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The production and transfer of spermatophores in three Asian species of *Luciola* fireflies

Adam South [a], Teiji Sota [b], Norio Abe [c], Masahide Yuma [d], Sara M. Lewis [a,*]

[a] Department of Biology, Tufts University, Medford, MA 02155, USA
[b] Department of Zoology, Graduate School of Science, Kyoto University, Kyoto, Japan
[c] Firefly Breeding Institute, Itabashi-Ward, Toyko, Japan
[d] Department of Environmental Solution Technology, Ryukoku University, Otsu, Japan

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ABSTRACT

During mating, many male insects transfer sperm packaged within a spermatophore that is produced by reproductive accessory glands. While spermatophores have been documented in some North American fireflies (Coleoptera: Lampyridae), little is known concerning either production or transfer of spermatophores in the aquatic *Luciola* fireflies widespread throughout Asia. We investigated this process in Japanese *Luciola lateralis* and *L. cruciata* by feeding males rhodamine B, a fluorescent dye known to stain spermatophore precursors. We then mated males with virgin females, and dissected pairs at various timepoints after mating. In both of these *Luciola* species, spermatophores were produced by three pairs of male accessory glands and were transferred to females during the second stage of copulation. Male spermatophores were highly fluorescent, and were covered by a thin outer sheath; a narrow tube leading from an internal sperm-containing sac fit precisely into the female spermathecal duct, presumably for sperm delivery. Both *L. lateralis* and *L. cruciata* females have a spherical spermatheca as well as a highly extensible gland where spermatophore breakdown commences by 24 h post-mating. Similar reproductive anatomy was observed for both sexes in *Luciola ficta* from Taiwan. These results suggest that nuptial gifts may play an important role in many firefly-mating systems.

1. Introduction

Before, during or after copulation, males in diverse insect taxa provide females with nuptial gifts in the form of male body parts, food items, or spermatophores produced by male accessory glands (Mann, 1984; Boggs, 1990, 1995; Vahed, 1998). Such nuptial gifts have the potential to increase a female's lifetime fecundity, and may thus select for polyandrous mating systems and post-copulatory female choice (Gwynne, 2008; Vahed, 2007). In certain insects, however, male seminal products have been shown to reduce female longevity (Chapman and Davies, 2004), suggesting they may have evolved through sexual conflict. In either case, it is clear that male nuptial gifts represent an important feature of insect mating systems, with far-reaching implications for the evolution of reproductive anatomy, physiology, and behavior in both sexes.

Nuptial gifts are likely to be of particular economic importance in the nutrient budgets of any insects that have non-feeding adults, since all reproductive activities are based on resources obtained by larval feeding. One such group is North American *Photinus* fireflies (family Lampyridae), in which males transfer spermatophores that are known to increase female lifetime egg production (reviewed by Lewis et al., 2004). However, little is known about nuptial gifts in *Luciola* firelies, a widespread Asian genus in which some species have aquatic larvae that feed primarily on freshwater snails (Ohba, 1984, 2004). Hayashi and Suzuki (2003) postulated spermatophore transfer in the Japanese Genji firefly, *L. cruciata* (Motschulsky), based on the morphology of male accessory glands, and observed multiple spermatophores in the reproductive tract of a female *Lucidina natsumiae* (Chujo and M. Sato). The only other spermatophore description for any Asian firefly was by Fu and Ballantyne (2006), who noted a spermatophore found within the reproductive tract of a field-collected female of the Chinese firefly, *Luciola leii* (Fu and Ballantyne).

In this study we describe in detail male and female reproductive anatomy for three *Luciola* firefly species: *Luciola lateralis* (Motschulsky), *Luciola cruciata*, and *Luciola ficta* (Olivier). In *L. lateralis* and *L. cruciata* we investigated the process of spermatophore production and transfer by feeding males rhodamine B, a fluorescent dye known to stain spermatophore precursors. We then allowed males to mate with virgin females, and dissected mated pairs at various timepoints after mating. Here we report the first description of spermatophore production and transfer for any Asian firefly species.

