

CORRELATED EVOLUTION OF FEMALE NEOTENY AND FLIGHTLESSNESS WITH MALE SPERMATOPHORE PRODUCTION IN FIREFLIES (COLEOPTERA: LAMPYRIDAE)

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The beetle family Lampyridae (fireflies) encompasses ~100 genera worldwide with considerable diversity in life histories and signaling modes. Some lampyrid males use reproductive accessory glands to produce spermatophores, which have been shown to increase female lifetime fecundity. Sexual dimorphism in the form of neotenic and flightless females is also common in this family. A major goal of this study was to test a hypothesized link between female flight ability and male spermatophore production. We examined macroevolutionary patterns to test for correlated evolution among different levels of female neoteny (and associated loss of flight ability), male accessory gland number (and associated spermatophore production), and sexual signaling mode. Trait reconstruction on a molecular phylogeny indicated that flying females and spermatophores were ancestral traits and that female neoteny increased monotonically and led to flightlessness within multiple lineages. In addition, male spermatophore production was lost multiple times. Our evolutionary trait analysis revealed significant correlations between increased female neoteny and male accessory gland number, as well as between flightlessness and spermatophore loss. In addition, female flightlessness was positively correlated with the use of glows as female sexual signal. Transition probability analysis supported an evolutionary sequence of female flightlessness evolving first, followed by loss of male spermatophores. These results contribute to understanding how spermatophores have evolved and how this important class of seminal nuptial gifts is linked to other traits, providing new insights into sexual selection and life-history evolution.

KEY WORDS: Bioluminescence, seminal nuptial gifts, flightlessness, life-history evolution, male accessory glands, neoteny, sexual selection, sexual signals

Neoteny occurs when shifts in regulatory timing cause reproductive traits to follow a normal developmental trajectory while somatic maturation is delayed, producing fully reproductive adults that nonetheless retain many juvenile characteristics (Gould 1977; Cicero 1988; Bocakova et al. 2007; Bocak et al. 2008). This process can generate highly modified adult phenotypes and dra-

matically alter life histories. Neoteny has been proposed as a key source of evolutionary innovation in both vertebrate and invertebrate lineages (Gould 1977; Raff 1996; Reilly et al. 1997; West-Eberhard 2003). In insects, such ontogenetic shifts can result in highly neotenic, larviform adults that lack wings (or in which wings are greatly reduced), and thus are unable to fly (Bocakova et al. 2007; Bocak et al. 2008; Cicero 2008). Because flight enables many essential tasks such as foraging, mate-finding,

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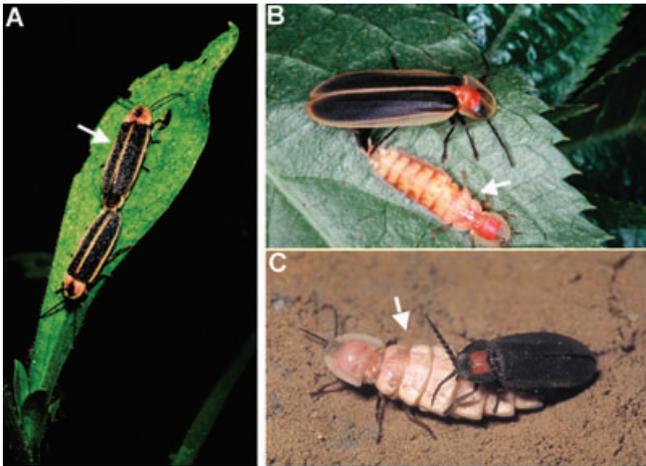


Figure 1. Range of neoteny exhibited by females (indicated by arrows) in various lampyrid fireflies. (A) *Photinus* sp. (neoteny score of 0). (B) *Diaphanes* sp. (neoteny score of 2). (C) *Pyrocoelia analis* (neoteny score of 2).

oviposition and dispersal, it is surprising that in many normally flight-capable insect groups, certain species have lost the ability to fly (e.g., Lepidoptera, Coleoptera, Hemiptera, Diptera, Orthoptera; reviewed by Roff 1994; Roff and Fairbairn 1991). The evolution of flightlessness in both insects and birds has attracted considerable attention (Darlington 1943; Roff 1986, 1990, 1994; Wagner and Lieberr 1992), yet few studies have attempted to explain why sex-specific loss of flight has evolved in particular groups. In Coleoptera, highly neotenic, flightless females have independently evolved in several lineages within the Series Elateriformia (Bocakova et al. 2007). This is a major beetle group consisting of ~40 families, including fireflies (Lampyridae), click beetles (Elateridae), net-winged beetles (Lycidae), soldier beetles (Cantharidae), and glowworm beetles (Phengodidae). Sexual dimorphism in the form of neotenic females is especially prominent within the Lampyridae (Fig. 1), where it is limited to particular species rather than being characteristic of large clades (McDermott 1964; Cicero 1988, 2008; Branham and Wenzel 2003; Jeng 2008). Jeng (2008) modified Cicero's (1988) system for classifying neotenic traits, and noted that high levels of neoteny involve strong reduction or absence of wings, which results in loss of flight ability.

Male nuptial gifts constitute a potentially important link between male and female energy budgets that could greatly influence resource allocation strategies within and between the sexes (Boggs 1990, 1995, 2009). Nuptial gifts are defined here as substances (principally nongametic) that are transferred from males to females during courtship or mating; this definition makes no assumptions concerning effects on female fitness, which can be either beneficial or detrimental. Such gifts encompass both oral nuptial gifts (absorbed through the digestive tract) and seminal

nuptial gifts (absorbed through the reproductive tract; see Boggs 1995; Vahed 1998; Gwynne 2008 for reviews). Spermatophores are sperm-containing packages (Davey 1960; Mann 1984) that constitute a major category of seminal nuptial gift (Thornhill 1976; Parker and Simmons 1989; Cordero 1996; Vahed 1998, 2007; Arnqvist and Nilsson 2000; Gwynne 2008; South and Lewis 2010). Spermatophores are manufactured by several distinct male accessory glands (Leopold 1976; Chen 1984; Happ 1984; Mann 1984), and in some cases spermatophores can account for between 10–33% of male body weight (Mann 1984; Rutowski et al. 1983; Svard and Wiklund 1989; Karlsson 1995). Thus, spermatophore production represents a costly investment for males in terms of allocation to reproductive tissue and manufacturing costs (Simmons 1990; Oberhauser 1988, 1992; Wedell 1994; Hughes et al. 2000; Wedell and Ritchie 2004; Ferkau and Fischer 2006; Vahed 1998).

Although nuptial gifts are widespread among insects, remarkably little is known about patterns of nuptial gift evolution for most taxa. One notable exception is a phylogenetic analysis of crickets and katydids (Orthoptera: Ensifera) conducted by Gwynne (1997) and based on morphological traits, which suggested that spermatophores were ancestral in this group. Several mechanisms have been proposed that favor the origin and selective maintenance of costly seminal gifts (Parker and Simmons 1989; Vahed 1998; Simmons 2001; Arnqvist and Nilsson 2000; Vahed 2007; Gwynne 2008). First and foremost, such gifts will only be selected and maintained when they provide a net fitness benefit to males by increasing the number of offspring that are sired by the gifting male. In numerous taxa, seminal nuptial gifts have also been demonstrated to provide net fitness benefits to females by increasing their lifetime fecundity (reviewed by Boggs 1995; Vahed 1998; Simmons 2001; Gwynne 2008; South and Lewis 2010). It has also been suggested that sexual conflict may be involved in the elaboration of male ejaculates into spermatophores, as these male seminal gifts may provide a way for males to manipulate female reproduction (Parker and Simmons 1989; Arnqvist and Nilsson 2000; Arnqvist and Rowe 2005). Among capital breeders, male nuptial gifts are particularly likely to be co-adapted with female reproductive activities (Boggs 1995, 2009). This is because capital breeders have limited adult food intake (Stearns 1992), so females must rely on stored reserves to support their reproductive activities. Thus, any additional input from nuptial gifts might represent important resources that can be used for reproduction.

