differences in mating behaviours among colour forms with frequency of occurrence in the population, thus potentially linking changes in reproductive behaviour to density dependence. Additionally, the possible influence of predation on frequencies of different colour morphs should be investigated. Regardless of the underlying mechanism that maintains these colour polymorphisms, our results challenge previous assumptions that the colour forms do not differ from each other in their reproductive behaviour.

**SUPPLEMENTARY MATERIAL**

Supplementary material is available at *Journal of Molluscan Studies* online.

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**REFERENCES**


SUPPLEMENTARY INFORMATION

**Figure S1.** Frequencies of mating role alterations undertaken by individuals of different colour forms of the *Chelidonura tsurugensis-sandrana* species complex when individuals of **A** yellow and white, **B** yellow and black and **C** white and black were paired in a mating trial. Note that the coloured bars show the proportion of colour forms which undertook the role of sperm donor (*n* = 80 pairings).