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First record of colour polymorphism in the Neotropical apple snail *Asolene platae*: inheritance mechanism and evidence for multiple paternity

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ABSTRACT

Asolene platae (Ampullariidae) is a dioecious freshwater snail with subaquatic gelatinous egg masses that dwells in the Río de la Plata basin (Argentina). The aim of this study was to describe the inheritance mechanism of the colour variations of the shell and soft parts of this snail, and to study their potential use as a genetic marker. The wild-type phenotype presents dark pigments in the soft parts and in shell bands, whereas the yellow phenotype lacks dark pigments in the soft parts and also most dark bands in the shell, except for a subsutural and a periumbilical band. The data showed that the lack of pigments in *A. platae* is a recessive homozygotic condition with a simple Mendelian inheritance mechanism. Females of the wild-type phenotype had a higher number of bands than the males. The pigment of the bands of both phenotypes is located in the calcareous matrix of the shell. Using the lack of pigments as a genetic marker we demonstrated the existence of biparental egg masses in *A. platae*, hitherto known in only one species within the Ampullariidae.

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Genetic marker; melanin; pigmentation; polyene; unbanded shell

Introduction

Asolene platae (Maton, 1811) is a South American freshwater snail that belongs to the family Ampullariidae and is naturally distributed in the Río de la Plata basin (Castellanos and Fernández 1976; Cowie and Thiengo 2003). It is a dioecious snail with internal fertilisation and deposits subaquatic gelatinous egg masses; males and females exhibit multiple copulation behaviour and females can lay hundreds of eggs in several egg masses after a single copulation (Tiecher et al. 2014). Contrary to previous studies (Castellanos and Fernández 1976) it has recently been considered conspecific with *Asolene pulchella* (Maton, 1838) (Hayes et al. 2009; Léon et al. 2014).

The general model of shell pigmentation in the Ampullariidae is light brown coloured with dark brown or black spiral bands (Estebenet et al. 2006); only a few species such as *Pomacea scalaris* (d'Orbigny, 1835) (Simone 2004) and *Pila virescens* (*Ampullaria virescens*, Deshayes in Bory de Saint Vincent, 1824) (Keawjam 1986) lack these bands or show other patterns, as in *Saulea vitrea* (Born, 1778) (Perera and Walls 1996). The cephalopodium and mantle of Ampullariidae (henceforth 'soft parts') are also heavily pigmented (Perera and Walls 1996; Yusa 2004). Colour variants to the general wild-type phenotype described above are known to exist in other species of *Pomacea* (e.g., Perera and Walls 1996; Coelho et al. 2012); however, only in *Pomacea canaliculata* (Lamarck, 1822) (Yusa 2004) and *Marisa cornuarietis* (Linnaeus, 1758) (Dillon 2003) have snails lacking dark pigments in the shell or body been thoroughly investigated. In both species the inheritance mechanism involved a single Mendelian gene, with the lack of dark pigment being a phenotype shown by the recessive homozygote. Besides the genetic basis of the colour variations of the shells and soft parts, the environment has an influence on the expression of these genes (Estebenet et al. 2006). In other molluscs evidence of effects of diet, temperature and light intensity upon shell and soft parts pigmentation has been found (e.g., Mitton 1977; Cowie and Jones 1985; Trevelyan and Chang 1987; Jordaens et al. 2001; Williams 2016). The colour polymorphism of *P. canaliculata* has proved useful to address fundamental questions on the biology of ampullariids as it was used by Yusa (2004, 2006) as a genetic marker to study the pattern of sperm use and the mechanism of sex determination.

During experimental work on the reproduction and life cycle of *A. platae* (Tiecher et al. 2014, 2015, 2016), several specimens without dark pigments and with conspicuous yellowish soft parts and shell were hatched in the laboratory. The object of the present work is to describe these colour variations and their inheritance mechanism and to use them to test whether multiple paternity occurs in this South American freshwater snail. We also investigated the relationship between the sex of the snails and the number of shell bands and the gaps that interrupt the continuity of some of these bands.

