Determinants of reproductive success across sequential episodes of sexual selection in a firefly

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Because females often mate with multiple males, it is critical to expand our view of sexual selection to encompass pre-, peri- and post-copulatory episodes to understand how selection drives trait evolution. In Photinus fireflies, females preferentially respond to males based on their bioluminescent courtship signals, but previous work has shown that male paternity success is negatively correlated with flash attractiveness. Here, we experimentally manipulated both the attractiveness of the courtship signal visible to female Photinus groenii fireflies before mating and male nuptial gift size to determine how these traits might each influence mate acceptance and paternity share. We also measured pericopulatory behaviours to examine their influence on male reproductive success. Firefly males with larger spermatophores experienced dual benefits in terms of both higher mate acceptance and increased paternity share. We found no effect of courtship signal attractiveness or pericopulatory behaviour on male reproductive success. Taken together with previous results, this suggests a possible trade-off for males between producing an attractive courtship signal and investing in nuptial gifts. By integrating multiple episodes of sexual selection, this study extends our understanding of sexual selection in Photinus fireflies and provides insight into the evolution of male traits in other polyandrous species.

Keywords: bioluminescence; courtship signal; Lampyridae; mating success; paternity success; spermatophore

1. INTRODUCTION

One of the principal forces driving the evolution of morphological, behavioural and physiological traits is sexual selection. This evolutionary phenomenon was first described by Darwin [1], who originally conceived this as a selective force that arises from differential mating success owing to intrasexual competition or intersexual choice. However, molecular methods of ascertaining paternity have revealed that females commonly mate with multiple males [2–5]. Therefore, a male’s reproductive success is determined by his ability to compete for and court females, to successfully mate, and to maintain paternity share when competing with other mating males. Morphological and behavioural traits traditionally considered as courtship signals might influence not only mating success, but also subsequent selection episodes such as male paternity success. Thus, a complete understanding of how sexual selection can drive trait evolution within polyandrous mating systems requires an integrative approach that encompasses courtship, pericopulatory (immediately before and during mating) and post-copulatory sexual selection episodes.

Different predictions have been made for the relationship between traits affecting male success across distinct episodes of selection. The phenotype-linked fertility hypothesis [6] predicts a positive association between male traits that mediate fitness across different selective episodes, and this relationship has some empirical support [7–10]. This could arise from a positive association between a male’s courtship signals and his fertilizing ability [11], or through reinforcement of initial female mating preferences via cryptic female choice [12]. Other work proposes a negative relationship between male success during pre- and post-copulatory sexual selection, which could be owing to trade-offs among male traits [13,14] or to sexual conflict [15]. Additional work is clearly needed to improve our understanding of how particular traits mediate male success during sequential episodes of sexual selection.

Across many animal taxa, males provide nuptial gifts to females during courtship and mating [16–18] and these gifts can potentially influence male paternity share. In many insects, males transfer their sperm in spermatophores, biochemically diverse packages that have been shown to influence male reproductive success [19–23]. Recent meta-analyses also show that spermatophore gifts can increase female fecundity [24,25]. Thus, variation in male nuptial gifts is likely to be an important factor influencing episodes of sexual selection.

Fireflies (Coleoptera: Lampyridae) are an especially interesting group for investigating how male traits influence reproductive success across distinct selection episodes. In Photinus fireflies, precopulatory sexual selection is based on a bioluminescent flash dialogue between flying males and stationary females [26] (reviewed in Lewis & Cratsley [27]). Females preferentially give flash responses to particular males based on temporal characteristics of male courtship signals [28–30]. Males that elicit higher response rates from females can locate females more quickly, and thus have higher mating success [31]. Furthermore, both sexes mate multiple times over their approximately two
week adult lifespan [32,33], and therefore a male's reproductive success will depend on both his mating and his paternity success. *Photinus* males produce an elaborate spermatophore that is transferred to females during mating [34]. Male-derived proteins are subsequently incorporated into developing oocytes [35], and females gain a fitness benefit from receiving multiple spermatophores via increased lifetime fecundity [36]. Gift production is costly for males, and spermatophore size declines across successive matings [37]. Males also vary in their post-copulatory reproductive success (based on their paternity share of offspring produced by doubly mated females [14,38]), and this might depend on spermatophore traits.

