



Experimental evidence of polyandry and prolonged copulations in *Photinus palaciosi*, a synchronous firefly with brachypterous females (Coleoptera: Lampyridae)

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Abstract. Males of some species of the firefly *Photinus* produce nutritious spermatophores (nuptial gifts) the production of which is associated with a specific reproductive morphology. In fireflies and other insects, the production of nuptial gifts favours the evolution of polyandry. On the other hand, in fireflies, wing reduction (brachyptery) in females is associated with the lack of nuptial gifts. *P. palaciosi* is a synchronous firefly with brachypterous females, which would indicate an absence of nuptial gifts and a monandrous mating system. However, the reproductive morphology of *P. palaciosi* is like that of species producing nuptial gifts, which indicates this species should be polyandrous. Here, the results of an experimental study designed to test if females of the synchronous firefly *Photinus palaciosi* are polyandrous or monandrous are reported. The duration of copula was determined because this trait is associated with absence/presence of nuptial gifts in fireflies: it is very short (few minutes) in species lacking nuptial gifts and long (hours) in gift-giving species. More than sixty percent of the females mated with several males and the duration of each copulation was long. Thus, these results, together with those of previous studies on the morphology of the reproductive system, strongly indicate that males produce spermatophores (which usually contain nutrients and are, thus, considered nuptial gifts), which provides a possible explanation of both polyandry and prolonged copulations in *P. palaciosi*.

INTRODUCTION

Fireflies are charismatic insects that recently have been used as a flagship group for conservation biology due to their susceptibility to habitat destruction and light pollution (Lewis et al., 2020), with some species, particularly those considered to be synchronous fireflies, being the focus of ecotourist activities (Lemelin et al., 2021; Lewis et al., 2021). Their charisma and fragility are, in part, a result of the fascinatingly diverse mating biology of the bioluminescent nocturnal species (Lewis & Cratsley, 2008). For example, while some fireflies occur at very low densities, synchronous fireflies are characterized by relatively short nightly mating periods (typically 1–2 h) during which very large numbers of flying males (usually thousands) produce bioluminescent signals, continuously or sporadically in synchrony (Lewis, 2016). Another intriguing feature of

fireflies is the variation in the degree of wing reduction in females (Lewis & Cratsley, 2008; South et al., 2011): whereas in many species females have well developed, fully functional wings, there are species in which females exhibit different degrees of wing reduction (a condition known as brachyptery) and cannot fly. Furthermore, in fireflies there is variation in ejaculates, with males of several species producing nutritious spermatophores that females use to increase their reproductive success (Lewis & Cratsley, 2008). The physiological trade-off between flight and reproduction led Lewis & Cratsley (2008) to propose that in fireflies with females that can fly selection would favour the evolution of nutritious spermatophores (hereafter, nuptial gifts) that would compensate in part for the resources invested by females in flying. The evolution of nuptial gifts, in turn, favours the evolution of polyandry in fireflies

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and other insects (Arnqvist & Nilsson, 2000; Rooney & Lewis, 2002; Lewis et al., 2004; Lewis & Cratsley, 2008). In contrast, Lewis & Cratsley (2008) predict that in species with brachypterous females, selection would favour the loss of nuptial gifts and, thus, the evolution of monandry.

Here the frequency of mating of brachypterous females of the synchronous firefly *Photinus palaciosi* (Zaragoza-Caballero, 2015), an endemic species in central Mexico, was recorded. Lewis & Cratsley's (2008) hypothesis predicts that females of the flightless *P. palaciosi* should be monandrous. On the other hand, van der Reijden et al. (1997) mention that in species of *Photinus* with nuptial gifts, males produce nutritious, spirally coiled spermatophores in spiral accessory glands; these nuptial gifts are then digested in an organ in the female reproductive tract known as spermatophore-digesting gland. Maquitico-Rocha & Carrillo-Ruiz (2019) describe that the reproductive system of *P. palaciosi* has a pair of spiral accessory glands in males and a structure in females that looks almost identical in shape and position to the spermatophore-digesting gland (although they call it a gland of the spermatheca). Thus, the morphology of the reproductive tract indicates the production of nuptial gifts and, consequently, polyandry in this species. The duration of copulation was also studied, as copulations involving nuptial gift transfer are expected to take longer (between one and several hours) than those of species that simply transfer sperm in seminal fluid (a few minutes). Van der Reijden et al. (1997) report this pattern for other species of *Photinus*.

