SEMINAL INFLUENCES: THE ROLE OF NUPTIAL GIFTS IN SEQUENTIAL EPISODES OF SEXUAL SELECTION

A dissertation submitted by Adam South

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ABSTRACT

Sexual selection drives the evolution of diverse traits that can prove advantageous in reproduction. Because females often mate with multiple males, traditional premating processes such as female choice and male-male competition have counterparts both during and after mating in the form of cryptic female choice and sperm competition. Therefore, it is critical to expand our view of sexual selection. The goal of this research was to investigate how nuptial gifts in the form of spermatophores operate in sequential episodes of sexual selection in several species of beetle. Examining how nuptial gifts affect male and female fitness components can provide insight into male trait evolution, patterns of female choice and potential sexual conflict.

The impact of receiving relatively different quantities of genital nuptial gifts on female fitness parameters across many arthropod taxa was examined using a meta-analysis. In response to greater amounts of nuptial gifts, females demonstrate enhanced fecundity but reduced longevity. In two studies, spermatophore size in *Photinus* fireflies is manipulated to evaluate the effect of variation in nuptial gift size on female fitness parameters and male reproductive success. One of these studies documents a significant effect on female post-mating lifespan of receiving a single large spermatophore relative to a single small spermatophore, but no significant effect on female reproductive output. In the other study, firefly males with larger spermatophores experienced dual benefits in terms of both higher mate acceptance and increased paternity share. Additionally, rigourous phylogenetic methods are utilized to demonstrate a link

between female flight and male spermatophore production in worldwide firefly species, with loss of female flight leading to a loss of male spermatophore production. An evaluation of the use of close chemical contact cues, cuticular hydrocarbons, to mediate communication in diurnal and nocturnal fireflies was done with gas chromatography. This analysis revealed undetectable levels of these cues in nocturnal species. Finally, proteomics and mass spectrometry were used to identify the seminal fluid proteome of male spermatophores in *Tribolium castanuem*. The results of these studies contribute a number of novel and broadly applicable insights into the nature and evolution of nuptial gifts in animal species.

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Seminal Influences: The role of nuptial gifts in sequential episodes of sexual selection

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Seminal Influences: The Role of Nuptial Gifts in Sequential Episodes of Sexual Selection

Chapter I. Thesis Overview

The courtship of animals is by no means so simple and short an affair as might be thought.

-Charles Darwin
The Descent of Man and Selection in Relation to Sex (1871)

The outcomes and implements of sexual selection have simultaneously delighted and puzzled generations of biologists. From rams battling with thunderous strikes of their heads, to sperm cells 20 times longer than the male *Drosophila* that produces it, to the twinkling bioluminescent signals of fireflies, the mysteries of sexual selection surround us. Like many ideas in evolution, Charles Darwin has profoundly influenced our current collective thinking on sexual selection. He laid the foundation for sexual selection research in his 1871 seminal treatise on the topic "The Descent of Man and Selection in Relation to Sex." He captures the essence of his theory with a definition that is largely still in use today:

"We are, however, here concerned only with that kind of selection, which I have called sexual selection. This depends on the advantage which certain individuals have over other individuals of the same sex and species, in exclusive relation to reproduction."

Darwin took great pains to distinguish sexual selection from natural selection on multiple grounds: Firstly, that it results from competition between members of the same sex and choice between members of the opposite sex; and secondly, it depends on variation in reproductive success rather than survival.

"This form of selection depends, not on a struggle for existence in relation to other organic beings or to external conditions, but on a struggle between

the individuals of one sex, generally the males, for the possession of the other sex. The result is not death to the unsuccessful competitor but few or no offspring."

Darwin divided sexual selection into two distinct, although not necessarily mutually exclusive categories: intrasexual (selection favoring traits that would aid in direct competition among members of same sex for access to the opposite sex) and intersexual (selection favoring traits that bestowed an advantage in attracting members of the opposite sex). However, prescient as Darwin might have been, his conception of sexual selection is notable for what it did not include: an understanding that mechanisms of competition and choice can extend after mating has already occurred. Darwin recognized the importance of competition and choice in securing a mating, but he never knew the critical importance of subsequent processes in securing fertilization. Molecular methods of ascertaining paternity have revealed that females across many taxa commonly mate with multiple males, which suggests that males are in reality competing for not only an opportunity to copulate with a female, but to fertilize her oocytes.

When females do mate with multiple males, events that occur just before and during copulation (pericopulatory) as well as following copulation (postcopulatory) can affect the proportion of the female's offspring sired by each mating male (often termed sperm precedence or paternity share). From the male perspective, these events are known as sperm competition and were first defined by Parker (1970) as "the competition between the sperm from two or more males for the fertilization of a given set of ova" but has subsequently been expanded to include any male mediated processes that can affect male paternity share

(Simmons 2001). From the female perspective, an appreciation that females can mediate biases in male paternity share after bouts of multiple mating was essentially unrecognized until Thornhill (1983). Eberhard's (1996) landmark review of cryptic female choice defined cryptic female choice as resulting "from a female-controlled process or structure that selectively favors paternity by conspecific males with a particular trait over that of others that lack the trait when the female has copulated with both types." Thus, since females can exhibit choice before, during and after mating, and males can compete for fertilizations without ever occupying the same space and time, it is incumbent upon the researchers of today to expand our view of sexual selection to encompass all possible episodes of sexual selection. This integrative perspective is necessary to gain a complete understanding of how total sexual selection can drive trait evolution within polyandrous mating systems.

To that end, this dissertation's primary focus is an examination of the role of nuptial gifts in sequential episodes of sexual selection in beetles, including several species of *Photinus* firefly and the red flour beetle, *Tribolium castaneum*. Potential episodes of selection considered in this dissertation research include precopulatory (courtship prior to contact), pericopulatory (immediately before or during mating) and postcopulatory (following copulation). The term nuptial gift encompasses a very wide range of objects and substances, but regardless are an integral part of the reproductive ecology of a diverse array of animals. In general, nuptial gifts are non-gametic materials that are transferred to the opposite sex during courtship and mating. Examples include items of prey, body parts,

hemolymph and secretions from accessory glands. The type of nuptial gift produced by the species in this dissertation are known as endogenous genital nuptial gifts (see Chapter II for an expanded definition). This category of gift is produced by secretory tissues in the male reproductive tract and is transferred into and absorbed by the female genital tract. The specific type of nuptial gift produced in the study species is a spermatophore (sperm containing package synthesized by male reproductive accessory glands).

The research in this thesis is presented in 7 chapters that detail investigations designed to provide insight into not only the role of nuptial gifts in fitness, but their evolutionary history. Chapter II is a review that introduces much of the general theory on the evolution of nuptial gifts and their role in the fitness of each sex while providing a framework for the remainder of the chapters. Chapter III adopts a meta-analytic approach to synthesize quantitatively extensive experimental work examining how male ejaculate quantity affects different components of female fitness across a broad range of arthropod taxa. Chapter IV presents the effects of a manipulation in size of a single spermatophore on female fecundity and life span in *P. obscurellus* fireflies. Chapter V largely focuses on male fitness in *P. greeni* fireflies across all three possible selective episodes. Both the attractiveness of the courtship signal and male spermatophore size were manipulated to determine how these traits might each influence mate acceptance and paternity share. Chapter VI investigates the existence of close contact chemical cues, cuticular hydrocarbons, in both nocturnal and diurnal fireflies, as these cues could be mediating mate choice between males and females in fireflies.

Chapter VII presents an in depth evolutionary analysis on a worldwide sampling of firefly species that examines potential macroevolutionary patterns between female flight, mode of sexual signaling and male nuptial spermatophore production. Chapter VIII presents the first proteomic-based investigation in any beetle species of one specific component of a male spermatophore, seminal fluid proteins. By examining spermatophores from many perspectives, these results contribute a number of novel and broadly applicable insights into the nature and evolution of nuptial gifts in animal species.

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Chapter II. The Evolution of Animal Nuptial Gifts

Rich gifts wax poor when givers prove unkind.

William Shakespeare
Hamlet, Prince of Denmark (3.1.101)

I. Introduction

A. What Are Nuptial Gifts?

Nuptial arrangements in many human cultures include gift-giving traditions (Medhi 2003, Cronk & Dunham 2007), and this behavior plays an important role in the mating systems of other creatures as well (Fabre 1918, Lack 1940, Thornhill 1976, Zeh & Smith 1985, Boggs 1995, Vahed 1998, 2007, Gwynne 2008). In species widely distributed across the animal kingdom, males transfer many different non-gametic materials to females during courtship and mating. Such materials can include lipids, carbohydrates, proteins, peptides, amino acids, uric acid, minerals, water, anti-predator defensive compounds, anti-aphrodisiac pheromones, and neuroendocrine modulators of recipient physiology. These nuptial gifts are an important aspect of reproductive behavior and animal mating systems (Thornhill & Alcock 1983, Andersson 1994). However, when compared to more conspicuous sexually selected traits such as male weaponry or ornamentation, such gifts have received relatively little attention from behavioral, ecological, and evolutionary research. Nuptial gifts heighten male reproductive investment, thus limiting male mating rates and altering courtship sex roles and sexual size dimorphism (Gwynne & Simmons 1990, Boggs 1995, Leimar et al. 1994). Selection acts on both gift-givers and receivers to shape nuptial gift structure and biochemical composition, as well as gift-giving behaviors. Not only

do nuptial gifts form the basis for dynamic coevolutionary interactions between the sexes, but they also link together male and female resource budgets (Boggs 1990). Because they are thus strategically poised at the intersection of nutritional ecology, sexual selection, and life history evolution (Boggs 2009), understanding the evolutionary origins and maintenance of nuptial gifts is of fundamental importance.

Animal nuptial gifts come in multitudinous forms (Figure 1), including food offerings, various male body parts, hemolymph, salivary gland secretions, seminal fluid, spermatophores (sperm-containing packages manufactured by male reproductive glands), and love darts. Many birds engage in courtship feeding, during which males provide prey to their own pair-bond partner or to extra-pair females (Lack 1940, Mougeot 2006). Scorpionfly males offer females dead insects or secretions from their enlarged, sexually dimorphic salivary glands (Thornhill 1981, Liu & Hua 2010). In some ground crickets, females imbibe hemolymph from a specialized spur located on their mate's hindleg (Gwynne 1997, Piascik et al. 2010). In numerous animals (including salamanders, molluscs, crustaceans, annelids, leeches, and most insects) males transfer biochemically diverse spermatophores to females during mating (Mann 1984). Nuptial gifts are not limited to animals with separate sexes, as during copulation many hermaphrodites inject chemicals that induce a physiological response in their partner (Koene & Maat 2001, Koene & Schulenburg 2005, Schilthuzien 2006, Michiels & Koene 2006). Neither is gift-giving an exclusively male behavior: in

heteropteran Zeus bugs, males feed upon glandular secretions provided by the female (Arnqvist et al. 2003).

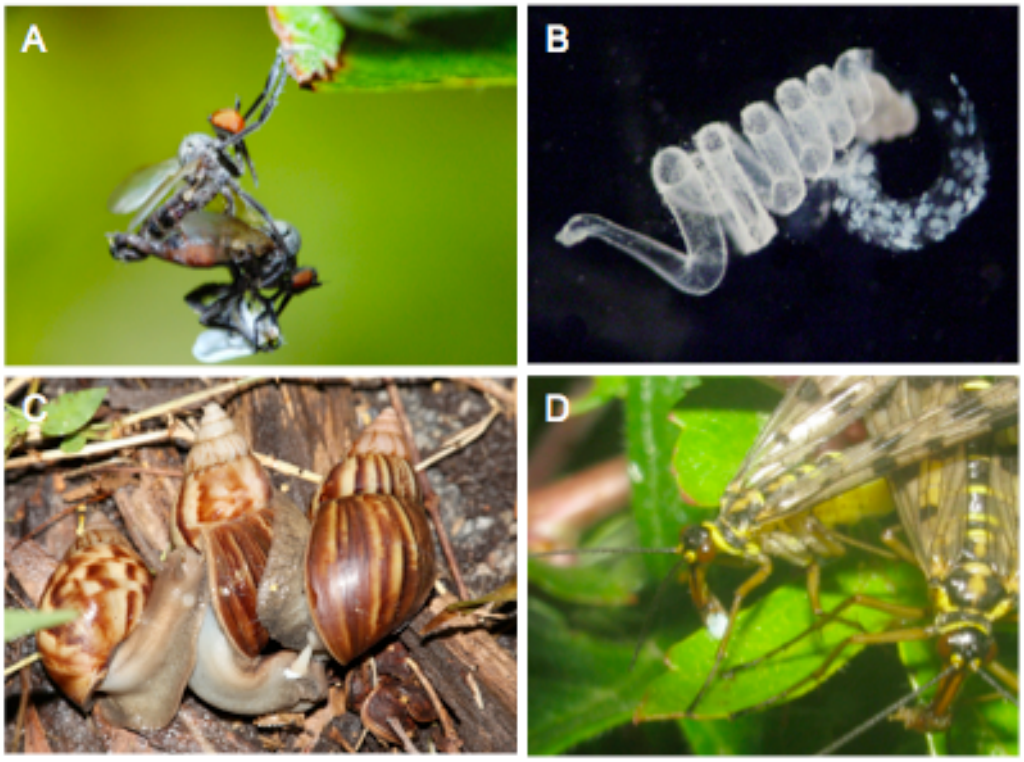

Figure 1. A sampler illustrating the extraordinary diversity of animal nuptial gifts: A) During mating, a female dance fly (Diptera: Empididae) feeds upon a dead insect provided by her mate (Photo by Rob Knell). B) Sperm rings are released from a spermatophore manufactured by male accessory glands in *Photinus* fireflies (Coleoptera: Lampyridae). C) During mating, hermaphroditic land snails (Gastropoda: Achatinidae) shoot their partner with a love dart that delivers mucus gland secretions (Photo by James Koh). D) A male scorpionfly (Mecoptera: Panorpodidae) secretes a white salivary mass that will be consumed by a female during mating (Photo by Arp Kruithof).

Clearly, if we intend to move beyond merely describing these traits to begin formulating and answering questions about how animal nuptial gifts have evolved, we will need to start with a carefully articulated, coherent definition that encompasses this remarkable diversity. In this review we begin by proposing such a definition, and then offer a conceptual framework for systematically classifying

nuptial gifts. We go on to discuss some ecological conditions and life history traits that might favor the evolution of nutritive nuptial gifts, *i.e*. those that contribute to female resource budgets. From the male perspective, gift-giving behavior will usually (but not always) provide a net benefit. We analyze these potential benefits by describing how nuptial gifts can increase male reproductive fitness over multiple selection episodes that take place before, during, and after copulation. As a case study, we describe previous work on the katydid *Requena verticalis* that has elucidated how gift-giving males benefit from this behavior. Rigorously testing the many scenarios that have been proposed about nuptial gift evolution requires a comparative phylogenetic approach, and we discuss results from three insect groups where such an approach has been applied (crickets and katydids, fireflies, and *Drosophila* fruitflies). We also summarize work on rates of evolutionary change in an important constituent of *Drosophila* nuptial gifts known as seminal fluid proteins. Finally, we suggest several directions for future research that promise to deepen our understanding of nuptial gift evolution.

B. Toward A Broader View of Nuptial Gifts

Before considering how nuptial gifts might have evolved, it is essential to clarify some relevant terminology. Previous studies have most often relied on *ad hoc* definitions of nuptial gifts, an approach reminiscent of the infamous "I know it when I see it" definition of hard-core pornography that Justice Potter Stewart used in his written opinion on the U.S. Supreme Court case Jacobellis v. Ohio (1964). The *Oxford English Dictionary* (1989) provides a legal definition of gift as "the transference of property or a thing by one person to another, voluntarily."

Further, in colloquial English the term "gift" generally implies some benefit for the recipient. However, because coevolutionary interactions between the sexes can continually alter costs and benefits for both givers and receivers, we contend that a broader view is essential for understanding the evolution of animal nuptial gifts. Within the scientific community, some researchers have limited the scope of nuptial gifts to encompass only "nutritive" gifts, i.e. those that contain male-derived substances used by females to sustain metabolic activities (e.g. Thornhill 1976, Boggs 1995, Andersson 1994, Gwynne 2008). Others have excluded from consideration any gifts that are not contained within a consolidated package (e.g. Thornhill & Alcock 1983). Again, we suggest that such restrictions may hinder progress toward the ultimate goal of understanding nuptial gift evolution.

As an alternative to this disjointed approach, we advocate the following definition (modified from South et al. 2011*b*, Lewis et al. 2011): *Nuptial gifts are materials beyond the obligatory gametes that are transferred from one sex to another during courtship or mating*. Importantly, this definition makes no assumptions concerning either: 1) how the gift currently affects fitness; thus, at certain times during its evolutionary trajectory, a gift might be beneficial, neutral, or detrimental to either sex, or 2) the presence or absence of gift-wrapping: thus, we include soluble proteins and other materials that are transmitted in seminal fluid or mucus as gifts, albeit unpackaged. In articulating this broad definition we hope to unify what have previously been disconnected lines of investigation. For example, the protein content of insect spermatophores is often used as a measure of gift quality (Wedell 1994, Bissoondath & Wiklund 1996, Cratsley et al. 2003).

This reasonable inference is based on female vitellogenesis being protein-limited (Wheeler 1996), coupled with evidence that male-derived amino acids are incorporated into female eggs and soma (e.g. Boggs & Gilbert 1979, Rooney & Lewis 1999). However, male seminal fluid in *Drosophila melanogaster* contains soluble proteins secreted by reproductive accessory glands and the male ejaculatory duct (Chapman 2008). Many of these seminal fluid proteins (SFP) have been identified and demonstrated to alter female reproduction by stimulating ovulation and oviposition, increasing sperm storage, and lengthening females' latency to remate (Avila et al. 2011, Wolfner 2009). However, because these proteins are transmitted in seminal fluid and are not encapsulated within a discrete package, traditionally they have not been considered as nuptial gifts (but see Vahed 1998, Markow 2002, Simmons & Parker 1989). Yet it is becoming clear that male spermatophores contain many of the same protein classes (Andres et al. 2006, 2008; Braswell et al. 2006; South et al. 2011a, Sonenshine et al. 2011) and these components may produce similar effects on females. It could perhaps be argued that including seminal fluid makes our nuptial gift definition overly broad. However, because various constituents of seminal fluid have been shown to exert diverse effects on both male and female fitness (Leopold 1976, Gillott 2003, Poiani 2006), such inclusion seems appropriate. Thus, we argue that drawing an arbitrary distinction between seminal products encased within a discrete package vs. unpackaged seminal products transferred in a liquid ejaculate may have inadvertently obscured basic similarities in gift composition and function, as well

as similarities in the evolutionary origin and maintenance of male reproductive accessory glands, the main gift-producing structures.

A key point is that this broad perspective on nuptial gifts allows for possible changes over evolutionary time in how gifts will affect the recipient's net fitness. While some degree of cooperation is required for sexual reproduction to occur, males and females have distinct reproductive interests (Trivers 1972, Parker 1979, Arnqvist & Rowe 2005). As a result, coevolutionary interactions between the sexes will cause nuptial gifts to evolve dynamically in a manner that alters the cost/benefit ratio of nuptial gifts for each sex. Thus, a nuptial gift that originates because it provides mutual fitness benefits to both sexes may evolve into a gift that reduces the recipient's net fitness, and *vice versa*.

In summary, even though some may find fault with our definition, there is an undeniable need for a more systematic approach to defining what exactly constitutes a nuptial gift. Furthermore, a broader definition such as the one we propose here will allow us to better track the changes in nuptial gift costs and benefits that are certain to occur over evolutionary time.

C. Classifying Nuptial Gift Diversity

In any contest, insects would surely emerge as the undisputed champions of gift diversity. For comprehensive insight into this fascinating diversity, readers are referred to excellent reviews by Boggs (1995) and Vahed (1998). Here, we highlight just a few notable patterns observed among insects before proposing a classification scheme that will encompass animal nuptial gifts.

First, gifts are conspicuously diverse, not only between different insect groups, but also within particular clades. For example, spermatophores are ubiquitous within the insect order Lepidoptera, yet they are absent in the Diptera and occur only sporadically within the Coleoptera (Davey 1960, Mann 1984). Within the beetle family Lampyridae (fireflies), some males pass elaborate spermatophores while firefly males of other species transfer free (unpackaged) ejaculates (Lewis & Cratsley 2008, South et al. 2011). Beyond spermatophores, orthopteran nuptial gifts have flowered into an especially impressive display of diversity (described in Section IV.A below).

A second notable pattern is that some groups show surprising plasticity in their gift-giving behavior. For example, male *Panorpa* scorpionflies (Mecoptera: Panorpidae) pursue alternative mating tactics using different gift types (Thornhill 1981, Sauer et al. 1998). In *P. cognata*, gift-giving behavior depends on a male's nutritional state: well-fed males secrete salivary masses that females consume during copulation, while low-nutrition males instead offer females a dead arthropod (Enqvist 2007b). Similarly, in several empidid dance flies (Diptera: Empididae) males optionally offer females either a dead prey insect or inedible tokens such as silk balloons or seed tufts (Preston-Mafham 1999, Vahed 2007).

Here we propose a taxonomy for animal gifts that we hope will facilitate mapping the landscape of nuptial gift evolution (for other classification schemes see Simmons & Parker 1989, Vahed 1998, Gwynne 2008). Table 1 presents four nuptial gift categories, with examples of relevant structures and behaviors from various taxa. One key distinction is based on the method of gift production. Thus,

we distinguish between *endogenous gifts* that are manufactured by males themselves and *exogenous gifts* that consist of externally procured food items such as seeds or prey that males gather and then transfer to females. Another important distinction is based on how gifts are absorbed by the recipient. Gwynne (2008) distinguished *oral gifts* that are taken in through the female digestive system (e.g. food items, spermatophylaces, hindwing secretions), from gifts we term *genital gifts* that are absorbed through the female reproductive tract: this includes both unpackaged secretions from male reproductive glands (conveyed in liquid seminal fluid) as well as those encased in discrete packages (spermatophores). We propose here another category consisting of *transdermal gifts* that are injected through the skin into the partner's body (e.g. snail love darts, intradermally implanted squid spermatophores, hypodermic insemination in leeches and bedbugs). Although nuptial gifts are often commingled together into a single category (e.g. Arnqvist & Nilsson 2000), we believe the distinctions drawn here will prove useful as a basis for future studies of the evolution of nuptial gift structure and composition. The primary reason for proposing this classification scheme is because very different predictions can be made about how various gift types might affect fitness of both sexes (see also Simmons & Parker 1989 and Section III below).

Table 1. A classification scheme that encompasses the diversity of non-gametic materials passed from one sex to the other during courtship and mating, with examples of each type of nuptial gifts.

Gift Production	**Gift Absorption**	**Nuptial Gift Examples**	**Taxonomic group & references**
Endogenous	*Oral*	Hemolymph from tibial spurs	Ground crickets (Piascik et al. 2010)
		Spermatophylax	Katydids & crickets (Gwynne 1997)
		Salivary secretions	*Panorpa* scorpionflies (Enqvist 2007*a*)
		Anal secretions	*Drosophila nebulosa* (Steele 1986)
		Metanotal secretions	Tree crickets (Brown 1997, Bussiére et al. 2005)
		Male body (sexual cannibalism)	Red-backed spider, mantids (Elgar & Schneider 2004)
Endogenous	*Genital*	Spermatophores	Salamanders, lepidopterans, molluscs, copepods, crabs, spiders (Mann 1984)
		Seminal fluid proteins	*Drosophila* spp. (Markow 2002, Wolfner 2007, Chapman 2008)
Endogenous	*Transdermal*	Love darts	Land snails (Koene & Schulenburg 2005)
		Setal gland injection	Earthworms (Koene et al. 2005)
		Intradermal spermatophore implantation	Squid (Hoving & Laptikhovsky 2005), leeches (Mann 1984)
		Haemocoelic injection of seminal fluid	Bedbugs (Stutt & Siva Jothy 2001)
Exogenous	*Oral*	Courtship feeding	Birds: kestrels, shrikes (Lack 1940, Mougeot 2006)
		Seeds	Lygaeid bugs (Carayon 1964)
		Insect prey	Hangingflies, scorpionflies (Thornhill 1981), empidid flies (Cumming 1994), *Pisaura* spiders (Austad & Thornhill 1986)

1. ***Exogenous oral gifts.*** These consist of food items that males capture or collect, so these are most likely to contain nutritive materials (defined as substances that contribute to female metabolic reserves). Thus, most exogenous oral gifts are predicted to deliver net fitness benefits to females, measured as increased lifetime fecundity. From the male perspective, these gifts are generally predicted to

increase male fitness across several selection episodes (reviewed in Vahed 1998, 2007, Gwynne 2008). First, because they can be assessed (visually or by gustation) prior to mating, exogenous oral gifts should affect a male's ability to attract and successfully mate with females. Second, because females remain stationary while feeding, food gifts may make it easier for males to initiate copulation. Third, because females feed on these gifts while copulating, such gifts are expected to increase both copulation duration and the quantity of sperm transferred.

