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Cloacal protuberances and extreme sperm production in Australian fairy-wrens

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SUMMARY

In many passerine species males have enlarged cloacal protuberances during the breeding season. One hypothesis for the evolution of cloacal protuberances posits that they are a response to sperm competition and thus predicts that both within and between species the size of the protuberance correlates with the number of sperm stored. Here we provide the first intraspecific test of this hypothesis. In Australian fairy-wrens (Aves: Maluridae) females regularly mate outside of their social group resulting in intense sperm competition among males. Male fairy-wrens develop enlarged cloacal protuberances, and in a study of three species, splendid fairy-wren, white-winged fairy-wren, and variegated fairy-wren, we found significant intraspecific correlations between the size of a male's protuberance and the stored sperm reserves in two of the three species. Males of these species had extreme numbers of sperm in their cloacal protuberances, up to 8.3 billion for splendid fairy-wrens, which should be available for a single ejaculate and is the most ever reported in an avian species. Studies of both captive and wild males showed that individuals can produce as many as 2 billion sperm per day. These data support the sperm competition hypothesis for the evolution of cloacal protuberances and highlight extreme sperm production as one possible outcome of sperm competition.

1. INTRODUCTION

Sperm competition occurs when sperm from different males attempt to fertilize the ova of a single female (Parker 1970). Factors influencing success in sperm competition are of interest because such factors may influence variance in reproductive success and, ultimately, intrasexual selection. Sperm competition is widespread in animals and may be responsible for the evolution of morphological and behavioural traits as extreme and exaggerated as those produced by direct physical combat among males (Parker 1970, 1984, 1990; Smith 1984; Eberhard 1985; Gomendio & Roldan 1991; Møller 1991; Birkhead & Møller 1992).

In birds, one trait thought to be the evolutionary result of sperm competition is the enlarged cloacal protuberance found in males of many passerines (Birkhead & Møller 1992; Briskie 1993). It has long been known that cloacal protuberances are partly formed by a pair of seminal glomera that act as the site of sperm storage in males (Wolfson 1952, 1954; Salt 1954) but the exact function of the protuberances has remained unclear. Three non-exclusive hypotheses have been proposed (as reviewed in Birkhead *et al.* 1991, 1993): (i) efficient copulation hypothesis; (ii) sperm size hypothesis; and (iii) sperm competition hypothesis. The sperm competition hypothesis is the

most directly testable of these hypotheses and is our focus here. This hypothesis states that in systems marked by high levels of sperm competition, selection should favor increased sperm production, either for multiple or more effective copulations, and that larger cloacal protuberances are an adaptation for the storage of larger numbers of sperm.

The sperm competition hypothesis predicts that across species, morphological or behavioural indices of sperm competition (e.g. testes size, sperm numbers, and copulation frequency) should covary with the relative size of the cloacal protuberance, and this prediction has received empirical support (Birkhead *et al.* 1993). On an intraspecific level, if cloacal protuberances function in sperm storage, the size of an individual male's protuberance should be positively correlated with the number of sperm stored there. In this study we examined this prediction in fairy-wrens (Aves: Maluridae) in Australia.

Fairy-wrens are small (8–10 g) insectivorous passerines, distributed throughout the Australasian zoogeographic region. The genus *Malurus* comprises 13 species, all of which are gregarious, highly social, and known or thought to exhibit cooperative breeding (Schodde & Weatherly 1982). Wren family groups vary in size, typically ranging from two to five individuals. Group composition varies across species,

but a typical group of splendid fairy-wrens consists of one behaviourally dominant male, one adult female, and several younger male offspring. Sperm competition resulting from extra-pair matings in fairy-wrens is intense. Paternity studies have shown that the majority of young in nests are fathered by males outside the social group (Brooker *et al.* 1990; Mulder *et al.* 1994) and genetic studies have shown extra-group fertilizations are frequent in all three populations included in this study (M. S. Webster, S. Pruett-Jones and E. M. Tuttle, unpublished data). Male *Malurus* wrens have large cloacal protuberances and extremely large testes that equal 5–10% of the mass of the individual male (Møller 1991; Birkhead & Møller 1992; Mulder & Cockburn 1993).