Because of well-established physiological trade-offs between flight and reproduction (Zera and Denno 1997; Harshman and Zera 2007), exploring the relationships between female flightlessness and reproductive traits of both sexes can potentially provide new insights into life-history evolution. Lewis and Cratsley (2008) predicted that female flightlessness would be associated

with an absence of male nuptial gifts based on differences in the proportional increase in female fecundity from male gifts between flight-capable versus flightless females. In species where females are capable of flight, female reproductive allocation is assumed to be limited by biomechanical constraints and by trade-offs with the structural and energy investments that are required for flight. In such species, positive selection is predicted for costly male gifts that can increase male fitness by making a large proportional contribution toward increasing female fecundity. In contrast, flightless females can maximize their allocation toward reproduction, as evidenced by their greater fecundity in comparison to flight-capable females (Roff 1986, 1990; Roff and Fairbairn 1991; Harshman and Zera 2007; Nespolo et al. 2008). Higher female fecundity would be expected to limit the proportional gain due to male gifts, raising the cost/benefit ratio of such gifts for males. Lewis and Cratsley (2008) thus predicted that in species with flightless females, selection would not favor the production of costly gifts. However, this predicted negative association between male nuptial gifts and female flightlessness has yet to be rigorously examined within a phylogenetic context.

Many species of fireflies are capital breeders; larvae are predaceous whereas adults of most species have degenerated mouthparts and do not feed (Williams 1917; Lloyd 1997). Males of many species possess multiple reproductive accessory glands that are used to manufacture elaborate spermatophores (van der Reijden et al. 1997; Lewis et al. 2004; South et al. 2008). Spermatophore production is costly to males, as *Photinus ignitus* spermatophore size and male mating rate decline over time (Cratsley et al. 2003). After they are transferred to females during mating, male spermatophores are stored within a specialized sac within the female reproductive tract where they are subsequently digested (van der Reijden et al. 1997). In the two firefly taxa that have been studied to date, radio-labeled amino acids derived from male spermatophores become incorporated into female oocytes (Rooney and Lewis 1999), and females that receive multiple spermatophores show significantly increased lifetime fecundity (Rooney and Lewis 2002). Therefore, existing evidence suggests that firefly spermatophores represent costly seminal gifts that positively affect female net fitness. Furthermore, previous work on fireflies supports an association between spermatophore production and female flight. One North American firefly (Wing 1989; Demary and Lewis 2007) and several Japanese species (Hayashi and Suzuki 2003) show reduced development of male reproductive glands, and females in these taxa are highly neotenic (either wingless or short-winged) and thus are incapable of flight. Finally, lampyrid species with highly neotenic, flightless females have both a greater complement of eggs and larger egg size compared to species with normal winged females (J.Z. Ho, pers. comm.).

Also potentially related to the life-history traits of female neoteny and male spermatophore production are the diverse signaling modes used during courtship in different firefly species. Although fireflies are renowned for their spectacular bioluminescent courtship flashes, many species within this family instead attract mates using female-produced pheromones or slowly modulated glows produced by either sex (reviewed by Lloyd 1997; Ohba 2004; Lewis and Cratsley 2008; Lewis 2009). Based on recent phylogenetic analyses (Branham and Wenzel 2003; Stanger-Hall et al. 2007) there seems to be a correlation between female flightlessness and the use of glows as a sexual signal, but this remains to be tested in a rigorous evolutionary trait analysis. Additionally, the potential association between sexual signal modes and neoteny remains to be investigated.

Fireflies are ideal for testing these evolutionary predictions based on the extensive occurrence of female neoteny in this group, as well as considerable previous work that has elucidated many aspects of their reproductive biology and sexual signaling systems. In this study, we used 32 species of fireflies from 24 genera to examine the evolutionary relationships among several key life-history features. We measured different levels of female neoteny and the number of male reproductive accessory glands as multistate characters, female flightlessness (associated with higher levels of neoteny) and male spermatophore production (presence/absence) as binary characters, and sexual signal mode as a multistate character. Using a recent molecular phylogeny of worldwide firefly species (Stanger-Hall et al. 2007), we conducted ancestral character state reconstruction as well as character correlation and independent contrast analyses on these traits to test the following specific predictions: (1) Male spermatophore production, nonneotenic females, and flight-capable females represent the ancestral lampyrid states. (2) Increasing degrees of female neoteny are correlated with decreasing numbers of male accessory glands. (3) Male spermatophore production is negatively correlated with female flightlessness. (4) The use of glows as a sexual signal is positively correlated with female flightlessness and increased levels of neoteny. We also examined the historical sequence of trait evolution using transition probability analysis for correlated binary traits to test whether female flight or male spermatophore production was lost first. Through this study, we hope to provide insight into previously unexplained patterns of female neoteny and male spermatophore production that might be broadly applicable across diverse taxa.

Materials and Methods

STUDY TAXA

Lampyrid species were chosen to provide a representative range of neotenic states for this analysis. Specimens of the following taxa (with collection location) were examined: *Lampyrus*

noctiluca, *Phosphaenus hemipterus*, and *Lamprohiza splendidula* (Belgium); *Diaphanes nubilis* (Taiwan); *Pyropyga nigricans* (Ohio, USA); *Pleotomus pallens* and *Microphotus angustus* (Texas, USA); *Phausis reticulata* (Tennessee, USA); *Ellychnia corrusca*, *Lucidota atra*, *Photuris versicolor*, *Pyractomena angulata* (Massachusetts, USA); *Photinus pyralis* (New Jersey USA); *Luciola cruciata* and *L. lateralis* (Japan). Information on the remaining species was obtained from Hayashi and Suzuki (2003) and F. Hayashi (pers. comm.).

CHARACTERIZING MALE REPRODUCTIVE TRAITS

Data on male reproductive anatomy and spermatophore production were obtained by dissection for 14 lampyrid species, with information for 18 additional species obtained from a study of Japanese fireflies by Hayashi and Suzuki (2003). Several lines of evidence were used to determine whether males produced spermatophores or instead transferred ejaculates containing free sperm. Mating experiments were conducted with live beetles for seven species (*P. pyralis*, *E. corrusca*, *P. angulata*, *P. versicolor*, *L. cruciata*, *L. lateralis*, and *P. reticulata*). Prior to mating, males were fed a solution of 40% sucrose and 1% 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; South et al. 2008), allowing us to visualize structures within the male reproductive tract responsible for producing spermatophore precursors. By dissecting pairs after mating, we were able to locate male spermatophores after they had been transferred to females (Supporting information). For those species where we only had access to preserved specimens, spermatophore production was inferred based on anatomical evidence, following Hayashi and Suzuki (2003). Previous work in several firefly species has established that spermatophore production is associated with multiple male accessory glands (van der Reijden et al. 1997; Lewis et al. 2004; South et al. 2008); fireflies that lack spermatophores show accessory glands that are reduced in both their number and size (Wing 1985; Demary and Lewis 2007). In addition, spermatophore production is associated with specialized female structures that receive the spermatophore internally (van der Reijden et al. 1997; Lewis et al. 2004; South et al. 2008). Thus, the anatomical evidence we used to infer spermatophore production was whether a species showed: (1) males with two or more pairs of reproductive accessory glands and (2) female reproductive tracts with spermatophore-receiving structures (in addition to a spermatheca for sperm storage). A single species (*M. angustus*) represented a borderline case with reduced male accessory glands, and female specimens were unavailable. For this species, we measured relative allocation to male reproductive accessory glands based on the percentage of total body dry mass following established methods (Demary and Lewis 2007). Because previous work has demon-

strated that male reproductive allocation was less than 0.1% in firefly species that lack spermatophores (Demary and Lewis 2007), any values below this level were assumed to indicate absence of spermatophores.