Previous work in *Photinus greeni* fireflies has demonstrated a negative relationship between a male's pre-copulatory courtship attractiveness and his subsequent post-copulatory paternity success [14]. Those males that were least attractive to females during courtship interactions nonetheless sired significantly more offspring compared with the most attractive males. Although this was a correlative study, it suggests the possibility of trade-offs between male traits affecting courtship attractiveness and other traits that influence paternity success, such as pericopulatory behaviours, spermatophore size or composition.

In this study, we build upon previous investigations of *P. greeni* fireflies to examine the relative importance of male flash attractiveness and spermatophore size in determining male close-range acceptance by females as well as paternity success in competitive mating situations. We experimentally manipulated flash attractiveness via photic playback, and also altered spermatophore size by manipulating male mating history. By using artificial signals, this experimental design allowed us to eliminate possible within-male trait correlations and isolate the effects of courtship signals and spermatophore-related traits. If male flash signals operate not only in the context of ensuring mating success, but also to increase paternity success, we predicted higher paternity share when females were exposed to more attractive courtship signals before mating. Independently manipulating male mating history allowed us to test the prediction that male paternity success was due to spermatophore-related traits. We predicted a positive relationship between spermatophore size and male reproductive success. In addition to measuring male paternity share, we also recorded pericopulatory behaviours and female mate acceptance after contact. Finally, we examined whether there were changes in sperm quantity between males' first and second spermatophores which might affect paternity success. This design thus allowed us to examine the separate effects of flash signals, pericopulatory behaviours and spermatophore size across multiple episodes of sexual selection. By spanning sequential episodes of sexual selection, this study provides novel insights into the evolution of male traits.

### 2. MATERIAL AND METHODS

#### (a) Study organisms and design

The effects of spermatophore size, flash signal attractiveness and pericopulatory behaviours on mate acceptance and paternity share were determined using *P. greeni* fireflies collected from Lincoln, MA, USA (46°26′ N, 71°18′ W). After collection (see the electronic supplementary material for details), virgin fireflies were weighed to the nearest 0.01 mg and maintained under a natural light cycle. Fireflies were housed separately in containers with access to water only, as adults of this species do not feed. Females were randomly allocated to one of four double-mating treatments. Each female was mated once to a male producing a large spermatophore and once to a male producing a small spermatophore after being exposed to a courtship signal that was either attractive or unattractive. All matings were conducted in the laboratory under a natural light cycle.

#### (b) Manipulating male flash signals

The courtship signal of *P. greeni* males consists of paired pulses separated by approximately 1.0–1.5 s [39]. Photic playback experiments using artificial flash signals covering the normal intraspecific range have shown that *P. greeni* females prefer male signals with a shorter interval between the two pulses [30,40,41]. Specifically, paired flashes with a 1.0 s interpulse interval (IPI) regularly elicit response flashes from *P. greeni* females, but females rarely respond to signals with a 1.4 s IPI [30]. Therefore, in the current study, we created artificial courtship signals that were either attractive to females (1.0 s IPI) or unattractive (1.4 s IPI) using a light emitting diode (LED) controlled by a programmable microprocessor. The LED (572 nm, Ledtronics Inc. Torrance, CA, USA) produced flashes that matched the wavelength of male *P. greeni* flashes [42]. Prior to mating, females were exposed to 25 artificial courtship signals that differed only in IPI depending on the treatment; pulse duration was held constant at 80 ms, with 10 s between consecutive signals.

#### (c) Manipulating male spermatophore size

Male spermatophore size was manipulated by controlling male mating history. In many insects, males transfer spermatophores that decrease in size with successive matings (Lepidoptera: [43–45]; Coleoptera: [46,47]; Orthoptera: [48]), a pattern that is especially prevalent in capital breeders such as *Photinus* fireflies. For example, in the related firefly *Photinus ignitus*, spermatophore weight decreases by 36 per cent between a males’ first and second matings [37]. Therefore, it is reasonable to assume that *P. greeni* males will produce relatively larger spermatophores during their first mating, and smaller spermatophores when they mate for a second time. In this experiment, we used virgin *P. greeni* males to obtain large (L) spermatophores, and used pre-mated males that were mated again the following night to obtain small (S) spermatophores.