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Materials and methods

All snails used in the experiments were descendants of individuals collected in the Lago Regatas (Parque Tres de Febrero, Buenos Aires, Argentina; 34°34′24″S, 58° 24′53″W). The adult snails were reared individually in 3 L aquaria and the hatchlings in groups in Petri dishes. All snails were kept in the rearing room at 25 °C, with tap water saturated with $CaCO_3$ that was renewed weekly and fed with lettuce *ad libitum* (Tiecher et al. 2014, 2015, 2016). The snails used in the present study belong to the third generation of a population of snails originally collected from the Lago Regatas and maintained in the laboratory for 3 years.

Four empty shells (two of each phenotype) of adult snails were treated either with sodium hypochlorite solution or with hydrochloric acid (Estebenet et al. 2006) to determine whether the pigments of the shell bands are located in the organic portion (periostracum) or in the calcareous matrix of the shell. The number of the bands and their discontinuities were observed in 81 shells of adult snails that had previously been sexed according to their copulatory behaviour or egg laying (Tiecher et al. 2014).

To study the inheritance mechanism of the soft parts and shell pigments in *A. platae*, controlled crosses between wild-type and yellow phenotypes were performed. The experimental individuals used in the parental (P) generation were snails belonging to a wild-type phenotype rearing pool (with unknown genotypes). A male and a female were put in the same 3 L aquarium and observed at 20 min intervals to confirm copulation (Tiecher et al. 2014). The females that were used in these crosses belong to the F1 and were virgin (reared in isolation after hatching) and after the first mating they were paired always with the same male. Controlled crosses between wild-type snails from the F1 were also performed and phenotypic proportions among F2 offspring were estimated. In addition, controlled crosses between two yellow phenotype males (from the F1) and wild-type females (from a rearing pool) were carried out. The egg masses laid by the females in the controlled crosses were observed under a stereoscopic microscope in order to determine the presence or absence of pigments in the eye, shell and soft parts of the embryos and hatchlings. The transparency of the jelly that forms most of the egg mass after about 8 days of development (Tiecher et al. 2014) allows observation of the embryos' phenotype.

In order to study the sperm use pattern in *A. platae*, seven virgin yellow phenotype females were used in controlled copulations. The snails used in the experiment were born in the laboratory and the yellow phenotype was employed as a genetic marker. The yellow females were paired in the first place with yellow males until they copulated once; after each female laid its first egg mass they were then paired with a wild-type male (with known heterozygote genotype) until they copulated.

Results

Two clearly different, discrete phenotypes in shell and soft parts pigmentation were observed. The wild-type snails exhibited dark pigments (dark brown or black) in the soft parts (mantle, foot, snout and eyes) and the shells (Figure 1A). The dark pigments of the shell are disposed in several bands of variable width: one subsutural, one periumbilical and two to eight medial bands.

The yellow phenotype had no dark pigments in the soft parts or bands in the shell, except for the dark coloured subsutural and periumbilical bands (Figure 1B–C). Both bands appeared in the teleoconch during intracapsular development, while the rest of the bands of the wild phenotype appeared mostly at

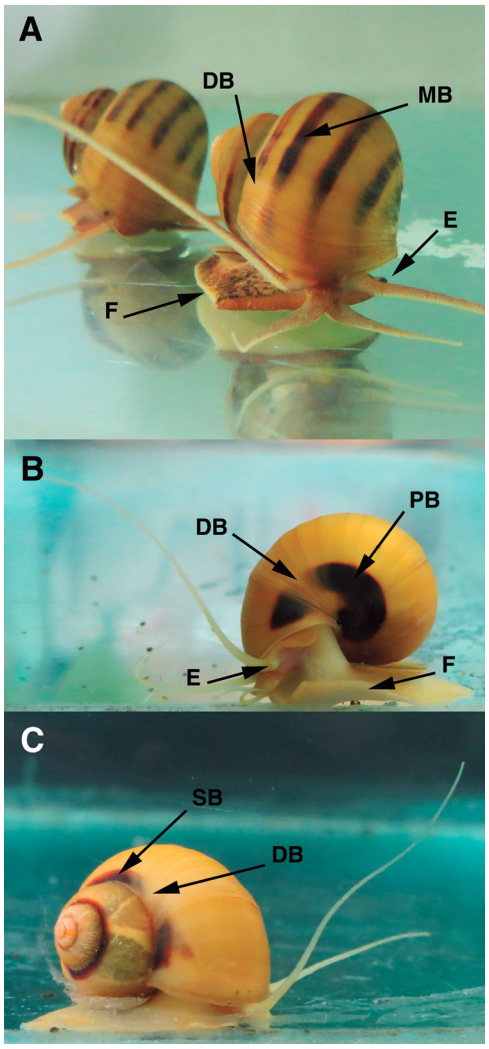

Figure 1. Pictures of *Asolene platae* showing soft parts and shells of both phenotypes. **A**, Individual of wild-type phenotype; **B**–**C,** individuals of yellow phenotype. DB—discontinued band; E—eye; F—foot; MB—medial band; PB—periumbilical band; SB—subsutural band.