2. *Endogenous oral gifts.* This category includes diverse materials that are secreted by male salivary, reproductive, and other glands, as well as parts or the whole of the male's body; these materials are then consumed by females before, during, or after copulation (reviewed by Boggs 1995, Vahed 1998, Elgar & Schneider 2004). Thus, in *Oecanthus nigricans* tree crickets (Orthoptera), females feed upon proteinacous secretions produced by dorsally-located male glands, while females of some true flies (Diptera) and scorpionflies (Mecoptera) consume male salivary secretions, and female *Allonemobius* ground crickets drink hemolymph from male hindleg spurs (Bidochka & Snedden 1985). Females in many katydids and crickets (Orthoptera) consume a spermatophylax, a gelatinous portion of the spermatophore produced by male reproductive glands. Many mantids and orb-weaving spiders engage in sexual cannibalism, where females kill and consume males either before or after insemination (Elgar & Schneider 2004); in both cases, the male body represents an endogenous oral gift under our

definition, even when it is given involuntarily (i.e. gifts can have a negative effect on male fitness).

Since they derive from such diverse sources, endogenous oral gifts are likely to have quite varied effects on females. Some endogenous oral gifts, such as hemolymph or male body parts, may closely resemble exogenous gifts of prey or other food items in contributing to females' nutrient budgets (Boggs 1995, Gwynne 2008). Rather than replicating whatever nutritional mixtures are available in the diet, however, glandular gifts have the potential to provide more targetted dietary supplements. These specialized glandular gifts might supply nutrients which are otherwise absent or limited in female diets, such as macronutrients (proteins, lipids, carbohydrates), micronutrients (sodium, zinc), or defensive compounds (cantharidin, pyrrolizidine alkaloids, cyanogenic glycosides). In cockroaches (Dictyoptera: Blattidae), males provide endogenous oral gifts that constitute an important nitrogen source for females and their eggs (reviewed by Boggs 1995, Vahed 1998). In many cockroaches, males accumulate uric acid in their accessory glands before packaging it into their spermatophore; after mating, females expel and eat the spermatophore. In other roaches, females feed directly on uric acid as it is secreted from male glands.

On the other hand, sexual conflict theory predicts male glandular gifts might evolve that benefit males even though they may adversely affect female net fitness (Rice 1998, Arnqvist & Nilsson 2000, Arnqvist & Rowe 2005). Through reciprocal sexual coevolution, an escalating arms race might then ensue in which females evolve the ability to metabolize or otherwise counteract manipulative

male substances (Eberhard 1996, Arnqvist & Nilsson 2000). However, it has been pointed out (Gwynne 2008) that such oral gifts might be less likely to contain manipulative substances because those would be subject to degradation while passing through the digestive tract. Thus, the category of endogenous oral gifts is diverse and includes nuptial gifts that may have positive, negative, or no effects on female fitness.

From the male perspective, when endogenous oral gifts (such as secreted salivary masses) can be inspected by females, they could resemble exogenous gifts that increase male mating success, copulation duration and possibly sperm quantity transferred during copulation. For example in spiders, sexual cannibalism that takes place after insemination can benefit the sacrificed male by prolonging copulation duration, thus increasing sperm transfer and male paternity share, in addition to increasing female fecundity and offspring survival (Andrade 1996, Elgar & Schneider 2004, Herberstein et al. 2011, Welke & Schneider 2012). For orally ingested glandular gifts, such as the orthopteran spermatophylax, males may be selected to incorporate phagostimulants that increase their gifts' gustatory appeal for females (Sakaluk 2000, Vahed 2007). Selection may also alter male gift composition to slow female consumption rates if this allows more time for males to transfer sperm. For example, in many crickets and katydids (Orthoptera: Ensifera) the male spermatophylax has a sticky, gelatinous consistency that prevents rapid ingestion by females (Vahed 2007).

3. ***Endogenous genital gifts.*** This category includes materials that are produced by secretory tissue in the male reproductive tract, transferred in seminal fluid or

spermatophores, and absorbed through the female genital tract. Although spermatophores may have originated to prevent sperm loss or dessication (Khalifa 1949, Davey 1960), in many animals these structures have become vastly elaborated (Mann 1984, Thornhill 1976). Many ideas have been proposed about the evolutionary origin of elaborated male ejaculates such as spermatophores. Wickler (1985) proposed that spermatophores originated as a way for males to prevent females from digesting sperm, as an adaptation secondary to intrasexual selection for greater sperm quantity. It has also been suggested that female choice, based on the quality or quantity of non-sperm ejaculate components, might have favored the elaboration of male ejaculates (Cordero 1996). Arnqvist & Nilsson (2000) proposed that elaborated male ejaculates represent "manipulative and sinister superstimuli" that evolved through sexual conflict over female remating rates. However, given the wide taxonomic distribution and diversity of endogenous genital gifts, it is unrealistic to expect a single explanation for their evolution. Rather, even a brief overview of gift constituents indicates that endogenous genital gifts have probably had multiple evolutionary origins and diverse trajectories.

Like orally-ingested glandular gifts, the products of male reproductive glands can also supply nutrients that are absent or limited within female diets. The geometric framework in nutritional ecology may provide a useful perspective for thinking about the evolution of nutritive nuptial gifts. This framework is based on locating an organism's nutritional requirements and dietary choices within a multidimensional resource space (Raubenheimer et al. 2009, Raubenheimer

2011). Importantly, rather than replicating nutritional mixtures that are available in the female diet, nuptial gifts could provide a vector that targets the female-specific requirements for vitellogenesis (Boggs 1990). Thus, selection may shape male glandular products to augment females' resources by providing them with entirely different nutritional mixtures compared to those gained through feeding. Empirical studies of numerous Orthoptera, Lepidoptera, and Coleoptera have demonstrated that diverse substances derived from endogenous genital gifts are incorporated into female somatic tissue and eggs; these substances include amino acids, zinc, phosphorus, and sodium transferred in male spermatophores (reviewed by Bogg 1995, Vahed 1998). For example, many lepidopteran males engage in puddling behavior on damp soil, dung, or carrion where they obtain sodium, which is a scarce nutrient for most folivores (Molleman 2010). Males accumulate this element in their reproductive glands, and transfer sodium-rich spermatophores during mating; in the moth *Gluphuisia septentrionis*, a single spermatophore contains >50% of the male's total body sodium content (Smedley & Eisner 1996). Females pass sodium along to their eggs, and in the skipper, *Thymelicus lineola*, such gifts enhance larval survivorship (Pivnick & McNeil 1987, but see Molleman et al. 2004). In addition, reproductive glands can serve as a reservoir for defensive compounds that males derive from dietary sources, and these compounds are later transferred to females within spermatophores or seminal fluid (reviewed by Vahed 1998). Thus, endogenous genital gifts can contain defensive compounds that protect the female or her eggs against predators or microbial attack; such gifts include cantharidin in *Neopyrochroa flabellata*

beetles (Eisner et al. 1996), pyrrolizidine alkaloids in *Utetheisa ornatrix* moths (Eisner & Meinwald 1995), cyanogenic glycosides in several *Heliconius* butterflies (Cardoso & Gilbert 2007), and vicilin-derived peptides in *Callosobruchus maculatus* cowpea beetles (Alexandre et al. 2011).

On the other hand, some components of endogenous genital gifts may reduce female fitness. In some male insects, reproductive accessory glands manufacture compounds that have diverse effects on female reproductive physiology and behavior (Eberhard 1996, Gillott 2003). In *Drosophila melanogaster,* for example, seminal fluid proteins have been shown to heighten female oogenesis and oviposition, increase sperm storage and utilization, and to reduce female re-mating rates and life span (reviewed by Chapman & Davies 2004, Ram & Wolfner 2007; Wolfner 2007; Chapman 2008). For most taxa, little is known concerning the nature of these secretions, although recent work has elucidated seminal fluid composition in *Aedes* mosquitoes (Sirot et al. 2008), *Gryllus* and *Allonemobius* crickets (Andres et al. 2006, Braswell et al. 2006), *Tribolium* flour beetles (South et al. 2011), and honeybees (Collins et al. 2006, Baer et al. 2009). In many species, male spermatophores contain anti-aphrodisiacs that reduce a female's likelihood of remating (*Tenebrio* beetles, Happ 1969; *Pieris napi* butterflies, Andersson et al. 2004; *Heliconius* butterflies, Estrada et al. 2011). Selection on males to reduce sperm competition risk favors inclusion of such substances, yet anti-aphrodisiacs can lower female net fitness if they depress remating rates below some female optimum.

Thus, endogenous genital gifts are complex mixtures that have likely been shaped by multiple selective forces. While it has been argued that male ejaculate composition will be selected primarily to manipulate female reproductive physiology and should carry a net fitness cost borne by its recipients (Arnqvist & Nilsson 2000, Arnqvist & Rowe 2005), it is clear that understanding the complex effects male ejaculates have on females will require a broad and balanced perspective.

4. Endogenous transdermal gifts. These nuptial gifts include male seminal and glandular products that are transferred and absorbed outside the female's digestive or reproductive systems. This happens during extragenital insemination in bedbugs (Stutt & Siva-Jothy 2001) and intradermal spermatophore implantation in deep-sea squid (Hoving & Laptikhovsky 2007). In the bedbug, *Cimex lectularius*, male ejaculates include (in addition to sperm) antioxidants, micronutrients, and anti-bacterial compounds (Reinhardt et al. 2009). Hypodermic injection of seminal products is particularly widespread among simultaneous hermaphrodites such as leeches, sea slugs, and polyclad flatworms (Michiels & Koene 2006). Another type of transdermal gift consists of allohormones, substances that induce a direct physiological response in the recipient (Koene & Matt 2001). These can be injected through the skin of a mating partner during copulation while sperm are being passed to the reproductive organs. This mode of delivery allows male products to bypass both digestive and reproductive tracts, where various gift components might get broken down. During copulation, hermaphroditic earthworms *Lumbricus terrestris* use their ventral copulatory setae

to inject their partner with setal gland products that induce sperm uptake and storage (Koene et al. 2005). A similar benefit for male function occurs in *Helix aspersa* land snails, which penetrate their partners with a calcareous dart coated with allohormones produced by a mucus gland; these substances inhibit sperm digestion and enhance sperm storage by the recipient (Koene & Schulenburg 2005, Schilthuzien 2006). As in other endogenous gifts, selection on transdermal gift production may favor the inclusion of compounds that benefit males yet are detrimental to female fitness.

II. Effects on Recipient Fitness

Empirical studies in numerous taxa have documented how male gifts affect several different female fitness components (including egg and clutch size, rate and timing of offspring production, longevity), as well as female net fitness (lifetime fecundity measured as the total number of eggs or offspring produced). Many studies have found that nuptial gifts can provide females with direct material benefits measured as an increase in the recipient's net fitness. Such evidence has been compiled and summarized by previous literature reviews for arthropod nuptial gifts (Boggs 1995, Vahed 1998, Rooney & Lewis 1999, Vahed 2007, Gwynne 2008) and for sexual cannibalism (Elgar & Schneider 2004), as well as by some meta-analyses (Arnqvist & Nilsson 2000, South & Lewis 2011).

Rather than recapitulating these synopses here, we simply advocate that the term nuptial gift be used in its broadest sense, *i.e.* independently of whether such materials currently exert a positive, a negative, or no effect on recipient net fitness (Figure 2). Others have used narrower terminology, using nuptial gifts to

mean only nutritive gifts or those that increase female fitness (*i.e*. falling within the upper-right cell of Figure 2). On the other hand, because male and female reproductive interests are not perfectly aligned, sexual conflict may drive the evolution of nuptial gifts that provide fitness benefits to males while reducing female net fitness. Arnqvist & Nilsson (2000) and Arnqvist & Rowe (2005) suggested that the term "Medea gift" (named after a mythological Greek sorceress who used a beautifully embroidered, poisonous robe to murder a rival) should be used for any gifts that reduce female net fitness (i.e. falling within the upper-left cell of Figure 2). However, because coevolutionary interactions are expected to create dynamic changes over time in gifts' cost/benefit ratios for their recipients, we believe such restrictive terminology is counterproductive to the goal of understanding nuptial gift evolution. One example of this shifting balance of costs and benefits is seen in the bedbug *Cimex lectularius*. Although traumatic insemination through the abdominal wall causes wounding that reduces female lifespan (Stutt & Siva-Jothy 2001), male ejaculates contain compounds that increase female net fitness via increased lifetime fecundity and oviposition rate, and delayed reproductive senescence (Reinhardt et al. 2009). One evolutionary scenario proposed by these authors is that male ejaculates were originally detrimental, and that subsequent female counteradaptations evolved to neutralize, and eventually reverse, these harmful effects. Alternatively, they suggest, male ejaculates may have positively affected female net fitness when they originated. While distinguishing between these evolutionary trajectories must await future phylogenetic studies coupled with ancestral trait reconstruction, it is clear that a

more holistic framework will be required to understand the evolution of nuptial gifts.

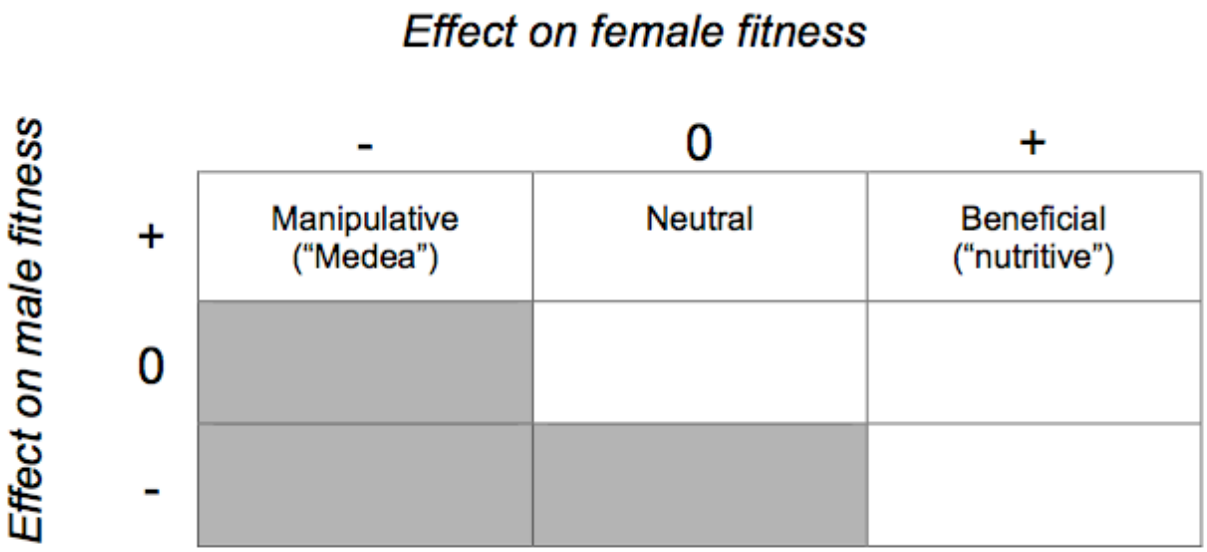

Figure 2. Our nuptial gift definition encompasses a range of possibilities for how nuptial gifts might influence male and female net fitness (benefit minus cost). Gifts that provide a net fitness benefit to males can have either negative, positive, or no effects on female fitness (top row). When gifts provide a net fitness benefit for females, they can be maintained whether or not males derive a fitness benefit; thus, sexual cannibalism would fall into the rightmost column (e.g. cases when the male is consumed before insemination would fall into the bottom right cell). The position of any gift is likely to shift over evolutionary time, as sexual interactions modify costs and benefits for each sex. (Gray areas indicate that evolutionary maintenance is unlikely as these gifts carry net fitness costs for one or both sexes).

Possible evolutionary trajectories leading to manipulative male gifts have been presented in detail by others (e.g. Eberhard 1996, Rice 1998, Sakaluk 2000, Arnqvist & Rowe 2005). Here we focus on circumstances that might lead to the evolution of nutritive nuptial gifts, i.e. those providing material benefits that directly increase fitness for the gift recipient.

Among the ways that nuptial gifts might increase a male's fitness relative to other males in the same population is by enhancing female fecundity relative to other females in the population. The enhanced fecundity hypothesis for paternal investment was proposed by Tallamy (1994), who suggested that male investment will evolve whenever males can provide materials whose availability constrains female reproductive output. While he mainly focused on postzygotic male investment (e.g. paternal brood care), Tallamy pointed out that this hypothesis should also apply to the evolution of nuptial gifts (*i.e.* prezygotic male investment). In addition, recent theoretical work has shown that depending on the degree of fecundity enhancement, male nuptial gifts can alter intersexual coevolutionary dynamics and lead to a stable evolutionary equilibrium with mutual fitness benefits for both sexes (Alonzo & Pizzari 2010).

In general, female reproduction will be resource-constrained because of the higher gametic investment by this sex (Trivers 1972); in oviparous organisms, all the nutritional resources required for embryogenesis must be contained within each egg. Female egg production is most often limited by protein availability (Wheeler 1996). In insects, as in most oviparous animals, oocyte development is fueled mainly by vitellogenin, a female-specific glycolipoprotein; insect eggs also contain lipids and some carbohydrates in the form of glycogen (Klowden 2007). Females need to obtain these macronutrients from larval feeding, from adult feeding, or from male nuptial gifts (Boggs 1990). Thus, the enhanced fecundity hypothesis predicts that selection for nuptial gifts will be influenced by the availability and quality of specific nutritional resources needed for female

reproduction. Resource availability will in turn depend on organismal life history traits, as well as on temporal and habitat variation within a particular species. Below we consider some combinations of ecological conditions and life-history traits that are expected to favor the evolution of nutritive gifts that enhance female fecundity through male contributions to female resource budgets (Boggs 1990, 1995). We do not discuss mating systems; while several studies have explored the relationship between nuptial gifts and polyandry (e.g. Karlsson et al. 1997), it is difficult to determine causal relationships between these two highly correlated features.

First we discuss some life history features that are expected to lead to female-specific resource limitation. These include: location of a species along the continuum between income and capital breeding, temporal dynamics of female oogenesis, and requirements for female dispersal and flight. Income breeders are those that fuel reproduction using current energetic income, while capital breeders support their reproduction with energy stores accumulated at an earlier life stage (Stearns 1992, Houston et al. 2007). In purely capital breeders, male nuptial gifts could provide resources to supplement reserves that otherwise would be depleted over a female's reproductive lifespan. One such example is *Photinus ignitus* firefly beetles (reviewed by Lewis et al. 2004, Lewis & Cratsley 2008), which are capital breeders that entirely lack adult feeding. Both sexes mate repeatedly over their two-week adult lifespan. Males manufacture a complex spermatophore from several reproductive glands, and spermatophore-derived proteins are allocated to females' developing oocytes. Females that receive multiple spermatophores gain

increased lifetime fecundity. In addition, a seasonal reversal in courtship roles occurs: late in the mating season when both sexes face depleted resource stores, females compete for access to gift-providing males and males selectively mate with more fecund females (Cratsley & Lewis 2005).

Nuptial gift evolution may also depend on interspecific life-history differences in the temporal dynamics of female oogenesis (Boggs 1990, 1995, 2009). In some insect taxa, adult females emerge with their entire complement of eggs already matured (e.g. mayflies), while in others (e.g. *Photinus ignitus* fireflies) females will continue to mature eggs throughout their reproductive lives (Jervis et al. 2005 compiled relevant data for many insects). When egg maturation is distributed over time, selection should favor male nuptial gifts that could enhance female reproductive output by replenishing resources.

A final life history trait that may alter selection for nuptial gifts relates to female mobility. If females must fly in order to locate food, mates or suitable oviposition sites, to disperse, or to escape predators, then wing-loading constraints may restrict how many mature eggs a female can carry at any point in time. In addition, females face a tradeoff between allocating resources to flight or oogenesis (Wheeler 1996, Boggs 2009). Lewis & Cratsley (2008) presented a conceptual model proposing that because flightless (wingless) females can devote all their resources to egg production, selection for nuptial gifts will be relaxed due to limited scope for any further increases in females fecundity. A recent evolutionary trait analysis in fireflies supported this predicted intersexual correlation between female flight ability and male nuptial gifts (South et al.

2011*a*; see Section V.B below). Females of ancestral fireflies most likely had wings, and received male nuptial gifts in the form of spermatophores. In several lineages, after females lost their flight ability (possibly driven by fecundity selection), males subsequently lost the ability to produce these nuptial gifts.

Selection for nuptial gifts should also be influenced by within-species variation in the availability and quality of specific resources required for female reproduction. When such resources are limited, females may increase their mating activity to gain access to nutritive nuptial gifts (Gwynne 1990, Boggs 1990). For example, in the pollen katydid *Kawanaphila nartee* (Orthoptera: Tettigonidae), scarcity of pollen (a protein-rich food source for both sexes) generates intersexual competition among females for access to endogenous oral gifts in the form of the male spermatophylax (Simmons & Bailey 1990). In the pollen-feeding butterfly *Heliconius cydno*, pollen load varies among females and is negatively correlated with number of matings, and thus nuptial gifts, that females acquire (Boggs 1990). In addition, many experimental studies have found that nuptial gifts provide larger fecundity increases when females are food-limited (Gwynne & Simmons 1990; reviewed by Boggs 1990, Gwynne 1991, 2008). Finally, Leimar et al. (1994) provided comparative data from butterflies suggesting that variation in available resources (rather than average) will increase selection for nuptial gifts. Similarly, another comparative study across butterfly species found increased polyandry with greater variation in female body size, again indirectly suggesting that species with more variable larval food resources might experience increased selection for nuptial gifts (Karlsson et al. 1997).

Thus, several ecological conditions and life history traits linked to female resource allocation are predicted to favor the evolution of fecundity-enhancing male gifts. Indeed, an entire suite of correlated life history traits seems likely to select for fecundity-enhancing nuptial gifts. Based on the connections outlined here between nuptial gifts, life history traits, and nutritional ecology, testing hypotheses about trait combinations that favor the evolution of nutritive nuptial gifts seems like an important and relatively unexplored research area. As Boggs (1995) pointed out nearly 20 years ago, we still need rigorous comparative phylogenetic studies focused on testing for evolutionary associations between nuptial gift presence (and type) and interspecific variation in resource conditions and life history traits.

III. Potential Gift Benefits for Males

Considerable evidence indicates that the collection and manufacture of nuptial gifts is costly for males (reviewed by Boggs 1995). In addition, males have been shown to strategically allocate their gifts depending on female reproductive status or age (e.g. Wedell 1992, Simmons et al. 1993, Sirot et al. 2011). While colloquial usage views gifts as something given voluntarily, in the case of sexual cannibalism there may also be involuntary gift-giving that carries a net fitness cost for males (Elgar & Schneider 2004, lower-right cell of Figure 2). Yet as mentioned above, in some species cannibalized males gain posthumous benefits through increased paternity share and decreased likelihood of the female remating (Andrade 1996, Herberstein et al. 2011: upper-right cell of Figure 2). In most cases, however, the male structures that produce nuptial gifts and the various

behaviors associated with gift-giving will only be maintained if they confer a net fitness benefit on males; that is, the gift-giving males must be able to sire more offspring compared to other males in the same population. These fitness advantages could accrue through higher mating success, increased paternity share relative to other males mating with the same female, and/or enhanced female fecundity compared to other females in the population.

In determining what specific benefits a male might derive from his gift-giving behavior, much previous work has been caught up in a largely unproductive semantic debate. For several decades many attempts were made to distinguish between two particular hypotheses for the origin and maintenance of male gifts (Alexander & Borgia 1979, Gwynne 1984, Sakaluk 1986, Simmons & Parker 1989, Vahed 1998). The mating effort hypothesis suggested that gifts function to ensure mating and sperm transfer, while the paternal investment hypothesis suggested that gifts function to increase the number or quality of the gift-giver's own offspring. However, attempts to sort nuptial gifts neatly into these two categories were unsuccessful, and a fatal terminological quagmire gradually developed (see also Wickler 1985, Simmons & Parker 1989, Simmons 1995, Vahed 1998, Gwynne 2008). Among the reasons for this failure was that these two hypotheses represent two non-independent gift functions (*i.e.* the latter depends on the former), and also that the paternity data necessary for empirical tests of the paternal investment hypothesis were lacking.