Accompanying these changes in spermatophore size, sperm quantity may also change across successive matings: decreased numbers of sperm have been reported for some taxa [49], while others show increases [50]. Based on the potential for sperm quantity to influence male paternity success, we compared sperm quantity between *P. greeni* males’ first versus second spermatophores. Twelve virgin males were each mated with two different virgin females on sequential nights. Each mating was interrupted after 45 min to ensure spermatophore transfer, after which females were frozen in 95 per cent EtOH. Females were dissected and male spermatophores were placed in 10 μl distilled water, then gently opened to ensure that all sperm were released. *Photinus* firefly sperm is packaged into bundles, each containing a fixed number of sperm [34] and sperm bundles were counted under 60× magnification (Olympus BX40, Olympus, Center Valley, PA, USA). Differences in the number of sperm bundles between males’ first and second
spermatophores were compared using a paired t-test (SPSS v. 18, SPSS Inc., Chicago, IL, USA).

(d) Experimental treatments: female double matings
Male mating success and paternity share were measured when females were mated to two different males on successive nights. Females were assigned to one of four treatments as described below (see the electronic supplementary material, figure S1). For treatment 1, we describe the procedures and introduce the notation used for the remaining three treatments. Treatment 1. Night 1: Attractive signal + Large spermatophore. Night 2: Unattractive signal + Small spermatophore (A + L → U; n = 10 females). On the first night, these females were shown attractive courtship signals (25 paired flashes with 1.0 s IPI) and were then mated to a large spermatophore male. After 24 h, these females were shown unattractive courtship signals (25 paired flashes with 1.4 s IPI) and were then remated to a male with a small spermatophore. Treatment 2. Night 1: Attractive signal + Small spermatophore. Night 2: Unattractive signal + Large spermatophore (A + S → U + L; n =11 females). Treatment 3. Night 1: Unattractive signal + Large spermatophore. Night 2: Attractive signal + Small spermatophore (U + L → A + S; n = 11 females). Treatment 4. Night 1: Unattractive signal + Small spermatophore. Night 2: Attractive signal + Small spermatophore (U + S → A + L; n = 10 females).

Thus, comparisons of treatments 1 versus 2 and treatments 3 versus 4 show effects of altering male spermatophore size, while comparing treatments 1 versus 3 and treatments 2 versus 4 shows the effect of altering courtship signals.

Experiments began at approximately 2000 h each night, when each female in a clear plastic container was placed 24 cm from the output LED and exposed to her assigned artificial courtship signal. Females perceived and gave flash responses to these artificial signals. After 25 signal repetitions, a single male was immediately (within 10 s) introduced into the container and placed near the female. In most cases, this prevented the male from emitting any courtship flashes of his own, and almost completely eliminated any courtship dialogue between the sexes. Once a male contacts a female, he dorsally mounts her and inserts his aedeagus into her genital opening (copulation stage I; [33]). Spermatophore transfer takes place during stage II of copulation, after the male swirls 180° to assume an abdomen-to-abdomen position with the female [34]. Successful copulations (those that reached stage II) were recorded and allowed to terminate naturally (copulations can last up to 8 h; [33]). If stage II copulation did not occur within 15 min, beetles were set aside and checked every 5 min to determine whether mating had occurred.

Female fireflies are known to remate at 24 h intervals in the field [33], so females in all treatments were presented with their second mating opportunity 24 h after their first mating; 77 per cent of females remated at this time. Of the remaining 10 females, nine remated at 48 h after their first mating and one remated at 72 h. We observed a total of 121 male–female pairs of which 28 failed to mate (13 involved virgin females and the remaining 15 were females that had already mated once). Experiments were continued until we obtained a minimum of 10 doubly mated females within each treatment.

Following their second mating, females were maintained in the laboratory on a natural light cycle until their death. Females were provided moss for oviposition only after their second mating (i.e. no egg laying occurred between matings), and eggs were collected at 2 days intervals and placed into sterile petri dishes with 1× phosphate-buffered saline. Eggs were incubated at 29°C until hatching, and first instar larvae were collected and frozen in 95 per cent EtOH at −80°C for later DNA extraction and paternity assignment (see below). Males and females were also frozen in 95 per cent EtOH. The total number of larvae that emerged from a given family varied between 0 and 103. Female fecundity (lifetime no. of offspring) was compared between the four treatments with a two-way ANOVA (SPSS, Inc.), with second mating male spermatophore size and courtship signal as fixed factors.