hatching or afterwards. The general colour of their shells and bodies was pale yellowish and areas with a higher number of mucous glands looked yellow. The absence of dark pigments allows the observation of the digestive gland and of the testis in the males through the shell (Figure 1C). In both phenotypes the operculum looks similar and shows no differences in colour or transparency (Figure 2A).

In the wild-type snails there was a significant difference between sexes in the mean number of bands ($t = 2.13$, $P = 0.036$), with females showing a slightly higher number (7.14 ± 1.13, mean $\pm$ SD) than males (6.52 ± 1.41) (Figure 3). Of all the wild phenotype adult snails studied ($n = 81$), 39.5% showed simultaneous discontinuities in all the medial bands, although in most of them the bands reappeared after some time; these discontinuities were also observed in the periumbilical and subsutural bands in the yellow phenotype snails (Figure 1A–C). In the case of females, 48.65% ($n = 37$) showed discontinuities in their bands while in the males only 31.82% ($n = 44$) presented them; however, the association between the discontinuity of the bands and the sex was not significant ($\chi^2 = 2.382$, $P = 0.123$).

The bands of the shell of both phenotypes (either medial, subsutural or periumbilical) remained unaltered after the treatment with sodium hypochlorite solution (Figure 2B); on the other hand, after dissolution of the shell with hydrochloric acid the periostracum showed no bands, indicating that the pigments are located in the calcareous matrix and not in the periostracum. After the treatment with sodium hypochlorite the yellowish background colouration of the shells disappeared from both phenotypes, showing that the background colouration is due to the periostracum.

Only the crosses that resulted in viable egg masses are reported (Figure 4). Three F1 egg masses were obtained from the copulation of a wild-type male and a wild-type female, with an overall average of 78% wild-type and 22% yellow hatchlings. Five crosses between wild-type males and F1 females produced six egg masses (F2) with a total average of 76% wild-type and 24% yellow hatchlings. Two crosses between F1 yellow males and wild-type females from the rearing pool produced all wild-type hatchlings. An F2 yellow female was crossed with a F1 yellow male (back-cross) and all the hatchlings from a single egg mass were of the yellow phenotype. The female that participated in the back-cross was later crossed with a wild-type male with heterozygote genotype and produced 42% wild-type and 58% yellow hatchlings (values from the three last egg masses; Figure 4).

In the study of sperm use, five out of seven controlled crosses performed ended in copulation, but only two females laid viable egg masses (Figure 5A). After 14 days female 1 copulated with its second mate, a wild-type male, and paternity was evident from the presence of 12.5% wild-type hatchlings in the first egg mass laid after the copulation and values around 42% attained after the laying of two egg masses. After 78 days female 2 copulated with its second mate (wild phenotype), and paternity was also noticeable in the first egg mass after the copulation but with a higher percentage of wild-type hatchlings (46.4%; Figure 5A). In the case of female 2 an

