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SHORT COMMUNICATION

Evidence for multiple paternity in broods of the green lynx spider *Peucetia viridans* (Araneae: Oxyopidae)

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Abstract. In the green lynx spider *Peucetia viridans* (Hentz 1832), the two openings of a mated female's epigynum are often sealed by copulatory plugs, sometimes with the two-pronged distal portion of the paracybium of a male palpus inserted in each opening and embedded in the plugs. The presence of copulatory plugs and paracymbia may prevent further mating by the female. However, not all mated females exhibit these structures, perhaps allowing some *P. viridans* females to mate with more than one male, despite the assertion of Whitcomb & Eason (1965) that females only mate once. We investigated this possibility by surveying the extent of multiple paternity in field-collected *P. viridans* broods from southern California. For adult females and their egg sacs, we determined the aspartate aminotransferase genotype for each mother and her spiderlings using allozyme electrophoresis in order to assess whether the progeny data best fit with a single male as the father. Two broods exhibited clear evidence of multiple paternity, verifying that multiple mating by females is possible in this species. Although most mothers of single paternity broods had one or both epigynal orifices blocked, some had no blockage at all, while the two mothers of multiple paternity broods had some kind of blockage to one or both orifices, suggesting that neither plugs nor inserted paracymbial processes are associated with a reduction in female remating.

Keywords: Multiple mating, polyandry, molecular marker, copulatory plug, paracymbial process

The green lynx spider *Peucetia viridans* (Hentz 1832) is the largest and commonest member of the family Oxyopidae, with a distribution throughout the southern United States, Mexico, and Central America (Brady 1964). It is a cursorial hunter that forages on prey commonly found on plants (Arango et al. 2000). Although little studied up to 1960, *P. viridans* has been the sole or partial focus of at least 25 papers since then, making it one of the best-characterized hunting spiders in North America.

While much is now known about the reproductive biology of *P. viridans*, one question that remains unresolved is whether adult females ever remate in the wild. This is especially significant given that *P. viridans* and *P. longipalpis* F.O. Pickard-Cambridge 1902 are the only oxyopids known to produce copulatory plugs (Suhm et al. 1996), structures which are commonly thought to delay and/or reduce the probability of female remating (Eberhard 1996). Brady (1964) was the first to note the presence of plugs in *P. viridans* females, as he found that the two openings of a mated female's epigynum were usually plugged with a hard, black material, often with the two-pronged distal portion of the paracybium of a male palpus inserted in each opening and embedded in the material. Brady stated that the black material must be deposited during or immediately after insemination, a suggestion possibly corroborated by Whitcomb & Eason's (1965) observation during a laboratory study of a large drop of shiny liquid on the epigynum of a female immediately following copulation that later disappeared.

Brady (1964) reasoned that the plugging of the female epigynum and the loss of the male paracymbial process should prevent further matings by both female and male (although since males possess two palpi, an individual male could potentially mate twice). However, in their study of mating behavior in *P. viridans*, Whitcomb & Eason (1965) found that each mating episode involves numerous copulations between female and male with both palps being inserted alternately into the epigynal openings. They also found that males mated freely on successive days, with one male having mated with three different females over consecutive days. In contrast, an individual female would only mate with one male and would actively reject subsequent male suitors. Of course, if the copulatory plug in *P. viridans* is indeed a device for impeding access to subsequent males as suggested by Brady (1964), perhaps assisted in this role by broken-off male paracymbia, why it is necessary if females never remate is unclear. However, since females of many species may be more reluctant to remate in captivity than in nature (Eberhard 1996), Whitcomb & Eason's (1965) assertion that female *P. viridans* mate only once may not be universally true, as it was based on laboratory observations.