2. METHODS

At Brookfield Conservation Park in South Australia we have studied three sympatric species, the splendid fairy-wren (*Malurus splendens melanotus*), white-winged fairy-wren (*M. leucopterus luconotus*), and variegated fairy-wren (*M. lamberti assimilis*) since 1992. During 1994 and 1995 we measured cloacal protuberances and collected sperm samples from males. The cloacal protuberance is shaped like a bulbous barrel projecting posterior to the cloacal opening. There is also a prominent triangular tip anterior to the cloacal opening. We took three measurements of the cloacal protuberance: length (L), which was the distance from the anterior portion of cloacal opening (not including the tip) to the posterior edge; depth (D); and width (W). Volume was calculated as: volume = $\pi \times (D/2 \times W/2) \times L$ (Briskie 1993; Mulder & Cockburn 1993).

Seasonal changes occur in the size of a male's cloacal protuberance (Nakamura 1990; Briskie 1993; Mulder & Cockburn 1993). Our study was done during the peak of breeding (mid-October through December) for each of the three species and the time period during which the protuberance should be at its maximum size.

Sperm samples were collected using a technique of cloacal manipulation modified from Quinn & Burrows (1936) as described in Wolfson (1960). Briefly, while maintaining upwards pressure on the bird's back near the area of the tail, the cloacal protuberance was repeatedly but gently squeezed until the sperm stored there was expelled. We refer to the samples collected as ejaculate samples. This manipulation technique did not cause the individuals any visible discomfort.

In an effort to determine how much of the sperm in a male's seminal glomera we were obtaining through the manipulation technique, we collected 13 males (six splendid, three white-winged, and four variegated fairy-wrens) after sampling and their seminal glomera were later dissected. The glomera were macerated in a known volume of avian physiological saline and the sperm counted using a hemocytometer as described below.

All sampled males were manipulated until an ejaculate sample was obtained or one minute had passed. The samples were collected in calibrated 10 μ l micro-capillary tubes and then diluted in 200 μ l of Minnesota Turkey Semen Extender (MTSE, Ogasawara & Sexton 1970). Samples were stored at 4 °C for 1–4 h before counting. Ejaculate samples greater than 10 μ l in volume were diluted a second time to facilitate counting.

All samples were mixed thoroughly before counting. We determined sperm density by standard counts with a hemocytometer that had a cell volume of 0.00078 mm³. Density was determined in ten cells on the hemocytometer for each individual. To verify our mixing procedure, we counted sperm in five aliquots (ten cells for each aliquot as above) for each of five male splendid fairy-wrens. For each of these five males, there was no significant variation in the sperm counts across aliquots (ANOVA, $p > 0.12$ in all analyses).

To quantify approximate rates of sperm production (see Amann 1981), we kept six adult males (five splendid fairy-wrens and one white-winged fairy-wren) in captivity and obtained ejaculate samples every 24–48 h. The birds were housed in small bird cages (0.35 m³) and fed mealworms *ad libitum*. To reduce stress during captivity, each male's known social mate was housed in a separate cage adjacent to the male's cage. Each male was thus physically separate from, but in visual and vocal contact with his social mate during captivity. After a maximum of four days in captivity, each male and his mate was returned and released on their territory.

In our analysis, ejaculate volume and sperm number were transformed (square root transformation) to normalize their distributions. For each male, only those data from the first capture and sampling were included in the analysis; data from recaptures were omitted. Additionally, males that did not produce an ejaculate sample or produced samples contaminated with feces were removed from the analysis of ejaculate volumes and sperm numbers (splendid, white-winged, and variegated fairy-wrens). Males that did not produce ejaculate samples commonly had swollen cloacal protuberances.

3. RESULTS

(a) Age and size-related variation in cloacal protuberances

Adult males of each species had large cloacal protuberances (see table 1). Within each species there was age-related variation in development of the protuberance. This variation was associated with plumage patterns in splendid and variegated fairy-wrens but was disassociated with plumage in white-winged fairy-wrens. Male splendid fairy-wrens attained part or full adult plumage at one year of age. All male splendid fairy-wrens in full adult plumage had a protuberance whereas one-year old males in part