* Corresponding author. Tel.: +1 617 627 3548; fax: +1 617 627 3805.
E-mail address: sara.lewis@tufts.edu (S.M. Lewis).

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2. Materials and methods

L. cruciata and *L. lateralis* adults used in this study were from Japan: the former is endemic to Japan, while the latter is widely distributed in Japan, Korea, and eastern Siberia (Ohba, 1984). These two species were reared by Dr. Norio Abe at the Firefly Breeding Institute in Itabashi Ward, Tokyo and originate from firefly populations in Fukushima and Tochigi, Honshu, Japan; a few *L. cruciata* males were field-collected in Katsuragawa, Otsu City, Shiga Prefecture. *L. ficta* adults were collected in Taiwan. To examine male and female reproductive anatomy, adults of each species were frozen at −20 °C in 70% EtOH until dissection in 1 × phosphate buffered saline (PBS).

Mating experiments were conducted with virgin *L. cruciata* and *L. lateralis* to determine if spermatophores were transferred and to characterize the time course of this process. Adults of *L. cruciata* and *L. lateralis* were kept in same sex groups, then separated into individual containers with moistened paper towel. Males were fed a 40% sucrose solution with rhodamine B, a thiol-reactive fluorescent dye that forms covalent bonds to proteins. This product is known to stain spermatophores (Sparks and Cheatham, 1973; van der Reijden et al., 1997), allowing us to visualize portions of the male reproductive tract responsible for producing spermatophore precursors and to track the location of male spermatophores within the female reproductive tract at various timepoints post-mating. Following rhodamine B exposure for 5–24 h, males were paired with females and monitored until mating occurred. Copulation in *L. lateralis* and *L. cruciata*, as in many other firefly species (Lewis and Wang, 1991) consists of two distinct stages; in stage 1 males mount females dorsally, while stage 2 begins after the male swivels around to face in the opposite direction in an abdomen-to-abdomen position. Mating pairs were monitored, and copulations were terminated by freezing at various timepoints after the beginning of stage 1 or 2 (ranging from 0 min to 24 h), after which pairs were stored in 70% EtOH until dissection in 1 × PBS. Reproductive tracts removed from males and females were observed with a Nikon SMZ1500 stereomicroscope equipped with an X-Cite 120 fluorescence illuminator, and photographed with an Insight 4 Mega-pixel Color Mosaic camera (Diagnostic Instruments, Michigan). For *L. lateralis* and *L. cruciata*, 11 males and 9 females of each species were dissected, while 4 of each sex of *L. ficta* were used. Wet weights were determined by removing specimens from alcohol and allowing them to air dry. Three spermatophores were dissected out of female reproductive tracts, placed on a pre-weighed plastic platter and kept in a dessicator at room temperature for 24 h. The body of the male whose spermatophore was collected from the female was placed on a pre-weighed aluminum foil platter and allowed to dry at 40 °C for 72 h. All structures were weighed on a MT 400 AT261 microbalance.

To confirm sperm presence, structures within the female reproductive tract, male seminal vesicles and spermatophores were separated on slides, stained with propidium iodide, a fluorescent DNA stain, and examined at 400 × on an Olympus BX40 fluorescence compound microscope. This procedure allowed us to determine the location of sperm within the female reproductive tract at various times post-mating.

In the rhodamine B mating experiments, only 2 of 5 *L. cruciata* pairs successfully mated, and these copulations did not progress beyond stage 1. For *L. lateralis*, 13 of 20 pairs successfully mated, and 6 of these progressed to stage 2. Because we had a greater number of *L. lateralis* specimens, and because this species appeared more conducive to mating in captivity, below we use *L. lateralis* to describe female and male reproductive anatomy, as well as the process of spermatophore transfer, and then present comparisons of *L. cruciata* and *L. ficta*.