Moving forward, we suggest that a more constructive approach will be to think about nuptial gifts as selection targets during several sequential episodes

that occur *before*, *during* and *after* mating (Figure 3). A similar approach was suggested by Gwynne (1997; his Table 6-1). For example, nuptial gifts may enhance a male's mating success by increasing his ability to attract females (episode 1) and to successfully copulate with them (episode 2). During mating, nuptial gifts may improve a male's insemination success (episode 3, measured as whether or not any sperm transfer occurs), or increase the number of sperm transferred (episode 4). After mating, nuptial gifts may increase the viability and storage of male sperm within the female reproductive tract (episode 5). In competitive mating situations (polyandry or polygamy), providing a nuptial gift may increase male paternity share (proportion of offspring sired by the gift-giving male) relative to other males mating with the same female (episode 6); this may occur through cryptic female choice favoring certain male or gift traits, or by increased sperm defense or offense. As discussed in Section II above, nuptial gifts may also increase a male's fitness by enhancing overall female fecundity (episode 7), as well as egg and offspring survival (episode 8), relative to that of other females in the population. Numerous experimental or observational studies of nuptial gift function have demonstrated that larger or more nuptial gifts lead to higher male fitness during one or more of these sequential selection episodes; we provide just a few examples below.

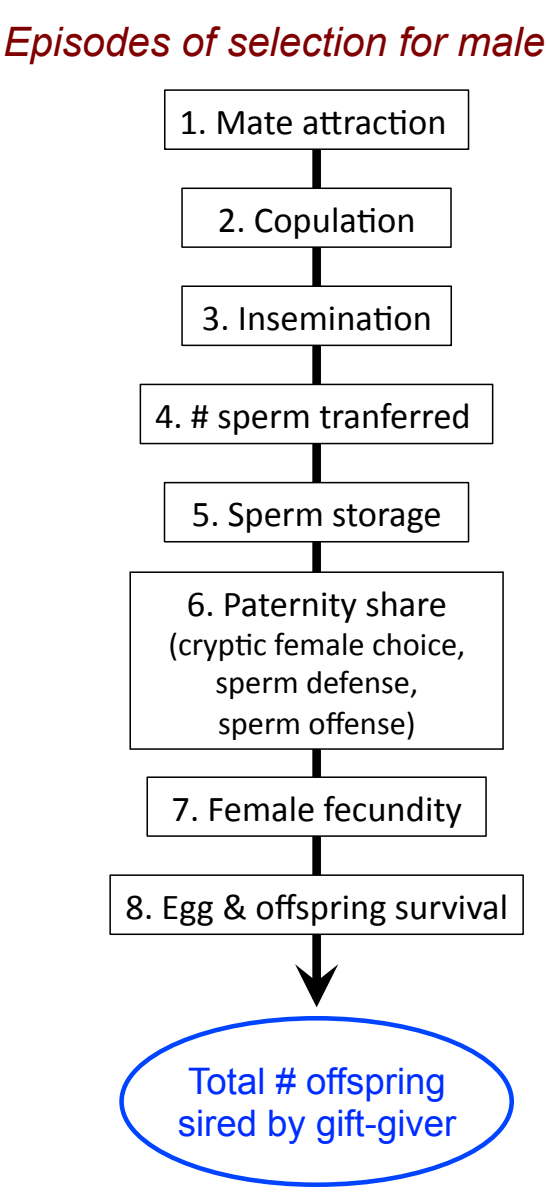

Figure 3. Potential fitness benefits gained by males from nuptial gifts across sequential selection episodes. Nuptial gifts may increase: mate attraction, copulation, or insemination success, quantity of sperm transferred or stored, and paternity share in competitive mating situations (gained through cryptic female choice, protecting paternity share when the female remates, and/or when the female has previously mated). Nuptial gifts may also provide fitness benefits to males by increasing overall female fecundity, egg or offspring survival, if on average such gifts increase the production and survival of the gift-giving male's own offspring.

Many oral nuptial gifts (both exogenous and endogenous) provide a benefit to males by attracting females and also by increasing the likelihood that females will copulate once they have been attracted. Among such gifts are the

edible and inedible gifts offered by male empidid dance flies (Preston-Mafham 1999, Lebas & Hockham 2005), prey and salivary secretions provided by *Panorpa* scorpionflies (Enqvist 2007a), and food regurgitated by *Drosophila subobscura* males (Steele 1986). Some oral gifts, such as male hindwings and hemolymph in *Cyphoderris* hump-winged crickets (Eggert & Sakaluk 1994), also facilitate successful insemination, as males can more readily accomplish sperm transfer when females hold still while feeding.

Oral nuptial gifts (both exogenous and endogenous) also function during mating to increase the quantity of sperm transferred to the female reproductive tract; these include the spermatophylax in several crickets and bushcrickets (Sakaluk 1984; reviewed by Gwynne 1997, Vahed 1998, 2007), prey gifts in *Bittacus* hangingflies (Thornhill 1976), and salivary secretions in *Panorpa* scorpionflies (Enqvist 2007a).

Other nuptial gifts benefit males during postcopulatory episodes of selection, including enhancing female storage of sperm that will later fertilize eggs. Acp36DE is a seminal fluid protein (endogenous genital) transferred by *Drosophila melanogaster* males that causes an increase in sperm numbers stored within the female reproductive tract (Qazi & Wolfner 2003). Similarly, hermaphroditic *Helix aspersa* garden snails penetrate their partner with a mucus-coated dart (endogenous transdermal gift) that increases sperm storage (Rogers & Chase 2001).

Many endogenous gifts (both oral and genital) also include materials that inhibit the female's mating receptivity (thus reducing the risk of sperm

competition) and/or increase her latency to remate (thus increasing sperm defense). Such receptivity-inhibiting materials include *Drosophila* sex peptide, unknown ejaculate components in *Requena verticalis* katydids (Gwynne 1986), non-fertilizing apyrene sperm in Lepidoptera (Wedell 2005), and salivary secretions of *Panorpa* scorpionflies (Enqvist 2007a). In addition, many spermatophores (endogenous genital) contain anti-aphrodisiacs that deter other males from approaching a mated female (e.g. Happ 1969, Estrada et al. 2011).

Although we lack information on male paternity share for most gift-giving taxa, nuptial gifts can also influence what proportion of offspring produced by a multiply-mated female gets sired by the gift-giving male. For example, larger salivary secretions (endogenous oral gifts) offered by male *Panorpa* scorpionflies increase male paternity share by increasing copulation duration (Sauer et al. 1998, Enqvist et al. 2007). Also, larger spermatophores (endogenous genital gifts) increase the paternity share of male *Photinus greeni* fireflies (South & Lewis, manuscript in revision). Many nuptial gifts (including exogenous oral, endogenous oral, and endogenous genital gifts) have been demonstrated to play a role in increasing female fecundity, either through nutritive contributions or allohormones that stimulate female ovulation or oviposition (see Boggs 1995, Eberhard 1996, Arnqvist & Nilsson 2000, Gwynne 2008, South & Lewis 2011). A central concern is that these gifts will provide a fitness benefit to males only if they increase the total number of offspring sired by the gift-giving male, yet the requisite information on offspring paternity is often not gathered.

As described in Section I.C.3 above, endogenous genital gifts can contain chemical defenses that protect a female and/or her eggs against predators, thus increasing offspring survival (e.g. cantharidin in spermatophore of male *Neopyrochroa* beetles [Eisner et al. 1996), pyrrolizidine alkaloids in *Utetheisa ornatrix* moths [reviewed by Eisner & Meinwald 1995])).

Thus, male costs incurred in manufacturing or procuring nuptial gifts are apparently outweighed by fitness benefits that can accrue during multiple selection episodes before, during and after mating. Endogenous nuptial gifts are especially likely to contain complex mixtures that will operate across across multiple selection episodes to increase male fitness. Also, nuptial gift composition and the associated male fitness benefits will shift dynamically over time due to coevolutionary interactions between the sexes.

IV. A Case Study of Male Benefits: *Requena verticalis*

A. Orthoperan Nuptial Gifts

Orthopteran insects (grasshoppers, crickets, and katydids) display a dazzling array of endogenously produced nuptial gifts (Figure 4); these include male body parts and glandular secretions that females absorb orally, genitally, or in some cases, both. Female *Pteronemobius* and *Allonemobius* (Gryllidae: Nemobiinae) ground crickets receive an endogenous oral gift by chewing on a modified hindleg spur and drinking the male's hemolymph (Mays 1971, Fedorka & Mousseau 2002). Similarily, female *Cyphoderris* hump-winged crickets (Tettigonioidea: Haglidae) drink hemolymph after feeding on the male's fleshy hindwings (Morris 1979, Dodson et al. 1983). In *Oecanthus* tree crickets

(Gryllidae: Oecanthinae), females consume secretions produced by dorsal glands on the male's thorax (Brown 1997); such metanotal gland feeding also occurs in many other orthopterans (see Vahed 1998). Many species with endogenous oral nuptial gifts also transfer a genital gift during mating in the form of a spermatophore (Vahed 1998, Gwynne 2001).

Figure 4. Diverse endogenous oral gifts are produced by males within the insect order Orthoptera (crickets, katydids, grasshoppers and their allies): A) After mating, a female Mormon cricket (*Anabrus simplex*) consumes the gelatinous spermatophylax portion of the male's spermatophore (Photo by Darryl Gwynne). B) A female tree cricket (*Oecanthus quadripunctatus*) feeds on the secretions from a male's metanotal gland (Photo by Kevin Judge). C) A female hump-winged cricket (*Cyphoderris*) feeds on a male's hindwings (Photo by David Funk).

In most species within the suborder Ensifera (katydids, crickets, and wetas), males produce spermatophores that can comprise between 2% to 40% of their total body weight. These two-part structures are produced by two distinct accessory glands: the smooth glands produce the small, sperm-containing ampulla, while the rough glands produce the larger, gelatinous spermatophylax (Gwynne 1997, 2001). During copulation, the ampulla tube is inserted into the female's genital opening, while the remainder of the gift is deposited externally. When the male departs after coupling, the female ingests the spermatophylax while sperm and associated seminal fluid drain from the ampulla into the female's

reproductive system. Once the female finishes consuming the spermatophylax, she removes and consumes whatever remains of the ampulla (Gwynne 1984, Sakaluk 1984, Gwynne et al. 1984).

The Australian katydid *Requena verticalis* (family Tettigoniidae) has been extensively studied as a model system for understanding the costs and benefits of nuptial gifts for both sexes. Thus, this species provides an excellent case study for illustrating the episodes of selection framework presented in section III above. Like other tettigonids, *R. verticalis* males produce a two-part spermatophore that is approximately 15-20% of total male body weight (Davies & Dadour 1989). The spermatophylax alone, (without the sperm-containing ampulla), comprises 78% of total spermatophore weight (Bowen *et al*. 1984). The spermatophylax is composed of 13.5% protein (Bowen et al. 1984). In this species both sexes mate multiple times and both courtship & mating are costly to males. The chirping acoustic signals that males use to attract females require an energetic investment that averages 3.2 kJ/hour (Bailey *et al*. 1993), and spermatophore production requires 1.1 kJ (Simmons *et al*. 1992a); together these two components make up approximately 70% of a male's daily energy budget (Simmons *et al*. 1992a). Nuptial gift costs also limit male mating frequency. After mating, males require 2.5 to 5 days (depending on diet quality) to manufacture another spermatophore before they can mate again (Davies & Dadour 1989; Gwynne 1990). Furthermore, when male diet is restricted, males invest less energy into courtship signals but nuptial gift production remains constant (Simmons *et al*. 1992a).

What fitness benefits might balance out these well-established costs of nuptial gifts for *R. verticalis* males? Here we expand on Gwynne's (1997) analysis of current gift function to examine the fitness benefits that males derive from spermatophylax production across multiple selection episodes that occur before, during and after mating.

B. Fitness Benefits to Males

1. Male insemination success and sperm numbers transferred

When *R. verticalis* females are deprived of a spermatophylax they will remove and eat the ampulla, effectively halting sperm transfer (Gwynne *et al*. 1984). Gwynne *et al*. (1984) demonstrated that while sperm drainage from the ampulla is completed within 3 hours, females take ~ 5 hours to eat the spermatophylax before moving on to consume the ampulla. In some other orthoperan species, in contrast, male spermatophylax size attains only the minimum necessary to ensure complete sperm drainage (e.g. the cricket *Gryllodes supplicans; Sakaluk* 1984). Thus, the *R. verticalis* spermatophylax serves to protect male ejaculates by insuring insemination and maximizing the number of sperm transferred (selection episodes 3 & 4 in Figure 3).

2. Paternity Share

Male nuptial gifts in *R. verticalis* also affect male paternity share postmating (Figure 3, episode 6). Laboratory studies indicate that *R.verticalis* generally show complete first-male sperm precedence; that is, the first male that mates with a virgin female will sire all of her offspring even when the female remates (Gwynne 1988a; Simmons & Achmann 2000). When females were given

a longer intermating interval and allowed to oviposit between matings, 2nd males gained ~20% paternity share (Gwynne & Snedden 1995). In addition, 1st males that had greater spermatophore mass gained higher paternity share. Radiolabeling studies show that amino acids derived from 2nd males become incorporated into eggs that were fertilized by the 1st male; thus, 2nd male gifts are allocated to offspring sired by another male. However, such cuckoldry may happen infrequently under natural conditions, as field estimates of female polyandry suggest that females in nature remate less frequently than in the lab (Simmons et al. 2007).

The high degree of first-male paternity seen in *R. verticalis* suggests that males should be selected to preferentially mate with virgin females, but males appear incapable of discriminating females' mating status (Lynam *et al*. 1992; Simmons *et al*. 1993, 1994). This may represent sexual conflict, with selection acting on females to hide their mating status to obtain the benefits provided by additional spermatophores (Simmons *et al*. 1994). However, Simmons *et al*. (1994) did find that males are able to discriminate among potential mates based on female age. By preferentially mating with younger females, males may increase their chance of mating with virgins and may thus gain higher paternity share.

Sperm competition theory predicts that males should strategically allocate their ejaculates depending on female mating status (Simmons 2001); when mating with a previously-mated female, males should maximize sperm number in the ampulla (to increase their sperm offense ability), but minimize spermatophylax

investment given the low probability of siring offspring. Instead, Simmons *et al*. (1993) found that when mating with young females, *R. verticalis* males transfer identical spermatophores regardless of female mating status; however, males transfer spermatophores with 50% more sperm and 25% less spermatophylax material when mating with older compared to younger females. Thus, *R. verticalis* males appear to strategically allocate their ejaculates when mating with older females to increase their sperm offense ability, and thus their potential paternity share.

Nuptial gifts produced by *R. verticalis* also affect male postcopulatory fitness by increasing sperm defense. Given the high cost of producing nuptial gifts, males should be selected to increase female latency to remate as a mechanism of reducing sperm competition. By experimentally manipulating ampulla attachment times, Gwynne (1986) demonstrated that the ampulla contains receptivity-reducing substances that act in a dose-dependent fashion, normally rendering females non-receptive for approximately 4 days. Substances in the ampulla also appear to negatively affect female longevity. Wedell *et al*. (2008) found that when females received the contents of 3 male ampullas (each without a spermatophylax), they had significantly shorter lifespans, and this negative effect was not counteracted by spermatophylax consumption.

3. Female Fecundity and Egg/Offspring Survival

Finally, nuptial gifts can also increase male fitness through effects on female fecundity and the survival of offspring sired by the gift-giving male (Figure 3, selection episodes 7 and 8). Radiolabeling experiments demonstrated that male

protein derived from the *R. verticalis* spermatophore is incorporated into the female's eggs (Bowen *et al*. 1984; Gwynne 1988a). Furthermore, females that consume more spermatophylaces produce more and heavier eggs (Gwynne 1984), and offspring from larger eggs had greater overwintering survival (Gwynne 1988b). If receiving spermatophylax nutrients directly benefits female fitness, nutrient-limited females would be expected to seek out matings to obtain additional nuptial gifts. Indeed, female *R. verticalis* females kept on a low quality diet remate more often than females kept on a high quality diet (Gwynne 1990).

By applying this framework in *R. verticalis*, we see that nuptial gifts increase male fitness across during several episodes of selection, ultimately increasing the number of offspring sired by the gift-giving male. A complete spermatophore (ampulla + spermatophylax) is necessary for insemination to occur (i.e. ejaculate protection), as otherwise the ampulla will be removed and eaten before sperm transfer. Presence of a spermatophylax increases the duration of ampulla attachment, and spermatophylax size exceeds that required for complete sperm transfer. Unidentified substances present in the male ampulla act to reduce female receptivity to additional matings, helping to ensure a male's paternity share relative to his rivals. Additionally, males discriminate against older females that have likely already mated as a mechanism to reduce incidence of cuckoldry. Spermatophylax consumption increases female fecundity, and has the potential to enhance fitness by increasing the number of offspring sired by the gift-giving male. Paternity success of second mating males increases if they mate with a female after she has had an opportunity to oviposit. Finally, spermatophylax

consumption increases egg size, which enhances survival of a male's offspring. Thus, this work on *R. verticalis* nicely illustrates how costly nuptial gifts might provide males with demonstrable fitness benefits measured across several sequential episodes of selection.

V. Phylogenetic Insights into the Evolution of Nuptial Gifts

Despite the key role that nuptial gifts play in the reproductive ecology of so many animals, surprisingly few studies have rigorously examined the evolution of nuptial gifts using a comparative phylogenetic approach. To thoroughly test the various evolutionary scenarios that have been proposed for nuptial gifts, it will be essential to map gifts and other relevant traits onto robust phylogenies developed for particular taxa. Using this approach will provide insight into the evolutionary sequence of gift trait transitions, and will also allow tests of correlated evolution between nuptial gifts, life-history, and ecological traits. Although to date relatively few studies have applied these methods, here we review work from three insect taxa where a comparative phylogenetic approach has provided insight into nuptial gift evolution: endogenous oral gifts within katydids and crickets (Ensifera: Orthoptera), correlated evolution of wingless females and male nuptial gifts in fireflies (Lampyridae: Coleoptera), and patterns of male ejaculate incorporation, as well as rates of seminal fluid protein evolution, in the genus *Drosophila* (Drosophilidae: Diptera).

A. Endogenous oral gifts in katydids & crickets

The first comparative phylogenetic study of nuptial gift evolution was presented by Gwynne (1995,1997, 2001), who examined the origins and

elaboration of edible glandular gifts within the orthopteran suborder Ensifera (katydids, crickets and their allies). Gwynne's (1995) phylogenetic reconstruction was based upon morphological characters, and suggested that the ancestral trait in this group was an exposed spermatophore (essentially a naked sperm–containing ampulla) that was deposited externally on the female genitalia (Figure 5). Female consumption of this unprotected spermatophore was hypothesized to be ancestral for all ensiferans, followed in the superfamily Tettigonioidea by the origin of the spermatophylax as an edible addition to the spermatophore. In the evolutionary branch leading to the family Gryllidae (true crickets), there were numerous origins of diverse glandular gifts consumed by females before and after mating, along with male spermatophylaces.

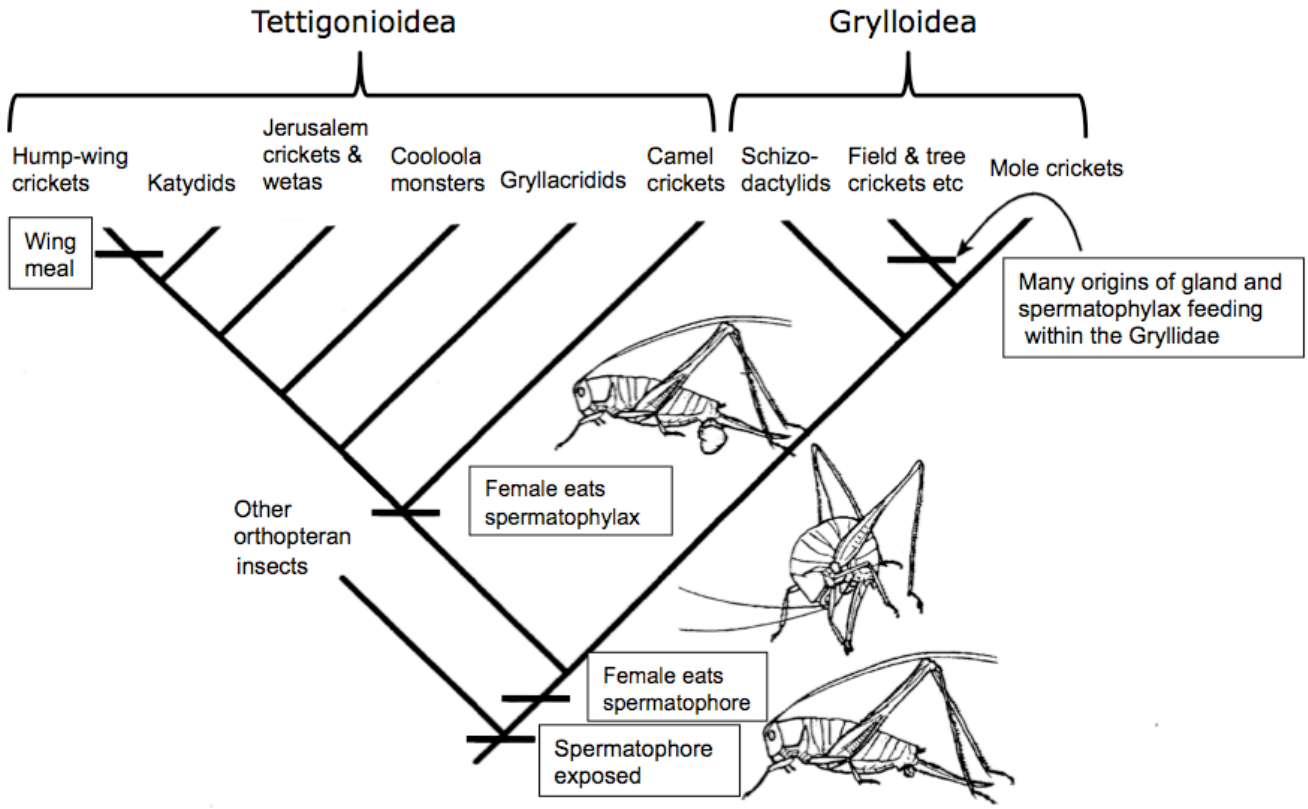

Figure 5. Proposed evolution of spermatophylax nuptial gifts within the orthopteran suborder Ensifera (two superfamilies indicated), with gift-related traits mapped onto the most parisimonous tree based on morphological characters (figure modified from Gwynne 2001).

A more detailed analysis (Gwynne 1995, 1997) showed a total of 11 origins of males producing endogenous oral gifts within the Ensifera; these included 3 origins of a spermatophylax, 4 origins of metanotal glands, 1 tibial gland and 3 others instances where females feed on other male body parts. This work also indicated several independent spermatophylax losses or size reductions; these occurred in some wetas (Stenopelmatidae; loss in *Deinacrida*, size reduction in *Hemideina*) and katydids (Tettigoniidae; loss in *Tympanophora, Decticita,* size reduction in *Neoconocephalus*). Interestingly, such losses were often associated with origins of other endogenous gifts, such as secretions from metanotal or tibial glands. Within the Grylloidea, Gwynne's analysis reveals that there were also 7 likely losses of nuptial gifts and 3 origins of postcopulatory mate guarding.

This analysis supports an evolutionary scenario in which males first used a simple, externally attached ampulla to transfer their sperm (for review see Gwynne 2001). Food limitation may have initially driven females to consume this proteinaceous package, leading to sexual conflict over ampulla attachment times. Selection on males to maximize sperm transfer could have lead to the origin of male reproductive glands that produced an additional spermatophore component, the edible spermatophylax. Thus, the spermatophlyax likely originated as an ejaculate protection mechanism, prolonging ampulla attachment times and allowing sufficient time for sperm to fully drain from the ampulla into the female reproductive tract. Further elaboration of the spermatophylax might have occurred if male gifts increased the number of offspring sired by increasing female fecundity and/or offspring survival. As females increased their mating

rates to obtain nutritional supplements from these oral gifts, male ejaculates (genital gifts) would have undergone selection to include compounds that suppress female receptivity to further matings, thus reducing sperm competition risk.

While this analysis provides considerable insight into the evolution of orthopteran nuptial gifts, additional work could increase taxon coverage and incorporate more detailed information on species' life history and ecological traits. Further studies could also help elucidate what conditions led to the spermatophylax loss seen across several ensiferan lineages, and what factors underlie the explosion of nuptial gift diversity seen among modern day Orthoptera.

B. Firefly spermatophores: Coevolution with female flight

Recent work on fireflies (Coleoptera:Lampyridae) also shows the power of a comparative phylogenetic approach, and offers new insights into how nuptial gift evolution is linked to other life history traits (South et al. 2011*a*). This analysis also allowed reconstruction of ancestral character states as well as the sequence of evolutionary transitions, and demonstrated trait coevolution between the sexes.