(e) Measuring pericopulatory behaviours
Because they occur in the dark, close-range male–female interactions that happen after contact but before copulation have not, to our knowledge, previously been described for any firefly species. These behaviours were videorecorded with a Sony TRV80 video camera under infrared illumination (Sony Nightshot, Tokyo, Japan). Filming started when males were first placed into the mating arena, and stopped once successful mating had occurred or after 15 min had elapsed. Digitized videos (30 frames per second) were analysed frame-by-frame using iMovie (Apple, Inc., Cupertino, CA, USA) to describe and quantify pericopulatory behaviours of both sexes (described in the electronic supplementary material).

In our behavioural analysis, we included unsuccessful matings only when we observed sex-specific rejection behaviours (see the electronic supplementary material, table S1 and figure S2). We excluded any pairs that failed to mate and four additional pairs where the male successfully mounted the female but was unable to successfully copulate despite females adopting a receptive posture. We used exact logistic regression to determine how female mate acceptance (yes or no) was affected by male pericopulatory behaviours, spermatophore size and artificial flash attractiveness (each as a categorical predictor) using SAS PROC Logistic (SAS Inc., Cary, NC, USA). We used conditional exact tests in this analysis owing to sparseness of data, as the usual asymptotic methods are unreliable for such datasets [51]. In addition, we assessed whether male pericopulatory behaviours changed between a male’s first and second matings using a Goodman–Kruskal test (STAT XACT v. 6, Cytel Inc., Cambridge, MA, USA).

(f) Measuring male paternity share
To determine paternity for offspring produced by doubly mated P. greeni females, we used random amplified polymorphic DNA (RAPD) markers [52] following the methods described in Demary & Lewis [14] (see the electronic supplementary material for details). RAPD markers require no prior knowledge of genomic DNA sequence [53] and have been used to assign paternity in multiple taxa when possible sires are known [54,55]. Paternity was determined for each larval offspring of doubly mated females based on the presence of polymorphic bands shared uniquely with either of the two potential fathers.

For females in each treatment, we calculated second-male paternity share (P2) as the proportion of offspring sired by this male. A total of 650 larvae were genotyped for this study. Some mating treatments had fewer than 10 families because we only included doubly mated females that produced greater than or equal to nine offspring. Final sample sizes were as follows: five families in treatment 1, 11 families in treatment 2, 10 families in treatment 3 and 10 families in treatment 4.
To separately examine the effects of male spermatophore size, courtship signal attractiveness and their interaction on male paternity, we used a generalized linear model approach [56], where the proportion of offspring sired by the second male was modelled using binomial errors and a logit link function using SAS PROC GenMod (SAS Inc.). In addition, we examined whether second-male paternity within each family ($P_2$) was influenced by other male morphological or behavioural traits. To do this, we used logistic regressions where $P_2$ was modelled as a binomial response variable (this was possible because 86% of families showed $P_2$ of either 0 or 1, and the five families showing mixed paternity were assigned the closer $P_2$ value). Two separate logistic regressions were run with second males’ body weight (continuous) and second males’ pericopulatory behaviour (categorical) as predictors.

3. RESULTS
(a) Female and male pericopulatory behaviours

Photinus greeni females showed specific behaviours associated with rejecting a male (electronic supplementary material, table S2), but the likelihood of female rejection was not significantly affected by a male’s pericopulatory behaviour (logistic regression, likelihood ratio $\chi^2_{6} = 8.79, p = 0.1855$). Male pericopulatory behaviours did not change between each male’s first and second matings ($2 \times 3$ tests of association for leg behaviours, Goodman–Kruskal estimate = 0.0058, $p = 0.9$; for antennal behaviours, Goodman–Kruskal estimate = 0.0010, $p = 1.0$).

(b) Spermatophore size and courtship signals: influence on male mating success

The likelihood that female $P.$ greeni fireflies would mate with a male differed significantly between treatments, with females significantly more likely to mate with virgin males that had larger spermatophores (figure 1, exact logistic regression, conditional exact test score = 6.11, $p = 0.0152$). Female mating status (virgin versus already mated) did not alter the likelihood of female acceptance (conditional exact test score = 2.40, $p = 0.1310$), and there was no effect of courtship signal attractiveness on the likelihood of female acceptance (conditional exact test score = 0.93, $p = 1.0$). Additionally, there was no interaction between the effects of spermatophore size and courtship signal attractiveness on mating success (conditional exact test score = 6.63, $p = 0.1310$).