All dissections were conducted in 1× phosphate buffered saline using a Nikon SMZ1500 stereomicroscope equipped with an X-Cite 120 fluorescence illuminator (Nikon Instruments, Melville, NY), and photographed with an Insight 4 Mega-pixel Color Mosaic camera (Diagnostic Instruments, Michigan). In addition to spermatophore presence/absence, the number of male accessory glands present (this varied from 0 to 5 pairs) was used in this analysis.

CHARACTERIZING NEOTENIC TRAITS AND FLIGHT ABILITY

The level of female neoteny shown by each species was based on retention of larval characters in adult females, using a modification of the classification scheme detailed in Jeng (2008), which in turn was based on Cicero (1988). The specific traits used to assess neoteny were: reduction (or loss) of hindwings and elytra, physogastrous abdomen, incomplete retention of adult pigmentation, unsclerotized integument, modification of abdominal and thoracic sclerites, modification of head and legs, and the presence of larval characters (pygopodium or tarsunguli). We assigned female neoteny scores ranging from 0 (no neotenic traits present) to 4 (highly neotenic) for analyses of continuous traits, based on Jeng (2008) (Fig. 1). Females that showed strong reduction or absence of hindwings and elytra (neoteny scores ≥ 2) were scored as flightless for analyses requiring discrete binary characters; females with lower neoteny scores were considered capable of flight. The only exception was *P. nigricans* (neoteny score = 1), a species that exhibits substantial interpopulation variation in wing length (Lloyd 1999) but shows no other neotenic traits. The specimens we examined were from a population with females that were brachypterous (reduced wings) and were incapable of flight.

CHARACTERIZING SEXUAL SIGNAL MODE

We scored sexual signal modes for extant taxa following Stanger-Hall et al. (2007) as follows: dark (nonluminescent), weak glows, strong glows, and discrete flashes (*Pyrocoelia discicollis* was scored as producing a weak glow based on Ohba 2004). In some species, sexual signal modes differ between the sexes (e.g., females glow but their males do not); in such cases we used female signal modes. Which sex is the primary signaler (initiates the male–female interaction) changes with different signal modes (Lloyd 1979; Branham and Wenzel 2003; Ohba 2004; Lewis 2009). In fireflies classified here as dark, females are the primary signalers and are either known (Lloyd 1972, 1999; De Cock and Matthysen 2005; Ohba 2004) or assumed to attract males by releasing pheromones. Females also tend to be the primary

signalers in fireflies that use glows to attract mates. In contrast, in fireflies that use discrete flashes as sexual signals, males are generally the primary signaler.

TAXON SAMPLING AND DNA SEQUENCE DATA

Phylogenetic hypotheses concerning species relationships were based on previously published Bayesian and maximum likelihood analyses using 18S, 16S, and *cox1* DNA sequences (Stanger-Hall et al. 2007). For the present analysis, this tree was pruned to yield a reduced phylogeny that included only the 32 taxa in this study (Table 1), and all traits were reconstructed on this reduced phylogeny. *Diaphanes formosus* used in the original phylogeny was not available, so we examined male reproductive traits for *D. nubilis*, a congener with an identical female neoteny score (Jeng 2008).

ANCESTRAL STATE RECONSTRUCTION

Maximum likelihood (Mk1 model: all changes equally probable, as implemented in Mesquite version 2.6: Maddison and Maddison 2009) was used to reconstruct ancestral character states and to plot the history of state changes onto the phylogeny. Each character was reconstructed separately using the original branch lengths. Results are presented (without branch length information) as “ball and stick” tree diagrams (Schluter et al. 1997), with the proportional likelihoods for each node (summing to 1.0) represented as a pie diagram.

CORRELATED TRAIT EVOLUTION

For male spermatophore production and female flight ability, states were coded as binary characters (present or absent). Number of male accessory glands (six states), female neoteny (five states), and sexual signal mode (four states) were coded as multistate characters.

To test for correlated evolution between the two binary traits, we used Pagel’s (1994) correlation analysis as implemented in Mesquite version 2.6 (Maddison and Maddison 2009). This analysis uses maximum likelihood to test whether the evolution (rate of change) of two binary (0,1) characters is independent (four-parameter model) or dependent (eight-parameter model). The observed likelihood ratios were tested for significance by running Monte Carlo tests using simulated data (100 iterations for 1,000 simulations). The likelihood ratio and the associated *P*-value are reported.

We treated the three multistate characters (four to six states) as continuous traits and tested for trait correlations using independent contrast analysis (Felsenstein 1985, as implemented in Mesquite version 2.6 and the PDTREE module version 1.14: Midford et al. 2008). This method requires verification that branch lengths are statistically adequate (Garland 1992; Midford et al. 2008). More specifically, branch lengths should be proportional to the expected variance for the character under study (Garland

et al. 2005). As suggested by Midford et al. (2008), we used different transformation methods and diagnostic checks to verify the adequacy of transformations for our data.

Diagnostics supported the use of Pagel’s (1992: contemporaneous tips and internode segments set equal) and Grafen’s (1989: contemporaneous tips, and each node set equal to one less than the number of tip species that descended from it) arbitrary branch length transformations. Both of these transformations produced statistically adequate branch lengths for all traits; we report the results based on Pagel’s (1992) transformation here (Grafen’s arbitrary branch lengths gave similar results).

We used a least-squares regression of positivized contrasts (PDTREE 1.14 module in Mesquite version 2.6) to test for significant correlation between two traits of interest (Garland 1992). To account for a soft polytomy (low resolution due to lack of data for *Cyphonocercus*, *Stenocladus*, and *Rhagophthalmus* and low Bayes support) in our phylogeny, the degrees of freedom (df) for the independent contrast analysis were reduced by 1 (Purvis and Garland 1993). The Pearson product-moment correlation coefficient *r* (computed through the origin) and its associated *P* value are reported.

EVOLUTIONARY SEQUENCE OF CHARACTER STATE TRANSITIONS

We used BayesDiscrete (www.evolution.rdg.ac.uk, Pagel and Meade 2006) to test the directionality of evolutionary trait transitions for two correlated binary traits: Female flight and male spermatophores. The model of correlated trait evolution can adopt four states, one for each state combination of the two binary traits (0,0; 0,1; 1,0; 1,1). In this model, the parameters are the eight possible transition rates q_{ij} (i = start state, j = end state) between these four states.

To account for phylogenetic uncertainty and uncertainty about the model of trait evolution we generated variants for our 32-taxon phylogenetic tree by running a reversible jump Markov chain Monte Carlo (RJ MCMC) analysis for 5,050,000 iterations with a burn-in of 50,000, a hyperprior distribution (exponential prior with a mean of 0.0 and ranging to 30), and a rate deviation of 10.0 (Pagel and Meade 2006). By sampling the chain every 100th generation, we created a posterior distribution of trait combinations with 50,000 sample points. We used this sample to determine the mean rate coefficients for all eight possible transitions (Fig. 6). For each rate parameter (q_{ij}) we calculated how often (% of sample) the rate coefficients were estimated to be zero (*Z*). We present *q* values (*q* values further away from zero represent more probable evolutionary transitions) and *Z* values (low *Z* values indicate more likely evolutionary transitions). Following Fitzpatrick et al. (2009), we considered transitions likely when $Z < 0.10$ (less than 10% of the iterations were assigned to zero), or when the transition parameter (*q* value) of a transition

Table 1. Trait values for the 32 lampyrid species used in this study.