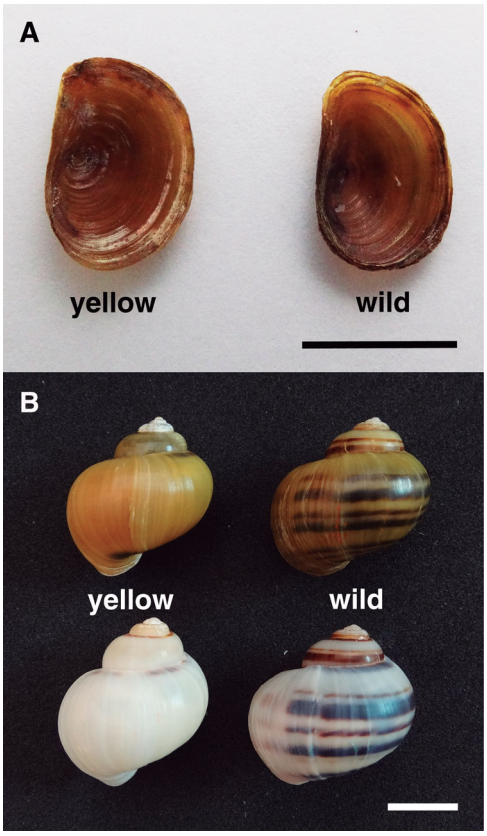

Figure 2. Opercula and shells of *Asolene platae*. **A,** Opercula of yellow and wild phenotypes; **B**, shells of both phenotypes shown untreated (upper) or treated with sodium hypochlorite (bottom). Scale bars = 1 cm.

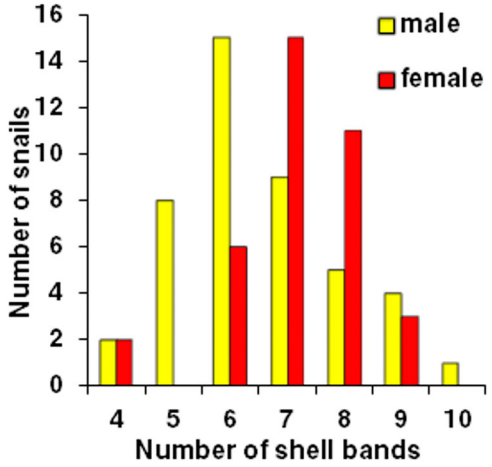

Figure 3. Number of shell bands in males and females of *Asolene platae*.

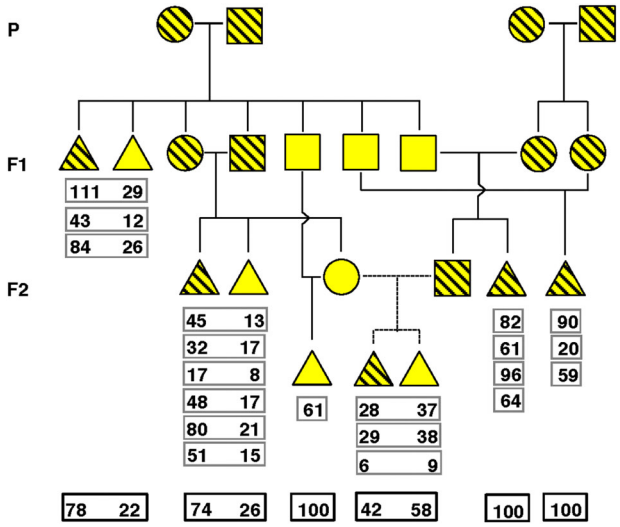

Figure 4. Scheme representing the crosses performed in *Asolene platae*. Circle—female; square—male; triangle—non-sexed progeny; striped forms—wild phenotype; forms with solid fill—yellow phenotype. The numbers inside grey boxes show the number of individuals of each phenotype in a single egg mass; values inside black boxes indicate the average of the phenotype percentage for all egg masses. Dashed lines indicate the crosses with the second male in the sperm use experiment.