(c) Spermatophore size and courtship signals: influence on male paternity share

Photinus greeni males with larger spermatophores sired a significantly greater proportion of females’ offspring than did males with small spermatophores (figure 2; generalized linear model, spermatophore size estimate = 2.72, likelihood ratio: $\chi^2_{2} = 12.41, p = 0.0004$). This effect was particularly pronounced when males producing large spermatophores were the second ones to mate (treatments 2 and 4; figure 2b,d). Within all experimental treatments, paternity showed a strikingly bimodal distribution (figure 2); when they mated with previously mated females, some males in each treatment sired all the subsequent offspring ($P_2 = 1$) while others sired none ($P_2 = 0$). Mixed-paternity broods were seen in only five out of 36 families.

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There was no significant effect of courtship signal attractiveness on male paternity share (figure 2; generalized linear model, courtship signal estimate = 0.153, likelihood ratio: $\chi^2_{3} = 0.03, p = 0.8559$), and there was no significant interaction between spermatophore size and signal attractiveness (interaction estimate = −0.936, likelihood ratio, $\chi^2_{2} = 28.28, p = 0.5955$). Also, male paternity share was not influenced by either male body weight (logistic regression; likelihood ratio: $\chi^2_{1} = 0.46, p = 0.4993$) or male pericopulatory behaviours (logistic regression; likelihood ratio: $\chi^2_{1} = 6.19, p = 0.1853$).

As expected, because every doubly mated female received one large and one small spermatophore, lifetime offspring production did not vary between experimental treatments (two-way ANOVA, spermatophore size: $F_{1,38} = 0.127, p = 0.723$, courtship signal: $F_{1,38} = 0.482, p = 0.492$, interaction of spermatophore size and courtship signal: $F_{1,38} = 3.593, p = 0.066$).

Sperm quantity declined significantly between $P.$ greeni males’ first and second spermatophores (figure 3, paired $t_{11} = 12.33, p < 0.005$; mean difference ± s.e. = 75.3 ± 6.1).

4. DISCUSSION

Theoretical models of sperm competition provide different theories for how males might invest into different phases of sexual selection. Parker [57] predicted a trade-off between investment in ejaculate quality and subsequent paternity success versus investment into other reproductive traits that modulate mating success. A number of empirical studies across a broad range of taxa document such a negative relationship [58–61]. However, Sheldon [6] advanced an alternative theory, the phenotype-linked fertility hypothesis. This theory predicts a concordance between investment into ejaculate and secondary sexual traits that
mediate mating success, a model that also has some empirical support [62–64]. Such concordance could arise because male courtship traits could covary with the traits that are responsible for paternity success. For example, male guppies (*Poecilia reticulata*) that are more attractive based on their coloration have greater fertilization success relative to rivals owing to superior sperm competitive ability [64,65]. However, many such studies are observational or correlative in nature. By experimentally manipulating male traits and including both pericopulatory and postcopulatory sexual selection episodes, our study provides insight into how traits can influence multiple episodes of sexual selection, as well as into potential trade-offs between traits.

**Figure 2.** Frequency histograms showing the effect of artificial courtship signal (attractive or unattractive) and spermatophore size (large or small) on second male paternity share ($P_2$ equals the proportion of offspring produced by doubly mated *Photinus greeni* females that were sired by the second mating male). Treatment descriptions indicate conditions for the second mating male: (a) unattractive courtship signal and small spermatophore; (b) unattractive courtship signal and large spermatophore; (c) attractive flash and small spermatophore; and (d) attractive flash and large spermatophore. Virgin females in all treatments were mated to two males at 24–72 h intervals.

**Figure 3.** Sperm quantity (number of bundles) contained within spermatophores produced by *Photinus greeni* males during their first versus second matings ($n = 12$ males).