Species	Female neoteny level	Female flight ability	No. of male accessory glands	Male spermatophore	Sexual signaling mode	References
<i>Photuris versicolor</i>	0	Yes	3	Yes	3—Flash	2,4,5
<i>Pleotomus pallens</i>	2	No	3	Yes	2—Glow	2,4,5
<i>Lampyris noctiluca</i>	2	No	2	Yes	2—Glow	2,4,5
<i>Pyrocoelia discicolis</i>	2	No	1	No	1—Pheromone/Glows	1,3,4,5
<i>Pyrocoelia fumosa</i>	2	No	1	No	1—Pheromone/Glows	1,3,4,5
<i>Pyrocoelia m. matsumurai</i>	2	No	2	No	1—Pheromone/Glows	1,3,4,5
<i>Diaphanes formosus</i>	2	No	1	No	2—Glow	2,4,5
<i>Pyrocoelia rufa</i>	2	No	1	No	2—Glow	1,3,4,5
<i>Pyrocoelia miyako</i>	2	No	0	No	2—Glow	1,3,4,5
<i>Pyrocoelia atripennis</i>	2	No	1	No	2—Glow	1,3,4,5
<i>Microphotus angustus</i>	3	No	1	No	2—Glow	2,4,5
<i>Ellychnia corrusca</i>	0	Yes	4	Yes	0—Dark	2,4,5
<i>Photinus pyralis</i>	0	Yes	4	Yes	3—Flash	2,4,5
<i>Pyropyga nigricans</i>	1	No	5	Yes	0—Dark	2,4,5
<i>Pyractomena angulata</i>	0	Yes	3	Yes	3—Flash	2,4,5
<i>Lucidota atra</i>	0	Yes	5	Yes	0—Dark	2,3,5
<i>Lucidina biplagiata</i>	0	Yes	3	Yes	0—Dark	1,3,4,5
<i>Lamprohiza splendidula</i>	2	No	2	No	2—Glow	2,4,5
<i>Phosphaneus hemipterus</i>	2	No	3	Yes	0—Dark	2,4,5
<i>Phausis reticulata</i>	3	No	2	No	2—Glow	2,4,5
<i>Cyphonocerus ruficollis</i>	0	Yes	3	Yes	1—Pheromone/Glows	1,3,4,5
<i>Stenocladus shiraki</i>	4	No	1	No	0—Dark	1,3,4,5
<i>Rhagophthalmus ohbai</i>	4	No	1	No	2—Glow	1,3,4,5
<i>Hotaria parvula</i>	1	Yes	3	Yes	3—Flash	1,3,4,5
<i>Luciola yayeyamana</i>	1	Yes	3	Yes	3—Flash	1,3,4,5
<i>Luciola cruciata</i>	0	Yes	3	Yes	3—Flash	2,4,5
<i>Luciola lateralis</i>	0	Yes	3	Yes	3—Flash	2,4,5
<i>Luciola kuroiwae</i>	0	Yes	3	Yes	3—Flash	1,3,4,5
<i>Curtos okinawana</i>	0	Yes	3	Yes	3—Flash	1,3,4,5
<i>Curtos costipennis</i>	0	Yes	3	Yes	3—Flash	1,3,4,5
<i>Pristolycus sagulatus</i>	0	Yes	2	Yes	0—Dark	1,3,4,5
<i>Drilaster axillaris</i>	0	Yes	3	Yes	0—Dark	1,3,4,5

References: ¹Hayashi and Suzuki (2003), ²This study, ³F. Hayashi (pers. comm.), ⁴Jeng (2008), ⁵Stanger-Hall et al. (2007).

with $Z > 0.10$ was higher than that of the lowest parameter (q) that was associated with a significant ($Z < 0.10$) transition.

Results

TRAIT ANALYSIS

Among the 32 firefly species included in our analysis, 18 species exhibited at least some degree of female neoteny, and females in 16 species lacked flight ability (Table 1). Males in 23 species had at least two pairs of reproductive accessory glands, and males in 20 of these species produced spermatophores. Most species with male spermatophores also showed low female neoteny; 17 of these species had neoteny scores of 0 or 1, whereas the remaining three species (*P. pallens*, *L. noctiluca*, and *P. hemipterus*) had

neoteny scores of 2. In all 12 of the lampyrid species lacking male spermatophores, the corresponding females were flightless (Table 1).

RECONSTRUCTING PATTERNS OF TRAIT EVOLUTION

Female neoteny was found to be a derived trait (Fig. 2). Ancestral state reconstruction revealed that the common ancestor had nonneotenic females (Fig. 2, proportional likelihood for neoteny score of 0 = 0.99) and thus these females were capable of flight (Fig. 3, proportional likelihood = 0.94). From this nonneotenic ancestor, there were at least seven independent increases (and no reductions) in female neoteny (Fig. 2), associated with at least five independent losses of female flight capability (Fig. 3).

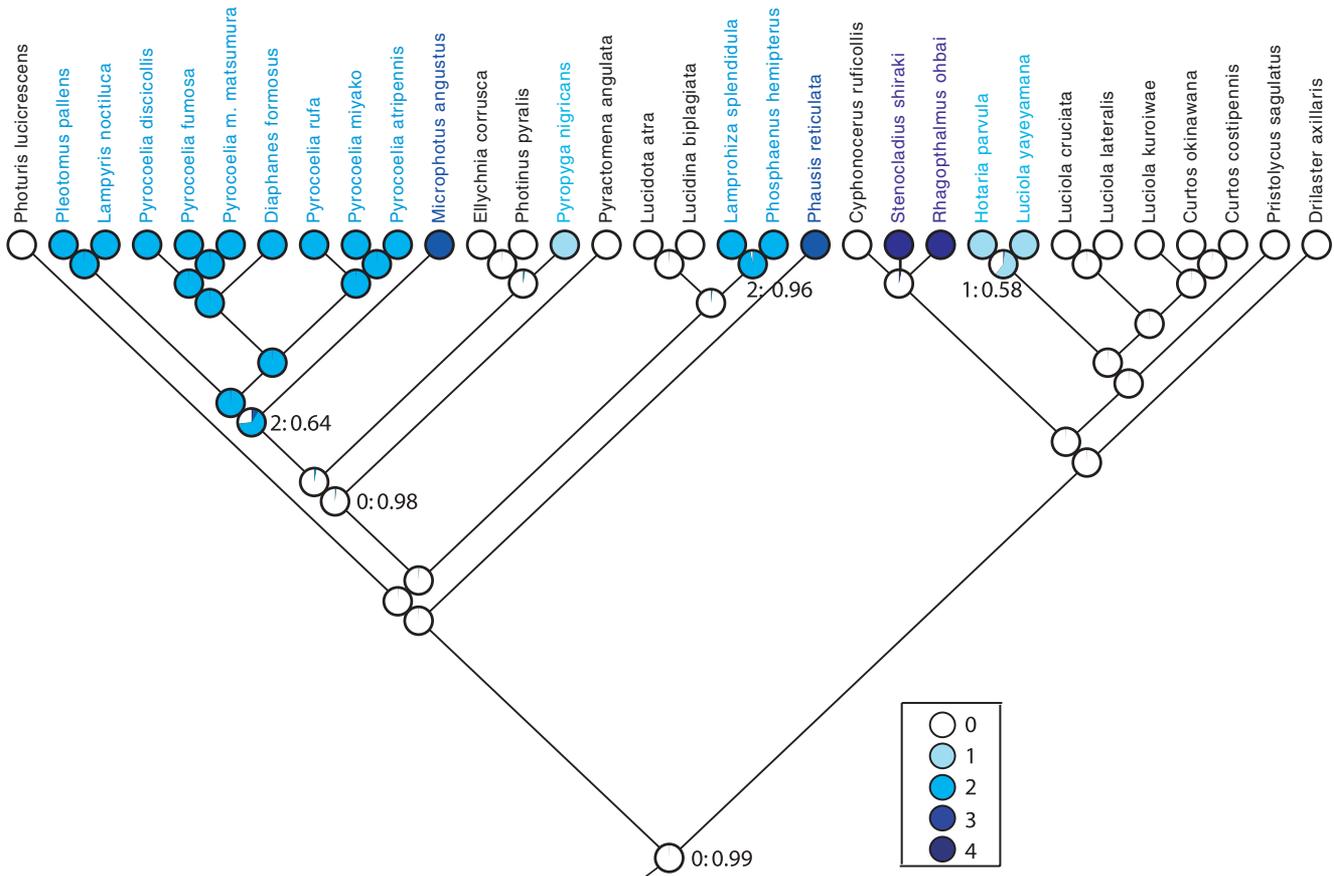


Figure 2. Maximum likelihood reconstruction of female neoteny for 32 firefly species. The pie charts indicate the ML support (proportional likelihood) for the ancestral state at each node, with values given at transition nodes.