3. Results

3.1. Female reproductive system

In the reproductive tract of *Luciola* females (Fig. 1A and B), the ovaries contained ovarioles with oocytes in many developmental stages, with mature oocytes occupying the lateral oviducts. These lateral oviducts converged into a common oviduct, which emerged posteriorly from the dorsal midpoint of the bursa copulatrix (BC). The BC was about 1.5 mm long and 0.5 mm wide with relatively thick walls and can be divided into two sections that are anterior and posterior to the common oviduct entrance. The posterior bursa has been termed the vagina by others (e.g. Ballantyne and Lambkin, 2006). The BC is also characterized by having two slightly curved, needle-shaped sclerotized plates embedded in the lateral walls. There was an additional small, sclerotized plate embedded in the dorsal wall of the BC near the entrance of the common oviduct (Fig. 1B). Two sclerotized valvifers of the ovipositor were attached to the posterior end of the BC, and the lumen narrowed slightly as the bursal wall thickened.

Located at the anterior end of the BC were two structures: a small, spherical spermatheca and a much larger spermatophore-digesting gland (SDG: Fig. 1B). The spermatheca was connected via a short duct to the dorsal side of the BC anterior to the

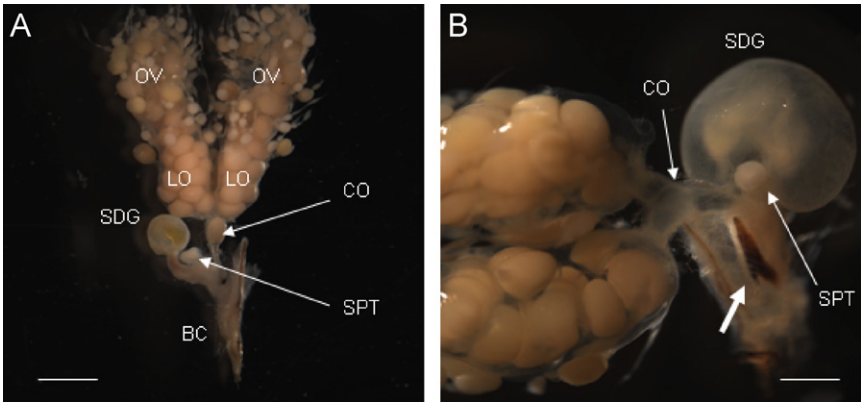

Fig. 1. Female reproductive anatomy in *Luciola* fireflies. Scale bars: 1 mm (A) Entire reproductive system of *L. cruciata* female: ovaries (OV) contain both mature and immature oocytes, and lead into the lateral oviducts (LO). The common oviduct (CO) enters the bursa copulatrix (BC) immediately adjacent to the spermatheca (SPT), the site of sperm storage and the spermataphore-digesting gland (SDG), the site of eventual spermatophore digestion. (B) Close-up of hemispherical spermataphore-digesting gland (SDG), common oviduct (CO) and smaller, spherical spermatheca (SPT) of *L. lateralis* female (sclerotized plate indicated by white arrow).

entrance of the common oviduct (Fig. 1A and B). Anterior to the spermatheca was a semi-spherical, thin-walled SDG, which eventually contained the spermatophore as it was being digested. Typically this structure was distended and fluid-filled, and in mated females also contained heterogenous granules from the male spermatophore; unmated females lacked these granules. Regardless of female mating status, each SDG also contained a small white mass of unknown origin. Posterior to the common oviduct, a small tubular gland of unknown function entered the dorsal side of the BC.

In *L. cruciata* and *L. ficta*, female reproductive anatomy was quite similar to that of *L. lateralis* (Table 1). In both species, the BC terminated in a hemispherical SDG, with a single spherical spermatheca connected via a short duct. Females in both *L. cruciata* and *L. ficta* had a sclerotized plate located in the bursal wall posterior to the common oviduct, but this structure was greatly reduced in *L. cruciata* females. Females in both species lacked the needle-like sclerotized plates that were embedded in the bursal walls of *L. lateralis* females.

3.2. Male reproductive system

In *L. lateralis* males, the paired testes were located at the anterior end of the abdomen (Fig. 2A and B) and each testis was thickly covered by fat body and connective tissue. The tubular vasa deferentia led to the seminal vesicles, which appeared as ovoid enlargements at the proximal ends of these ducts. Sperm within the seminal vesicles were packaged into sperm bundles, each consisting of 50–80 sperm. The seminal vesicles emptied into the ejaculatory duct at its junction with the accessory glands.