Whitcomb & Eason (1965) also reported on the frequency of the copulatory plug, as did Exline & Whitcomb (1965), who also provided data on the frequency of inserted paracymbia. Whitcomb & Eason (1965) found plugs in all mated females they examined, but not in any virgin females. In contrast, Exline & Whitcomb (1965) stated that not all mated females exhibit this covering, noting that it can be easily removed and is probably sometimes lost during egg-laying. They found that in a sample of approximately 20 mated females, 10 had at least one male paracybium embedded in the plug. Among the remaining 10, the plug was missing altogether or did not contain a paracybium. Like Exline & Whitcomb (1965), we have found that the presence of plugs and paracymbia in the epigyna of mated *P. viridans* females is variable in both laboratory-mated and wild females, with almost half of a field-collected sample of females with egg sacs from southern California (n = 54, 2004) having neither plugs nor paracymbial processes in their epigynal orifices (unpublished data). Thus, since plugs and paracymbia are often absent as obstacles in the epigyna of mated *P. viridans* females, some females may be physically capable of mating with more than one male. Given this possibility, the purpose of this study was to search for cases of multiple paternity in field-collected *P. viridans* broods using a genetic approach, since such broods would result from individual females having had more than one male sexual partner.

From October through December 2007, we collected 29 adult *P. viridans* females with their respective egg sacs from six sites in southern California (population abbreviation and sample size are

indicated in parentheses): Los Angeles Co.—Kenneth Hahn State Recreation Area (HSR, 18), Yvonne Burke Sports Complex (HBF, 2), Ernest Debs Regional Park (DEB, 3), Robert Bernard Biological Field Station, Claremont (BFS, 1); San Diego Co.—Crest Canyon Preserve, Del Mar (CC, 4), Carmel Valley Road, Del Mar (CVR, 1). In the laboratory, we assigned adult females and their respective egg sacs unique identification numbers. Each female was microscopically examined for the presence of epigynal plugs, their condition noted, and any retained male paracymbial processes were recorded. Females were then frozen at −85° C pending genetic analysis. The egg sacs were maintained separately in small plastic tumblers until spiderlings had emerged from each sac, typically within 2–4 weeks of collection. Up to 60 randomly chosen spiderlings from each brood were then placed individually into 1.5 ml microcentrifuge tubes marked to match the identification number of their respective mothers. These brood samples were also then frozen at −85° C.

We used allozyme electrophoresis as our molecular paternity assessment technique, given its cost-effectiveness for large samples. Procedures for horizontal starch gel electrophoresis generally followed Ramirez (1990) and used gels that were 12.5% starch (StarchArt). We homogenized individual spiderlings directly in their 1.5 ml microcentrifuge tubes along with 20 µl of deionized water using a hand-held microcentrifuge tube pestle. This homogenate material was centrifuged at 484 G's at 0–4° C for 5 min to separate extracted proteins from cellular debris and was then frozen at −85° C until needed for gel loading. The adult females were homogenized individually in 15 ml Corning centrifuge tubes with an approximately equal volume of deionized water using a motorized grinding pestle. These homogenates were centrifuged at 12,100 G's at 0–4° C for 15 minutes. Following centrifugation, the relatively greater volume of liquid supernatant for each female was transferred into multiple 0.5 ml microcentrifuge tubes, which each contained sufficient material for one gel run; these tubes were then frozen at −85° C pending gel loading. Since our study involved destructive processing of the specimens, no vouchers were retained.

During a related study, we found that five loci are regularly polymorphic in *P. viridans* populations (aspartate aminotransferase, AAT-1,-2, E.C. 2.6.1.1; glucosephosphate isomerase, GPI, E.C. 5.3.1.9; lactate dehydrogenase, LDH, E.C. 1.1.1.27; phosphoglucomutase, PGM, E.C. 2.7.5.1) (Commission on Biochemical Nomenclature 1979). Unfortunately, three of these loci (AAT-2, GPI, PGM) presented problems of poor resolution of multiple alleles and/or inconsistent staining during test gel runs, and so were not used. The two remaining loci (AAT-1, LDH) are both diallelic systems that resolve well in adults, but since LDH could not be consistently scored in spiderlings during preliminary testing, probably due to a low level of enzyme concentration in their homogenates, only AAT-1 (a cationic locus) was used in this study. The recipe for AAT was based on Manchenko (1994), and it was resolved using the Continuous Tris-Citrate I buffer system of Selander et al. (1971). Females and their respective brood samples were run side-by-side on the same gels to aid in recognizing band homologies during genotype assignment. Agreement between observed genotypic proportions and Hardy-Weinberg expectations was evaluated for the adult females by calculation of exact significance probabilities using the BIOSYS-2 (Black 1997) computer program.