Maximum likelihood reconstructions revealed that male spermatophore production was the ancestral state (Fig. 3, proportional likelihood = 0.99). Loss of spermatophore production occurred subsequently in at least four lineages, with no reversions. Ancestral lampyrid males most likely used three paired reproductive accessory glands to produce spermatophores (Fig. 4, proportional likelihood = 0.98). In our taxon sample the number of male accessory glands independently increased twice (to four or five pairs), and also decreased four or five times (to two or one pairs). In addition, there were two independent reversals where male accessory gland number increased from a single pair to either two or three pairs. In the case of *Pyrocoelia m. matsumurai*, such a reversal led to males having two pairs of accessory glands and losing spermatophore production (Table 1, Figs. 3 and 4). The other reversal occurred in the common ancestor of *P. pallens* and *L. noctiluca*; males in both extant species produce spermatophores, the former with three gland pairs and the latter with two gland pairs. Thus, with the exception of *L. noctiluca*, all taxa in which males transfer spermatophores show ≥ 3 pairs of accessory glands (Table 1, Figs. 3 and 4).

Analysis of this taxon sample indicated that light was not used for sexual communication in the lampyrid ancestor (Fig. 5;

see Stanger-Hall et al. 2007 for more extensive taxon sampling); instead, pheromones were most likely used for mate attraction (proportional likelihood of dark signaling mode = 0.76). From this ancestral state, sexual communication involving strong bioluminescent glows evolved four times: three times from dark ancestors and once from an ancestor that used bioluminescent flashes. Weak bioluminescent glows evolved twice, once as a reversal from strong glows (*P. discicollis* group), and once from a dark ancestor (*Cyphonocerus ruficollis*; see also Stanger-Hall et al. 2007).

CORRELATED TRAIT EVOLUTION

Ancestral state reconstruction for female flight ability (proportional likelihood = 0.94) and spermatophore production (proportional likelihood = 0.99) showed remarkable congruence (Fig. 3), and our analysis showed strong support for correlated evolution between the loss of male spermatophores and female flightlessness (Pagel’s test of correlated evolution; likelihood ratio = 8.21, $P < 0.001$). Similar results were obtained from the independent contrast analysis examining correlated evolution between female neoteny level (Fig. 2) and male accessory gland number (Fig. 4, $r = -0.6146$, $F_{1,29} = 18.21$, $P < 0.001$).

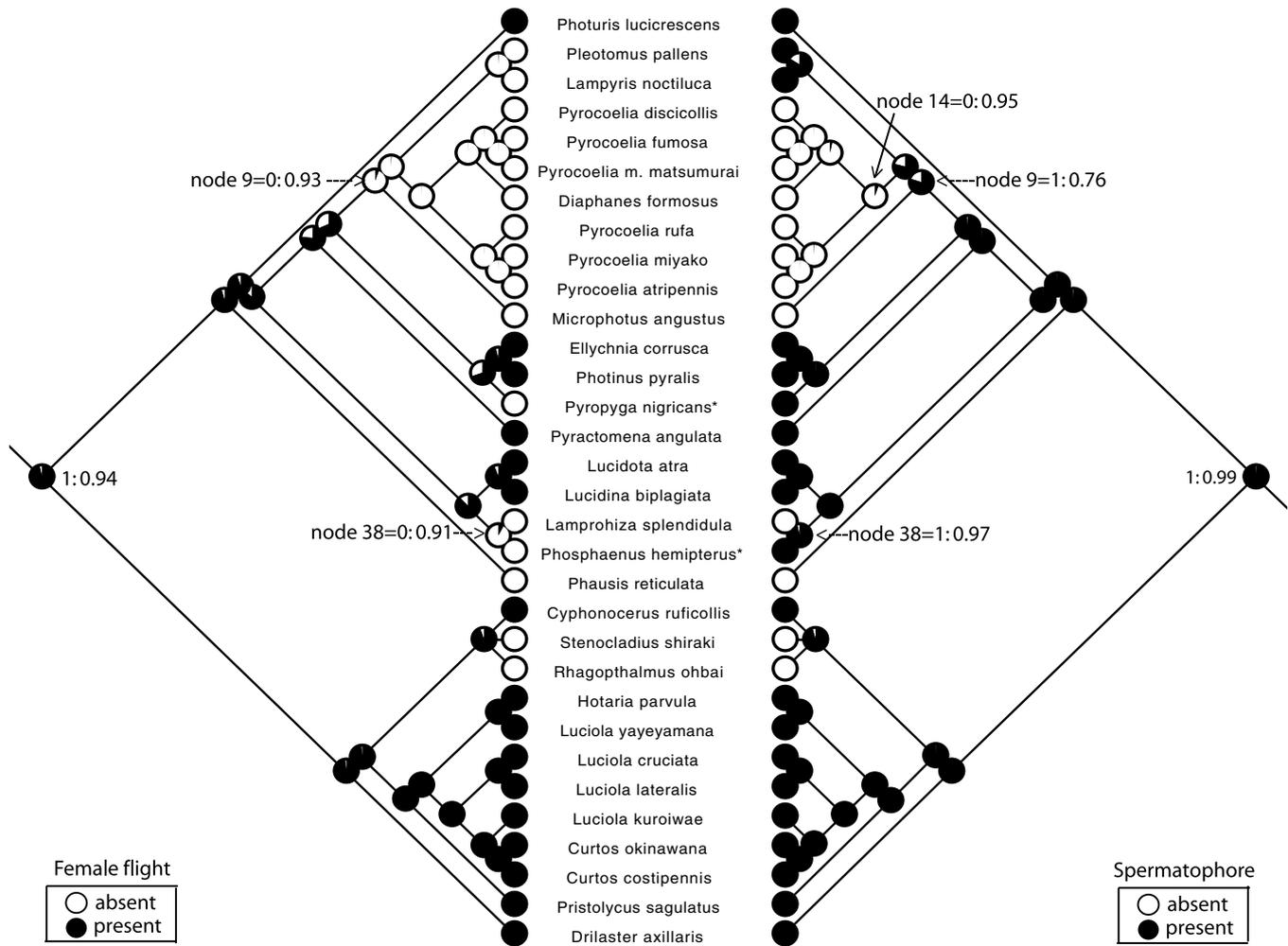


Figure 3. Maximum likelihood reconstructions of female flightlessness (left) and spermatophore production (right). The pie graphs indicate the ML support (proportional likelihood) for the ancestral states at each node, with values given at transition nodes. Numbered nodes with arrows are discussed in the text.

In contrast, tests of correlated evolution showed no significant association between female neoteny level and sexual signaling mode (Independent contrasts: $r = 0.016$, $F_{1,29} = 0.008$, $P = 0.928$). However, as evident in Figure 5, there was a highly significant positive correlation between female flightlessness and use of female glows as primary sexual signal (Pagel's test of correlated evolution; likelihood ratio = 4.0837, $P < 0.0001$). For example, the transition to female flightlessness that occurred in the common ancestor of *Microphotus*, *Pyrocoelia*, and *Diaphanes* (node 9 in Fig. 3) also represents a shift to glowing females from flashed light signals (Fig. 5, proportional likelihood = 0.94). Similarly coinciding with the loss of female flight, *Rhagophthalmus*, *Phausis*, and *Lamprohiza* each independently evolved strong female glows as sexual signals; in these cases such glows evolved from dark ancestors. Interestingly, although flightless *Lamprohiza* females attract males with a strong glow, flightless females in their sister taxon *Phosphaenus* use pheromones rather than light as sexual signals (De Cock and Matthyssen 2005; De Cock 2009).