The most conspicuous structures in the reproductive tract of *L. lateralis* males were three pairs of bilaterally symmetrical accessory glands (Fig. 2A and B) that entered the ejaculatory duct at a common point. The most central were the paired curled glands, which were tapering, slightly twisted glands arranged longitudinally in the abdominal cavity; these glands were approximately 2.2 mm long and measured 0.3 mm at their widest point. In males that had injested rhodamine B dye, these curled glands were the most fluorescent portion of the reproductive system (Fig. 2B), and dissections of virgin males revealed spermatophore precursors located within these glands. Near the curled glands were two additional pairs of tubular accessory glands. The thin-walled long glands were approximately 5.3 mm long and narrow, widening distally to about 0.35 mm and ending in a bulbous mass of white, spongy tissue; these glands contained heterogeneous granules similar to those found in spermatophores that had been transferred to females (see below). The short

Table 1
Reproductive characters for three species of *Luciola fireflies* based on the following sample sizes: *L. lateralis* and *L. cruciata* = 11 males, 9 females; *L. ficta* = 4 males, 4 females. Wet masses reported as means (±1 SE)

Species	Number of male accessory glands	Spermatophore present?	SDG present?	Number of SPT	Mean wet weight of males (mg)	Mean wet weight of females (mg)
L lateralis	Three	Yes	Yes	One	25.15(±0.983)	34.17(±2.43)
L. cruciata	Three	Yes	Yes	One	65.95(±7.51)	171.25(±10.98)
L. ficta	Three	Yes	Yes	One	18.5(±0.912)	32.63(±7.24

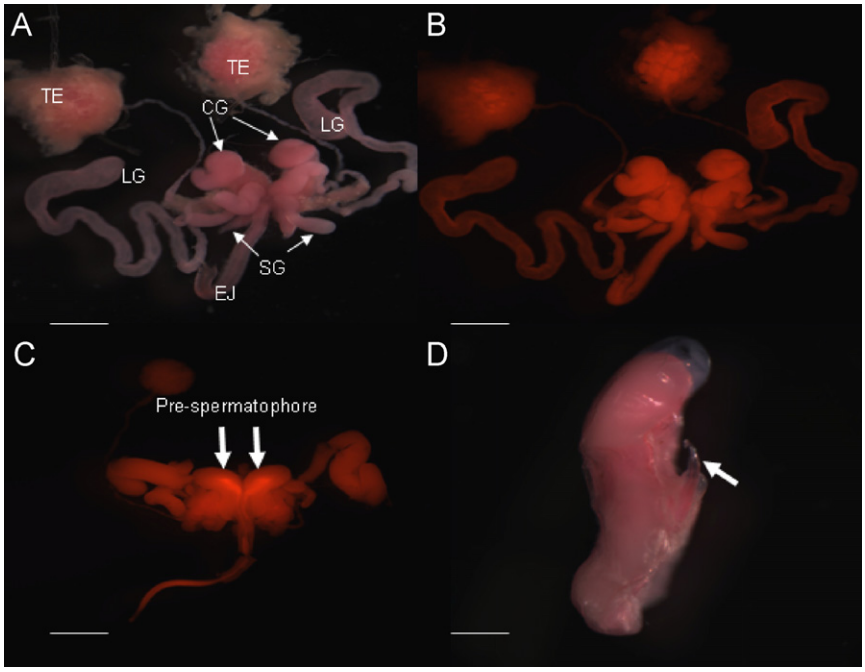

Fig. 2. Male reproductive anatomy and spermatophore structure in *Luciola* fireflies. Scale bars for panels A and B: 0.5 mm, panel C: 1 mm, panel D: 0.25 mm. (A) Reproductive system of *L. lateralis* male in visible light. (B) Same specimen under fluorescence illumination showing rhodamine B staining. Labeled structures: LG—long accessory glands, CG—curled glands, SG—short accessory glands, TE—testes, EJ—ejaculatory duct. (C) Reproductive system of a male *L. cruciata* interrupted during Stage 1 mating under fluorescence illumination showing rhodamine B staining of the pre-spermatophore emerging from the curled glands into the ejaculatory duct. (D) Intact spermatophore dissected from the reproductive tract of a *L. lateralis* female. Sperm delivery tube is indicated by white arrow.

accessory glands (about 0.82 mm long and 0.23 mm wide) were also thin-walled, and contained a slightly opaque fluid. In some specimens, both the long and short glands showed slight fluorescence, suggesting that they may also produce proteins.