Table 1. *Cloacal protuberances and sperm samples in male fairy-wrens. Shown are the mean values \pm standard error. Sample sizes are shown in parentheses*

species of fairy-wren	body mass	cloacal protuberance volume/(mm ³)	ejaculate sample volume/(μ l)	sperm number ($\times 10^{-6}$)	sperm length/(μ m)
splendid	9.1 \pm 0.1 (56)	162.6 \pm 6.9 (59)	8.2 \pm 0.8 (59)	907.6 \pm 127.3 (59)	83.1 \pm 0.2 (52)
white-winged	7.7 \pm 0.1 (27)	180.8 \pm 12.4 (28)	9.8 \pm 0.9 (28)	1651.6 \pm 236.8 (28)	83.0 \pm 0.4 (19)
variegated	8.2 \pm 0.1 (37)	73.3 \pm 2.9 (39)	2.0 \pm 0.3 (39)	180.6 \pm 41.7 (39)	86.3 \pm 0.3 (17)

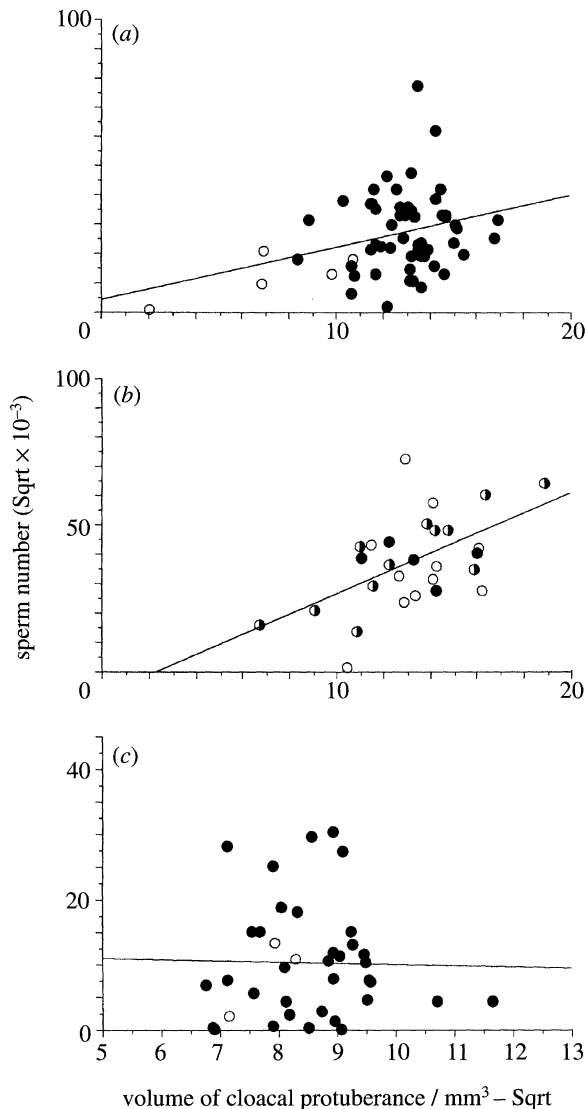


Figure 1. Regression of volume of cloacal protuberance versus sperm number in: (a) male splendid fairy-wrens (filled = adult plumage; hollow = partial plumage); (b) male white-winged fairy-wrens (filled = adult plumage; half-filled = partial plumage; hollow = brown plumage); and (c) male variegated fairy-wrens (filled = adult plumage; hollow = partial plumage). The regression is statistically significant in splendid and white-winged fairy-wrens. See text.

plumage either did not have a protuberance or had a very small one (differences in volume of protuberance between partly plumed males having a protuberance and fully plumed males statistically significant, ANOVA, $F_{1,57} = 30.00$, $p < 0.001$). In variegated fairy-wrens partial or adult plumage in males was attained at two years of age and only males in full adult plumage had enlarged protuberances. In white-winged fairy-wrens, males did not attain part or full adult plumage until after two years of age. In stark contrast to the other two species, however, all male white-winged fairy-wrens at least one year of age, regardless of plumage condition, had enlarged protuberances. In this species, the cloacal protuberances of female-plumaged males did not differ significantly in volume from those of partly plumed or fully plumed males (ANOVA, $F_{2,25} = 0.03$, $p = 0.969$).

Size of the cloacal protuberance did not vary with body mass of the male. Within each species, a regression

of individual male body mass versus length of the protuberance was not statistically significant ($p > 0.07$ in each regression).

