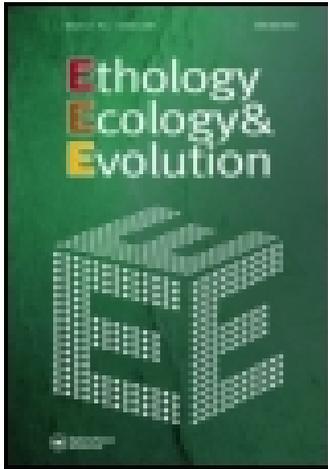


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An indirect approach to study sperm precedence in a subsocial spider

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An indirect approach to study sperm precedence in a subsocial spider

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The subsocial spider *Anelosimus* cf. *studiosus* has a sex ratio biased toward females, but unexpectedly males perform ritualised fights to gain first access to females. Usually females will mate first with the winner male, but they can remate with loser males, remaining as satellite. We tried to discover why loser males wait to mate with a copulated female, instead of looking for virgin females. We considered the first male as a winner and the second as a loser, without allowing them to engage in fights. The objective of the present study was to determine sperm priority, by performing double copulations, with normals and males sterilised with gamma radiation (2000 rad). They were randomly assigned to one of the three experimental groups (IN, NI or NN). In the group IN, 13 females mated with irradiated males and 24–48 hr later with normal males. In the group NI, 12 females mated with normal males and 24–48 hr later with irradiated males, and in the control group NN, 12 females mated with 2 normal males. We opened the egg sac and counted the number of embryos and eggs. In the IN and NN groups we obtained 80% successful egg sacs and 75% in NI. We did not find differences in the total number of eggs laid per egg sac (eggs + embryos) or between the total number of embryos, among the three groups. The results suggest that first and second males would have similar percentages of paternity. Future studies will focus on morphology and mechanisms of female choice in this species.

KEY WORDS: subsocial spider, sperm precedence, sterile-male technique, sperm competition, cryptic female choice.

INTRODUCTION

Traditionally, in dioicous species with sexual reproduction there is a sex that invests more and is expected to be more selective (typically females) and a sex (typically

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males) that competes for access to the choosy sex (DARWIN 1871; ANDERSSON 1994). The different morphological and behavioural characteristics among competing males can affect their fertilisation success by affecting competition for mates and/or female choice (ANDERSSON 1994). Males can increase their paternity success by locating immature females and guarding them until they achieve sexual maturity, as a way to ensure being the first to inseminate the female (COHN et al. 1988; ELGAR 1998). However, after copulation, females can mate again with other males. Therefore paternity can be shared with two or more males. This creates the scenario for post-copulatory sexual selection mechanisms such as sperm competition (PARKER 1970) and cryptic female choice (THORNHILL 1983; EBERHARD 1985). Sperm competition occurs when males continue competing for egg fertilisation inside the female reproductive tract. On the other hand, in a polyandrous mating system, females have many ways to control paternity, during and after copulation, such as dumping sperm, failing to transport sperm to storage organs or fertilisation sites, collaborating with the male in making genital plugs, or remating with another male (EBERHARD 1985, summarised in EBERHARD 1996). Sexual selection operating through post-copulatory female selection drives male sexual behaviour and the morphology of copulatory organs in insects, arachnids, and others taxons (EBERHARD 1985, 1996).

Spiders possess many structures and features that make them appealing models for the study of post-copulatory sexual selection mechanisms, such as sperm competition and cryptic female choice (EBERHARD 2004). In most entelegyne spiders the genitalia are paired. Females have two independent genital pores, connected to two independent spermathecae via an insemination duct (FOELIX 1996). In the same way, males have two secondary copulatory organs inside the pedipalps, which are intromitted separately and alternately into the female's genital pores (FOELIX 1996). In many spider species, females mate with more than one male in one reproductive period, and also they can store viable sperm for long periods, perhaps establishing a sperm priority pattern (ELGAR 1998). The males are able to mate with more than one female, although generally they cannot monopolise them because the female lifespan is longer than the males (AUSTAD 1984; FOELIX 1996). Females usually live much longer, since they must still lay eggs and build egg sacs, and in some species also exhibit brood care (FOELIX 1996). AUSTAD (1984) stated that in spiders the morphology of the female spermathecae would determine the tactics that males would follow to obtain sperm priority upon fertilisation. He established two types of spermathecae: "conduit" and "cul de sac". The author concludes that the "conduit" spermathecae (with two separated ducts, one for insemination and one for fertilisation) will favour first male sperm priority, while the "cul de sac" spermatheca (one unique duct) will favour last male sperm priority. AUSTAD (1984) assumed that sperm inside female spermathecae is stratified. Then, when sperm priority favours the first male that mates with a female, it would be expected that males mature before females, as generally occurs in spiders, and perform precopulatory guards of sub-adult females (AUSTAD 1982; JACKSON 1986; TOFT 1989; WATSON 1990; DODSON & BECK 1993; EBERHARD et al. 1993). Males can also perform ritualised fights for access to virgin females (AUSTAD 1983; SUTER & KEILEY 1984; TOFT 1989; NIELSEN & TOFT 1990), copulating with them shortly after they moult to adulthood (AUSTAD 1982; TOFT 1989). Nevertheless, when sperm priority favours the last male, males perform postcopulatory guarding, and mate guarding and fights among males occur shortly before egg laying (ELGAR & BATHGATE 1996; FAHEY & ELGAR 1997; ELGAR 1998; FROMHAGE & SCHNEIDER 2005). The sperm used by females to fertilise the eggs after re-mating is generally expressed as the proportion of descendants assigned to the second male in achieving copulation (UHL 2000). In double mating trials, the proportion of eggs assigned to the second male is called P2 (BOORMANN & PARKER 1976).