MATERIAL AND METHODS

P. palaciosi lives in the mountains of Central Mexico (Zaragoza-Caballero, 2012; López-Palafox et al., 2020) and has a reproductive season from late May to early August. The daily courtship period lasts between 90 and 120 min, starting at approximately 20:30 h. This study was done at "Piedra Canteada", a communal property whose main business is ecotourism, which is located in the municipality of Nanacamilpa de Mariano Arista, Tlaxcala state, Mexico. As expected, the number of synchronous signalling fireflies in this area is very high. An experimental approach was used because in the field the females are widely spaced and the marking of the delicate wingless females, necessary for the long-term monitoring of individual females, is potentially damaging. Between July 12 and July 19, 2019, we located and observed signalling females until they mated in the field, the couples were then collected (by cutting the twig or leaf on which they were mating) and kept isolated in one-litre transparent plastic containers with covers. When the nightly courtship period ended, the containers with individual mating pairs were transported (by car) to a temporary laboratory located about 30 min from the display site, where they were kept in the dark. The duration of copulation was determined by periodically checking each pair using a red light. After copulation ceased, the pairs were kept in the containers with a piece of fresh apple (previous observations indicated that fireflies use peeled apple or cucumber as a source of liquid) and the females were exposed on subsequent nights to wild males at the site where the mating pairs were collected. The males that mated with the experimental females the previous night were released the following night at the display site. These males flew away soon after the containers were opened, thus it is very unlikely that the same male remated with the same female. The fe-

males were exposed by uncovering their containers and gently placing them near the tip of a twig of about half the height of the container. The females started to glow, sometimes before their containers were uncovered. The females were continuously observed from a distance of about 50 cm to determine if they mated with one of the males that courted them and to prevent them from escaping (however, four females escaped). If a female mated, the container with the mating pair was covered and then processed as described above for females collected in copula. Since the sample sizes were relatively small, non-parametric statistics were used and all tests were done using the software available on the website www.socscistatistics.com.

RESULTS

Twenty-seven mating females were collected in the field. These females were subsequently exposed to males in the field on between one and five consecutive nights (median $[Q_{25\%}-Q_{75\%}] = 3$ [2–4]; Fig. 1), and they produced bioluminescent signals and mated between zero and four times (median $[Q_{25\%}-Q_{75\%}] = 1$ [0–2]; Fig. 1). Seventeen of these females (63%) mated at least once (i.e. they were polyandrous), ten mated with three or four males and one with five males. Fifteen of the 17 females mated a second time the night after being collected (i.e. they mated on two consecutive nights). The results for the four females that were lost during exposure to males on either the first or second night, of which only one mated (once), were included in the mating calculations. Two females mated, the afternoon before their first exposure, with the same male they were mating with when collected, both of these females mated a second time with a different male, the results of which were also included in the mating calculations. The correlation between the number of nights the females were exposed to males and the number of times they mated was positive and statistically significant (Spearman correlation: $r_s = 0.54$, $p = 0.003$, $n = 27$; Fig. 1), which suggests that the