Bioluminescent signals are also absent in flightless *Pyropyga* and *Stenocladus* females. *Cyphonocerus* is the only taxon in our analysis that retained nonneotenic females with full flight ability and weak glows.

RECONSTRUCTING HISTORICAL SEQUENCES OF TRAIT EVOLUTION

The evolution of female neoteny with a concomitant loss of flight ability occurred at least five times in our taxon sample, as did the loss of male spermatophores (Figs. 2 and 3). Based on the maximum likelihood values of ancestral nodes it appears that female neoteny and neoteny-induced flightlessness preceded spermatophore loss in at least three instances. For example, in the lineage leading to *Microphotus*, *Diaphanes*, and *Pyrocoelia* fireflies, the change from nonneotenic females to neoteny level 2 occurred somewhere between nodes 8 and 9 (Fig. 2: node 9, proportional likelihood of neoteny level 2 = 0.64); because flight is based on neoteny level, flight was also lost

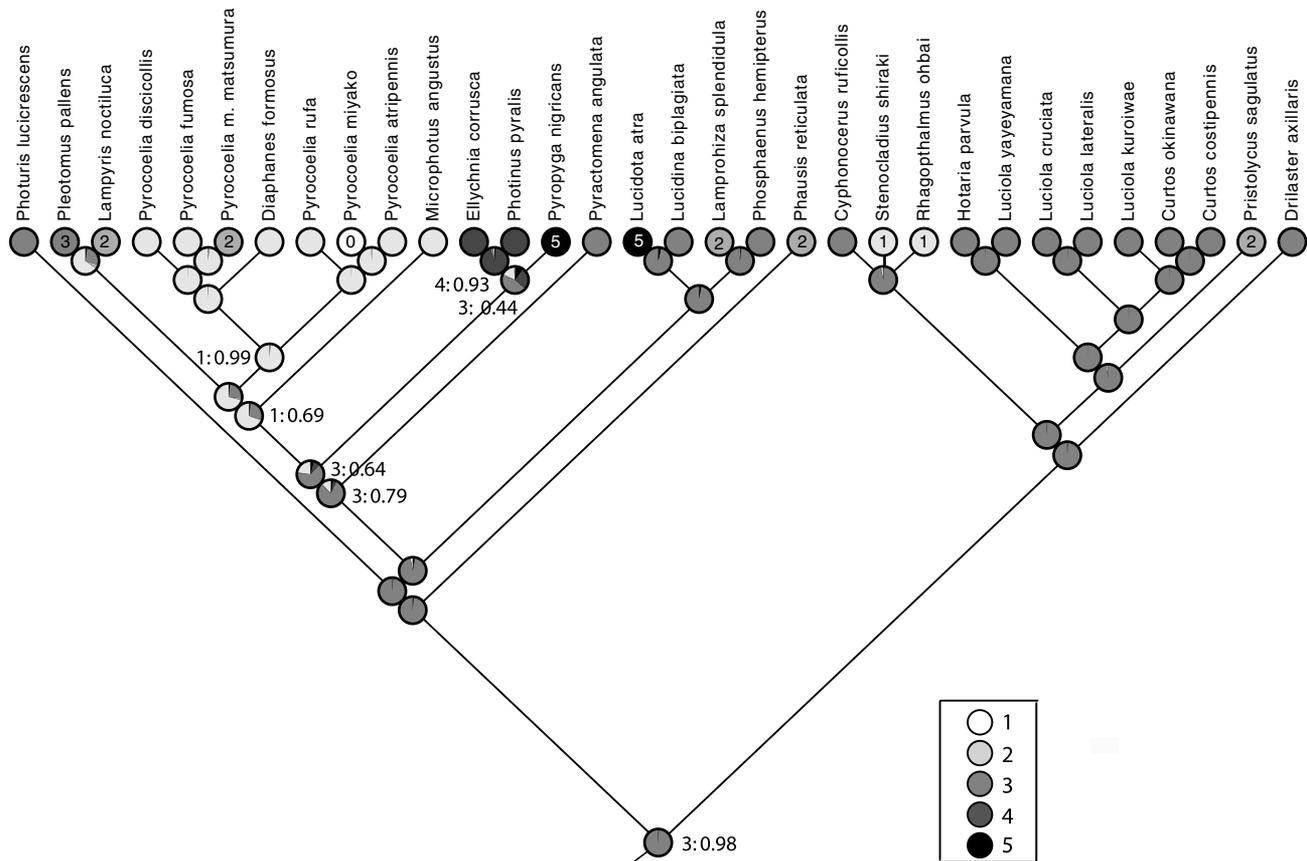


Figure 4. Maximum likelihood reconstruction of the number of male accessory glands for 32 firefly species. The pie graphs indicate the ML support (proportional likelihood) for the ancestral state at each node, with values given at transition nodes.

at the same node (Fig. 3; at node 9, proportional likelihood of flightlessness = 0.93). However, the first putative ancestor to lack male spermatophores appeared later (Fig. 3: node 14, proportional likelihood of spermatophore absence = 0.94). Similarly, an increase in female neoteny and associated flight loss also occurred in the lineage leading to the common ancestor of *L. splendidula* and *P. hemipterus* (Fig. 2, node 38 in Fig. 3: proportional likelihood of female flightlessness = 0.091). This was followed in *L. splendidula* by a reduction in male accessory glands (Fig. 4) and spermatophore loss (Fig. 3), yet male accessory glands and spermatophores were retained in *P. hemipterus*. Females of *P. nigricans* are neotenic (Level 1) and flightless, but males of this flightless population still produce spermatophores (Fig. 3). Finally, *P. reticulata*, *S. shirakii*, and *R. ohbai* all show increased female neoteny, associated loss of flight, and absence of spermatophore production (Fig. 3) without indication of their evolutionary sequence. Thus, in three of four instances the evolution of female neoteny and associated flightlessness is supported at nodes basal to those where male spermatophore production was lost, indicating that spermatophore production ceased only after females started to become neotenic.

This conclusion is supported by transitional probability analysis (Fig. 6). The most likely evolutionary transition from the

ancestral state with female flight and male spermatophores was through an intermediate state of flightless females and male spermatophores ($Z = 0.023$); this was followed by the transition to loss of male spermatophores ($Z = 0.002$). In contrast, transition from the ancestral state through the alternate intermediate was considered extremely unlikely (Fig. 6, $Z > 0.90$). Finally, once both flight and spermatophores were lost, reversals to either intermediate were unlikely (Fig. 6, both $Z > 0.70$).