Anelosimus (Theridiidae) is one of the few spider genera that contain solitary, subsocial and social species. *Anelosimus* cf. *studiosus* is a subsocial spider common in Uruguay, considered closely related to *A. studiosus* (Hentz), but its taxonomic status is still under revision (I. AGNARSSON pers. comm.; VIERA et al. 2007). This species is a non-territorial, periodically social spider (AVILÉS 1997) with dispersion stages due to the noticeable intolerance between adult females (VIERA et al. 2007). The presence of adult males next to sub-adult females has been observed in the field, where they are probably guarding females to ensure being the first to inseminate them (ALBO et al. 2007; VIERA et al. 2007). These observations suggest first male sperm priority. However, ALBO et al. (2007) observed that if two males are simultaneously exposed to a virgin female they engage in escalated and ritualised fights. The female tends to copulate first with the winning male, but she can also accept copulation with the losing male, which remains hidden as a “satellite” male. These facts suggest that the second male would also fertilise eggs and obtain a percentage of paternity, even though he copulated with an inseminated female. The objective of this study was to determine sperm priority patterns in *Anelosimus* cf. *studiosus*, by using the sterile male technique (BOORMANN & PARKER 1976). For this species this is the first time that male sterilisation by gamma radiation has been used, which is also the first time for the genus.

MATERIAL AND METHODS

Study species

Anelosimus cf. *studiosus* is a subsocial spider common in Uruguay which lives in low branches of perennial trees, in nests formed by a female with one or two broods (VIERA et al. 2007). The reproductive period ranges between October and November (spring), and the egg-laying period occurs during the latter month. Descendants emerge during December (summer of the southern hemisphere) (VIERA et al. 2007).

Juvenile and sub-adult individuals of *A. cf. studiosus* were collected from a single population from Montevideo, Uruguay (34°51'29"S, 56°10'14"W), during the reproductive season (July–October). In the laboratory, individuals were housed in groups of 25–35 individuals (males separated from females), in plastic Petri dishes (9.0 cm diameter, 1.7 cm height) and maintained with moistened cotton wool. All spiders were fed ad libitum with *Drosophila* spp. (Diptera) and *Tenebrio* sp. larvae (Coleoptera) and the average temperature was 23.9 °C (\pm 1.79 SD, range: 13–29 °C). Individuals were checked daily and recently moulted adults were removed from communal groups and housed individually in small Petri dishes (3.5 cm diameter, 0.9 cm height) in similar conditions to those cited above.

For setting up the sterile male technique, three experimental groups were created by irradiating males with different doses: 2000 ($n = 26$), 4000 ($n = 29$) and 6000 ($n = 25$) rads, performed with a cobalt γ -emitter. Males irradiated with each dose were exposed to virgin females, and the development of sexual behaviour was registered by one or two observers. As a control group, normal males (non-irradiated) were exposed to virgin females ($n = 14$) and behaviour was similarly registered. Egg sacs obtained from each group were opened after 16 days, when embryos reach their first instar (according to VIERA et al. 2007), and descendants were counted. Irradiated males did not generate descendants, while all normal males did so. Therefore, we determined that a dose of 2000 rads was enough to stop postembryonic development in the egg instar. Consequently, we used males irradiated with a dose of 2000 rads.

We maintained the irradiated individuals in the same laboratory conditions mentioned above. Once individuals reached sexual maturity, they were randomly assigned to one of the three experimental groups and individuals were used once.