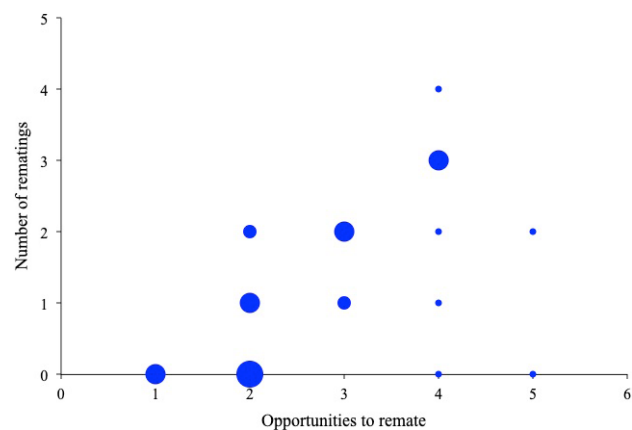


Fig. 1. Relation between the numbers of nights that females of *Photinus palaciosi* fireflies collected in copula were exposed to males in the wild (= Opportunities to remate) and the number of times they mated. Their relatively long duration of copulation prevents females from mating more than once per night. The correlation between these two variables was positive and statistically significant (Spearman correlation: $r_s = 0.54$, $p = 0.0034$, $n = 27$). The size of the blue circles indicates the number of females: the smallest circles correspond to one female, the largest to five and the two intermediate sizes to two and three females.

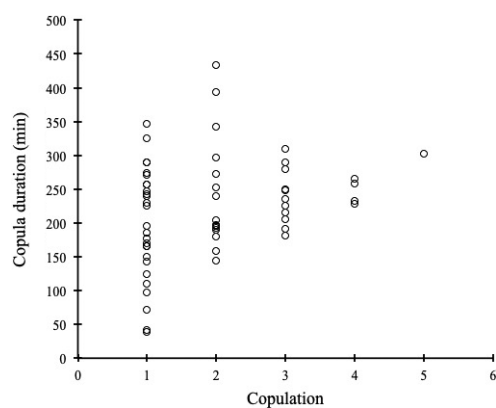


Fig. 2. Duration of copulation of consecutive copulations recorded for female *Photinus palaciosi*. Duration of copulation increased significantly in consecutive copulations (Spearman correlation, $r_s = 0.29$, $p = 0.028$, $n = 59$). First copulations tended to be shorter than subsequent copulations (Mann Whitney $U = 302.5$, $z = 1.963$, $p = 0.05$, $n_{1st} = 27$, $n_{2nd-5th} = 32$)

fraction of females that mated several times and the number of matings per female were underestimated.

Duration of copulation was relatively long (median [$Q_{25\%}$ – $Q_{75\%}$] = 228 [180.5–267.25] minutes, $n = 59$) and variable (minimum–maximum: 39–434 minutes; Fig. 2). There was a statistically significant negative correlation between the duration of copulation and the time at which it started (Spearman correlation: $r_s = -0.34$, $p = 0.009$, $n = 58$; Fig. 3); excluded from this test was one copulation that started in captivity at 7 PM (i.e. about 1.5 h before the beginning of courtship in the field), which was the second longest copulation (394 min). Duration of copulation increased significantly in consecutive copulations (Spearman correlation: $r_s = 0.29$, $p = 0.028$, $n = 59$; Fig. 2). This correlation reflects in part the fact that first copulations of experimental females (i.e. those starting before they were collected as mating pairs) tended to be shorter (Mann Whitney $U = 302.5$, $z = 1.963$, $p = 0.05$) than those recorded for the mated females that were subsequently exposed to males in the field (first copulations: median [$Q_{25\%}$ – $Q_{75\%}$] = 195 [146.5–257], $n = 27$; pooled second to fifth copulations: 234 [195–274.75], $n = 32$; Fig. 2).