In *L. ficta* and *L. cruciata* males, reproductive structures were quite similar to those of *L. lateralis* (Table 1). Male accessory glands were virtually identical in all three species, with only slight variations in the long accessory glands; the distal swelling in the long glands of *L. ficta* and *L. cruciata* males was not as pronounced as in *L. lateralis*.

3.3. Spermatophore structure and transfer

In several *L. lateralis* pairs where copulation was interrupted during stage 1, spermatophore precursors were visible emerging from the curled gland and merging in the ejaculatory duct to begin spermatophore formation. Out of the 4 pairs interrupted during Stage 1, none had transferred the spermatophore. Instead, spermatophore transfer appears to take place during stage 2 of copulation (Fig. 3A and B).

Several intact spermatophores were dissected from the reproductive tracts of *L. lateralis* females that had mated with rhodamine B dyed males (Fig. 2D). Three of these spermatophores were weighed, and their mean percentage of the total dry male body mass was 1.4% ($\pm$0.1 SE). The spermatophore consisted of an outer membranous sheath surrounding a spongy matrix which contained granules resembling the contents of the curled and long accessory glands. In turn, this matrix surrounded an inner sac containing sperm bundles. This inner sperm sac emptied through a lightly sclerotized tube that emerged from the spermatophore at a 45° angle. Overall, the male spermatophore in *L. lateralis* resembles a mitten (Fig. 2D), with the tube emerging from the sperm sac representing the thumb, and the inner sperm sac located in the mitten's palm.

This spermatophore structure appears to function in delivering sperm directly into the female's spermatheca. For all mated *L. lateralis* females that were killed between 1 and 9 h following the initiation of copulation stage 2 ($n = 4$), the male spermatophore was always positioned partially in the BC and partially in the SDG, with the spermatophore thumb extending up into the spermathecal duct (Fig. 3C and D). However, in a single *L. lateralis* female frozen 24 h following the initiation of copulation stage 2, the male spermatophore was located entirely within the female's SDG, which was extremely distended. At this timepoint the male spermatophore was still intact, but the membranous outer sheath had begun to disintegrate.

In 2 out of 4 field-collected *L. ficta* females, spermatophores resembling those of *L. lateralis* were dissected from their reproductive tracts. Because none of the *L. cruciata* pairs progressed beyond stage 2 of copulation, we were unable to directly observe spermatophore transfer in this species. However, dissection of a *L. cruciata* male frozen during stage 1 revealed a spermatophore forming in the ejaculatory duct (Fig. 2C), as we had also observed in *L. lateralis*.

4. Discussion

This study adds considerably to our knowledge of reproductive anatomy and spermatophore transfer in *Luciola* fireflies, a genus widespread throughout Asia. Hayashi and Suzuki (2003) dissected males of 20 species of Japanese fireflies, and reported