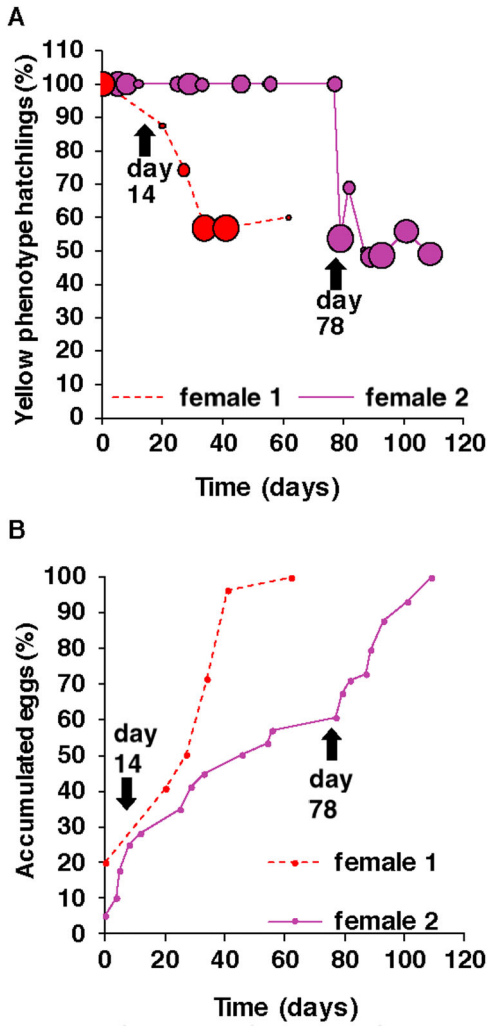

Figure 5. Sperm use experiment of *Asolene platae*. **A**, Percentages of yellow phenotype hatchlings; the size of the spheres show the number of hatchlings of each egg mass; **B**, percentage of accumulated eggs of each female. The arrows indicate the day in which each female copulated with its second mate, a wild-type male.

increment in the egg laying rate was registered after the copulation with the second male (Figure 5B).

Discussion

The general lack of pigmentation in the shell and soft parts in some individuals of *A. platae* is the first record in the genus *Asolene* and only the third at generic level within the nine currently recognised genera of the Ampullariidae (Hayes et al. 2015). The yellow phenotype specimens present a yellowish colouration of soft parts and shell, but nevertheless show dark subsutural and periumbilical bands. Except for these bands, the pattern in *A. platae* is similar to that described for *P. canaliculata* (yellow snails without dark pigments in both soft parts and shell; Yusa 2004) and *P. bridgesii* (Reeve, 1856) (yellow shell and white soft parts; Perera and Walls 1996). It is noteworthy that, in *M. cornuarietis*, a species that is genetically closer to and probably congeneric with *A. platae* (Hayes et al. 2015), Dillon (2003) described a 'golden' variety with unbanded yellowish shell but with wild-type soft parts, indicating that the pigmentation of soft parts and shell is, genetically at least, partly decoupled.

In addition to its utility as a genetic marker (see below), the absence of dark pigments in the soft parts and shell allows for an early sexing since the whitish testicle is easily observed through the translucent shell of the yellow phenotype. This is important in *Asolene* snails which lack external shell and operculum dimorphism (Tiecher et al. 2014, 2016). Early sexing has been very useful in different research lines on *Pomacea* spp. (e.g., Yusa, 2006; Tamburi and Martín 2008).

After the removal of periostracum with sodium hypochlorite the bands of both phenotypes remain

unaltered and the periostracum of dissolved shells lacks bands. These data indicate that the dark pigments of the shell are located in the calcareous matrix (Estebenet et al. 2006). On the other hand, the yellowish background colouration of the shell almost completely disappeared after the chemical treatment, showing that it is due to the outermost organic layer of the shell, the periostracum, as in *P. canaliculata* (Estebenet et al. 2006). In *A. platae* there is no difference between the opercular pigmentation of the wild-type and yellow phenotypes. In *P. canaliculata* (Tiecher, pers. obs.), *P. bridgesii* (Perera and Walls 1996) and *Marisa cornuarietis* (Dillon 2003), the opercula in the 'yellow' or 'golden' phenotypes also seem to have the same colouration as in the wild-type phenotypes. This evidence suggests that the light brownish colour of the operculum is solely due to the conchiolin that forms it, as in the case of the periostracum.

The locations of the pigments of bands and the background colouration of the shell in *A. platae* correspond to those described for *P. canaliculata* (Estebenet et al. 2006). However, in *A. platae* different shell bands appear at different times in development: during intracapsular development in the case of the subsutural and periumbilical bands present in both phenotypes and after hatching in the medial bands (as occurs with those of the wild-type phenotype of *P. canaliculata*; Estebenet et al. 2006). On the other hand, the dark pigments of medial bands and soft parts may be under the control of the same gene whilst those of subsutural and periumbilical bands are not. Many variants of soft parts and shell colouration have been described in the ampullariids, but the presence in *A. platae* of two groups of shell bands with different genetic controls and with different ontogenetic patterns represents the first record in the family. Kozminsky (2014) concluded that in *Littorina obtusata* (Linnaeus, 1758) the purple and the yellow pigments of the shell are regulated by at least two separate genes, and also reported that the pigments controlled by different genes appear at different times during embryonic development.