Based upon Tallamy's (1994) enhanced fecundity hypothesis and Boggs' (1990) female allocation model, Lewis & Cratsley (2008) developed a conceptual model to explore the evolution of nuptial gifts in lampyrids as a function of female allocation tradeoffs between flight and reproduction. Because nuptial gifts can link together male and female resource budgets, they have the potential to alter the allocation strategies used by both sexes. Thus, selection for nuptial gifts

might depend on female reproductive allocation, which in turn depends on allocation to other activities, including flight. If females do not require flight ability, fecundity selection can act to maximize female reproductive allocation; in this case, because female reproductive output is already at its maximum (E_{max} in Boggs 1990), male nuptial gifts will have limited scope to further enhance female fecundity. On the other hand, when female reproductive allocation is constrained by the energetic and biomechanical demands of flight, nuptial gifts could provide larger proportional fecundity increases for females. Therefore, this model predicts that nuptial gifts would not be selected in species with flightless females. Fireflies present an opportunity to test this relationship, as they demonstrate variation in not only nuptial gift-giving, but also in female flight ability.

As fireflies are capital breeders and both sexes mate multiply, nuptial gifts can have major fitness consequences for both sexes (Lewis and Cratsley 2008). Firefly nuptial gifts consist of spermatophores (endogenous genital gifts) that are manufactured by several accessory glands and transferred to females during mating (Lewis et al. 2004). Some female fireflies possess a specialized reproductive sac to receive and break down the spermatophore after sperm are released into the female spermatheca (van der Reijden et al. 1997). Radiolabeling experiments in *Photinus* fireflies have shown that spermatophore-derived proteins are incorporated into the female's developing oocytes (Rooney & Lewis 1999), and male gifts benefit females by increasing their lifetime fecundity (Rooney & Lewis 2002). Gift production is costly for males, as spermatophore size declines across successive matings in *Photinus* (Cratsley et al. 2003). Among the 2000

extant species of firefly worldwide, spermatophores are present in some (van der Reijden *et al*. 1997, South *et al*. 2008, 2011), yet absent in others (Hayashi and Suzuki 2003, Lewis *et al*. 2004). Those species that lack spermatophores show reduced male accessory glands and females do not have a spermatophore-receiving sac (Demary & Lewis 2007, South *et al*. 2011). What accounts for such interspecific variation in nuptial gifts?

Fireflies also exhibit extensive interspecific variation in life history traits. In some fireflies, females have greatly reduced wings and as a result are flightless, while in other species both sexes have normal wings and can fly (Jeng 2008). Hayashi & Suzuki (2003) first proposed that female wing reduction might be negatively associated with male nuptial gifts in Japanese fireflies. Thus, the existing variation in both spermatophore production and female flight within the Lampyridae provided an opportunity to test Lewis & Cratsley's (2008) model and to examine whether this life history trait could help explain how nuptial gifts are distributed across fireflies.

South *et al*. (2011) performed a phylogenetic analysis of the relationship between spermatophore production and female flightlessness within the Lampyridae (Figure 6). These two traits were measured in 32 taxa and mapped onto a lampyrid molecular phylogeny constructed by Stanger-Hall *et al*. (2007). Ancestral state reconstruction revealed it was highly likely that firefly males originally produced spermatophores, but these nuptial gifts were subsequently lost in 4 separate lineages (Figure 6, right). This reconstruction also revealed that ancestral fireflies had flight-capable females, and females then lost their flight

ability at least 5 times (Figure 6, left). Furthermore, this work revealed a remarkably congruent pattern between male nuptial gifts and female flight, with the correlated loss of both female flight and male gifts occurring in many lineages. This congruence (statistically confirmed by Pagel's test of correlated evolution) demonstrated coevolution between two traits expressed in different sexes. Finally, transitional probability analysis demonstrated that first females lost their flight ability, subsequently followed by male spermatophore loss.

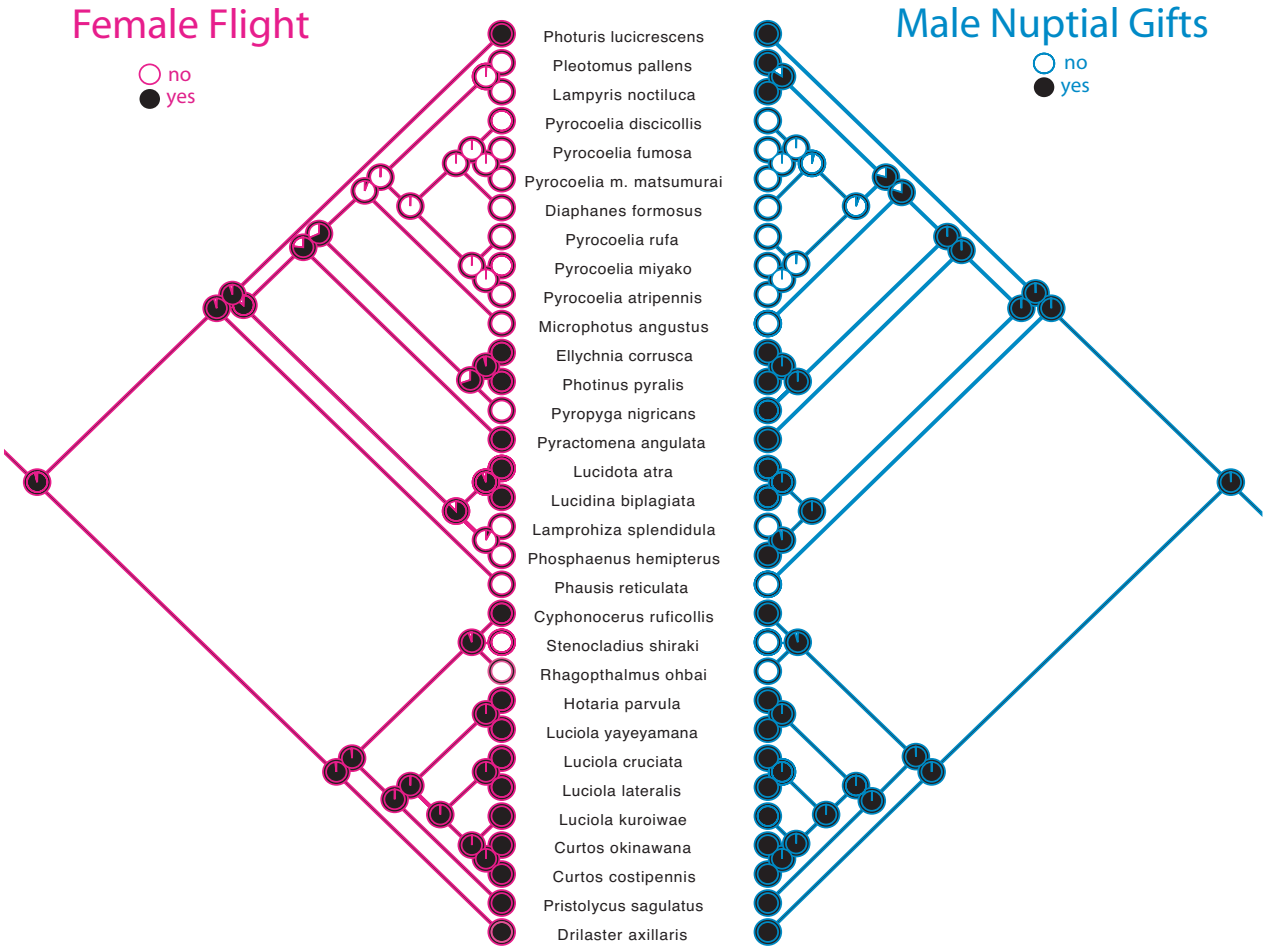

Figure 6. Firefly beetles (Coleoptera: Lampyridae) show correlated evolution (Pagel's test, $P < 0.001$). between male nuptial gifts (spermatophores) and female flight ability (based on presence of functional wings). For 32 worldwide firely species, these two traits were mapped onto a lampyrid molecular phylogeny based on 18S, 16S, and *cox1* DNA sequences (Stanger-Hall et al. 2007). For each trait, pie charts at each node indicate the proportional likelihood support for ancestral states (figure modified from South *et al*. 2011).

Thus, female flight ability provides a compelling explanation for observed patterns of nuptial gifts in fireflies, but what selected for female flight loss in the first place? Based upon considerable evidence demonstrating that flightless females can allocate more to reproduction, the most likely explanation for female-specific flight loss is selection for increased fecundity. Thus, these results strongly support the conclusion that male nuptial gifts are co-adapted with patterns of female reproductive allocation, at least in fireflies. These results could be broadly applicable to other capital breeders, and could help explain patterns of nuptial gift evolution in other taxa. Further studies are needed to see whether variation in other ecological and life history traits associated with resource allocation can provide additional insights into nuptial gift evolution.

C. Female incorporation of ejaculate-derived proteins in *Drosophila* fruit flies

In our taxonomy of nuptial gifts, the category of endogenous genital gifts explicitly includes seminal products that are transferred in a liquid ejaculate; this occurs in many Diptera, including *Drosophila* fruit flies. *Drosophila* species vary widely in several aspects of their mating systems, including: female remating latency, male ejaculate composition, mating behavior, and the degree to which substances from male ejaculates are incorporated into female tissue (Markow & Ankey 1984, Pitnick *et al*. 1997, Markow 2002, Markow & O'Grady 2005). Studies mapping reproductive traits onto a *Drosophila* phylogeny provide insight in the evolutionary history of these unpackaged nuptial gifts.

For 34 species of *Drosophila,* Pitnick *et al*. (1997) used radiolabelled amino acids to determine how much protein transferred in male ejaculates was incorporated into female ovarian or somatic tissue. *Drosophila* species showed dramatic variation in the degree to which females incorporated male-derived proteins (Figure 7). Females in most species, including those in the *melanogaster* group, showed no incorporation into their ovarian tissue and about half showed no incorporation into somatic tissue. However, *Drosophila* species within the *subpalustris* group showed substantial incorporation into somatic tissue, and those within the *mojavensis* cluster showed substantial incorporation of male-derived protein into both somatic tissue and oocytes.

By mapping these data onto a molecular phylogeny, Pitnick *et al*. (1997) showed that incorporation of male-derived protein into female somatic tissue has independently evolved multiple times (Figure 7). In the *mojavensis* cluster, high levels of ovarian incorporation were also seen to accompany high incorporation into somatic tissue. This phylogeny also reveals some degree of lability, as incorporation into both tissue types seems to have been subject to both gains and losses. Thus, this work provides evidence that multiple *Drosophila* groups have evolved male ejaculates that contribute to female somatic maintenance or reproduction.

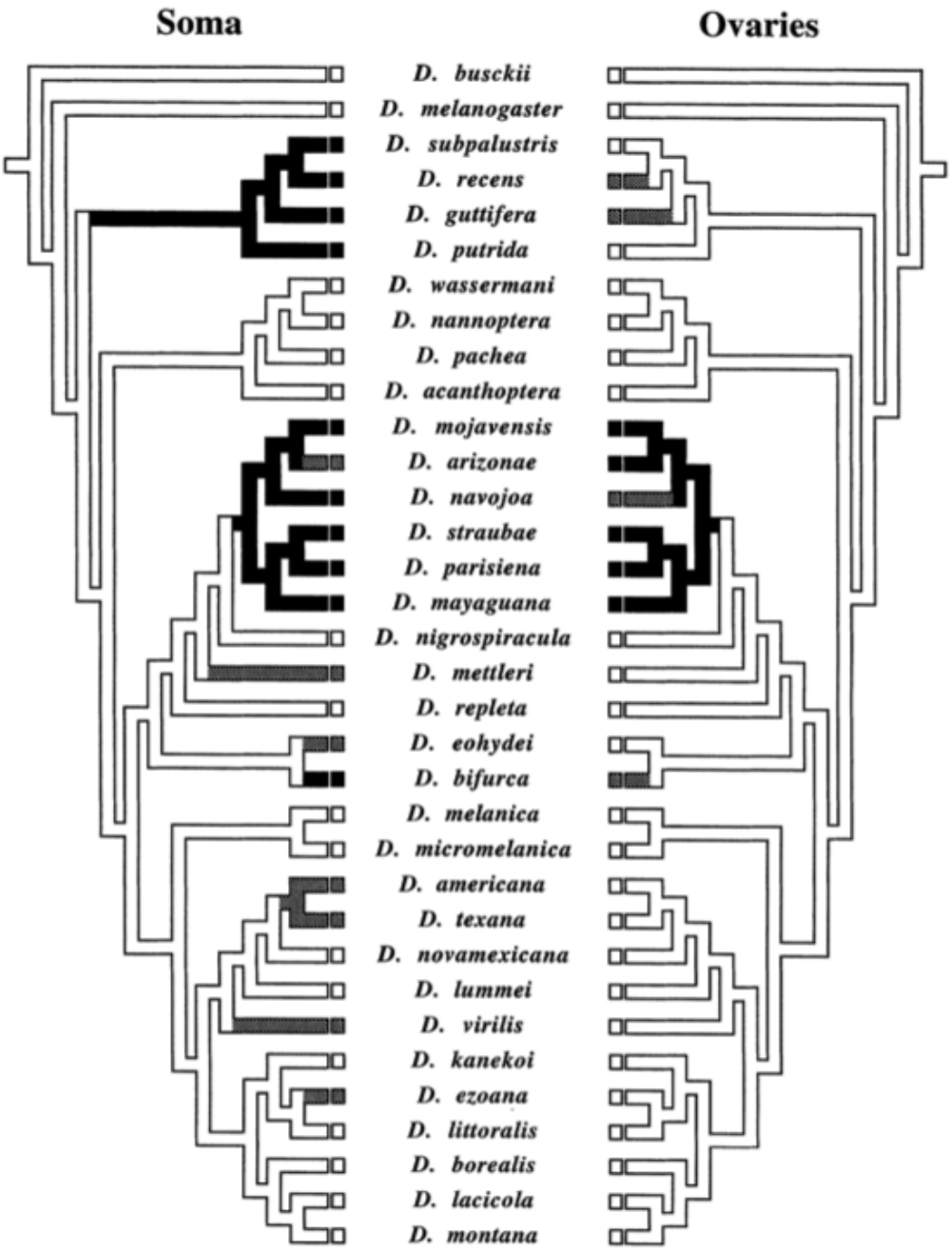

Figure 7. Phylogenetic distribution of nutritive male ejaculates in 34 species of *Drosophila* fruitflies (Diptera: Drosophilidae). Shading indicates the degree to which females have incorporated ^{14}C-labelled proteins derived from male ejaculates into their somatic tissue and ovaries 6-8 h after mating. White bars indicate no female incorporation (0-50 corrected DPM), grey bars indicate a small degree of female incorporation (51-100 DPM), and black bars indicate substantial incorporation of male-derived protein by females (>100). Figure from Pitnick *et al*. (1997).

As pointed out by Pitnick *et al*. (1997), these patterns of female ejaculate incorporation might be related to differences among species in their nutritional ecology, as the host resources exploited by *Drosophila* vary widely in quality (Markow & O'Grady 2008). Species in the *mojavensis* group (*D. mojavensis*, *navojoa*, *D. straubae*, *D. parisiena* & *D. mayaguana, D. arizonae*) all breed and feed on necrotic cactus (see Markow & O'Grady 2005, 2008), which contains lower levels of both nitrogen and phosphorus compared to the fruit hosts used by the *melanogaster* group (Markow *et al*. 1999). In addition, by manipulating nutritional content of the cactus host, Brazner *et al*. (1984) showed that *D. mojavensis* are likely to undergo frequent nutritional stress. Markow *et al*. (1990) showed that *D. mojavensis* females kept on low quality diets experienced enhanced fecundity from the receipt of male ejaculate, and suggested that nutritive ejaculates are more likely to evolve when adults are subject to nutrient limitation. Consistent with the enhanced fecundity hypothesis (Tallamy 1994), females in some cactophilic, and presumably nutrient-limited, *Drosophila* species show a high degree of male ejaculate incorporation (Pitnick *et al*. 1997). In addition, Markow *et al*. (2001) found that in *Drosophila nigrospiracula*, a cactophilic species subject to larval phosphorus limitation, mated females incorporate phosphorus derived from male ejaculates into oocytes. Therefore, variation in resource availability among *Drosophila* species may be one factor in the evolution of nutritive male ejaculates that contribute to female somatic maintenance and reproduction.

The presence of nutritive male ejaculates also shows strong phylogenetic correlations with other features of *Drosophila* mating systems (Markow 2002). Across 21 *Drosophila* species, Markow (2002) found strong congruence between female mating frequency and exaggerated male ejaculates (these include nutritive male ejaculates). Additionally, Markow (2002) suggested that the evolution of nutritive male ejaculates may have been preceded by higher female remating rates. However, because so many reproductive traits covary with mating systems, additional work is needed to test the sequence of these evolutionary transitions. The extensive knowledge base for *Drosophila* concerning host use, life histories, and reproductive traits makes this a compelling system for examining specific factors that promote the evolution of endogenous genital gifts.

In summary, in this section we present previous work that has taken a comparative phylogenetic approach to decribe and test hypotheses about nuptial gift evolution. To rigorously test the various evolutionary scenarios that have been proposed for nuptial gifts, it will be essential to map gifts and other relevant traits onto robust phylogenies developed for particular taxa. We emphasize how valuable it is to include life history and ecological traits in such evolutionary analyses. This approach should provide insight into the evolutionary sequence of gift transitions, and will also allow formal tests of correlated evolution between male gifts and other traits that can influence their evolutionary trajectory.

D. Evolutionary Rates of *Drosophila* Seminal Proteins

Drosophila male ejaculates are certainly the most well-characterized of all endogenous nuptial gifts. Their non-sperm components comprise a complex

cocktail of molecules produced by male accessory glands and secretory tissues in the male ejaculatory duct. While many different types of molecules are transferred within *Drosophila* male ejaculates, research has focused on seminal fluid proteins (SFPs). Once transferred, these SFPs engage in dynamic molecular interactions within the female reproductive tract, and this sexual interplay is likely to influence SFP evolution. Because these molecules have been so well-studied, research on SFP evolutionary rates can contribute to a broader understanding of nuptial gift evolution.

Nearly 150 different SFPs have been identified from the ejaculate of *D. melanogaster* males, and these proteins initiate many physiological and behavioral changes within mated females (reviewed by Avila *et al*. 2011). Significant changes in female gene expression are seen 1-3 hours following the receipt of ejaculate, and are maximized at 6 hours post-mating. Conformational changes of the female reproductive tract allow for sperm storage, and the oviduct shows increased innervation and enhanced formation of myofibrils (Adams & Wolfner 2007, Kapelnikov *et al*. 2008). Specific SFPs are necessary for female sperm storage and release, while others improve sperm survival (Xue & Noll 2000, Ravi Ram & Wolfner 2007). Male SFPs increase female egg production and ovulation (Heifetz *et al*. 2000, Ravi Ram & Wolfner 2007), initiate the formation of a mating plug (a gelatinous mass containing sperm; Lung & Wolfner 2001, Bretman *et al*. 2010), and cause females to actively reject courting males. Female activity levels also increase following mating, with increased foraging

(Carvalho et al. 2006) and 70% less sleep (Isaac et al. 2010), possibly leading to shorter lifespans for mated females (Wigby & Chapman 2005, Isaac *et al*. 2010).

Notably, rapid evolution of genes encoding male SFPs has been documented in *Drosophila* as well as other taxa (Clark *et al*. 2006, Vacquier 1998, Swanson & Vacquier 2002). Comparisons between *D. melanogaster* and *D. simulans* demonstrated high rates of non-synonymous nucleotide substitution in SFP genes compared to non-SFP genes (Swanson *et al*. 2001). Sequence comparisons between *D. melanogaster* and *D. pseudoobscura* of 52 SFP-encoding genes from male reproductive accessory glands detected only 58% conserved as true orthologs (Mueller *et al*. 2005). Such rapid and dynamic evolution of SFPs is likely due to postcopulatory sexual selection (Swanson & Vacquier 2002, Panhuis *et al*. 2006, Clark *et al*. 2006). Sperm competition (Birkhead & Moller 1998), cryptic female choice (Eberhard 1996) and sexual conflict (Parker 1979) may all contribute to a coevolutionary arms race between and within sexes over control of reproductive outcomes.

Comparisons between *Drosophila* species can be used to test the prediction that SFP evolution will proceed more rapidly when postcopulatory sexual selection is more intense. Mating systems and reproductive ecology differ dramatically between species in the *repleta* group and those in the *melanogaster* group. *D. repleta* males transfer a nutritive ejaculate, and females remate more frequently (Markow & Ankey 1984, Pitnick *et al*. 1997, Markow 2002). In addition, many *repleta* species show an insemination reaction, consisting of an opaque mass that develops within the female vagina after mating. This is thought

to prevent females from remating, thus protecting the male's nutritional investment from cuckholdry by rival males (Markow & Ankney 1984, 1988). Based on these differences in reproductive ecology, species in the *D. repleta* group appear subject to more intense postcopulatory sexual selection and thus are predicted to show faster rates of SFP evolution compared to *D. melanogaster*. Supporting this prediction, several studies have shown that SFP genes expressed by male accessory glands in the *repleta* group evolve more rapidly than those in the *D. melanogaster* group (Wagstaff & Begun 2005, Wagstaff & Begun 2007, Almedia & DeSalle 2009). In the *repleta* group, SFP genes also show high rates of gene duplication, which is suggested to facilitate adaptive protein evolution (Ohno 1970, Walsh, 2003). Thus, *repleta* SFPs appear to be undergoing rapid evolution, potentially due to differences in their reproductive ecology.

Consistent with the prediction that sexual coevolution is responsible for rapid evolutionary changes in male gifts, some female reproductive proteins in the *repleta* group also show rapid adaptive evolution, and gene duplication has also been important in the evolution of these proteins (Kelleher *et al*. (2007). Of particular interest are several digestive proteases, which Kelleher *et al*. (2007) suggest might play a role in breaking down the mating-induced insemination reaction. Interestingly, male ejaculates in *D. mojavensis* contain protease inhibitors (Wagstaff & Begun 2005), two of which have also experienced lineage-specific gene duplication events (Kelleher *et al*. 2009). The reproductive tract of female *D. arizonae* (a close sister species to *D. mojavensis*) shows exceptionally high proteolytic activity that is negatively regulated by mating (Kelleher &

Pennington 2009). Taken together, these results from different *repleta* species suggest active sexually antagonistic coevolution around the insemination reaction, with male protease inhibitors acting to prevent male ejaculate components getting broken down by female proteases.

Thus, rapid evolution of *Drosophila* nuptial gifts appears to be driven by a complex sexual interplay taking place at the molecular level. While some male-derived proteins are incorporated into female oocytes and somatic tissue, other seminal fluid proteins may have evolved to counter defenses mounted by females to prevent male manipulation. Further exploration of these dynamic sexual interactions should provide many insights into the constantly shifting balance between the costs and benefits of nuptial gifts.

VI. Conclusions and Future Directions

Animal nuptial gifts take multitudinous forms, and their evolutionary stories promise to be just as diverse. In this overview, we have tried to offer a fresh perspective on the evolution of animal nuptial gifts. We argue for a broader definition of nuptial gifts that can accommodate anticipated lability of nuptial gift structure and function arising from coevolutionary interactions both between and within the sexes. By systematically classifying nuptial gifts according to how they are produced (endogenous vs. exogenous) and how they are absorbed by the recipient (oral, genital, or transdermal), we hope to establish a robust framework for testing predictions about how gifts influence both male and female fitness. Rather than attempting to place potential benefits gained by gift-giving males into the falsely dichotomous categories of parental investment vs. mating effort, we

illustrate how nuptial gifts might enhance male fitness across multiple selection episodes that occur before, during, and after mating. Finally, we highlight some studies that have greatly advanced our understanding by using comparative phylogenetic methods to examine how nuptial gifts and associated life history traits have changed over evolutionary time.

We hope this foundation will inspire future research efforts to enhance our understanding of nuptial gift evolution. Despite many advances, there remain several areas that clearly call out for more focused research efforts:

- We have detailed morphological descriptions of the glands which are responsible for manufacturing many endogenous gifts (e.g. Leopold 1973, Liu & Hua 2010). In many taxa, nuptial gifts are the combined productions of multiple glands, yet much work remains to fully characterize these glandular products. Transcriptome studies of gene expression within gift-manufacturing glands will provide insight into differences and similarities in their gene products and associated functions. For example, to what extent has convergent evolution occurred between those male reproductive glands that produce oral vs. genital gifts, or between reproductive and salivary glands?