DISCUSSION

The results indicate that females of *P. palaciosi* are polyandrous. First, already mated females signalled when exposed to males during the nightly display period. Second, a large proportion of the females were observed to mate at least twice (63%) and more than a third (37%) mated with more than two males (Fig. 1). As there is a positive correlation between the number of nights the females were exposed to males and the number of times they mated and this experiment was carried out about a month after the beginning of the mating season some of the females could have mated before, which possibly accounts for why 10 females did not mate again and indicates it is possible that the frequency of mating and the average number of times females mated were underestimated. Thus, these results are not in accordance with the prediction that fireflies with

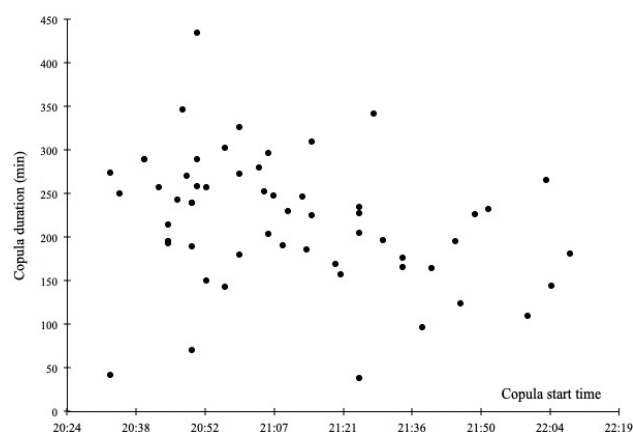


Fig. 3. Relation between the time of night at which copulations started and their duration. The correlation between these two variables was negative and statistically significant (Spearman correlation: $r_s = -0.34$, $p = 0.0094$, $n = 58$).

brachypterous females are monandrous. This prediction follows from Lewis & Cratsley's (2008) hypothesis that nuptial gifts (i.e. nutritious spermatophores), that favour the evolution of polyandry in fireflies and other insects (Arnqvist & Nilsson, 2000; Rooney & Lewis, 2002; Lewis et al., 2004; Lewis & Cratsley, 2008) are unlikely to be produced by species with brachypterous females because there is no trade-off between reproduction and flight. The phylogenetically based comparative study of South et al. (2011) provides statistical support for Lewis & Cratsley's (2008) hypothesis. However, South et al. (2011) also report there are some species (four out of 16) with flightless females and males that produce nuptial gifts (Lewis & Cratsley, 2008: see their Table 1), like *P. palaciosi*. Unfortunately, there is no data on whether these four species are polyandrous, like *P. palaciosi*.

Is there any evidence that the males of *P. palaciosi* produce nuptial gifts? Two lines of evidence support this hypothesis. First, the transfer of nuptial gifts in association with polyandry is reported in several species of *Photinus* in North America (reviewed in Lewis & Cratsley, 2008). The nutritious spermatophores of *Photinus* fireflies are produced by four pairs of accessory glands, one of which is a pair of spiral glands (van der Reijden et al., 1997) where a “major component”, called the prespermatophore (Hayashi & Suzuki, 2003), is produced. Maquitico-Rocha & Carrillo-Ruiz (2019) report that males of *P. palaciosi* have four pairs of accessory glands and the first pair is spirally shaped, which suggests this species produces nuptial gifts.

Second, the prolonged copulations recorded for *P. palaciosi* are typical of fireflies producing nuptial gifts (Wing, 1985; van der Reijden et al., 1997; Rooney & Lewis, 2000; Faust, 2010; cf. Wing, 1984). Wing (1985) suggest that prolonged copulations could be due to the time it takes to transfer a complex ejaculate or a form of mate guarding, although both hypotheses are not mutually exclusive. The fact that the time needed to transfer an spermatophore is shorter than the duration of copulation in several species (Wing, 1985; van der Reijden et al., 1997; Fu & Ballan-

tyne, 2021) and the existence of apparently adaptive variation in duration of copulation as a function of operational sex ratio in two species (Lewis & Wang, 1991; see also Wing, 1984), support the hypothesis that prolonged copulations are a form of mate guarding. In *P. palaciosi* the duration of the daily courtship period is 90–120 min, whereas the median duration of copulation is almost twice as long as the maximum duration of this period (228 min) (Fig. 2). In conclusion, the results presented indicate that *P. palaciosi* is polyandrous and the evidence provided by this study and previous research (Maquitico-Rocha & Carrillo-Ruiz, 2019) indicates that males produce nuptial gifts, which could account for polyandry in *P. palaciosi*.

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