Discussion

Using phylogenetic methods and rigorous tests of correlated evolution, this study has revealed correlations among the life-history traits of female neoteny, neoteny-induced flightlessness, male spermatophores, and sexual signaling mode in fireflies. Although several hypotheses have been previously proposed concerning the separate evolution of each of these traits, this is the first study to examine the evolutionary associations among these life-history and reproductive traits. As predicted, we found strong evidence supporting correlated evolution between female neoteny, female flightlessness, and loss of male spermatophore production. Although there was no overall association between female neoteny

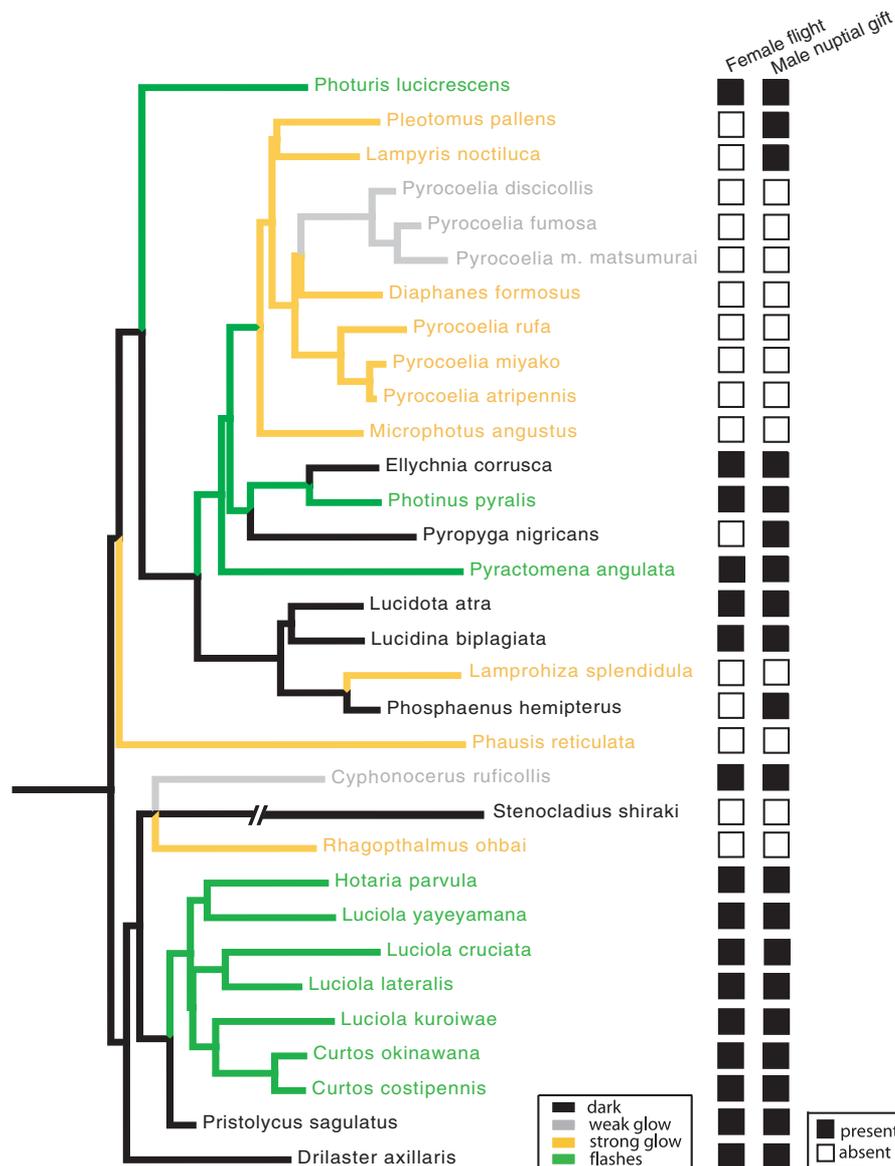


Figure 5. Phylogenetic relationships (from Stanger-Hall et al. 2007) among 32 firefly species indicating signaling mode used for sexual communication, and character matrix for female flight ability and male spermatophores.

and sexual signal mode, correlated trait analysis confirmed the predicted association between female flightlessness and females glowing as a sexual signal. By demonstrating correlated evolution between these sex-specific traits, these results provide novel insights into how selection acting on developmental and physiological traits in one sex can influence reproductive allocation in the other sex, and shed new light on life-history evolution within this beetle family.

Neoteny emerged as a key life-history feature in our analysis. Our results provide strong support for a lampyrid ancestor with nonneotenic females, followed by several independent origins of female neoteny. Furthermore, our results indicate that female neoteny increased monotonically within several lineages once it had arisen; there were no reductions in neoteny level.

These results are consistent with previous studies based on morphological phylogenies that also have supported multiple origins of neoteny (Jeng 2008) and flightlessness (Branham and Wenzel 2003) within this family. Recent molecular phylogenetic analyses of other beetle families within the Series Elateriformia also support multiple origins of neoteny (Bocakova et al. 2007; Bocak et al. 2008). Thus, considerable evidence now refutes an early argument for a single basal origin of neoteny in this group (Crowson 1972; former Cantharoidea now Elateroidea). Neoteny is associated with reduced mobility (Gould 1977), and neotenic lycid beetles show reduced geographic ranges compared to their sister taxa (Bocak et al. 2008). The repeated evolution of female-specific neoteny within the Lampyridae is likely to have been driven by selection for increased fecundity, based on the

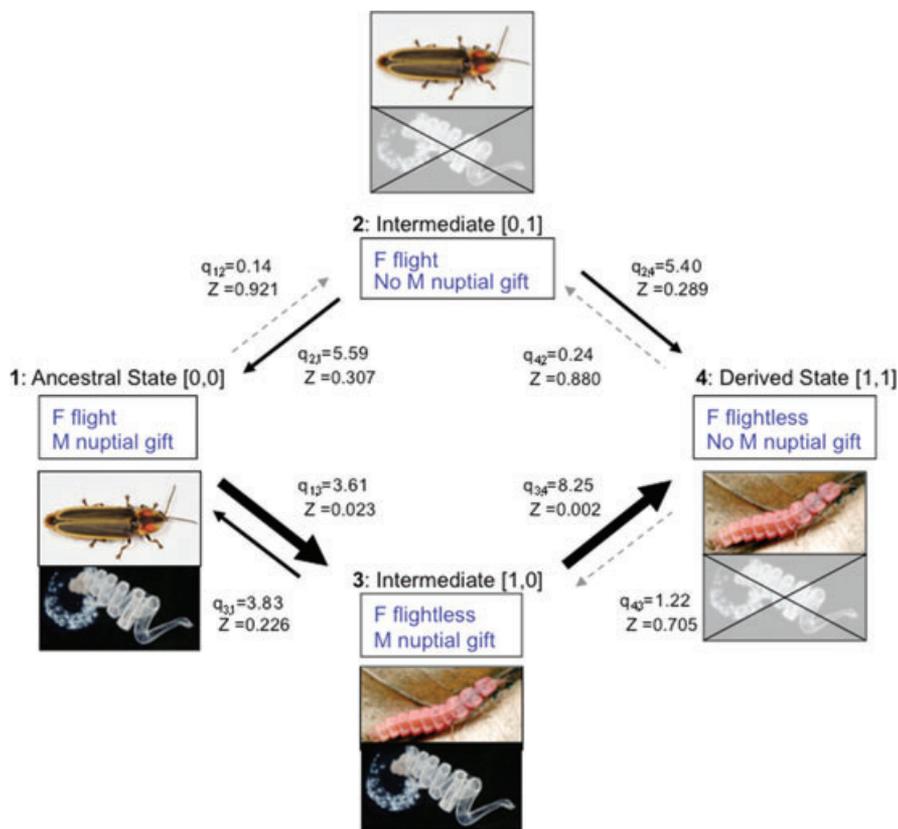


Figure 6. Possible evolutionary transitions between ancestral and derived states for female flight and male spermatophores. For each transition, we report both the q value (mean transition parameter between these two states) and the Z value (proportion of iterations that were assigned zero), each based on 50,000 iterations. q values farther from zero and Z values closer to zero represent more probable evolutionary transitions. Evolutionary transitions with highest probabilities ($Z < 0.10$, indicating that $< 10\%$ of the iterations from the RJ MCMC were assigned to zero) are shown with wide solid arrows. Transitions between $Z = 0.22$ and $Z = 0.31$ and q -values higher than the lowest q -value of a transition with $Z < 0.10$ were also considered likely (thin solid arrows). Transitions with $Z > 0.70$ and low q values were considered unlikely (dashed arrows).

reasonable assumption that neotenic females show higher allocation to reproduction. This assumption is supported by considerable evidence for physiological and allocation trade-offs between flight and reproduction (Roff and Fairbairn 1991; Boggs 2009). Reproductive benefits associated with flightlessness have been documented in many insect taxa, with greater wing muscle histolysis correlated with higher egg production (Roff 1986; Roff and Fairbairn 1991; Nespolo et al. 2008). In insect species that exhibit wing dimorphism, flightless morphs show higher fecundity than flight-capable morphs (Roff and Fairbairn 1991; Nespolo et al. 2008). Maas and Dorn (2003) described a single mosaic winged/wingless *L. noctiluca* female, and reported that the ovary on the wingless side had three times more oocytes than the winged side. Thus, fecundity selection provides a likely explanation for the multiple origins and monotonic increases in neoteny (this study; Bocak et al. 2008) that have occurred within fireflies and other related groups.