- In considering selection for nutritive nuptial gifts, the geometric framework developed for nutritional ecology (Raubenheimer et al. 2009, Raubenheimer 2011) provides a powerful tool for testing whether male gifts evolved to support female reproduction. Does selection shape male glandular products to provide novel nutritional mixtures that will supplement females' dietary resources, *i.e.* do such gifts act as vectors that specifically target the

requirements of vitellogenesis? We need more detailed biochemical analyses of different types of nuptial gifts to test many of the predictions laid out here.

- Most importantly, there is a compelling need for additional phylogenetic analyses of nuptial gift traits that can provide insight into the evolutionary origin and maintenance of nuptial gifts across different taxonomic groups. Continuing to examine evolutionary patterns within the Orthoptera will be especially interesting, because their nuptial gift types are so variable. Phylogenetic analysis would also be worthwhile in the Lepidoptera, where reconstructing ancestral character states could shed light on possible trajectories of spermatophore evolution. Finally, because nuptial gifts lie at the intersection of nutritional ecology, sexual selection, and life history evolution, testing informed predictions concerning evolutionary associations between nuptial gifts and relevant ecological and life history traits is of fundamental importance.

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Chapter III. The influence of male ejaculate quantity on female fitness: a meta-analysis

ABSTRACT

Although the primary function of mating is gamete transfer, male ejaculates contain numerous other substances that are produced by accessory glands and transferred to females during mating. Studies with several model organisms have shown that these substances can exert diverse behavioural and physiological effects on females, including altered longevity and reproductive output, yet a comprehensive synthesis across taxa is lacking. Here we use a meta-analytic approach to synthesize quantitatively extensive experimental work examining how male ejaculate quantity affects different components of female fitness. We summarize effect sizes for female fecundity (partial and lifetime) and longevity from 84 studies conducted on 70 arthropod species that yielded a total of 130 comparisons of female fecundity and 61 comparisons of female longevity. In response to greater amounts of ejaculate, arthropod females demonstrate enhanced fecundity (both partial and lifetime) but reduced longevity, particularly for Diptera and Lepidoptera. Across taxa, multiply mated females show particularly large fecundity increases compared to singly mated females, indicating that single matings do not maximize female fitness. This fecundity increase is balanced by a slight negative effect on lifespan, with females that received more ejaculate through polyandrous matings showing greater reductions in lifespan compared with females that have mated repeatedly with the same male. We found no significant effect size differences for either female fecundity or longevity between

taxa that transfer sperm packaged into spermatophores compared to taxa that transfer ejaculates containing free sperm. Furthermore, females that received relatively larger or more spermatophores demonstrated greater lifetime fecundity, indicating that these seminal nuptial gifts provide females with a net fitness benefit. These results contribute to our understanding of the evolutionary origin and maintenance of non-sperm ejaculate components, and provide insight into female mate choice and optimal mating patterns.

INTRODUCTION

Although the primary function of mating is gamete transfer, male ejaculates also contain numerous other substances that are produced by reproductive accessory glands (Leopold, 1976; Chen, 1984; Gillott, 1996, 2003; Simmons, 2001; Poiani, 2006). Recent work has identified many of the specific non-sperm components of ejaculates, which include numerous seminal fluid proteins, compounds with immunostimulant and antibiotic properties, as well as antipredator chemical defences (see Gillott, 2003; Poiani, 2006; Wolfner, 2007, for reviews). When transferred to the female, this complex cocktail of non–sperm materials is likely to have profound fitness implications for both sexes.

In arthropods, these substances not only assist in delivering and provisioning sperm, but also have diverse physiological and behavioural effects on females: reducing the likelihood of re-mating, stimulating egg production and oviposition, initiating sperm storage and/or release, and shortening lifespan (Chapman, 2001; Chapman & Davies, 2004; Wolfner, 2007; Chapman, 2008).

Such effects have been most extensively studied in *Drosophila melanogaster* (reviewed in Wolfner, 2002, 2007, 2009) because of the powerful genetic and molecular techniques available for this model organism. Documented effects of *Drosophila* seminal fluid proteins (SFPs) on females include increased egg production (Soller, Bownes & Kubli, 1999), stimulation of ovulation and oviposition (Heifetz *et al*., 2000), decreased receptivity to re-mating (Manning, 1962; Chen *et al*., 1988; Chapman *et al*., 2003), elevated sperm storage (Tram & Wolfner, 1999) and sperm utilization (Tram & Wolfner, 1999), altered feeding behavior (Carvalho *et al*., 2006), and decreased lifespan (Fowler & Partridge, 1989; Chapman *et al*., 1995). SFPs mediate a characteristic short-term response to mating shown by *D. melanogaster* females (Kalb, DiBenedetto & Wolfner, 1993), and some are also essential for a more persistent long-term response (Liu & Kubli, 2003; Peng *et al*., 2005; Ravi Ram & Wolfner, 2007). SFPs have also been implicated as being crucial mediators of *Drosophila melanogaster* sperm competition (Harshman & Prout, 1994; Prout & Clark, 2000; Wigby *et al*., 2009).

Although numerous studies have been conducted in arthropods to elucidate fitness costs and benefits, considerable debate persists about how male ejaculates affect female fitness. Some authors have argued that male ejaculates enhance female fitness by providing nutrients to females or offspring (Boggs, 1990; Vahed, 1998; Gwynne, 2008), while others have proposed that male ejaculates produce a net decrease in female fitness by manipulating female reproduction (Arnqvist & Nilsson, 2000; Gillott, 2003; Vahed, 2007; Wolfner, 2007). Previous attempts to synthesize extensive empirical results include

narrative reviews and meta-analyses that have focused primarily on the fitness effects of polyandry (Ridley, 1988; Simmons, 2005; Arnqvist & Nilsson, 2000). However, these syntheses have largely ignored other factors that also affect ejaculate quantity; such factors include male mating status (virgin or previously mated) and repeated matings by a single male. Other reviews have focused solely on the fitness effects of male nuptial gifts (Boggs, 1990, 1995; Vahed, 1998, 2007; Gwynne, 2008). The term "nuptial gift" encompasses a wide range of structures that are transferred to females during mating, and which may have either positive or detrimental effects on female fitness. Nuptial gifts can be divided into oral gifts, which are absorbed through the digestive tract (e.g. captured prey, secretions of male salivary and other glands), and seminal gifts (i.e. spermatophores) that are absorbed through the reproductive tract. As pointed out by Gwynne (2008), these two categories of nuptial gift may differ in how they affect female fitness. Because seminal gifts provide males with direct access to the female reproductive tract (Sakaluk, Avery & Weddle, 2006), they may be more likely to include manipulative substances that provide a benefit to males at a cost to female fitness (called "Medea gifts" by Arnqvist & Nilsson, 2000). A meta-analysis by Arnqvist & Nilsson (2000) included a comparison of how polyandry affects female fitness between species with and without nuptial "feeding". Importantly, this study pooled species with oral gifts and seminal gifts together and thus could not test directly the Medea hypothesis prediction that male seminal gifts should reduce female fitness. In addition, because this previous meta-analysis was focused on polyandry several other important factors were not

assessed, including distinguishing between polyandrous matings and repeated matings with the same male.

In the present study we conducted a comprehensive meta-analysis to quantify how male ejaculate quantity affects female fitness, which has not previously been done. We focused specifically on male ejaculated substances, defined here as any male-derived material transferred through genital contact to the female (oral gifts were excluded). Male ejaculates include both free ejaculates and spermatophores, which consist of sperm packaged within a structure manufactured by male reproductive glands (Mann 1984). We restricted this meta-analysis to arthropods based on the extensive empirical literature from this group. Meta-analysis provides a powerful tool that can objectively synthesize previous research results, allowing treatment effects to be quantified and effect sizes compared across multiple studies (Hedges & Olkin, 1985; Gurevitch & Hedges, 1993; Nakagawa & Cuthill, 2007). Previous meta-analyses have provided insight into several topics in behavioural ecology and evolution (Arnqvist *et al*., 1996; Gontard-Danek & Moller, 1999;Vollestad, Hindar & Moller, 1999; Arnqvist & Nilsson, 2000; Torres-Vila & Jennions, 2005). Using this meta-analytic approach, our goal was increased understanding of selective forces, such as sexual conflict, driving the evolution of reproductive traits and behaviors, including male spermatophores, female polyandry, and mate choice.

In this meta-analysis we evaluated several factors that could potentially influence the magnitude or direction of male ejaculate effects on female fitness. These factors include: (1) whether males transfer spermatophores or free

ejaculates - if spermatophores represent delivery vehicles for manipulative compounds, we predict that receiving more ejaculate should reduce female fitness to a greater extent in spermatophore-producing species compared to those with free ejaculates. (2) Different experimental designs commonly used to evaluate effects, to help identify particular design artifacts and weaknesses. (3) Taxonomic affiliation (insect orders), which allowed us to examine whether different taxa show similar patterns in how females respond to ejaculate quantity. (4) Effects of polyandry *versus* repeated matings with the same male - this comparison yielded insight into the importance of direct *versus* indirect benefits, as only the former would provide indirect fitness benefits to females.

META-ANALYSIS METHODOLOGY

(1) Selection criteria

We conducted a literature search for relevant studies by querying *Scopus*, *ISI Web of Science*, and *Google Scholar* using keyword combinations that included polyandry, mating rate, spermatophore, nuptial gift and ejaculate size paired with female fitness or fecundity. We also included studies that were referenced in previous narrative reviews (Boggs, 1995; Ridley, 1988; Vahed, 1998) and meta-analyses on related topics (Arnqvist & Nilsson, 2000).

To be included, studies needed to meet several additional criteria: (1) The study included ≥ 2 treatments comparing females that received larger *versus* smaller quantities of ejaculated substances based on differences in known or inferred number of matings, or based on matings conducted with males that differed in their mating history. (2) The study reported at least one measure of

female fitness based on fecundity (egg or offspring production); longevity was sometimes also reported. (3) The study reported means, some measure of variability and sample sizes for each group somewhere in the text, tables, or figures. We found 84 studies published from 1962 to 2008 that met all of these criteria; 80 of these studies were conducted on insects, while four were conducted on other arthropods (details of these studies are included in an online appendix, see Section VIII).

(2) Methods

We classified each study according to several factors that could potentially influence how male ejaculate size affects female fitness, including whether males transfer sperm in spermatophore packages or in free ejaculates, and taxonomic affiliation (Order). In addition, we also categorized studies based on five experimental designs commonly used to investigate effects of mating on female fitness. We classified these designs into comparisons of either: (1) Nonvirgin males *versus* virgin males*:* females received single ejaculates from either previously-mated or virgin males. (2) Small *versus* large spermatophore: females received single spermatophores from either virgin males or previously-mated males. (3) Single mating *versus* multiple matings: females received ejaculate from either a single mating or from multiple (≥ 3) matings. (4) Single mating *versus* double matings: females received male ejaculate from either a single mating or from two matings. (5) Less *versus* more ejaculate: treatments differed in how much exposure females had to males, but the mating rate and number of matings was unknown (e.g. females that were exposed to a single male

for 24 h likely achieved fewer matings compared with females exposed to a single male for 48 h. For treatments in which females mated more than once, we further distinguished between studies in which females were assigned polyandrous matings (≥ 2 matings with different males) *versus* repeated matings (≥ 2 matings with the same male). Finally, we also distinguished between studies that measured fecundity measured over a female's entire lifetime or only part thereof. Whenever it was reported we used female lifetime fecundity, clearly the most comprehensive fitness measure, but many studies reported only partial fecundity. Although reproductive timing may also constitute an important aspect of female fitness (Brommer, Merila & Kokko, 2002; Reinhardt, Naylor & Siva-Jothy, 2009), most published studies did not include such data.

Based on the included studies, we obtained data for 130 comparisons of female fecundity and 61 comparisons of female longevity (some studies yielded more than one comparison). For each comparison we calculated Hedges' *d*, an unbiased weighted estimate of effect size that is typically used with continuous response variables and categorical predictors (Nakagawa & Cuthill, 2007). Hedges' *d* is calculated as the difference between a control and experimental group measured in standard deviation units (Gurevitch *et al*., 1992). In this meta-analysis, the control was always defined as the group in which females received less male ejaculate (either through fewer matings or receiving a smaller spermatophore or ejaculate), while the experimental group was defined as the group in which females received more male ejaculate. Hedges' *d* was separately

calculated for female fecundity (based on either egg or offspring production) and female longevity.

We used MetaWin 2.1 (Rosenberg, Adams & Gurevitch 2000) to calculate mean effect sizes weighted by sample size, and ran an initial analysis combining all studies to determine whether the overall effect sizes for female fecundity and longevity differed significantly from zero. We then examined differences in mean effect size for each factor described above using categorical random effects models, which incorporate both sampling error and a random component as contributors to effect size variation (Hedges & Olkin, 1985: Rosenberg *et al*., 2000). In addition to mean effect sizes, we also report 95% confidence intervals calculated using a bias-corrected bootstrapping approach (with 1,000 replicates used for re-sampling), along with the appropriate test statistics Q_t (total heterogeneity) and Q_b (between-group heterogeneity). If a significant overall difference was detected between groups, then we examined differences in mean effect sizes between specific groups using randomization tests based on 1,000 replicates. We consider Hedges' $d \sim 0.2$ to represent a small treatment effect, $d \sim 0.5$ to be a moderate effect, and $d \sim 0.8$ to be a large effect (Cohen, 1988; Moller & Jennions, 2002; Hagen, Connelly & Schroeder, 2007; Morris *et al*., 2007; Nakagawa & Cuthill, 2007; Sara 2007).

We used several methods to check for publication bias, which arises when published studies are biased towards reporting significant differences. For each analysis, we calculated Rosenthal's fail-safe number and generated funnel plots using MetaWin (Rosenberg *et al*. 2000). Rosenthal's fail-safe number represents

how many unpublished studies with effect sizes equal to zero would be required to negate an effect size significantly different from zero at the $\alpha = 0.05$ level of significance. Funnel plots provide a graphical check for publication bias (Wang & Bushman, 1998; Gurevitch & Hedges, 1999), and have been used widely as a method for ascertaining bias in meta-analyses (Gurevitch *et al*., 1992; Arnqvist *et al*., 1996; Gontard-Danek & Moller, 1999; Vollestad *et al*., 1999). Both approaches indicated that no systematic sampling bias was evident for data on female fecundity. However, for female longevity these two methods for detecting bias differed: Rosenthal's fail-safe number was not robust, although the funnel plot indicated a lack of bias for these data.

RESULTS

(1) Overall effects on longevity and fecundity

When data were combined across all arthropod studies, treatments in which females received relatively more male ejaculate showed effect sizes that were significantly different from zero (*i.e*. the 95% confidence intervals exclude 0) for both female longevity and fecundity (Fig. 1). Across all studies, fecundity showed a significant increase when females received more male ejaculate (Fig. 1; Hedges' $d = 0.4542$, 95% CI: 0.3373 to 0.5658, d.f. = 129, $Q_t = 181.3$, $P = 0.002$).

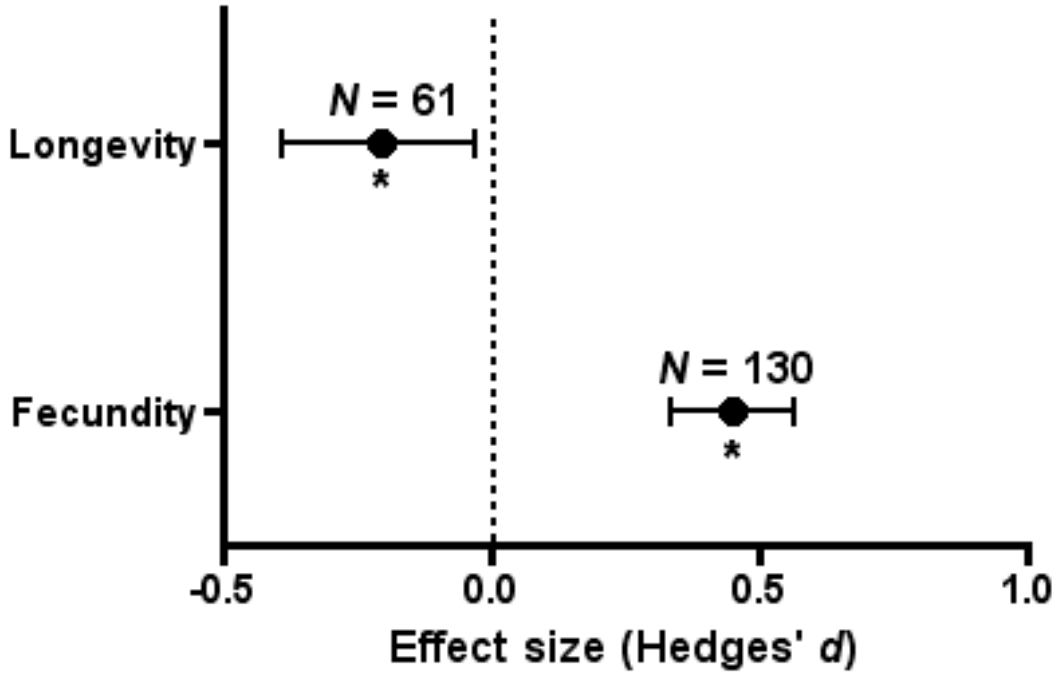

Figure 1. Mean effect size (Hedges' *d* with 95% CI) for arthropod studies that examined the effects of receiving relatively more male ejaculate on female fecundity and longevity. Asterisks indicate effect sizes significantly different ($P < 0.05$) from zero (dotted line).

This positive effect of male ejaculate quantity on female fecundity was remarkably consistent across insect orders; all five orders showed mean effect sizes for fecundity that were significantly greater than zero (Table 1). Also, when we compared studies in which female fecundity was measured over only part of each female's adult lifetime with measurements over her entire lifetime, significantly positive effect sizes were seen for both fecundity measures (Fig. 2), with no significant difference between them ($Q_b = 0.904$, d.f. = 1, $P = 0.341$).

Table 1. Mean effect sizes (Hedges' *d* with 95% CI) and number of comparisons (*N*) classified by insect order that have examined the effects of receiving relatively more male ejaculate on female longevity and fecundity (effect sizes only reported for groups with ≥ 5 comparisons).

Order	Fecundity Effect size (95% CI)	Longevity Effect size (95% CI)
Orthoptera	0.5816 (0.2899 to 0.8960) *N* = 12	0.3612 (-0.1364 to 0.7542) *N* = 7
Lepidoptera	0.2646 (0.1073 to 0.4752) *N* = 38	-0.2560 (-0.5812 to -0.0088) *N* = 22
Heteroptera	0.3603 (0.0556 to 0.8131) *N* = 8	Insufficent data
Diptera	0.6605 (0.3702 to 1.0158) *N* = 23	-0.4501 (-0.6910 to -0.2330) *N* = 6
Coleoptera	0.4847 (0.2273 to 0.7168) *N* = 31	-0.3914 (-0.8241 to 0.0416) *N* = 16

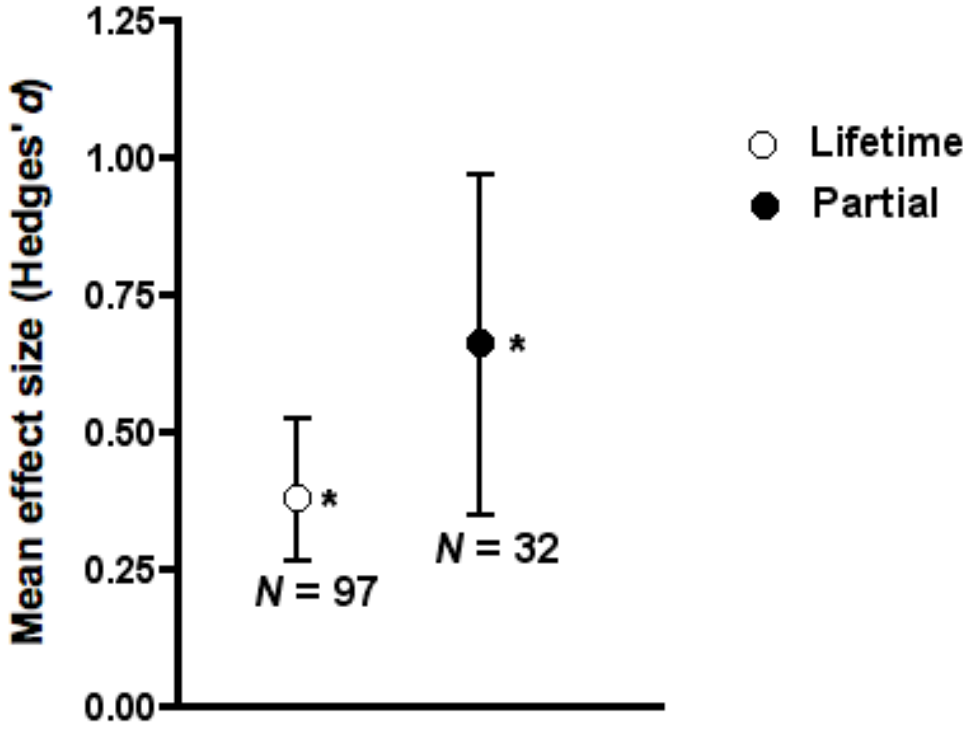

Figure 2. Mean effect sizes (Hedges' *d* with 95% CI) of arthropod studies that examined the effects of receiving relatively more ejaculate on female fecundity categorized according to whether female fecundity was measured over female's entire lifespan or only a portion of it. Asterisks indicate effect sizes significantly different ($p < 0.05$) from zero.

However, male ejaculate showed opposite effects on female longevity; when data were combined across all studies, longevity decreased significantly when females received more male ejaculate (Fig. 1; Hedges' d = -0.2034, 95% CI: -0.3770 to -0.0200, d.f. = 60, Q_t = 82.5, P = 0.029). This decrease in female lifespan was most pronounced in Diptera and Lepidoptera, as mean effect sizes for longevity were significantly less than zero in these two insect orders (Table 1).

(2) Differences between spermatophores and free ejaculates

When we compared taxa in which male ejaculate is packaged into spermatophores *versus* taxa where males transfer ejaculates containing free sperm, we found no significant difference in average effect size between these groups when females received more ejaculate (Fig. 3); this was true for both female longevity (Q_b = 1.3, d.f. = 1, P = 0.262) and fecundity (Q_b = 0.4, d.f. = 1, P = 0.533). When we compared lifetime fecundity for females that received relatively larger or more spermatophores across 30 species (Fig. 3), we found an overall positive effect size (Hedges d = 0.3320, 95% CI: 0.1987 – 0.4807, N = 55 comparisons). This positive effect of larger/more spermatophores on lifetime fecundity was found for Coleoptera, Lepidoptera and Orthoptera (remaining orders had < 5 comparisons).

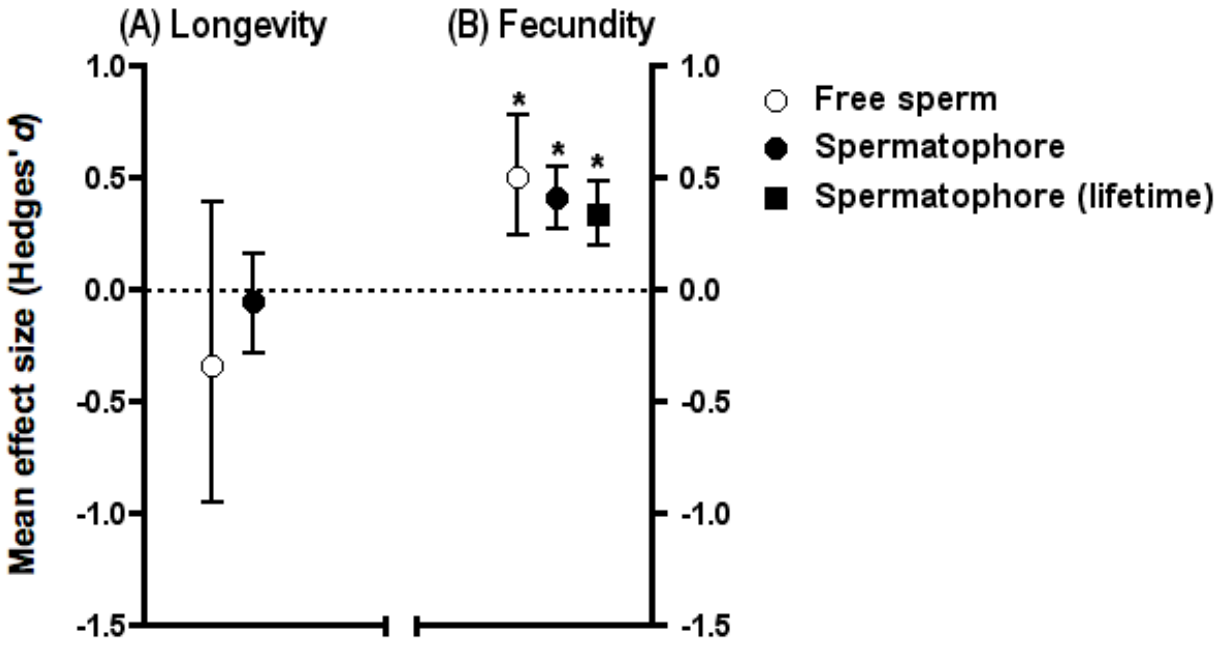

Figure 3. Mean effect sizes (Hedges' *d* with 95% CI) for arthropod studies that examined the effects of receiving relatively more male ejaculate on: (A) female longevity (species in which the ejaculate is packaged into a spermatophore $N = 39$, species with free sperm $N = 10$), (B) female fecundity (species in which the ejaculate is packaged into a spermatophore $N = 67$, species with free sperm $N = 36$). Also shown is the effect size for lifetime fecundity for species transferring spermatophores ($N = 55$). Asterisks indicate effect sizes significantly different ($P < 0.05$) from zero (dotted line).