The dark medial bands in *A. platae* were highly variable in number and width. In *P. canaliculata* a lower number of wider shell bands was observed in deeper waterbodies but the mechanism involved is not clear (Galan et al. 2015). On average, the shells of *A. platae* females showed more bands than those of males although there is a broad overlap. In the family Ampullariidae, in which sexual dimorphism in many secondary traits is common (Hayes et al. 2015), the colour of soft parts and shell banding has not been recorded as dimorphic, except for *M. cornuarietis* in which the soft parts of females are darker (Demian and Ibrahim 1972).

Although the pigmentation of soft parts and shell in *A. platae* is under genetic control, environmental or physiological conditions may have influence on its expression. Environmental factors such as food (Jordaens et al. 2001), temperature (Cowie and Jones 1985) and light intensity (Mitton 1977; Trevelyan and Chang 1987) influence the colouration intensity of the shells of different molluscs. However, although in the present study the snails were all reared under constant conditions (food, temperature and photoperiod), the discontinuity and reappearance of all the shell bands of some snails were observed in different stages. Although these changes were not related to a snail's sex or reproductive activity, they seem to have an individual physiological basis.

Crosses performed between yellow phenotypes of *A. platae* produced only yellow progeny while crosses between wild-type phenotypes produced either a homogeneous wild-type progeny or a mixed progeny (c. 75% wild-type and 25% yellow phenotypes, when both parents also come from a mixed progeny). Crosses between a yellow and a wild-type phenotype produced either an all wild-type progeny or a 50:50 mixed progeny (when the wild-type parent originated from a cross between a yellow and a wild-type parent). Based on these controlled crosses we can conclude that the gene that encodes the dark colouration of the soft parts and the medial shell bands segregates under a simple Mendelian model, where the yellow phenotype is given by a recessive homozygote genotype and the wild-type corresponds to a dominant homozygote genotype or to a heterozygote genotype, as happens in *M. cornuarietis* (Dillon 2003) and *P. canaliculata* (Yusa 2004).

In each of *A. platae* and *P. canaliculata* the control of soft parts and shell band pigmentation by one gene is noteworthy since the pigments responsible for the dark colours of these two parts of the snail are usually different. For instance, the dark pigment of the soft parts (mantle and cephalopodium) is probably melanin as in other gastropods (Fox 1983; Margry and van Ooijen 2011), although there are no specific studies in ampullariids. On the other hand, the pigments of the shell are polyenes in several species of *Pomacea* and in *M. cornuarietis* (De Oliveira et al. 2013). Furthermore, two of the bands of *A. platae* shells are apparently controlled by a different genetic mechanism that is already expressed during intracapsular development and perhaps they are chemically different from the other bands. Up to four different pigments have been reported in caenogastropod shells, each of them likely to be under the control of a different genetic system (Kozminsky 2014).

The pattern of sperm use was markedly different between the two females that deposited egg masses. In both homozygote yellow females the paternity of the heterozygote second male was evident in the first egg mass produced after the copula. In female 2, which laid egg masses for 78 days until it copulated with the second male, the paternity of the latter

abruptly reached 42% of the progeny. In female 1, in which only 17 days passed between the first and second copulation, 20 days were needed to reach a similar percentage of paternity from the second male. A female that copulates only once can deposit up to nine egg masses during 82 days without a new copulation (Tiecher et al. 2014), indicating that the female seminal receptacle empties or perhaps that the sperm loses viability. Notwithstanding, for female 1 the percentage of wild-type progeny never reached the values of 50% that would be expected if the sperm of the heterozygote second male totally replaced that of the first mate, a recessive homozygote. In *A. platae* females the sperm replacement seems to be affected by the number of days and the number of egg masses deposited between the first and second copulations. Although limited by the difficulties of rearing *A. platae* (age at maturity 1 year, high post-hatching mortality and strong cannibalism of egg masses; Tiecher et al. 2014, 2016), the evidence from biparental egg masses indicates the existence of sperm competition in the species, which hitherto is only known for *P. canaliculata* within the Ampullariidae (Yusa 2004).

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Disclosure statement

No potential conflict of interest was reported by the authors.

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