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accepted as mates. Although previous studies have also found that seminal nuptial gifts can influence both male mating success and paternity success [19–23], possible mechanisms for how male spermatophore size might affect mate acceptance are unclear. Although females clearly rejected certain males, female acceptance of *P. greeni* males based on their pericopulatory behaviours seems unlikely, as we found no behavioural differences between males’ first and second matings. In the moth *Utetheisa ornatrix*, females choose among males during close-range courtship on the basis of a pheromonal signal that is correlated with chemical defence titres within the male spermatophore [67]. Although similar close-range chemical cues might allow *Photinus* females to distinguish between virgin and previously mated males, studies to date provide no evidence for signalling in *Photinus* fireflies via either cuticular hydrocarbons [68] or volatile pheromones [69]. Because *Photinus* fireflies are chemically defended [70], it is tempting to speculate that firefly pericopulatory mate acceptance might be based on signals correlated with lucibufagin content of male spermatophores, but this remains to be explored.

**(b) Influence of male courtship signals is limited to precopulatory female choice**

Previous work on *Photinus* fireflies has shown that in the field, a male’s mating success is determined primarily by how attractive his courtship flash is to females (reviewed by Lewis & Cratsley [27]). In *P. greeni*, females preferentially respond to courtship signals with faster pulse rates by emitting their own response flashes [30]. *Photinus* males use these female response flashes to locate females [26], and males that can elicit more female responses have higher mating success [28]. In the current study, however, we found that *P. greeni* male courtship signals have no direct influence on later sexual selection episodes, as they did not affect either the likelihood of female acceptance after contact or males’ paternity share.

Demary & Lewis [14] found a negative relationship in *P. greeni* between a male’s attractiveness based on his courtship signal and his subsequent paternity share. To explore this relationship further, the current study used artificial courtship signals to control for other possible differences among males. Because our results show no direct effect of courtship signal on male paternity success, taken together, these findings suggest that males may be subject to energetic trade-offs constraining them either to produce an attractive, fast-pulsed courtship signal or to invest in larger nuptial gifts. This adds to a growing body of evidence suggesting trade-offs between secondary sexual traits that mediate mating success and ejaculate quality (e.g. plumage in red-backed fairy-wrens *Miliarus melanopthalmus*, [59]; pheromones and dominance behaviour in Australian field crickets; *Teleogryllus oceanicus* [71], and level of sexual ornamentation in guppies *Poecilia reticulata*, [58]).

**(c) Bimodal distribution of paternity share**

In the current study, the vast majority of females produced offspring that were sired solely by either their first mate (*P₂ = 0*) or their second mate (*P₂ = 1*); very few broods showed mixed paternity. Such starkly bimodal distributions of *P₂* have now been documented across many taxa (reviewed by Simmons [4]; *Poecilia reticulata* guppies [72]; *Ephippiger ephippiger* bushcrickets [73]; *Teleopsis dalmanni* stalk-eyed flies [74]; butterflies and moths [75]; and *Cadra cautella* moths [66]). Despite this ubiquity, the mechanisms generating such bimodal paternity share are not well understood. In *T. dalmanni*, Corley et al. [74] suggest that differences in male fertility, patterns of sperm usage and ejaculate expenditure as a function of female reproductive value could explain extreme variations in paternity share. A recent study on *Teleogryllus commodus* field crickets [76] suggests a role for both sperm competition and cryptic female choice in determining reproductive success, highlighting the complexity of these post-copulatory interactions.

Bimodality of *P₂* could also be influenced by male-derived substances such as seminal fluid proteins, which are important in sperm competition (see [77]). In *P. greeni* fireflies, another mechanism generating bimodal paternity share might involve different sperm storage organs for housing first versus second males’ sperm. *Photinus greeni* females have two sperm storage chambers, and differences in stored sperm viability have been documented between these [78]. The idea that females might shunt sperm to different storage sites has been previously suggested as a mechanism for females to retain control over paternity [79,80]. Females that choose their mates based on spermatophore size might gain direct benefits through enhanced fecundity and longevity. If females can assess spermatophore size after mating, they may be able to bias fertilizations towards males with larger spermatophores. Such post-copulatory choice could provide females with indirect benefits, as spermatophore size has been found to be heritable in some insects [81]. Thus, female choice at different episodes could provide either direct or indirect benefits. Additional work is needed to examine how cryptic female choice and sperm competition might interact to determine patterns of paternity.

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