(3) Differences due to experimental design

Many different experimental designs have been used to assess how relatively more male ejaculate influences female fitness, so we compared average effect sizes among these common study designs (Fig. 4). For female longevity, effect sizes did not differ significantly among study designs ($Q_b = 3.4$, d.f. = 4, $P = 0.488$). However, significantly negative effect sizes were seen in only two study designs (Fig. 4A): (1) virgin males *versus* non-virgin males, and (2) a single mating *versus* multiple (≥ 3) matings. For female fecundity, three study designs

showed significantly positive effect sizes (Fig. 4B): (1) virgin males *versus* non-virgin males, (2) single *versus* multiple matings, and (3) single *versus* double matings. In addition, studies comparing single *versus* multiple matings had fecundity effect sizes that differed significantly from studies in which females were singly-mated to males producing small *versus* large spermatophores (Q_b = 7.4, d.f. = 1, P = 0.006). When studies compared experimental treatments that differed in how much exposure females had to males but failed to control mating rates (less *versus* more in Fig. 4B), measured effect sizes for female fecundity varied widely.

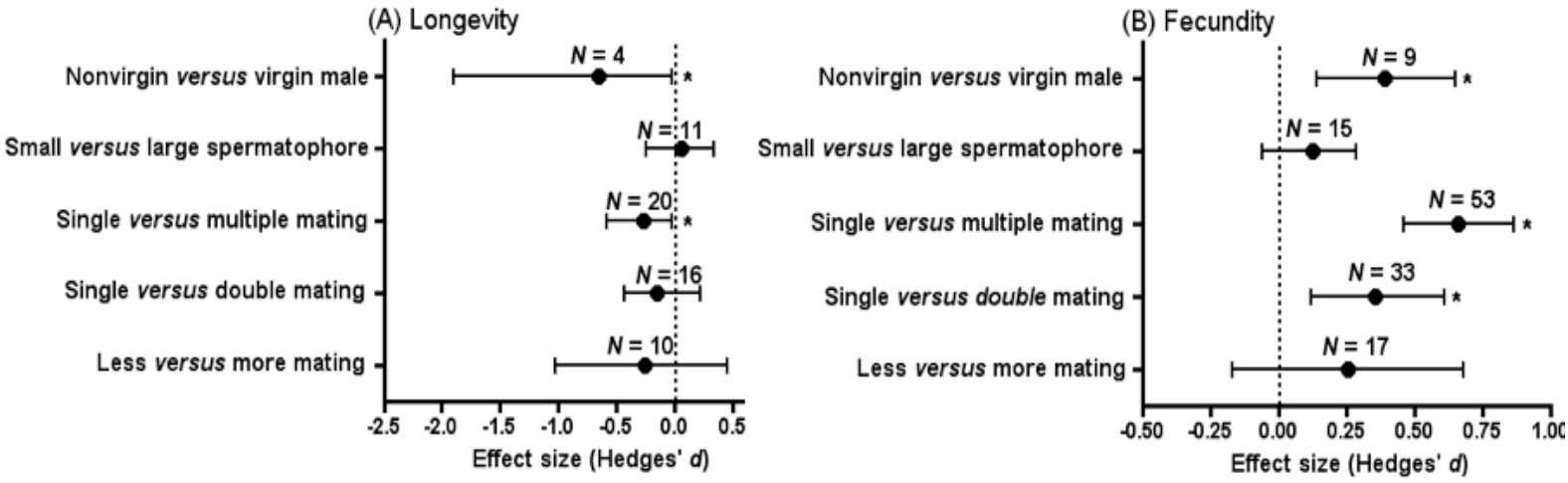

Figure 4. Mean effect sizes (Hedges' *d* with 95% CI) classified by experimental design for arthropod studies that have examined the effects of receiving relatively more male ejaculate on: (A) female longevity, (B) female fecundity. Asterisks indicate effect sizes significantly different ($p < 0.05$) from zero (dotted line). For details of experimental categories see Methods (Section II.2).

(4) Multiple matings: polyandrous versus repeated matings

We further examined two variations on study designs in which singly-mated females were compared to multiply-mated females. When we compared

studies in which females mated multiple times to different males (polyandrous matings) *versus* multiple times to the same male (repeated matings), we found significantly positive effect sizes for female fecundity using both designs (Fig. 5B), with no significant difference between them ($Q_b = 0.9$, d.f. = 1, $P = 0.34$). For longevity (Fig. 5A), however, these two multiple mating designs yielded significantly different effect sizes ($Q_b = 6.5$, d.f. = 1, $P = 0.011$), and only polyandrous matings showed a mean effect size that was significantly less than zero.

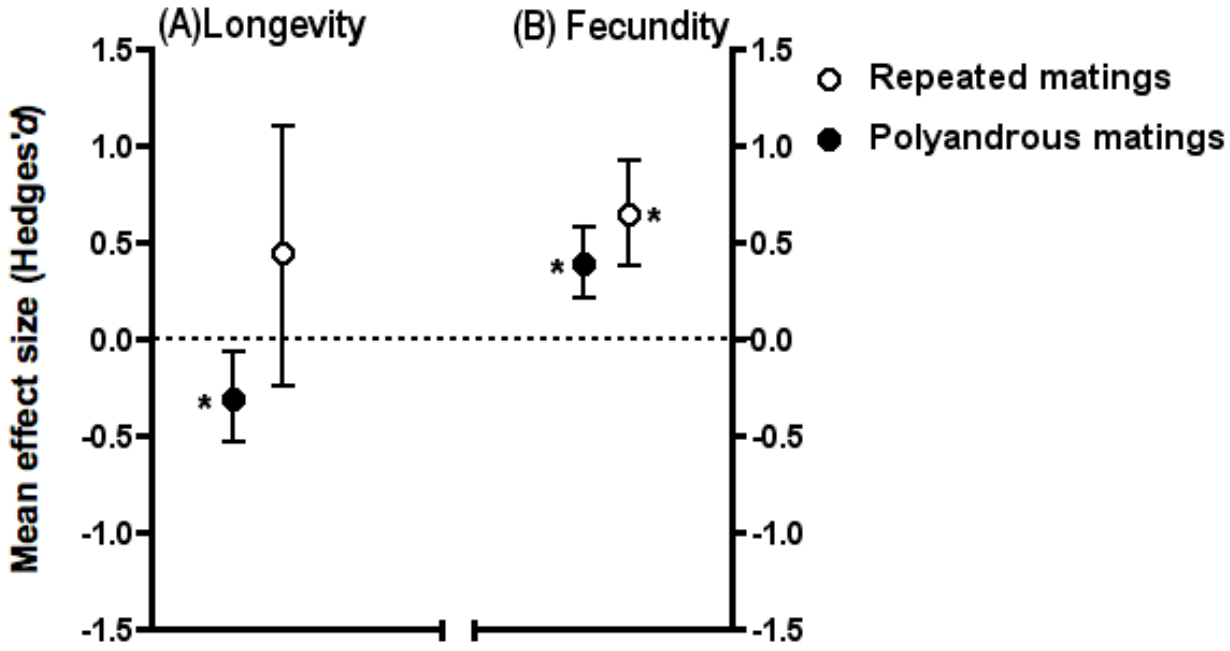

Figure 5. Mean effect sizes (Hedges' *d* with 95% CI) for arthropod studies that have examined the effects of receiving relatively more ejaculate on: (A) female longevity (polyandrous matings $N = 34$, repeated matings with the same male $N = 7$), (B) female fecundity (polyandrous matings $N = 66$, repeated matings with the same male $N = 7$). Asterisks indicate effect sizes significantly different ($P < 0.05$) from zero (dotted line). Open circles in the figure represent repeated matings, closed circles in the figure represent polyandrous matings.

DISCUSSION
(1) Overall effects on longevity and fecundity

These results reveal a general pattern that male ejaculates produce opposite effects on different female fitness components. Within several insect orders, positive effect sizes indicate that fecundity of arthropod females is significantly increased when they receive relatively more ejaculate. However, receiving more ejaculated substances often decreased female lifespan, as shown by significantly negative effect sizes. For the subset of studies that measured lifetime fecundity (59 species), significantly positive effect sizes demonstrate that females gain a net fitness benefit from receiving more ejaculate. It would be worthwhile for future studies to focus on measuring lifetime fecundity, and also to report data concerning the timing of reproductive events (Brommer *et al*., 2002; Reinhardt *et al*., 2009).

These general results are consistent with work in *D. melanogaster* that has detailed how specific male seminal fluid proteins affect female behaviour and reproduction. Females that receive male ejaculate lacking Acp70A (a 36-amino-acid peptide also known as sex peptide) have greater longevity (Wigby & Chapman, 2005), are more willing to re-mate, and produce fewer eggs (Chen *et al*., 1988 Soller *et al*., 1999; Chapman *et al*., 2003; Liu & Kubli, 2003) Another SFP, ovulin, induces ovulation in the first 24 h following copulation (Herndon & Wolfner, 1995; Heifetz *et al*., 2000), while Acp62F has been linked to shorter female life span (Lung *et al*., 2002; Mueller, Page & Wolfner, 2007). We still have much to learn about the functional role played by specific components within male ejaculates in most species, although recent work has begun to

characterize seminal fluid proteins in other taxa (yellow fever mosquito, *Aedes aegypti*, Sirot *et al*., 2008; *D. mojavensis*, Kelleher *et al*., 2009).

(2) Differences between spermatophores and free ejaculates

We distinguished between species in which males transfer free ejaculates *versus* spermatophores, which are categorized as seminal nuptial gifts (Gwynne 2008). For both categories, our meta-analysis revealed that more male ejaculate significantly increased female fecundity, and that effect sizes did not differ between these groups. This result differs from a previous meta-analysis that focused on polyandry: Arnqvist & Nilsson (2000) found that insects with nuptial feeding (this included both oral and ejaculated nuptial gifts) showed more pronounced positive effects of polyandry on both egg production and longevity compared to groups lacking nuptial feeding. One explanation for these differing results may be that orally ingested nuptial gifts are more likely to be nutritive in function, whereas seminal nuptial gifts might be more likely to contain specialized compounds targeted to receptors within the female reproductive tract (Poiani, 2006; Gwynne 2008). However, our meta-analysis results demonstrate that rather than reducing female fitness, seminal nuptial gifts instead have a significantly positive effect on female lifetime fecundity. This result thus argues against spermatophores arising through selection for more elaborate male ejaculates that enhance the delivery of manipulative compounds to females.

(3) Differences due to experimental design

Our results also show that different study designs can dramatically alter measured effect sizes. Only two study designs showed both significant positive

effects on female fecundity coupled with significant negative effects on female longevity: virgin males *versus* non-virgin males, and a single mating *versus* multiple matings. Also, the wide discrepancy in effect sizes from studies in which the mating rate was not controlled (Fig. 4B) suggests that this is not an effective design for measuring how male ejaculates affect female fitness.

(4) Multiple matings: polyandrous *versus* repeated matings

Our results indicate that polyandrous females across many arthropod taxa show increased fecundity as compared to females with only a single mating. This overall fitness benefit of polyandry is consistent with Arnqvist & Nilsson's (2000) result showing higher offspring production for multiply mating females. Possible advantages of polyandry for females have been discussed extensively (e.g. Thornhill & Alcock, 1983; Choe & Crespi, 1997; Yasui, 1998; Hasson & Stone, 2009), and include both direct benefits (replenished sperm supply, ejaculate nutrients, enhanced paternal care, *etc*.) and indirect benefits (increased offspring fitness, avoidance of genetic incompatibility, increased genetic diversity) to females. While indirect benefits have attracted much attention (Yasui, 1998), our results support Arnqvist & Nilsson's (2000) suggestion that the near-ubiquity of female polyandry might be explained solely on the basis of direct benefits.

When we considered polyandrous and repeated matings separately, we found that both categories of multiple mating resulted in increased female fecundity, but only polyandrous matings significantly decreased female longevity. This suggests that interactions among ejaculates from multiple males may have costs for females. It is also possible that repeated matings by a single male might

deplete any longevity-reducing components of male ejaculates, such that females with polyandrous matings receive a higher dose of these ejaculate components.

(5) The impact of seminal nuptial gifts on female fitness

Spermatophores are seminal nuptial gifts produced by male accessory glands in many invertebrate taxa (Mann, 1984). Some debate has arisen over how these seminal products affect female fitness (Vahed, 2007; Gwynne, 2008). Several reviews have highlighted the negative impact on females of nuptial gifts, because they can reduce female mating rates below optimum levels (Arnqvist & Nilsson, 2000; Arnqvist & Rowe, 2005; Vahed, 2007). However, other reviews have suggested that nuptial gifts generally increase female fitness by providing direct benefits (Gwynne, 2008).

Our meta-analysis results demonstrate a net fitness benefit to females from seminal nuptial gifts, as across 30 species, females receiving larger/more seminal nuptial gifts showed positive effect sizes for lifetime fecundity. These results are consistent with several other lines of evidence suggesting that seminal nuptial gifts provide females with a net fitness benefit. In many species, seminal nuptial gift components are incorporated into female oocytes or utilized for defensive purposes, resulting in enhanced fecundity and increased lifespan (e.g. Boggs 1990, 1995; Brown, 1997; Vahed, 1998; Rooney & Lewis, 1999, 2002). Therefore, our meta-analysis results indicate that seminal nuptial gifts provide females in many taxa with a lifetime fecundity benefit in spite of associated longevity costs.

Although seminal nuptial gifts currently appear to provide a net fitness benefit to females in many arthropod species, they may have originated *via* sexual conflict to benefit males at the expense of female fitness (see Arnqvist & Rowe, 2005; Gwynne 2008 for reviews). Male accessory glands may have originated to produce compounds that exploit female sensory pathways and limit female re-mating rates (Arnqvist & Nilsson, 2000; Sakaluk, 2000; Arnqvist & Rowe, 2005; Fedorka & Mousseau, 2002, Enqvist 2007). Females may have then responded by evolving ways to cope with these compounds and derive a direct benefit by using them for somatic maintenance or reproduction (Arnqvist & Nilsson, 2000; Fedorka & Mousseau, 2002; Arnqvist & Rowe, 2005). Thus, the evolutionary origins of seminal gifts remain unclear, although in general they currently function to increase female fitness.

CONCLUSIONS

(1) A comprehensive meta-analysis was conducted for studies on arthropods that have examined how male ejaculate quantity affects female fitness; 84 studies of 70 species yielded a total of 130 comparisons of female fecundity and 61 comparisons of female longevity. Across most arthropod taxa, females show significantly higher fecundity (both partial and lifetime) after receiving more male ejaculate. However, greater ejaculate quantity had the opposite effect on female lifespan, particularly for Diptera and Lepidoptera.

(2) The overall effect size for the subset of 59 species that measured lifetime fecundity indicated that females gained a net fitness benefit from receiving more ejaculate, as their lifetime fecundity increased. Studies comparing

multiply-mated to singly-mated females showed particularly large fecundity increases, indicating that single matings do not maximize female fitness. Therefore, these results support the hypothesis that non-sperm components of male ejaculates provide a direct benefit to females.

(3) The positive effect of multiple matings was seen whether females mated polyandrously (with different males) or repeatedly (with the same male). However, polyandrous females showed reduced longevity compared to females that had repeated matings, suggesting that interactions among multiple males ejaculates might have costs for females.

(4) Our results also have implications for the evolution of seminal nuptial gifts. Our meta-analysis results showed greater lifetime fecundity for females that received relatively larger or more spermatophores, demonstrating that seminal nuptial gifts provide a net fitness benefit that is likely to offset any potential longevity costs. Additionally, we believe that seminal and oral nuptial gifts should be considered separately when evaluating their effects on female fitness.

(5) We suggest that future empirical studies on this topic should control for past mating history of both sexes, record the exact number of copulations, and monitor lifetime egg and offspring production as well as female longevity. We also advocate more focused efforts to identify and characterize the functional role of particular non-sperm components within male ejaculates in species beyond *Drosophila*, as this will provide valuable insights into the evolutionary dynamics of sexual selection.

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Supplementary Information.

Table containing data used in calculation of effect sizes. For mating treatment: 1 = virgin *versus* non-virgin male, 2 = small spermatophore *versus* large spermatophore, 3 = single *versus* multiple mating, 4 = single *versus* double mating, 5 = less *versus* more mating. For ejaculate: 1 = free sperm, 2 = spermatophore, 3 = unknown. For duration of experiment: 1 = lifetime, 2 = partial, 3 = not clear. For repeated *versus* polyandrous: 0 = not clear or not applicable, 1 = polyandrous, 2 = repeated. The control group is defined as the group in which females received less male ejaculate; the experimental group is the group receiving more ejaculate.

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Chapter IV. Effects of male ejaculate on female reproductive output and longevity in *Photinus* fireflies.

ABSTRACT

In many insects, nuptial gifts in the form of spermatophores have been shown to increase female fecundity and to contribute to female somatic maintenance. Examining how variation in male spermatophore size affects female fitness components can provide insight into the evolution of nuptial gifts as well as potential conflicts between the sexes. Here we present an experimental study on *Photinus obscurellus* (LeConte, 1851) fireflies in which we altered spermatophore size by manipulating male mating history and examined effects on female offspring production and longevity. Females were randomly allocated to one of two mating treatments in which they mated once with a male producing either a large or a small spermatophore. We found that male spermatophore size had no significant effect on lifetime fecundity or daily reproductive rates of female *P. obscurellus* fireflies, but females that received a larger spermatophore showed a tendency toward longer post-mating lifespans. These results suggest a direct benefit to females from nuptial gifts and also reveal the potential for synergistic effects on multiple facets of female fitness.

INTRODUCTION

In holometabolous insects, nutrients that adult females can allocate to reproduction might come from three sources: larval stores, adult dietary intake and nutrients that are transferred from males during mating. (Boggs 1990, 2009). Male-derived nutrients are predicted to be of particular economic importance in capital breeders, which include species that lack adult feeding (Boggs 1990, 1997). Spermatophores (sperm-containing packages manufactured by male accessory glands; Mann 1984) comprise a major category of nuptial gifts that are deposited and absorbed in the female reproductive tract (Lewis et al. 2011). Although numerous studies have been conducted in insects to elucidate fitness costs and benefits, considerable debate persists about how such male ejaculates affect female fitness. Some authors have argued that male ejaculates enhance female net fitness (Boggs 1990; Vahed 1998; Gwynne 2008), while others have proposed that male ejaculates produce a net decrease in female fitness by manipulating female reproduction (Arnqvist and Nilsson 2000; Gillott 2003; Vahed 2007; Wolfner 2007). In many insects, nuptial gifts have been shown to increase female fecundity (egg or offspring number), and also to contribute to female somatic maintenance (reviewed by Boggs 1990, 1995; Gwynne 1997, 2008; Vahed 1998). Therefore, examining how variation in male spermatophore size affects female reproductive output and longevity provides insight into the evolution of nuptial gifts as well as potential conflicts between the sexes.

During mating, males of several species of nocturnally active *Photinus* fireflies transfer a spirally coiled, protein-rich spermatophore to the female (van der Reijden et al. 1997). Following sperm release, the male spermatophore disintegrates over several days within a specialized female reproductive organ known as the spermatophore digesting gland. Within a few days after mating, male-derived protein is incorporated mainly into developing oocytes, and also into female somatic tissue (Rooney and Lewis 1999). Because adults do not eat in most *Photinus* species, vitellogenesis depends on both larval food stores and input from male spermatophores. Previous work has shown that receiving more male spermatophores increases lifetime offspring production by *Photinus* females (Rooney and Lewis 2002). When *P. ignitus* (Fall, 1927) females mated with three different males, their lifetime fecundity was 73% greater than that of singly-mated females, while female longevity was unchanged.

In addition to multiple matings, females may also encounter considerable variation in ejaculate (spermatophore) size received from a single mating. In many taxa, males transfer spermatophores that decrease in size with successive matings (Davies and Dadour 1989, Svard and Wiklund 1989; Royer and McNeil 1993, Savalli and Fox 1999; Wilson et al. 1999). In addition, *P. ignitus* males show an average reduction of 36% in spermatophore mass between a male's first and second mating (Cratsley et al. 2003). Therefore, it is also of interest to see how variation in spermatophore size, not just number, influences female fitness. In a previous study of *P. ignitus* fireflies, Rooney and Lewis (2002) found that mated females showed no significant difference in fecundity when they received

large vs. small spermatophores from a second male. However, this study included potential extraneous variation because females were collected throughout their mating season, and thus their prior mating history was unknown.

Here we present an experimental study on *P. obscurellus* fireflies in which we manipulated male mating history to alter spermatophore size, and then examined effects on female offspring production and longevity. In this study, we maximized our ability to detect any changes by using virgin females. We predicted that females receiving larger spermatophores would exhibit enhanced fecundity and longevity relative to females receiving smaller spermatophores.

MATERIALS AND METHODS

To examine effects of spermatophore size on female reproductive output and longevity, we collected *P. obscurellus* in Lincoln, MA (42°26′N, 71°18′W) at the beginning of their breeding season. The nightly mating period for this species lasts ~ 2 h with only 1 mating occurring per night (Lloyd 1966). To obtain virgin fireflies, the field site was monitored nightly for 1-2 weeks before the anticipated emergence date of *P. obscurellus* (these fireflies are inactive during the day). Once they emerge, both sexes emit easily-detected bioluminescent courtship flashes during a courtship flight period that lasts between 45 min and 2 hours. Fireflies are protandrous, and over the first several nights we were able to collect all the emerging males. In the absence of any signaling males, we could still locate and capture newly-emerged females because they will respond to simulated male flashes. This method maximizes the likelihood of obtaining virgins of both sexes. Beetles were weighed to the nearest 0.1mg and housed individually; no

food was provided because *P. obscurellus* has non-feeding adults, as do most *Photinus* species (Lloyd 1997).

Females collected each night were randomly allocated to one of two mating treatments in which they mated once either with a virgin male (thus receiving a large spermatophore), or with a male that had mated within the previous 24 h (thus receiving a small spermatophore). Males in the latter treatment were collected as virgins (see above for description) but then mated in lab prior to their use in the experimental matings. Previous work in numerous insects has found that virgin males transfer larger spermatophores compared to previously-mated males (Lepidoptera: Oberhauser 1988; Svard and Wiklund 1989; Royer and McNeil 1993; Hiroki and Obara 1997; Lauwers and Van Dyck 2006; Coleoptera: Eady 1995; Savalli and Fox 1999; Wilson et al.1999; Orthopetera: Davies and Dadour 1989). A decline in male spermatophore weight after mating has also been confirmed for *P. ignitus* fireflies (Cratsley et al. 2003), a sympatric, ecologically similar firefly that also lacks adult feeding. Therefore, based on previous work it seems reasonable to assume that spermatophore weight is also altered by male mating history in *P. obscurellus*. Spermatophore composition may also change, but this possibility has not been examined.

Most (>70%) females assigned to both treatments had successfully mated within 24 h of collection, and all but one female had mated within 48 h. After mating, females were given moss as an oviposition substrate and eggs were collected every 2 *d* until females died. Eggs were incubated at 29°C until they hatched. For each female, we recorded lifetime fecundity (total number of larvae

produced), daily reproductive rate (number of larvae produced per day), and post-mating lifespan.