A major finding here is the evolutionary correlation between neoteny-induced female flightlessness and loss of spermatophore

production by males, which matched the predicted negative association between these traits (Hayashi and Suzuki 2003; Lewis and Cratsley 2008). We found strong support that spermatophores were present in the lampyrid ancestor, along with nonneotenic females. Within several lineages, when female neoteny increased, it was followed by a reduction in male reproductive glands and associated spermatophore loss. This result supports the idea that male spermatophore production is co-adapted with patterns of female reproductive allocation (Boggs 1990, 1995), at least for these capital breeders. One explanation for the loss of male spermatophores following the evolution of female neoteny is based on differences in the proportional fecundity increases that spermatophores might provide in species with flight-capable versus flightless females. Spermatophore production represents a costly investment for males in general (Parker and Simmons 1989; Simmons 1990; Oberhauser 1988, 1992; Wedell 1994; Hughes et al. 2000; Wedell and Ritchie 2004; Ferkau and Fischer 2006; Vahed 1998), and for firefly males in particular (Cratsley et al. 2003). Selection for spermatophores requires a net fitness

benefit for males (this may occur by increasing the proportion of offspring sired relative to other males and/or by enhancing the number of offspring produced by females). In two firefly species with flight-capable females, *E. corrusca* and *P. ignitus*, females receiving multiple spermatophores show lifetime fecundity increases of 73% and 41%, respectively (Rooney and Lewis 2002). Male spermatophores have also been shown to increase lifetime fecundity of females in many other insects (reviewed by Boggs 1995; Vahed 1998; Simmons 2001; Gwynne 2008; South and Lewis 2010), although to our knowledge no studies have been conducted on species with flightless females. Because flightless females should be able to maximize their allocation toward reproduction, they may have limited scope for any further increases in their lifetime fecundity. If the proportional fecundity gain from spermatophores is lower than their cost, this should lead to reduction and eventual loss of male spermatophore production (Boggs 1990; Lewis and Cratsley 2008).

An alternate explanation assumes that spermatophores provide a net fitness benefit to males by increasing their paternity share relative to other males mating with the same female (e.g., by reducing female remating probability or increasing a male's sperm competitive success). Male spermatophores might then be lost if transitions to female flightlessness were associated with a shift to monandrous mating systems, as intrasexual selection would be relaxed due to complete paternity assurance. However, what little is currently known concerning mating systems of neotenic fireflies fails to support this explanation: although neotenic *Photinus collustrans* females are monoandrous (Wing 1989), neotenic females in several *Pyrocoelia* species are polyandrous (A. South, unpubl. data; X. Fu, pers. comm.).

It is important to note that these explanations are not mutually exclusive, because the cost/benefit of male spermatophores is likely to change over evolutionary time (Parker and Simmons 1989; Simmons 2001). Distinguishing between these two explanations for the observed loss of male spermatophores subsequent to the evolution of female neoteny in fireflies will require further experiments across multiple taxa to document mating systems and to determine the effects of male spermatophores on female lifetime fecundity, particularly in taxa with highly neotenic females. Female neoteny might be part of a larger syndrome (sensu Agrawal 2007) involving many correlated reproductive traits. Such traits include not only mating systems, but also female reproductive schedules (i.e., semelparity vs. iteroparity) and egg maturation patterns (Jervis et al. 2005; Boggs 2009). For example, neotenic *P. collustrans* females are semelparous with most eggs fully mature at adult emergence (Wing 1989). Collecting this information for additional firefly species will greatly improve our understanding of such reproductive syndromes. It would be especially instructive to study the four extant taxa (*P. pal-lens*, *L. noctiluca*, *P. nigricans*, and *P. hemipterus*) where

spermatophores have been retained even though females are flightless.

In this study, we also found a significant correlation between female flightlessness and females producing bioluminescent glows to attract males; in 14 of 16 species with flightless females, females glow. It is possible that such glows are simply a consequence of neotenic female development, as all lampryid larvae are capable of bioluminescent glows. Alternatively, the glows exhibited by flightless (and thus vulnerable) adult females may have evolved as aposematic signals to warn potential predators of chemical defenses (Sagegami-Oba et al. 2007; Bocak et al. 2008).

Because increasing neoteny has led to the evolution of flightless females several times, it is worth considering what factors might release females from needing to fly. Environmental stability leading to habitat persistence is the classic explanation offered for the evolution of flightlessness (Darlington 1943; Roff 1986, 2002; Roff and Fairbairn 1991). This explanation is supported by recent work examining evolutionary patterns of flight loss in relation to food habit in Silphinae beetles (Ikeda et al. 2008). In this group, the evolution of flightlessness in both sexes was associated with a shift in food resources from vertebrate carcasses to soil invertebrates, which are spatially more predictable. In the case of female-specific neoteny, this explanation may be recast in terms of oviposition sites; flightless females with limited mobility require access to predictable habitat suitable for oviposition. Consistent with this explanation is Lloyd's (1999) observation that in the wing-polymorphic lampryid *P. nigricans*, those populations with brachypterous (short-winged) individuals occur in habitats with permanent moisture, implying more predictable access to oviposition sites. Further work is needed to examine the relationship between habitat stability and female-specific neoteny. Others have noted that flightlessness in insects appears to be associated with high altitudes (Bocak et al. 2008; Jeng 2008). For example, among the highly diverse Taiwanese lampryid fauna, female neoteny and flightlessness are found in many of the high altitude winter fireflies, which are active at low temperatures (Jeng 2008). This suggests neoteny may be a sex-specific adaptation to some physiological constraint, such as limits on metabolic rates, or ecological opportunity, associated with low temperatures. Because of their reduced mobility, neotenic females would seem especially vulnerable to predators, and it is possible that effective anti-predator chemical defenses may have facilitated the evolution of neoteny, as suggested by Bocak et al. (2008). Many lampryids are known to be distasteful to predators and defensive steroids have been identified from some species (see Lewis and Cratsley 2008 for review). Future studies exploring variation in firefly chemical defenses across species, sexes, and life stages might therefore provide additional insights into the evolution of neoteny.

By investigating correlations among multiple life-history traits in both sexes, we have gained a better understanding of how selective forces have shaped observed phenotypes. This study reveals an evolutionary relationship between female neoteny and loss of male spermatophore production in firefly beetles. Loss of female flight appears to have evolved multiple times within this group, followed by the loss of male spermatophores. To expand our understanding of this evolutionary process, further studies exploring additional life-history features such as mating system (monandry, polyandry) reproductive strategy (iteroparous, semelparous), relative allocation to reproductive structures, and chemical defense are needed. By helping to explain macroevolutionary patterns as well as to propose functional linkages among traits, such studies have the potential to yield new insights into sexual selection and life-history evolution in fireflies and other insects.

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Supporting Information

The following supporting information is available for this article:

Figure S1. Reproductive systems in three lampyrid firefly species, showing evidence used to assess spermatophore production.

Supporting Information may be found in the online version of this article.

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