(b) Cloacal protuberances and sperm storage

Volume of the cloacal protuberance differed significantly among the species (ANOVA, $F_{2,123} = 52.99$, $p < 0.001$). Splendid and white-winged fairy-wrens had the largest cloacal protuberance volumes and did not differ significantly from each other (Fisher's PLSD, $p = 0.1051$). Variegated fairy-wrens had significantly smaller protuberances than either of these other species (Fisher's PLSD, $p < 0.001$ in both comparisons).

Males of each species had large numbers of sperm in their cloacal protuberances (table 1). This was a function both of the large volumes of ejaculate samples (table 1) and high concentrations of sperm per sample (mean sperm concentration equaled 108.07×10^6 sperm μl^{-1} for splendid fairy-wrens, 159.22×10^6 sperm μl^{-1} for white-winged fairy-wrens, and 99.81×10^6 sperm μl^{-1} for variegated fairy-wrens; differences across species significant, ANOVA, $F_{2,123} = 4.59$, $p = 0.012$). Total numbers of sperm in the ejaculate samples ranged from 1.59×10^6 to 6084.43×10^6 for splendid fairy-wrens, 1.93×10^6 to 5295.31×10^6 for white-winged fairy-wrens, and 0.002×10^6 to 936.09×10^6 for variegated fairy-wrens. On recapture, one male splendid fairy-wren had 8.3 billion sperm in his ejaculate sample.

Larger ejaculate samples contained more sperm (splendid fairy-wren $r^2 = 0.567$, $p < 0.001$; white-winged fairy-wren $r^2 = 0.635$, $p < 0.0001$; variegated fairy-wren $r^2 = 0.201$, $p < 0.004$). The direct relation between volume of the cloacal protuberance and sperm number was significant within splendid (figure 1, $r^2 = 0.083$, $p < 0.027$, sperm number = 1.775 (volume of cloacal protuberance) + 4.615) and white-winged fairy-wrens (figure 1, $r^2 = 0.275$, $p < 0.025$, sperm number = 3.430 (volume of cloacal protuberance) - 7.767). The relation was not significant in variegated fairy-wrens (figure 1, $r^2 = 0.0004$, $p > 0.904$, sperm number = -0.166 (volume of cloacal protuberance) + 1.173).

(c) Comparison of ejaculate samples and macerated glomera samples

For the 13 males we dissected after sampling, the sperm obtained in the ejaculate samples represented 47.0% of the total number of sperm contained in the seminal glomera (table 2). By species, the values were 62.4% for splendid fairy-wrens, 31.9% for white-winged fairy-wrens, and 35.4% for variegated fairy-wrens.

Combining the sperm counts in the ejaculate samples with those from the seminal glomera provides a total sperm count for these males. The average total sperm counts for the three species were: splendid fairy-wren = 2755.66×10^6 ($n = 6$; range = 380.17×10^6 to 9141.88×10^6), white-winged fairy-wren = 3498.44×10^6 ($n = 3$; range = 777.16×10^6 to 5991.24×10^6), variegated fairy-wren = 517.09×10^6 ($n = 4$; range = 140.62×10^6 to 764.62×10^6).

Table 2. Comparison of sperm counts in ejaculate samples versus seminal glomera for individual male fairy-wrens

species	individual	volume of ejaculate sample/ μl	sperm counts ($\times 10^{-6}$)		percent of total in ejaculate samples
			ejaculate sample	seminal glomera	
splendid	303	7.00	329.01	51.16	86.5
	304	14.00	1453.17	51.16	96.6
	305	12.50	1768.03	306.92	85.2
	368	9.50	1107.93	667.69	62.4
	410	4.33	382.91	8758.97	4.2
	414	11.00	649.32	1007.69	39.2
white-winged	140	11.50	2316.24	3675.00	38.7
	142	15.75	669.79	3057.11	18.0
	144	13.25	303.31	473.85	39.0
variegated	275	9.50	115.28	376.92	23.4
	279	4.00	0.62	140.00	0.4
	286	3.50	638.08	126.54	83.5
	291	1.25	229.40	441.54	34.2