To determine whether a single male was unlikely to account for the genetic diversity of a female's brood, we used a significant deviation from a Mendelian ratio among progeny genotypes as our criterion. We identified the paternal alleles present in each brood by inspection of the progeny and maternal genotypes and then used this single paternal genotype along with the maternal genotype to determine an expected Mendelian progeny distribution. We tested the observed genotype numbers against the expected numbers using a Chi-square test with Yates continuity correction for small sample size (X^2_c), as implemented in the E-Z Stat 1.0.1 (Towner 1999) statistical analysis program.

The frequencies of the A and B alleles at the AAT-1 locus were 0.862 and 0.138, respectively, for the adult females and genotype numbers did not differ significantly from Hardy-Weinberg expectations. The 29 broods of *P. viridans* yielded 1337 spiderlings, which were genotyped at the AAT-1 locus along with their respective mothers (mean brood sample = 46, range = 15–60). In 17 broods, the mothers and spiderlings were all of the same genotype (AA), making it impossible to test for deviations from Mendelian ratios among progeny genotypes. The 12 remaining broods contained two or three offspring genotypes and among these, two (HSR-160, HSR-179) showed significant deviations from expected Mendelian ratios (Table 1), indicating that a single male was unlikely to account for the observed ratios in each case. Since multiple mating episodes involving males of the same genotype cannot be detected using a single diallelic locus, the frequency of multiple mating (2/12 = 16%) reported here may be an underestimate, especially given the limited number of broods analyzed. Nonetheless, we have verified that *P. viridans* females sometimes mate with more than one male, contrary to Whitcomb & Eason's (1965) assertion that females do not remate.

Among the 12 females whose broods contained multiple genotypes suitable for paternity assessment, the presence and state of the epigynal plug was quite variable, as was the presence of male paracymbial processes (Table 1), consistent with the findings of Exline & Whitcomb (1965) and our prior observations in southern California. Of the 10 females whose broods provided no evidence of multiple paternity, 7 (CC-17, DEB-330, DEB-343, HSR-141, HSR-143, HSR-144, HSR-176) possessed a complete copulatory plug in one or both of their orifices, sometimes accompanied by a paracymbial process, while the remaining three (CC-15, HSR-148, HSR-177) had neither plugs nor paracymbial processes in their orifices (Table 1). As for the two females whose brood genotypes indicated multiple paternity (HSR-160, HSR-179), HSR-160 had complete plugs in both orifices and HSR-179 had a partial plug in one orifice and no plug in the other, while neither possessed paracymbia in their epigynal orifices (Table 1). Thus, although most females classified as "singly inseminated" had one or both of their orifices blocked, three had no blockage at all, while both of the multiply inseminated females had some kind of blockage to one or both orifices.

In this study, we found clear evidence for the occurrence of multiple paternity in *P. viridans* offspring from southern California. At a minimum, 16% of the broods that were suitable for paternity assessment had been multiply sired. The fact that some level of female remating and multiple paternity is possible in this species indicates that sperm competition is a component of sexual selection in *P. viridans*. A widespread adaptive response for paternity assurance given sperm competition is the formation of copulatory plugs (Wigby & Chapman 2004), so their production in *P. viridans* is understandable. Moreover, since parts of the male palp often break off within the female during mating in several groups of spiders (Eberhard 2004; Huber 2005), where they can act as impediments to sperm transfer by subsequent males (e.g., Fromhage & Schneider 2006), the paracymbial process of *P. viridans* males may similarly serve as a copulatory plug when lodged in a female. However, both copulatory plugs and palpal structures in the female genital tract are less than 100% effective at preventing female remating in many spiders (references in Huber 2005; Schneider et al. 2005), perhaps partly due to female efforts to counteract male monopolization if they can benefit from polyandry (Hosken et al. 2009). More generally, plugs of any sort are not expected to be absolute barriers since selection would then favor male avoidance of nonvirgin females and plugging would be selected against (Eberhard 1996). As for *P. viridans*, while we cannot know how many of the plugs and paracymbial processes we observed were the result of first matings, the fact that both multiply inseminated females had some kind of blockage to their epigynum and three females classified as singly inseminated had no blockage at all