In each group, one virgin female was exposed and mated consecutively with two males, in the female's dish. In the group IN, females first copulated with an irradiated (I) male, and 24–48 hr later with a normal (N) male. The group NI was composed of females that mated first with a normal male and 24–48 hr later with an irradiated male. Finally, as a control group, the NN group was composed of females that copulated first with a normal male and 24–48 hr later with another normal male. Therefore, if there is sperm mixing within female spermathecae we will expect to obtain a P2 (proportion of eggs assigned to the second male) value near 50%, whereas if there is a sperm priority pattern favouring the first or the second male, then the P2 value will be low or high, respectively. Trials were followed by direct observation during a maximum period of 2 hr. Every male was used once, and the females were used twice. We recorded courtship and copulation duration. If none of the individuals performed sexual behaviour during the first 15 min or mating did not occur during the first 30 min, the trial was concluded and the male was replaced by another one. If the male inserted any palp in any female side, we considered these matings in the analysis. If we did not observe sexual activity during 15 min after copulation, the experience was considered finished. If females did not accept the second male between 24 and 48 hr, they were not considered for the analyses. Copulated females were fed *ad libitum* with *Drosophila* spp. Once females laid egg sacs, they were opened on day 16 (according to VIERA et al. 2007, see above) and we counted the number of eggs + embryos and their degree of development.

The results were analysed using Past Statistical Program Version 1.82 (HAMMER et al. 2001). When necessary, data were log transformed before the analyses and if data did not show a normal distribution, non-parametric statistics were applied. Descriptive statistics are presented as mean \pm standard deviation. We compared courtship and copulation duration, and duration of first and total palp insertions between first and second matings with different males and between each experimental group and control groups using the *t*-test and Mann–Whitney *U*-test (Table 1). In order to examine differences between the sexual performances of irradiated and normal males, we used Spearman correlations to test whether there was any relationship between copulation duration and the number of offspring. We used Spearman correlations to search for relationships in the DCI/DCN index (copulation duration of irradiated males over copulation duration of normal males) and the index number of eggs/number of embryos.

Table 1.

Courtship duration (a), first insertion (b) and total insertion durations (c) (min) in the first and second mating exposures in the three experimental groups (IN, NI, NN).

	First mating exposure (mean)	Second mating exposure (mean)	<i>n</i>
(a) Courtship duration			
Group IN	19.18 \pm 23.07 (0.4–86.4)	13.11 \pm 13.43 (0.2–49.3)	13
Group NI	24.63 \pm 17.81 (0.5–69.6)	21.91 \pm 24.98 (1.7–91.7)	12
Group NN	18.88 \pm 17.87 (0.9–69.6)	13.7 \pm 16.79 (0.5–45.8)	12
(b) First insertion duration			
Group IN	21.83 \pm 17.62 (0.6–52.4)	24.43 \pm 24.22 (1.1–94.2)	13
Group NI	18.83 \pm 17.94 (0.2–50.8)	29.52 \pm 30.86 (0.9–106.9)	12
Group NN	26.47 \pm 17.99 (0.2–47.7)	14.96 \pm 17.00 (1.1–59.00)	12
(c) Total insertion duration			
Group IN	57.63 \pm 26.94 (22.2–99.6)	43.45 \pm 32.63 (4.7–115.6)	13
Group NI	57.98 \pm 25.21 (11.6–90.9)	46.31 \pm 30.44 (2.3–106.9)	12
Group NN	60.41 \pm 22.37 (25.8–86.9)	58.08 \pm 35.61 (12.1–132.0)	12

RESULTS

Courtship and mating behaviour

We obtained 37 trials in which the first and second copulations were successfully completed, NN (12), IN (13) and NI (12). No significant differences between courtship duration in the first and second encounters in IN ($t_{paired} = 0.66$, $P = 0.52$), NI ($T = 51$, $P = 0.35$), NN ($t_{paired} = 1.0$, $P = 0.34$) groups were found. Duration of the first palpal insertion at the first and second encounters did not show significant differences in the IN ($t_{paired} = -0.45$, $P = 0.66$), or the NI ($t_{paired} = -1264$, $P = 0.23$) or the NN ($T = 58$, $P = 0.136$). Copulation duration at the first encounter (ANOVA: $F = 0.05$, $P = 0.96$, $df = 36$) and the second (ANOVA: $F = 0.68$, $P = 0.51$, $df = 36$) did not show significant differences within the three groups.