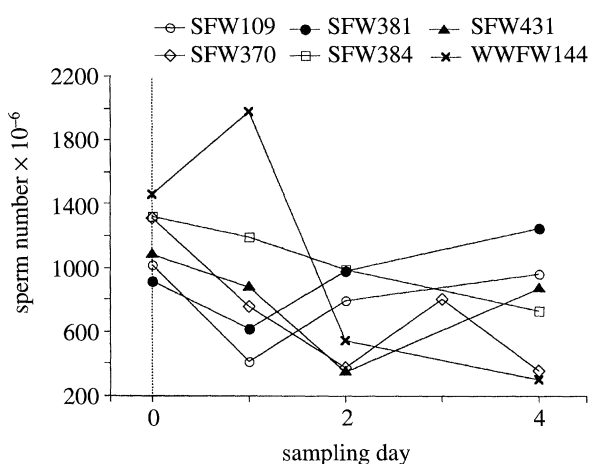


Figure 2. Sperm counts in ejaculate samples of six captive males (five splendid fairy-wrens (SFW) and one white-winged fairy-wren (WWFW)) sampled on consecutive days.

(d) Sperm production

The data on estimated daily sperm production are illustrated in figure 2. For the five male splendid fairy-wrens, the mean sperm count on the day of capture was 1126.60×10^6 sperm. Over the next 2 d, the mean sperm counts of these males were: 771.54×10^6 on day 1 (24 h after initial capture), and 692.99×10^6 on day 2. Only one male was sampled on day 3, but all five males were again sampled on day 4 and they had a mean sperm count of 835.50×10^6 . Additional data on sperm production in splendid fairy-wrens comes from two wild males each caught and sampled on two consecutive days. Male SW001 had a sperm count of 456.62×10^6 on 10 November 1994 and 721.81×10^6 on 11 November. Male SW228 had a sperm count of 6084.43×10^6 on 11 November 1995 and 2012.23×10^6 on 12 November. Combining all samples of sperm production over a 24 h period (14 samples from the five captive and two wild males) yields an average daily sperm production rate for splendid fairy-wrens of $801.22 \times 10^6 \pm 435.46 \times 10^6$ s.d. Expressed relative to body mass this value is 88.05×10^6 sperm per gramme of body mass.

The male white-winged fairy-wren held in captivity

had a sperm count of 1974.59×10^6 on day 1 (24 h after initial capture and sampling) and 538.95×10^6 on day 2. This male was not sampled on day 3, but on day 4 he had a sperm count of 303.31×10^6 .

(e) Sperm size

Sperm size varied significantly across the three species (table 1; ANOVA, $F_{2,88} = 36.65$, $p < 0.0001$). These differences were the result of interspecific differences in lengths of both the tail of the sperm (ANOVA, $F_{2,87} = 31.24$, $p < 0.0001$) and the acrosome (ANOVA, $F_{2,88} = 6.70$, $p < 0.0001$) but not the head of the sperm (ANOVA, $F_{2,87} = 1.86$, $p = 0.1613$). The variegated fairy-wren had the lowest number of sperm reserves but had significantly longer sperm than either of the other two species (Fisher's PLSD, $p < 0.0001$ for both comparisons, splendid versus variegated and white-winged versus variegated).

Sperm size also varied significantly across individual males within each species. For each of the three species there was significant variation across individuals in each of the sperm measurements (total length, head length, acrosome length, and tail length, ANOVA, $p < 0.0004$ for each analysis). This individual variation did not, however, covary with sperm numbers; within each species there was no relation between length of a male's sperm and the number of sperm in his ejaculate sample ($p > 0.146$ for each regression).

4. DISCUSSION

The sperm reserves and sperm densities in cloacal protuberances documented here for fairy-wrens are, relative to body size (per gramme of body mass), larger than any previously reported value for either birds or mammals (Møller 1988*a,b*, 1989; Birkhead & Møller 1992). The maximum sperm counts we obtained for splendid fairy-wrens, 8.3 billion from an ejaculate sample and 9.1 billion from an ejaculate sample combined with glomerular tissue, are also absolutely greater than any reported value for birds (Møller 1988*a*; Birkhead & Møller 1992). Massive sperm reserves could function in two ways. First, they would allow a high copulation rate without depletion, and in

interspecific comparisons, large sperm reserves and large cloacal protuberances have been shown to correlate with high copulation rates (Birkhead *et al.* 1993). Alternatively, if the sperm reserves were completely used in a single copulation, they could increase a male's chance of paternity (Birkhead *et al.* 1995; Colegrave *et al.* 1995).