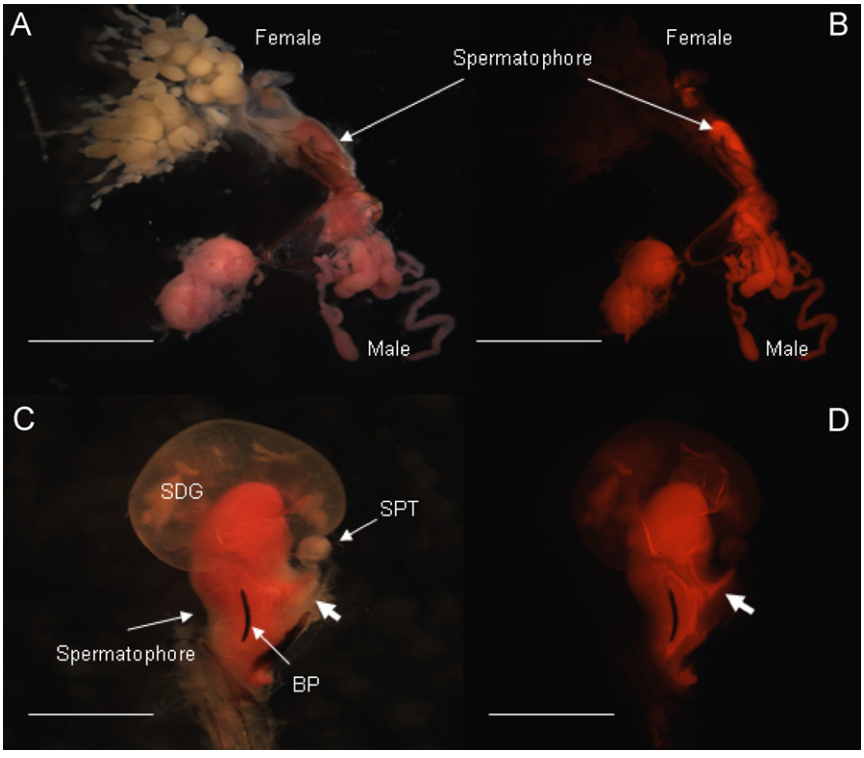

Fig. 3. Process of spermatophore transfer in *Luciola lateralis* fireflies. Scale bars for panels A and B: 2 mm, panels C and D: 1 mm. (A) Reproductive tracts of male (lower right) and female (upper left) in copula, with spermatophore in the process of being transferred into the female bursa copulatrix (taken in visible light). (B) Same specimen under fluorescence illumination showing rhodamine B staining of male reproductive tract and male spermatophore inside female. (C) Close-up of female reproductive tract taken in visible light, showing male spermatophore (pink, mitten-shaped structure) located partly within the female's spermatophore-digesting gland (SDG) and partially in the BC. Sperm delivery tube (indicated by white arrow) leading from the internal sperm sac is positioned within the female's spermathecal duct. Needle-shaped sclerotized plate is visible embedded in bursal wall (labeled BP). (D) Same specimen under fluorescence illumination showing rhodamine B stained male spermatophore with sperm delivery tube marked with white arrow.

pre-spermatophores from 12 of these, including *L. cruciata*, based on male internal anatomy. Fu and Ballantyne (2006) described a spermatophore in a field-collected female of *L. leii*. Our study provides a comprehensive description of male and female reproductive anatomy in *L. lateralis* and *L. cruciata*, as well as describing the time course of spermatophore transfer in *L. lateralis*. We also observed the early stages of spermatophore formation in a mating pair of *L. cruciata*, and documented the presence of spermatophores in field-collected females of *L. ficta*.

Males of these three Asian *Luciola* species show similarities to several North American *Photinus* fireflies for which spermatophore structure, transfer, and fate was described by van der Reijden et al. (1997). In both of these lampyrid genera, sperm coming from the testes are packaged into sperm bundles, and males have multiple pairs of reproductive accessory glands (three in *Luciola* spp., four in *Photinus* spp.) that are involved in spermatophore production. The main body of the spermatophore appears to be produced by the largest accessory glands (curled glands in *Luciola*, spiral glands in *Photinus*), as these contain spermatophore precursors that are very similar in shape to portions of the final spermatophore. In addition, the strong rhodamine B fluorescence observed in these glands suggests they produce spermatophore components with a high-protein content. Our findings may also provide insight into *Luciola* mating systems. In a comparison of 4 *Photinus* species, only those known to exhibit polyandrous mating systems were characterized by males with multiple accessory glands (Demary and Lewis, 2007). Thus, our results suggest that female multiple mating may also occur in the three *Luciola* species studied here.