Copulation duration in irradiated males and number of offspring (eggs), considering males from both IN and NI groups ($r = 0.03$, $P = 0.91$, $n = 25$), or normal males and number of offspring, considering males from both the IN and the NI groups ($r = 0.31$, $P = 0.26$, $n = 25$), did not show a relationship. Considering all the males, we did not find a significant relationship between copulation duration and the total number of offspring (eggs + embryos) within the three groups ($r = -0.32$, $P = 0.128$, $n = 25$). We did not find any relation between DCI/DCN index (copulation duration of irradiated males over copulation duration of normal males) and the index number of eggs/number of embryos ($r = 0.35$, $P = 0.20$, $n = 25$). Nor did we find a significant correlation between the duration of copulation of the first male and the duration of copulation of the second male, in any of the experimental or control groups (IN: $r_2 = -0.15$, $P = 0.64$, NI: $r_2 = 0.09$, $P = 0.78$, NN: $r_2 = 0.02$, $P = 0.95$).

Males did not show a preference for inserting first with the right or the left palp, considering all the groups together ($\chi^2 = 0.46$, $P = 0.5$, $n = 34$) or separately ($\chi^2 = 2.68$, $P > 0.2$, $n_{in} = 13$, $n_{ni} = 11$, $n_{nn} = 10$). In three cases it was not possible to determine which palpal organ was inserted first so the corresponding data were eliminated.

Twenty-eight copulated females laid egg-sacs from a total of 37 (Table 2). No significant differences were found among the total number of eggs laid per egg sac (eggs + embryos) among the three groups ($F = 0.94$, $P = 0.41$, $df = 22$), or among the total number of embryos between the three groups ($H = 3.86$, $P = 0.15$, $n = 20$).

Table 2.

Number of egg sacs laid in each experimental group, distinguishing between viable egg sacs and non-viable (defective) egg sacs.

Groups	IN ($n = 13$)	NI ($n = 12$)	NN ($n = 12$)
Total number of ♀♀	10	8	10
No. of ♀♀ that laid viable egg sacs	8	6	8
No. of ♀♀ that laid defective egg sacs	2	2	2
% viable egg sacs	80	75	80
% defective egg sacs	20	25	20

P2 analyses

We found a very wide P2 range in both NI and IN groups (P2 = 0–1 for IN, P2 = 0–0.84 for NI). The P2 average in both groups (0.49 ± 0.41) did not show a priority pattern in either of the groups. However, there were cases in which the first male had 100% of the offspring and the second male had 0% (Table 3).

Proportional adjustments

We made a proportional adjustment of paternity values because in the NN group we found clutches with non-viable eggs (based on DRENGSGAARD & TOFT 1999). Under laboratory conditions, females copulated by two normal males had a natural clutch loss of 25% (Table 3). Therefore the highest success of males in the NN group was 75%. This percentage was used as the 100%, within the adjustment for our sample. In this way viable percentages of embryos in each group were adjusted and the reproductive success of normal males was higher than that of irradiated males (Table 4).

Table 3.

Percentages of eggs and embryos in IN and NI experimental groups.

IN		NI	
% of eggs	% of viable embryos	% of eggs	% of viable embryos
0.0	100.0	8.1	91.9
6.6	93.3	84.0	16.0
36.0	64.0	0.0	100.0
100.0	0.0	44.4	55.6
3.8	96.2	41.5	58.5
100.0	0.0	78.6	21.4
100.0	0.0	82.4	17.6
9.1	90.9		
100.0	0.0		

Table 4.

Proportional adjustments of the number of offspring in each experimental group, corrected by the normal percentage of viable embryos and non-viable eggs of copulations with normal males performed under laboratory conditions.

Group	<i>n</i>	Total number of offspring (eggs + embryos)	Total number of viable embryos	Viable eggs (%)	Proportional adjustment (%)
IN	8	253	129	50.98	67.63
NI	7	212	105	49.52	65.7
NN	8	199	150	75.37	100

DISCUSSION

We did not find a sperm priority pattern in *Anelosimus* cf. *studiosus* that favoured the first or the second male to achieve copulation. The results disagree with the hypothesis of sperm priority for the first male, despite the observations of males guarding sub-adult females and performing ritualised fights to be the first to achieve copulation (ALBO et al. 2007). On the other hand, these results agree with the observations of satellite males waiting to copulate with recently mated females (ALBO et al. 2007). According to AUSTAD's hypothesis (1984) first male sperm priority should be expected in this species, because *A. cf. studiosus* presents two separated ducts (one for insemination and another for fertilisation) coinciding with a "conduit" spermatheca (LEVI 1956).