There is circumstantial evidence to suggest that in fairy-wrens the sperm reserves function in the latter manner, as massive ejaculates for a single or few copulations. First, fairy-wrens are not known to have high rates of copulation, in fact behavioural observations suggest exactly the opposite (Brooker *et al.* 1990; Rowley & Russell 1990). Additionally, the volumes of the ejaculate samples we obtained are comparable to volumes of actual ejaculates for other bird species of similar size (Gee & Temple 1978; Møller 1988*b*). Nevertheless, the densities of sperm in fairy-wren species are so large that even if the ejaculate samples represent reserves for a number of copulations, the number of sperm transferred to females would still be massive. For example, in the zebra finch (*Taeniopygia guttata*) males transfer, on average, between 0.17×10^6 and 5.29×10^6 sperm during a single copulation (Pellatt & Birkhead 1994; Birkhead *et al.* 1995). Male white-winged fairy-wrens have, on average, 300–1000 times this amount of sperm in their cloacal protuberances.

Across the three species, the sperm we obtained in the ejaculate samples comprised about half of all of the sperm in a male's seminal glomera. If the males transfer during copulation the amount of sperm we obtained, the remaining sperm in the glomera could then be moved to the distal portion of the glomera and be ready for other copulations the following day. Alternatively, if males actually ejaculate more sperm than that which we obtain by manipulation, the sperm counts of the three species would be proportionately even larger than the values reported here. In zebra finches, Birkhead *et al.* 1995 found that 84% of the sperm in the seminal glomera was available for ejaculation and that the other 16% of sperm remained in the glomera after copulations ceased.

The fact that our ejaculate-sample sperm counts do not represent all of the sperm the seminal glomera of males does not diminish the correlation between sperm number and protuberance size. Unless there is significant variation across males in the proportion of their total sperm obtained through our manipulation technique (which because of small sample sizes we cannot at present determine), the relation between size of cloacal protuberance and sperm numbers would be the same as we have reported even though our sperm counts are only a percentage of the total sperm available.

Birkhead *et al.* (1995) document that sperm counts in ejaculates of male zebra finches are dependent on the time since their previous copulation. This may also be the case in fairy-wrens. There was a slight reduction in sperm counts in the captive males from the date of capture to the samples on days 1 and 2. Then, after no sample on day 3, there was an increase in the mean counts on day 4 (see §3). This relation obviously

introduces a large component of variance in a field study such as ours, where we generally do not know when males have copulated, and would tend to obscure a relation between size of cloacal protuberances and sperm numbers. The fact that we did observe such a correlation suggests either that male fairy-wrens recover their sperm numbers quickly after copulation, or that copulations are generally rare and that most males have near maximal numbers of sperm in their cloacal protuberances. We suspect that both conditions are true in the species we studied.

The interspecific differences in sperm number and sperm length observed in our study may represent a trade-off between sperm numbers and sperm length (see Parker 1982, 1993). Alternatively, the differences may reflect differences in the number and lengths of sperm storage tubules in females of the three species (Briskie & Montgomerie 1992). Besides having smaller sperm counts, variegated fairy-wrens also differed from splendid and white-winged fairy-wrens in not exhibiting the correlation between sperm number and size of cloacal protuberance. These differences are likely the result of differing dynamics of sperm competition in variegated fairy-wrens. As cloacal protuberance size is correlated with testes size (Birkhead *et al.* 1993), and testes size is correlated with levels of extra-pair paternity (Møller & Briskie 1995), we might predict that sperm competition is less intense in variegated wrens than in splendid and white-winged fairy-wrens.

The results of this study support the sperm competition hypothesis for the development of large cloacal protuberances in passerine birds. In fairy-wrens, the frequency of extra-pair paternity is higher than in any other socially monogamous bird species (Brooker *et al.* 1990; Mulder *et al.* 1994) and this should produce intense sperm competition among males (Birkhead & Møller 1992; Møller & Briskie 1995). We interpret the extreme sperm counts and sperm production rates in fairy-wrens as evolutionary responses to such sperm competition. It is clear that an understanding of sperm reserves and sperm production capabilities in these species is critical to a complete understanding of their mating systems.

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