To investigate the effects of male spermatophore size on female fitness components, we used analysis of covariance (ANCOVA) with female mass included as a covariate. We checked that there was no initial difference in body mass for females assigned to the two treatments (separate variances *t*-test, $t = 0.923$, df = 17.9, $p = 0.368$). We also checked that data conformed to assumptions of normality and homogeneity of variances, and checked the ANCOVA assumption of homogeneity of regression slopes (Enqvist 2005). In addition to statistical results, we also report effect sizes, which we calculated as Hedges' *d*, an unbiased weighted estimate of effect size that is typically used with continuous response variables and categorical predictors (Nakagawa and Cuthill 2007). Hedges' *d* is calculated as the difference between a control and experimental group measured in standard deviation units (Gurevitch et al. 1992). We also conducted parametric survival analysis with female body weight as a covariate to examine how spermatophore size affected female post-mating lifespan (JMP 9, SAS Institute, Cary, NC).

RESULTS

We found that male spermatophore size had no significant effect on the lifetime fecundity of female *P. obscurellus* fireflies (Figure 1: ANCOVA for spermatophore size $F(1,18) = 1.527$, $p = 0.232$, effect size = 0.53, ANCOVA for female weight covariate, $F(1,18) = 47.1828$, $p < 0.0001$). Daily reproductive rates were also nearly identical between females that received a large spermatophore

(mean ± 1 SE = 4.16 ± 0.44 larvae/day, n = 11) and females that received a small spermatophore (4.16 larvae/day ± 0.46, n = 10, ANCOVA for spermatophore size $F(1,18) = 0.0005$, $p = 0.995$, effect size = 0.0, ANCOVA for female weight covariate, $F(1,18) = 47.6244$, $p < 0.0001$). However, females that received a larger spermatophore had a marginally significant increase in post-mating lifespan, gaining about 2d in an average lifespan of approximately 12 d (Figure 2: ANCOVA for spermatophore size $F(1,18) = 4.459$, p=0.049, effect size = 0.87, ANCOVA for female weight, $F(1,18) = 0.7212$, $p = 0.4069$). Parametric survival analysis yielded similar results, showing a marginally insignificant effect of spermatophore size on female longevity (likelihood ratio $x^2 = 3.69$, p = 0.0546) and no significant effect of female weight (likelihood ratio $x^2 = 0.34$, p = 0.5567).

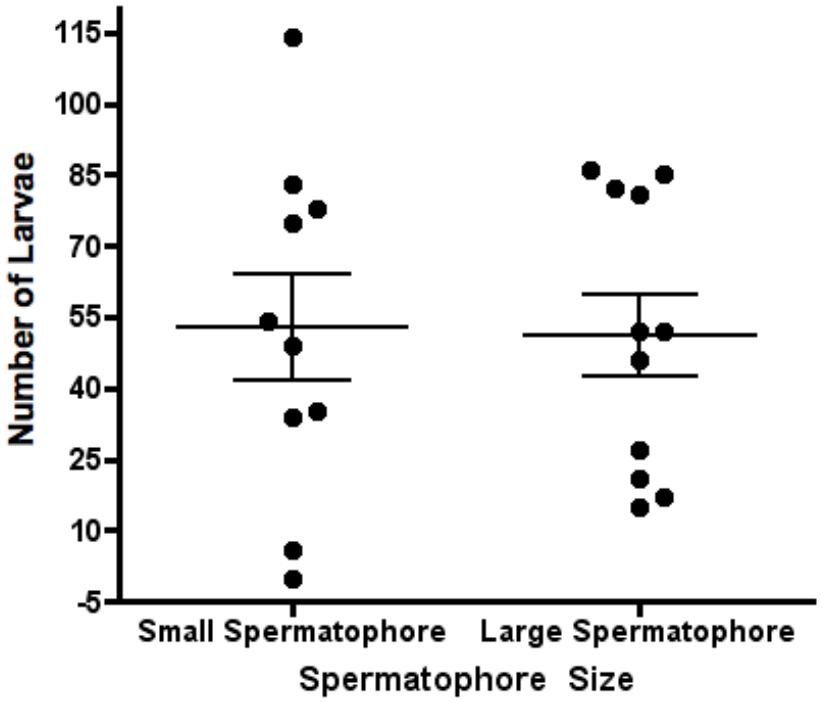

Figure 1. Lifetime fecundity as number of larvae produced by female *Photinus obscurellus* that mated once either with a virgin male (thus receiving a large spermatophore, n=11), or with a male that had mated within the previous 24 h (thus receiving a small spermatophore, n=10). Dots indicate data for individual females, horizontal lines indicate mean ± 1 SE .

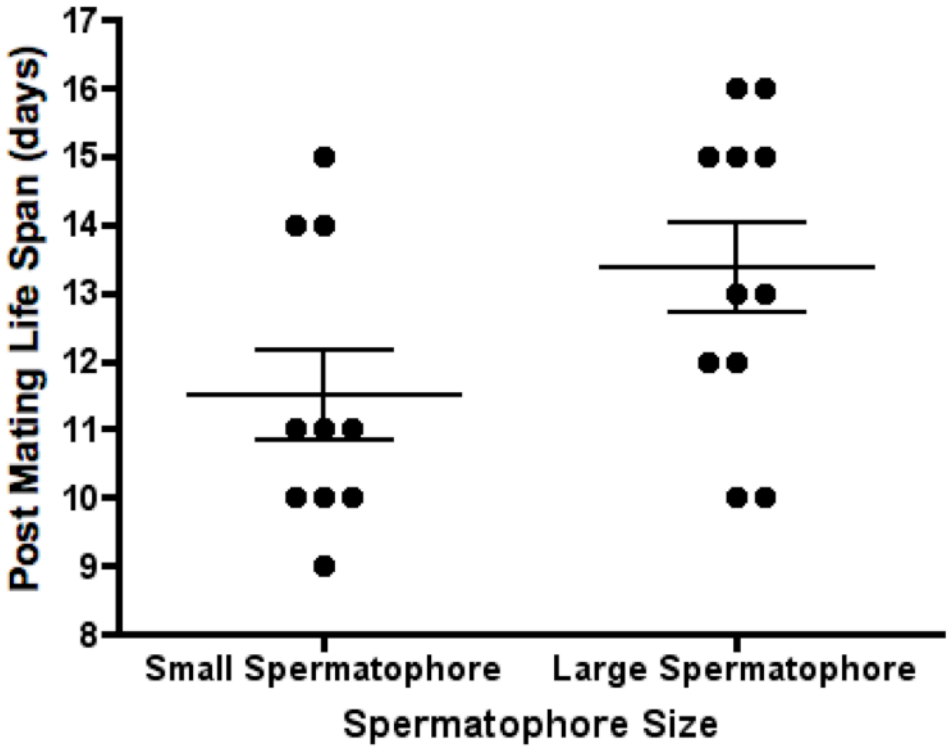

Figure 2. Post mating life span of female *Photinus obscurellus* that mated once either with a virgin male (thus receiving a large spermatophore, n=11), or with a male that had mated within the previous 24 h (thus receiving a small spermatophore, n=10). Dots indicate data for individual females, horizontal lines indicate mean ± 1 SE.

DISCUSSION

This study found no difference in lifetime reproductive output between *P. obscurellus* females based on differences in male ejaculate size received during a single mating. This contrasts with the significant 73% increase in female lifetime fecundity documented for triply-mated compared with singly-mated *P. ignitus* females (Rooney and Lewis 2002). Thus, while variation in male ejaculate quantity or composition may influence female fecundity, at least in *P. obscurellus* this effect appears too subtle to detect based on variation in single spermatophores.

However, females that had mated with virgin males and thus had received larger spermatophores showed a ~ 16% increase in longevity. Although this effect was on the borderline of statistical significance at the a=.05 level, this possible longevity benefit from male spermatophores is consistent with previous work on *Photinus* fireflies. When *P. ignitus* females that had previously mated were mated again to males with either large or small spermatophores (manipulated via mating history as in this study), there was no significant effect on either female fecundity or longevity (Rooney and Lewis 2002); however, those females that received larger spermatophores showed a 12% increase in longevity. In addition to studying once-mated females, the current study provided a methodological advantage because we were able to control for differences in prior mating history of both sexes by using virgin females and males. This is likely to have reduced extraneous variation, and thus may have improved the ability of the current study to detect potential differences.

These results provide some insight into the possible evolution of nuptial gifts in *Photinus* fireflies. Under natural conditions, the distinct benefits that females derive from larger spermatophores and multiple mating may complement one another. Under artificially created lab conditions, the experimental females in our study were not given the opportunity to remate. However, because in field populations *Photinus* females typically remate on subsequent nights (Lewis and Wang 1991), even a small increase in longevity should also increase the number of mates (and thus the number of ejaculates) a female gains, resulting in higher lifetime fecundity (Rooney and Lewis 2002). These combined benefits are

expected to select for female choice of males with larger spermatophores, as well as for female polyandry (reviewed in Lewis and Cratsley 2008). Possible benefits to males producing larger spermatophores might include increasing female re-mating latency, as well as increasing their paternity success relative to a female's ensuing mates. However, these putative male benefits have yet to be examined.

Other experimental studies in a variety of insects demonstrate a variety of effects of male nuptial gifts on female net fitness (reviewed in Boggs 1995; Vahed 1998; Arnqvist and Nilsson 2000; Gwynne 2008; South and Lewis 2011). It has been proposed that nuptial gifts may have originated via sexual conflict to benefit males at the expense of female net fitness (see Arnqvist and Rowe 2000, 2005; Vahed 2007; Gwynne 2008 for review). Male reproductive accessory glands could have originated to produce compounds that exploit female sensory pathways and induce a greater latency period between matings (Arnqvist and Nilsson 2000; Sakaluk 2000; Fedorka and Mousseau 2002; Arnqvist and Rowe 2005; Enqvist 2007). Females may have then responded by evolving ways to cope with these compounds and derive a direct benefit by using them for somatic maintenance or reproduction (Arnqvist and Nilsson 2000; Fedorka and Mousseau 2002; Arnqvist and Rowe 2005). Thus, although it seems likely that the effects of nuptial gifts on the fitness of both sexes will change over evolutionary time, in many insects they currently appear to function to increase female fitness (South and Lewis 2011).

Studies to date on *Photinus* fireflies suggest that male nuptial gifts can exert synergistic effects on multiple components of female fitness: longer female

lifespan suggested by the current study leads to increased probability of females remating and thus obtaining additional ejaculates shown to increase fecundity (Rooney and Lewis 2002). Such fitness benefits would be predicted to favor female choice of males that can provide larger spermatophores, although additional study is needed to explore possible correlated traits that females might use as the basis for choice. Because male gifts are selected through their fitness consequences for both sexes, they may profoundly influence both precopulatory and postcopulatory sexual selection. Additionally, changes in nuptial gift size during male lifetimes in *Photinus* species can potentially alter courtship behavior and mate choice. Further study in this suite of species could reveal unique insights into coevolution between nuptial gifts and other life-history parameters.

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

Abstract

Because females often mate with multiple males, it is critical to expand our view of sexual selection to encompass pre-, peri- and postcopulatory episodes to understand how selection drives trait evolution. In *Photinus* fireflies, females preferentially respond to males based upon 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 *P. greeni* 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 behaviors 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 behavior 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.

INTRODUCTION

One of the principal forces driving the evolution of morphological, behavioral 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 due 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 behavioral 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 postcopulatory 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 postcopulatory sexual selection, which could be due 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 dialog between flying males and stationary females (26, reviewed in 27). Females preferentially give flash responses to particular males based upon 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 life span (32,33), and therefore a male's reproductive success will depend upon 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 postcopulatory reproductive success (based on their paternity share of offspring produced by doubly-mated females 38,14), and this might depend on spermatophore traits.

Previous work in *Photinus greeni* fireflies has demonstrated a negative relationship between a male's precopulatory courtship attractiveness and his subsequent postcopulatory paternity success (14). Those males that were least attractive to females during courtship interactions nonetheless sired significantly more offspring compared to 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 behaviors, 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 behaviors 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 behaviors, 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.

MATERIALS AND METHODS

(a) Study organism and design

The effects of spermatophore size, flash signal attractiveness and pericopulatory behaviors 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 supplementary materials 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 1 of 4 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 ~1.0 to 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 (40,41,30). 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 an LED controlled by a programmable microprocessor. The LED (572 nm, Ledtronics Inc. Torrance, CA) 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 *P. ignitus,* spermatophore weight decreases by 36% 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 spermatophores (L), and used pre-mated males that were mated again the following night to obtain small spermatophores (S).

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 (e.g. 50). Based on the potential for sperm quantity to influence male paternity success, we compared sperm quantity between *P. greeni* males' 1st *vs.* 2nd 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% EtOH. Females were dissected and male spermatophores were placed in 10 uL 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 60x magnification (Olympus BX40, Olympus, Center Valley, PA). Differences in the number of sperm bundles between males' 1st vs. 2nd

spermatophores were compared using a paired t-test (SPSS v. 18, SPSS Inc. Chicago, IL.).

(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 also supplementary material Figure 1). 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+S; 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 large-spermatophore male. After 24 h, these females were shown unattractive courtship signals (25 paired flashes with 1.4 s IPI), and then were 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 vs. 2 and Treatment 3 vs. 4 show effects of altering male spermatophore size, while comparing Treatments 1 vs. 3 and 2 vs. 4 shows the effect of altering courtship signals.

Experiments began at ~ 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 dialoging 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 swivels 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% of females remated at this time. Of the remaining 10 females, 9 remated at 48 h after their first mating, and 1 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 remaining15 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 in 2 d intervals and placed into sterile petri dishes with 1x phosphate buffered saline. Eggs were incubated at 29 C° until hatching, and first instar larvae were collected and frozen in 95% EtOH at – 80 C° for later DNA extraction and paternity assignment (see below). Males and females were also frozen in 95% EtOH. The total number of larvae that emerged from a given family varied between 0 and 103. Female fecundity (lifetime # of offspring) was compared between the four treatments with a two-way ANOVA (SPSS, Inc.), with 2nd mating male spermatophore size and courtship signal as fixed factors.

(e) Measuring pericopulatory behaviors

Because they occur in the dark, close-range male-female interactions that happen after contact but before copulation have not previously been described for any firefly species. These behaviors 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 analyzed frame-by-frame using iMovie (Apple, Inc., Cupertino, CA) to describe and quantify pericopulatory behaviors of both sexes (described in supplementary materials).

In our behavioral analysis, we included unsuccessful matings only when we observed sex-specific rejection behaviors (see supplementary Table 1 and

Figure 2). We excluded any pairs that failed to make contact and 4 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 behaviors, spermatophore size, and artificial flash attractiveness (each as a categorical predictor) using SAS PROC Logistic (SAS Inc., Cary, NC). We used conditional exact tests in this analysis due to sparseness of data, as the usual asymptotic methods are unreliable for such data sets (51). In addition, we assessed whether male pericopulatory behaviors changed between a male's first vs. second matings using a Goodman Kruskal tests (Stat Xact version 6, Cytel Inc., Cambridge, MA).

(f) Measuring male paternity share

To determine paternity for offspring produced by doubly-mated *P. greeni* females, we utilized Random Amplified Polymorphic DNA (RAPD) markers (52) following methods described in 14 (see 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 (e.g., 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 (P_2) 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 ≥ 9 offspring. Final samples sizes were as follows: 5 families in Treatment 1, 11 families in Treatment 2, 9 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 modeled using binomial errors and a logit link function using SAS PROC GenMod (SAS Inc., Cary, NC). In addition, we examined whether second-male paternity within each family (P_2) was influenced by other male morphological or behavioral traits. To do this we used logistic regressions where P_2 was modeled as a binomial response variable (this was possible because 86% families showed P_2 of either 0 or 1, and the 5 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 behavior (categorical) as predictors.

RESULTS

(a) Female and male pericopulatory behaviors

P. greeni females showed specific behaviors associated with rejecting a male as a mate (Supplementary Table 2), but the likelihood of female rejection was not significantly affected by a male's pericopulatory behavior (logistic regression, likelihood ratio $x^2 = 8.79$, df = 6, p = 0.1855). Male pericopulatory behaviors did not change between each male's first and second matings (2 X 3

tests of association for leg behaviors, Goodman-Kruskal estimate = 0.0058, p= 0.9; for antennal behaviors, 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 males that had larger spermatophores (Figure 1, exact logistic regression, conditional exact test score = 6.11, p = 0.0152). Female mating status (virgin vs. 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

P. 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 x^2=12.41, df = 32, p= 0.0004). This effect was particularly pronounced when large-spermatophore males were the second ones to mate (Treatments 2 & 4: Figure 2b & 2d). 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 5 out of 36 families.

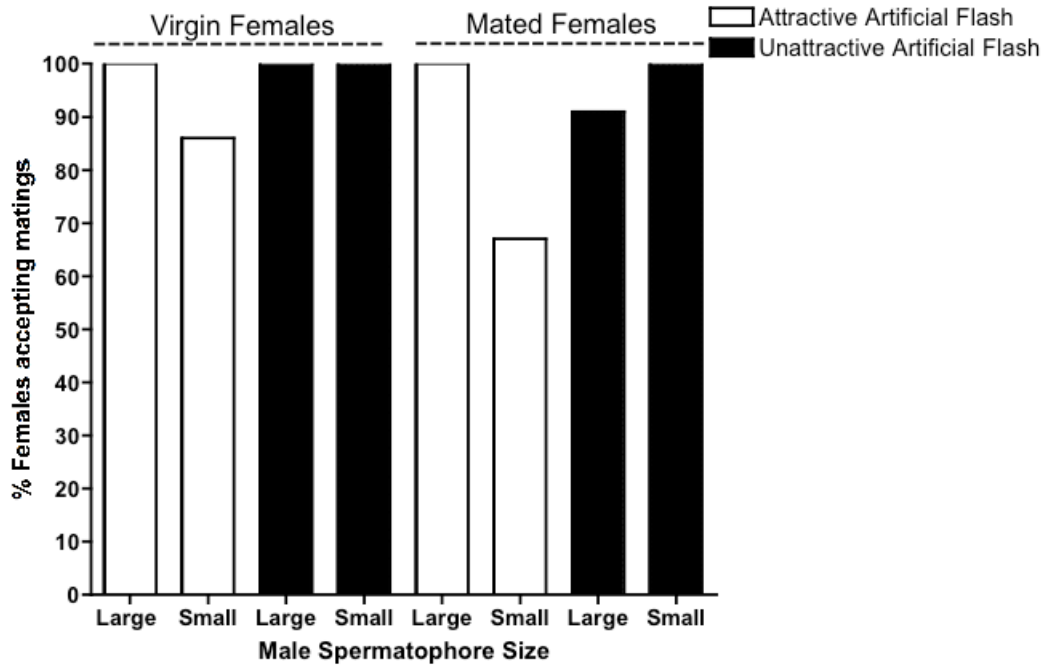

Figure 1. Percentage of females accepting *Photinus greeni* males as mates depending on male spermatophore size (large vs small), artificial flash signal attractiveness (unattractive vs attractive), and female mating status (virgin vs mated). Total number of pairs observed was 102).

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 x^2=0.03, df = 32, p= 0.8559), and there was no significant interaction between spermatophore size and signal attractiveness (interaction estimate =-0.936, likelihood ratio x^2=0.28, df = 32, p= 0.5955). Also, male paternity share was not influenced by either male body weight (logistic

regression; likelihood ratio x^2= 0.46, df = 1, p = 0.4993) or male pericopulatory behaviors (logistic regression; likelihood ratio x^2= 6.19, df = 5, p = 0.1853).

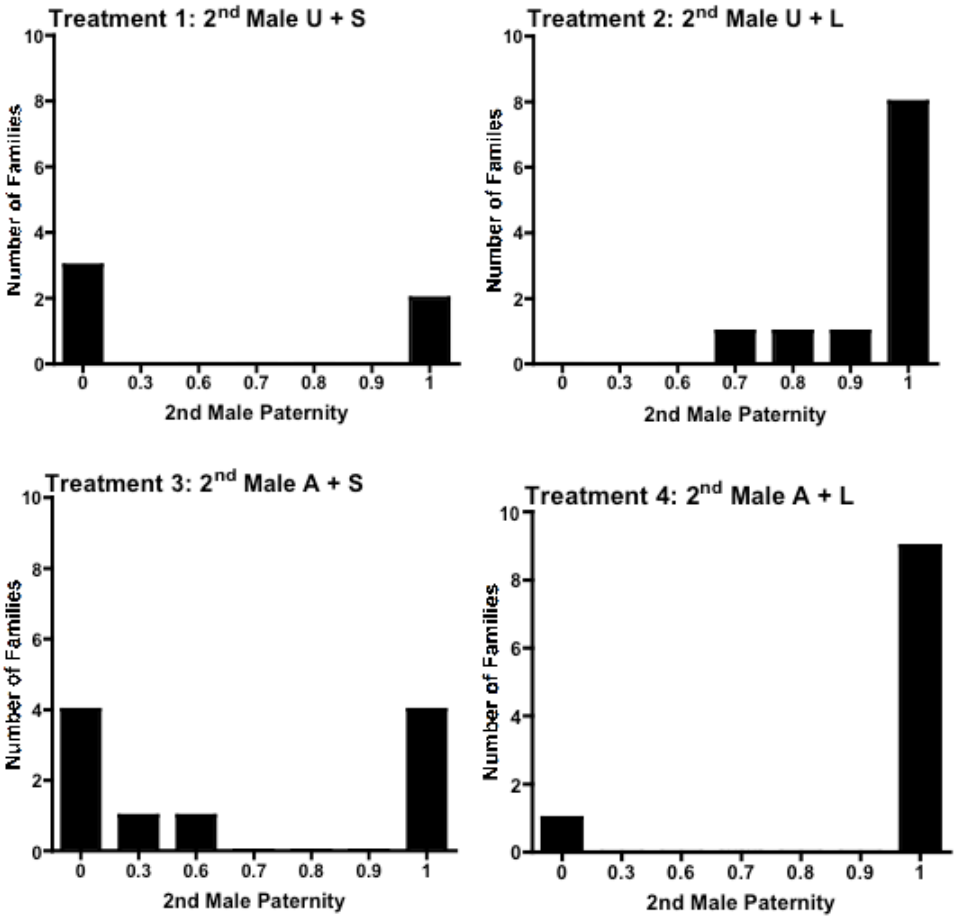

Figure 2. Frequency histograms showing the effect of artificial courtship signal (A = attractive, U = unattractive) and spermatophore size (L = large, S= small) on second male paternity share (P_2 = proportion of offspring produced by doubly mated *Photinus greeni* females that were sired by the second mating male). Virgin females were mated to 2 males at 24-72 hours intervals (treatment descriptions indicate conditions for the second mating s). A. Treatment 1 (U+S)- unattractive flash and small spermatophore; B. Treatment 2 (U+L)- unattractive flash and large spermatophore; C. Treatment 3 (A+S)- attractive flash and small spermatophore; D. Treatment 4(A+L)- attractive flash and large spermatophore.

As expected, because every doubly-mated female received one large and one small spermatophore, lifetime offspring production did not vary between experimental treatments (2-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 = 12.33, df = 11, p < 0.005; mean difference ± SE = 75.3 ± 6.1).

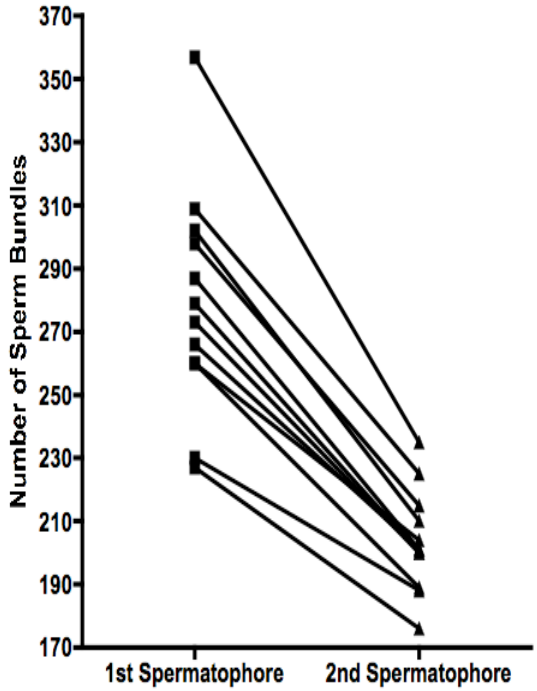

Figure 3. Sperm quantity (number of bundles) contained within spermatophores produced by *Photinus greeni* males during their 1st vs. 2nd matings (n= 12 males).

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 vs. 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 due 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.

(a) Spermatophore size influences male paternity share and pericopulatory success

This study demonstrates that in *P. greeni* fireflies, male spermatophore size positively affects two distinct episodes of sexual selection. Relative to males with smaller spermatophores, males with large spermatophores gained fitness benefits through increased paternity share, and also through their higher likelihood of successfully mating once they contacted a female. A likely mechanism for the effect on paternity share is that larger spermatophores contain more sperm, which could provide a numerical advantage in sperm competition (4). Males with larger

spermatophores had higher paternity share regardless of whether they were a female's first or second mate. Thus, it seems that large spermatophores provide a benefit not only in sperm offense, but also in sperm defense, a pattern also documented in the almond moth, *Cadra cautella* (66).