However, possessing two independent ducts does not ensure that sperm precedence is for the first male (UHL 2000). *Nephila edulis* (Nephilidae) is an entelegyne spider that has the insemination duct located very close to the fertilisation duct (UHL & VOLLRATH 1998) and sperm priority corresponds to the last male that achieves copulation (SCHNEIDER et al. 2000). Considering several cases that do not agree with AUSTAD's hypothesis, UHL (2000) raises the use of the terms 'entelegyne' and 'haplogyne', regardless of taxonomic classification, because species classified as haplogynes can function as entelegynes and vice versa. Thus, even if *A. cf. studiosus* is taxonomically classified as entelegyne, it might behave as haplogyne if insemination and fertilisation ducts are juxtaposed. Further studies will focus on spermatheca morphology and location of insemination and fertilisation ducts.

On the other hand, the average percentage of P2 (50%) agrees with the hypothesis of shared paternity, based on the presence of satellite males waiting to copulate with recently mated females (ALBO et al. 2007; VIERA & ALBO 2008). This could be due to the mixture of sperm (BIRKHEAD & MØLLER 1998), favouring the existence of sperm competition (PARKER 1970) and/or cryptic female choice (THORNHILL 1983; EBERHARD 1985, 1996). The assumption that there is a mixture of sperm implies that all matings, regardless of their order, could be successful (WEST & TOFT 1999). Variation in sperm priority patterns could be a consequence of the variance of first and second copulation durations (DRENGSGAARD & TOFT 1999). However, in *A. cf. studiosus* we did not find differences between the duration of copulation in first and second male–female encounters.

The observed range of P2 (0–100%) shows that there is a high variability in the percentage of paternity assigned to each male. Even with or without sperm mixing, this variability could be driven by cryptic female choice, such as dumping sperm, failing to transport sperm to storage organs or fertilisation sites, collaborating with the male to make genital plugs, or remating with another male (EBERHARD 1985, summarised in EBERHARD 1996). WEST & TOFT (1999) suggested for *Tetragnatha extensa* that sperm priority patterns favouring the second male can be explained by assuming variability in the way that females store and pack the sperm from different males. Moreover, SNOW & ANDRADE (2005) determined that the reproductive success of *Latrodectus hasselti* (Theridiidae) males is significantly influenced by how the sperm is stored in females' spermathecae. These authors noted that sperm priority for the first male was the predominant pattern when two males inseminated the same side of the spermathecae, while there was a shared paternity pattern when both spermathecae were inseminated (SNOW & ANDRADE 2005). In *Leucauge mariana* (Tetragnathidae) and *Pholcus phalangioides* (Pholcidae) female glandular secretions seem to shoot the sperm by a chemical activation process (UHL 1994; EBERHARD & HUBER 1998), therefore females would be able to control the success of males' fertilisation by activating the stored sperm selectively (EBERHARD 1996).

On the other hand, the occurrence of cryptic female choice does not exclude the co-occurrence of sperm competition. In some spiders, copulation duration is directly related to paternity success (ELGAR 1998; SCHNEIDER et al. 2006). In these cases, polyandrous females can exert choice of future paternity of their offspring by controlling copulation duration and regulating the amount of sperm transferred to the spermathecae (ELGAR 1998). However, we did not find variations in copulation durations in *A. cf. studiosus*, so this parameter would not affect males' success. However, we did not compare copulation characteristics, such as number of insertions per copulation, body vibrations, or palpal drumming. In *Latrodectus hasselti* ANDRADE (1996) found a very wide range of P2. For this species most of the variation was explained by the second male copulation duration, relative to the first male copulation duration. The paternity of the second male was correlated positively with copulation duration and negatively with copulation duration of the first male (ANDRADE 1996). Nevertheless, our results showed that copulation duration in *A. cf. studiosus* was not related to paternity (offspring number) in irradiated males or in normal males. Moreover, copulation duration of the second male was not correlated with copulation duration of the first male, in any of the groups.

The absence of differences in courtship durations, duration of first insertions, total copulation duration, and the total number of eggs laid per egg sac among the three groups suggests that radiation doses were appropriate and did not affect males' behaviour or the competitiveness of irradiated sperm. However, with proportional adjustment we found that sperm from irradiated males fertilised less than the sperm of normal males. Nevertheless, these results do not alter the fact that there is no sperm priority for the first or the second male.

The present results are a first approach to assessing paternity in *A. cf. studiosus*, but further analyses are necessary. It would be interesting study other aspects of benefits to the satellite males in addition to the percentage of paternity finding here. We did not consider in this study other benefits for the males of remaining at the nest instead of seeking out other mating opportunities, such as avoiding or diminishing predation risks, energetic costs, or the probability of finding virgin or copulated receptive females; in further studies we will try to study these aspects. Behavioural, morphological and physiological studies are necessary too to improve our knowledge of the sexual selection mechanisms, such as sperm competition, cryptic female choice or both, operating in this subsocial spider species.

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