Table 1.—Distribution of AAT-1 genotypes among *Peucetia viridans* spiderlings from 12 brood samples, along with maternal epigynal configurations. The expected brood genotype distributions (in parentheses) assume Mendelian inheritance of two alleles (A, B) at the AAT-1 locus and appropriate genotypes for the unknown male parents based on the paternal alleles evident in each brood. The Chi-square test with Yates correction (X^2_c) evaluates the hypothesis that a single male inseminated each female; superscript ^{M} designates females determined to be multiply inseminated. Scoring of the female epigyna is the same for both the left and right epigynal orifices (LO, RO): ○ = copulatory plug absent; ● = complete plug present; ◍ = partial plug present; ⌊ or ⌋ = male paracymbial process in left or right orifice.

Female	Female genotype	Presumed male genotype	Spiderling genotypes AA	AB	BB	X^2_c	P	Epigyna LO	RO
CC-15	AB	AA	32 (30)	28 (30)	---	0.150	0.699	○	○
CC-17	AA	AB	21 (20)	19 (20)	---	0.025	0.874	●⌋	●
DEB-330	AA	AB	16 (15.5)	15 (15.5)	---	0.000	1.000	●	○
DEB-343	AB	AB	18 (15)	28 (30)	14 (15)	0.546	0.761	●⌋	○
HSR-141	AA	AB	34 (29.5)	25 (29.5)	---	1.085	0.298	●	●
HSR-143	AB	AA	10 (12.5)	15 (12.5)	---	0.641	0.423	◍	●⌋
HSR-144	AB	AA	33 (30)	27 (30)	---	0.417	0.519	●⌋	●⌋
HSR-148	AB	AA	32 (30)	28 (30)	---	0.150	0.699	○	○
HSR-160^M	AB	AB	30 (15)	26 (30)	4 (15)	22.046	<0.001	●	●
HSR-176	AB	AA	25 (24)	23 (24)	---	0.021	0.885	○	●⌋
HSR-177	AB	AB	3 (4)	10 (8)	3 (4)	0.586	0.746	○	○
HSR-179^M	AA	AB	53 (30)	7 (30)	---	33.759	<0.001	◍	○

suggests that neither plugs nor inserted paracymbial processes are associated with a reduction in female remating.

While the frequency of multiple paternity in *P. viridans* populations may be greater than that indicated by our limited data set, it is possible that the frequency may not be considerably greater due in part to changes in mate availability associated with a seasonal shift toward a female-biased sex ratio, as documented for a *P. viridans* population in Mérida, México (Arango et al. 2000). Thus, females reaching adulthood later in the year at this site presumably had access to fewer male suitors, a pattern also seen with the orb-weaving spider *Nephila clavipes* (Linnaeus 1767) due to a similar sex ratio shift (Higgins 2000). If a seasonal, female-biased sex ratio shift also occurs in southern California *P. viridans* populations, this may be one reason why even more broods did not exhibit evidence of multiple paternity in this study, particularly those whose mothers possessed neither plugs nor paracymbial processes (CC-15, HSR-148, HSR-177, Table 1).

The analysis of mating and parentage in *P. viridans* would greatly benefit from the future use of one or more hypervariable molecular markers applied to females, their spermathecal contents, and broods (e.g., Simmons et al. 2007) because such an approach would estimate both female remating rates and patterns of sperm utilization in natural populations. In addition, since copulatory plugs are often externally visible when they are present in the epigynum, if females were inspected for the presence of plugs on a daily basis (e.g., Kim & Choe 2007) prior to such genetic analyses, it might also improve our understanding of the frequency, persistence, and influence of this structure on opportunities for multiple mating by females.

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