Female reproductive anatomy in these three *Luciola* species is also similar to that described for two genera of North American lamyprids by van der Reijden et al. (1997) and Rooney and Lewis (2000). Females have a single, spherical spermatheca, although this is much smaller in *Luciola* females compared to *Photinus* and *Ellychnia* (*Photinus greeni* (Lloyd) females have two spermathecae; Demary, 2005). Fu and Ballantyne (2006) report similar female reproductive anatomy for *Luciola leii*. A distinctive feature characterizing female reproductive systems in the three lampyrid genera studied to date, *Luciola*, *Photinus*, and *Ellychnia*, is the presence of a specialized gland in which male spermatophores are internally digested.

This study reveals a novel method of delivering sperm from the spermatophore into the female spermatheca in *L. lateralis*. In *Photinus* and *Ellychnia* fireflies, sperm bundles are released from the anterior end of the spermatophore while it is positioned within the female spermatheca, which can expand greatly to receive the spermatophore (van der Reijden et al., 1997). However, in *Luciola* females the spermatheca is smaller, and is connected to the BC by a narrow duct. When the spermatophore is transferred to the female reproductive tract, it is positioned so that its thumb enters the spermathecal duct, allowing sperm rings to be transferred from the sperm sac directly into the spermatheca. It is possible that females might influence sperm movement into or out of the spermathecae through movements of muscles or bursal plates, but this remains to be investigated. Fu and Ballantyne (2006) also propose a similar function for a median oviduct plate in female *Luciola leii*, a structure they suggest could influence sperm release from the spermatheca.

Our results indicate that multiple male accessory glands in these *Luciola* fireflies each contribute seminal products that are incorporated into spermatophores. In many insects, male accessory gland products have been shown to have beneficial effects on female fitness by increasing longevity or lifetime fecundity (Boggs, 1995; Vahed, 1998). In other insects, male seminal products have detrimental effects on female fitness (Wolfner, 2002; Chapman and Davies, 2004; Vahed, 2007, Gwynne, 2008). Based on the number and complexity of *Luciola* male accessory glands, diverse seminal products with multiple functions are likely, and further work to characterize the nature and function of these products would be worthwhile.

Two major life-history factors that may influence the evolution of nuptial gifts are adult feeding habits and sexual size dimorphism. When adult dietary input is limited, male nuptial gifts may represent important contributions to female nutrient budgets (Boggs, 1990, 1995). As in *Photinus* spp. fireflies, many *Luciola* spp. also do not feed as adults (Hayashi and Suzuki, 2003; Fu and Ballantyne, 2006), suggesting the possibility that male spermatophores provide a nutritional supplement for *Luciola* females. In *Photinus ignitus* (Fall), male spermatophores have been shown to provide a net benefit to females: 62% of radiolabelled spermatophores proteins transferred to females later appeared in female oocytes (Rooney and Lewis, 1999), and triply-mated females had 73% greater lifetime fecundity compared to singly-mated females (Rooney and Lewis, 2002).

In addition to adult feeding habits, sexual size dimorphism may also play a role in nuptial gift evolution, because such dimorphism could influence the potential for any male nutrient contributions to increase female fecundity (Lewis and Cratsley, 2008). Hayashi and Suzuki (2003) postulated that spermatophore production in fireflies may be linked with a high degree of sexual dimorphism and female flightlessness, with spermatophores being absent in species that have larviform, apterous (wingless), or brachypterous (short-winged) adult females. Consistent with this hypothesis, spermatophores have been found in several species of North American lampyrids that are not sexually dimorphic, while being absent in *Photinus collustrans* (LeConte), a species with larviform females (Lewis et al., 2004). In this study, we found spermatophore production in three *Luciola* species with moderate sexual size dimorphism, although females of all three species are still capable of flight; size dimorphism (measured as ratios of average female:male wet mass) ranged from 1.4 to 2.6 for *L. lateralis* and *L. cruciata*, respectively. Future studies to determine the net effect that male spermatophores have on the lifetime fitness of *Luciola* spp. females will be of considerable interest, and will provide a more complete understanding of the relationship between sexual dimorphism and nuptial gift evolution.

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