This study also demonstrated that *P. greeni* males that had not previously mated, and thus would transfer relatively larger spermatophores, were significantly more likely to be 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 behaviors seems unlikely, as we found no behavioral 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 defense titers 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 signaling in *Photinus* fireflies via either cuticular hydrocabons (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 upon 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 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 *Malurus melanocephalus*, 71; pheromones and dominance behavior in Australian field crickets; *Telogryllus oceanicus*, 72; 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 ($P_2 = 0$) or their second mate ($P_2=1$); very few broods showed mixed paternity. Such starkly bimodal distributions of P_2 have now been documented across many taxa (reviewed by 4; *Poecilia reticulata* guppies, 73; *Ephippiger ephippiger* bushcrickets, 74; *Teleopsis dalmanni* stalk-eyed flies, 75; butterflies and moths, 76; *Cadra cautella* moths 66). Despite this ubiquity, the mechanisms generating such bimodal paternity share are not well understood. In *T. dalmanni*, Corley *et al*. (75) 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 (77) suggests a role for both sperm competition and cryptic female choice in determining reproductive success, highlighting the complexity of these postcopulatory interactions.

Bimodality of P_2 could also be influenced by male-derived substances such as seminal fluid proteins, which are important in sperm competition (see 78 for review). In *P. greeni* fireflies, another mechanism generating bimodal paternity share might involve different sperm storage organs for housing first vs. second males' sperm. *P. greeni* females have two sperm storage chambers, and differences in stored sperm viability have been documented between these (79). 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 (80,81). If females can assess spermatophore size, postcopulatory female choice may bias fertilizations towards males with larger spermatophores. As

spermatophore size has been found to be heritable in some insects (e.g. 82), females could experience both direct and indirect benefits as a consequence. Additional work is needed to examine how cryptic female choice and sperm competition might interact to determine patterns of paternity.

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Supplementary materials for:

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

MATERIALS & METHODS

Firefly Collection

Photinus fireflies cannot be reared in the laboratory so to obtain virgin male and female fireflies for this study, we monitored the field site nightly for 1-2 weeks before the anticipated emergence date of *P. greeni* (these fireflies are inactive during the day). Once they emerge, both sexes emit easily-detected bioluminescent courtship flashes during a 20 min courtship period. Fireflies are protandrous, and over the first several nights we were able to collect all the emerging males. In the absence of any signaling males, we could still locate and

capture virgin females because they readily respond to simulated male flashes. This method maximizes the likelihood of obtaining virgins of both sexes.

Measuring Paternity Share

To determine paternity for offspring produced by doubly-mated *P. greeni* females, we utilized Random Amplified Polymorphic DNA (RAPD) markers (52) following methods modified from Demary & Lewis (14). These markers have previously been used for both *P. ignitus* and *P. greeni* fireflies to assign paternity to larval offspring of females that had mated to two known males (38,14).

Genomic DNA was extracted from adult tissue after removing the elytra, wings, legs and exoskeleton. Adult tissue was then ground with sterile disposable pestles and gDNA was extracted using a DNeasy Blood and Tissue kit (Qiagen, Germany). First instar larvae were individually ground with pestles prior to gDNA extaction. Following DNA extractions, RAPD PCR reactions were conducted. Each RAPD reaction (25 uL total volume) contained: 2.5 uL of 10X buffer plus magnesium and 5X Master Taq respectively, 3.0 uL RAPD primer (10 uM), 5.0 uL DNTPs (10 mM) 8 uL DNA (approximately 5 ng) 0.5 uL extra magnesium (25 mM) and 0.5 uL Master Taq polymerase. A Perkin Elmer Cetus thermocycler (Perkin Elmer, Waltham, MA) was programmed for one cycle at 94°C, 20 cycles at 94°C for 30 sec, 36.4°C for 1 min, and 72°C for 1 min followed by a 4°C soak. PCR products were run out on 1.5% agarose gels and stained with ethidium bromide. Gels were visualized and photographed using Quantity One software (Bio-Rad, Hercules, CA).

Paternity was determined for each larval offspring based on the presence

of polymorphic bands shared uniquely with either of the two potential fathers. We were able to definitively assign paternity to nearly all offspring using one to four RAPD primers per family; any larval offspring that could not be positively attributed to either of the two males was excluded from P_2 calculations (the total number of excluded larvae for all treatments was less than 10). Paternity assignments were confirmed for a subset of larvae using duplicate runs with the same or with different RAPD primers. All of the offspring rerun with the same primer yielded identical paternity assignments.

RESULTS

Pericopulatory behaviors

Few descriptions exist of firefly behaviors that take place after contact but before intromission, as these interactions generally take place in the dark. Copulations are typically initiated by males approaching stationary females. After contact, a male dorsally mounts the female, grasps the sides of her abdomen with his legs, and explores her elytra and pronotum with his maxillary palps and antennae. Once positioned, the male extrudes his aedeagus and attempts to achieve intromission. For this to occur, a female must remain quiescent and curve her abdomen towards the mounting male.

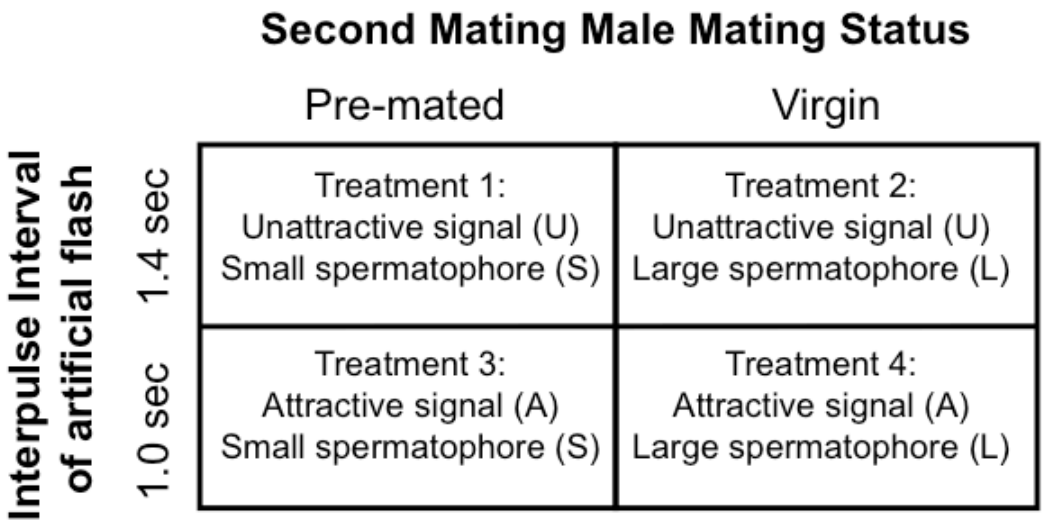
In this experiment, videorecordings of *P. greeni* male-female pairs under infrared illumination allowed detailed analysis of male and female behaviors immediately before mating, and their impact on mate acceptance or rejection (Supplementary Table 1, Figure 2). Males generally approached females from the side or rear, and their first contact was typically male antennation of the female elytra. As males mounted females, they quivered and drummed their antennae

across the female's elytra, pronotum and/or antennae; these antennal movements were often accompanied by side-to-side head motion. Males also commonly brushed their maxillary palps across the junction between the female's pronotum and her elytrae. Simultaneously, males grasped the female with their front legs, often stroking the sides of the female's elytra or pulling on her abdomen with the other legs. After achieving intromission, males continued to antennate the female's elytra, pronotum and antennae until they turned tail-to-tail for the second stage of copulation (33).

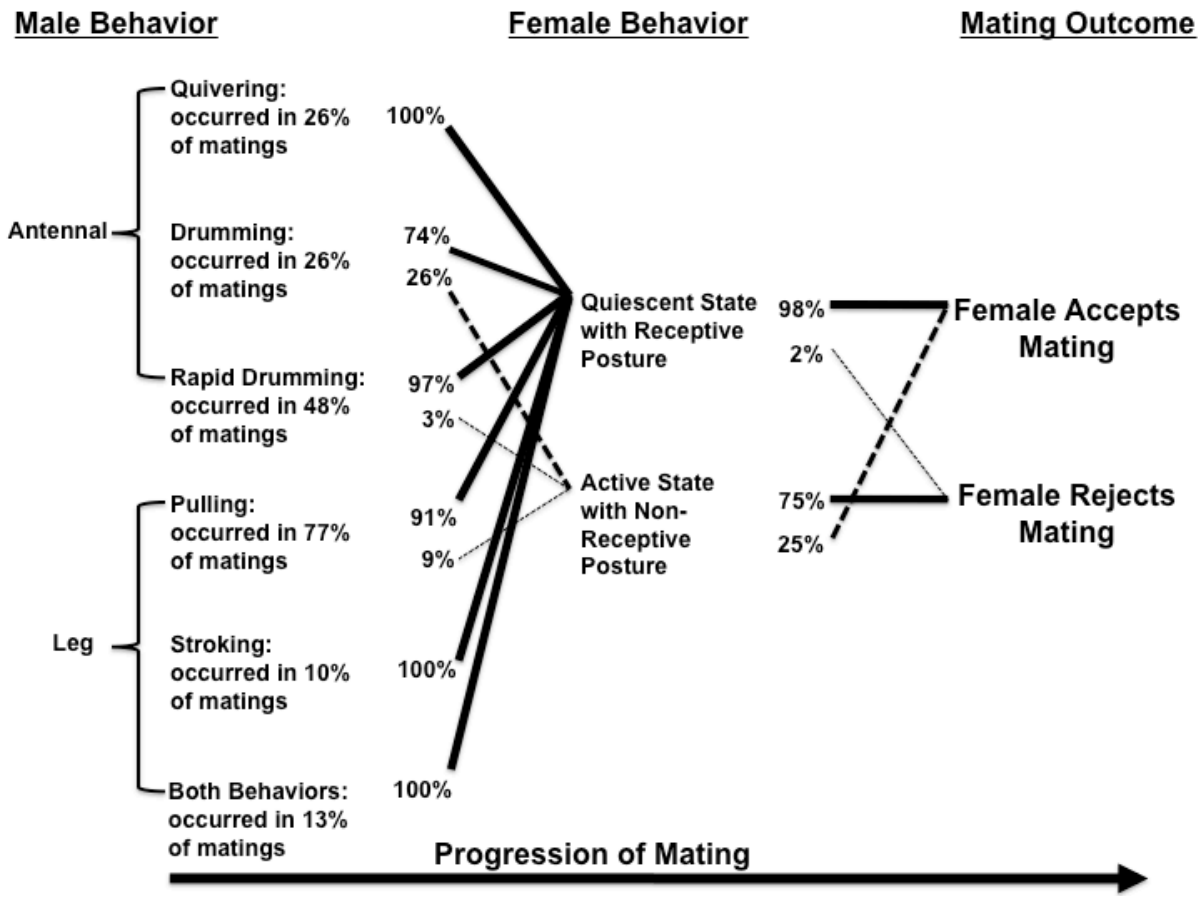
Females rejected males in 9 of 121 pairs (see Supplementary Table 1), and males walked away after contacting the female in 2 out 121 pairs. Generally, before females accepted a male's mating attempt they became quiescent (no body movement). Females also depressed their antennae against the substrate and curved their abdomen dorsally towards the male; together these behaviors were defined as a receptive posture. When females were both quiescent and assumed a receptive posture, mate acceptance followed 98% of the time (Supplementary Figure 2). In contrast, in other pairs even after the male had mounted the female continued active walking, moved her antennae, and kept her abdomen depressed against the substrate. When females exhibited this active state and non-receptive posture, it led to mating failures 75% of the time (Supplementary Figure 2).

Supplementary Table 1. Pericopulatory behaviors exhibited after contact but before intromission by male and female *Photinus greeni* fireflies.

Male Behavior	**Classes and Description of Behavior**
Antennal	1) Rapid Drumming – Rapidly moving within full range of motion (1 cycle/0.1 sec) 2) Drumming – Moving within the full range of motion (1 cycle/0.2 sec) 3) Quivering – Moving less than full range of motion
Leg	1) Both Stroking and Pulling 2) Stroking 3) Pulling
Female Behavior	**Classes and Description of Behavior**
Activity	1) Quiescent – Body and parts are stationary 2) Active – Body and parts are in motion
Antennal	1) Depressed – Pressed flat against substrate 2) Lifted – Off substrate and/or moving
Abdominal Curvature	1) Curved – Abdomen bent towards mating male 2) Uncurved – Abdomen held straight or pressed against substrate
Rejection	1) Walking away from male after first contact 2) Pressing abdomen against substrate to prevent intromission 3) Walking with male mounted on her elytra



Supplementary Figure 1. Schematic of experimental design used to examine effects of artificial flash attractiveness and male spermatophore size on second male paternity success (P_2) in *Photinus greeni* fireflies. Females in each of four treatments were allowed to mate twice (descriptions shown only for each female's second mating). Prior to each mating, females were exposed either to 25 artificial flash signals that were previously shown to be unattractive (1.4 sec interpulse interval) or 25 attractive (1.0 sec interpulse interval) signals. Females were subsequently mated either to pre-mated males (small spermatophore) or to virgin males (large spermatophore).



Supplementary Figure 2. Temporal sequence of male and female behaviors that occur after contact but before intromission in *Photinus greeni* fireflies, and their impact upon mating outcome. Solid lines represent the most frequent behavioral sequences.

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Chapter VI. Examining the role of cuticular hydrocarbons in firefly species recognition

ABSTRACT

During animal courtship, multiple signals transmitted in different sensory modalities may be used to recognize potential mates. In fireflies (Coleoptera: Lampyridae), nocturnally active species rely on long-range bioluminescent signals for species, sex, and mate recognition, while several diurnally active species rely on pheromonal signals. Although in many insects non-volatile cuticular hydrocarbons (CHC) also function in species and sex discrimination, little is known about the potential role of CHC in fireflies. Here we used gas chromatography to characterize species and sex differences in the CHC profiles of several North American fireflies, including three nocturnal and two diurnal species. Additionally, we conducted behavioral bioassays to determine whether firefly males (the searching sex) were differentially attracted to extracts from conspecific vs. heterospecific females. Gas chromatography revealed that nocturnal *Photinus* fireflies had low or undetectable CHC levels in both sexes, while diurnal fireflies showed higher CHC levels. No major sex differences in CHC profiles were observed for any firefly species. Behavioral bioassays demonstrated that males of the diurnal firefly *Ellychnia corrusca* were preferentially attracted to chemical extracts from conspecific vs. heterospecific females, while males of the remaining species showed no discrimination. These results suggest that while CHC may function as species recognition signals for

some diurnal fireflies, these compounds are unlikely to be important contact signals in nocturnal *Photinus* fireflies.

INTRODUCTION

Animal courtship signals and responses play a key role in species, sex and mate-quality recognition. Courtship interactions potentially involve multiple signals transmitted in several different sensory modalities (Candolin 2003; Hebets & Papaj 2005; Partan & Marler 2005). Signal generation can involve multiple organs, and complex signaling behaviors can evolve together as a unit (Hebets & Papaj 2005). For example, courtship by male *Schizocosa* wolf spiders depends on both visual and vibratory signals (Uetz & Robets 2002; Hebets 2005), and males in the Hawaiian *Drosophila* species complex solicit copulations using chemical, tactile, visual and vibrational signals (Greenspan & Ferveur 2000; Boake 2005). Thus, a comprehensive understanding of animal communication requires examining the potential for information to be transmitted across multiple signaling modalities.

Insect cuticular hydrocarbons (CHC) are secreted from a variety of glands and accumulate on the external surface of the exoskeleton. These low volatility lipids function in protection against desiccation (Gibbs 1998; Singer 1998; Howard & Blomquist 2005; Barbour *et al*. 2007), and have also been shown to act as contact signals for species, kin, and mate recognition in numerous social insects (reviewed by Howard 1993; Howard and Bloomquist 2005; Dani 2006; Monnin 2006) as well as in many solitary insects, including *Drosophila* (Ferveur 2005), *Laupala* (Mullen *et al*. 2007) and *Gryllus* crickets (Tregenza & Wedell

1997), *Cataglyphis niger* ants (Lahav *et al*. 1998), *Glossina* tsetse flies (Carlson *et al*. 2005), and many beetles (Peschke 1987; Page *et al*. 1990; Johannson & Jones 2007; Stoeffler *et al*. 2007). Recent work on *Laupala* crickets has demonstrated both interspecific and sex differences in CHC composition, and suggests that acoustic signals are used for long-range mate attraction while cuticular hydrocarbons function in close-range species discrimination (Mullen *et al*. 2007).

Within the family Lampyridae, firefly species differ considerably in the signal modalities used during courtship (Lloyd 1979; Ohba 2004; Lewis & Cratsley 2008). In many nocturnally active species, photic signals consisting of bioluminescent flashes or glows are used to attract mates, while mate attraction in some diurnally active species has been shown to involve volatile pheromonal signals (Lloyd 1972; De Cock & Matthysen 2005). Most North American *Photinus* fireflies are nocturnal, and males broadcast precisely-timed bioluminescent signals to elicit female flash responses. *Photinus* males fly in search of perched females, making their final approach by walking to contact the stationary females. Temporal characteristics of *Photinus* courtship flash signals convey information concerning species identity, sex, and mate quality (Lloyd 1966; reviewed by Lewis & Cratsley 2008). There is no evidence that volatile pheromones are important in nocturnal *Photinus* fireflies, as flash responses from females in airtight containers readily attracted conspecific males (Lloyd 1966). The possibility that contact chemical cues may function in *Photinus* pre-mating reproductive isolation was first suggested by Lloyd (1966). In two sympatric *Photinus* species with similar flash signals, Lloyd noted that males were attracted

to flash responses from heterospecific females, yet males subsequently rejected such females after contact. Further behavioral observations indicate that after initial contact with a female, *Photinus* males vigorously antennate the female and pass their maxillary palps over the female's pronotum and elytra (unpublished data). Insect maxillary palps and antennae function as olfactory and gustatory receptors capable of distinguishing chemosensory signals (Chapman 1998). These observations suggest the possibility that while nocturnal *Photinus* fireflies rely on flash signals for long-range mate location, they may also use contact chemical signals for close-range species and/or sex discrimination.

Although diurnal fireflies are generally presumed to use chemicals for mate attraction, considerably less is known about their courtship signals (but see Shibue *et al*. 2000). Field experiments have shown that in several diurnal fireflies including *Lucidota atra*, females produce volatile pheromones that attract males (Lloyd 1972, De Cock & Matthysen 2005). In another diurnal firefly, *Ellychnia corrusca*, males use their maxillary palps to examine females before copulation (Rooney & Lewis 2000). Shibue *et al*. (2004) characterized CHC profiles for several firefly species, and found greater CHC diversity in a diurnal species compared to nocturnal species, although few individuals were examined and *Photinus* fireflies were not included in this study. No studies have explored the potential role of CHC for species recognition in diurnal fireflies.

This study was conducted to assess whether cuticular hydrocarbons may play a role in species or sex recognition in fireflies. We conducted behavioral bioassays to determine whether firefly males (the searching sex) were

differentially attracted to chemical extracts from conspecific vs. heterospecific females. Additionally, we used gas chromatography to characterize species differences in CHC profiles between three nocturnal and two diurnal North American firefly species, as well as to examine possible sex differences and individual variation in CHC profiles.

METHODS

Beetle Collection and Maintenance

The nocturnal fireflies *Photinus greeni* and *P. obscurellus* were collected in Lincoln, MA (42°26´N, 71°18´W), and *P. ignitus* were collected in Lancaster, MA (42°46´N, 71°67´). Diurnal fireflies *Lucidota atra* and *Ellychnia corrusca* were collected in Holderness, NH (43° 74´N, 71° 59´ W) and in Belmont, MA (42 °39´N, 71°17´W) respectively. All *Photinus* fireflies were kept individually in plastic cups with moist paper towel, while *L. atra* and *E. corrusca* beetles were kept in single-sex groups in mesh cages. All beetles were kept at room temperature and on a natural light cycle.

CHC extraction for bioassays and Gas Chromotography

To collect CHC from *P. greeni,* and *P. obscurellus,* we rubbed cotton held by a toothpick (both washed in hexane and then autoclaved) 75 times over each beetle's pronotum and elytra, and CHC were isolated using standard techniques (Turillazzi *et al*. 1998; Sumana *et al*. 2005). The cotton was placed in 500 uL dichloromethane and sonicated for 20 minutes, after which the cotton was removed and the sample allowed to evaporate. Samples were resuspended in 15 uL pentane, and test extracts were made by pooling extracts from several

individuals to control for individual variation. To collect CHC for bioassays with *E. corrusca* and *L. atra* beetles, we switched to the more efficient method of removing both elytra from each firefly and soaking them in 500 uL dichloromethane (CH_2Cl_2) for 20 minutes. Samples were evaporated and re-suspended in 100 uL GC capillary grade heptane and vortexed for 10 seconds. This more efficient method was used to extract CHC for GC characterization. Gas chromatographs comparing samples collected with both methods were similar.

Gas chromatography analysis of CHC profiles

Cuticular hydrocarbon composition was analyzed with a Hewlett Packard 6980 gas chromatograph with a flame-ionization detector using standard techniques modified from Shibue *et al*. (2004). An Agilent HP-5 column (30 m x 0.319 mm x 0.25 μm) coated with nonpolar (5% phenyl)-methylpolysiloxane was used with the following temperature program: 50°C initial temperature, a 20°C/min ramp, 280°C final temperature with a 10 minute hold, and a 3 minute post run. Helium was the carrier gas and the program was performed in splitless mode. Final detector temperature was set at 300°C. Four uL of each sample was injected, and we analyzed multiple individuals of each species and sex (*P. greeni* - 4 males, 15 females; *P. obscurellus* – 8 males, 5 females; *P. ignitus* – 9 males, 12 females; *L. atra* – 3 males, 2 females; *E. corrusca* – 39 males, 39 females). In each group of samples, we included at least one quality-control extract (following the identical protocol without adding elytra); this allowed us to identify and disregard any non-beetle contaminants. Multiple GC runs of the same extract

confirmed that CHC profiles were highly repeatable. Insect cuticular hydrocarbons, including those previously identified from fireflies, have been shown to be between 21 and 35 carbons in length (Stanley-Samuelson & Nelson 1993; Shibue *et al*. 2004), and were identified as compounds with a retention time greater than 11 minutes based on a heneicosane ($C_{21}H_{44}$) standard. To concisely summarize differences in CHC profiles between the two diurnal firefly species, *L. atra* and *E. corrusca*, we used principal component analysis on the correlation matrix of relative peak areas (for this statistical analysis, we only included compounds with relative peak areas >6, as these could be readily distinguished from background peaks that were also present in quality controls). Nocturnal *Photinus* fireflies were not included in this analysis due to their low/undetectable levels of CHC. Principal component analysis was conducted using JMP 5.0 (SAS Inc., Cary NC).

Behavioral bioassays

To examine whether chemical signals may be used by firefly males for species recognition, we conducted behavioral assays in which we measured male response to extracts from conspecific vs. heterospecific females. We focused on male discriminatory abilities because in all of these firefly species, males approach females, make contact, and initiate copulation. For nocturnal fireflies, we conducted bioassay trials to determine whether males of *P. greeni* (n=11 males) and *P. obscurellus* (n = 8 males) were differentially responded to extracts from *P. greeni* compared to *P. obscurellus* females. These two locally sympatric *Photinus* species are morphologically indistinguishable in terms of

body size, shape, and coloration; they differ only in male genitalic structure and flash behavior (Green 1956; Lloyd 1969). Therefore, our bioassays were designed to test whether males could distinguish between conspecific and heterospecific females solely on the basis of chemical cues. Chemical extracts (prepared as described above) from females were applied to filter paper; presenting chemical extracts on filter paper is a well-established method that has been used successfully to examine discriminatory abilities of many insects, including *Argas* ticks (Leahy *et al*. 1973), *Lariophagus* parasitic wasps (Steidle & Ruther 2000), *Ixodes* deer ticks (Allan & Sonenshine 2002), *Tenebrio* beetles (Bryning *et al*. 2005), *Piezodorous* stink bugs (Borges *et al*. 2007), *Callosobruchus* weevils (Nojima *et al*. 2007), and *Lonomia* moths (Zarbin *et al*. 2007). For each firefly species, bioassays were conducted during the appropriate mating period in either the field or the laboratory (at 24-26°C).

Bioassays for nocturnal fireflies species were conducted by placing each male in a 200 cm^3 transparent chamber containing two 1 cm^2 squares of Whatman filter paper placed 7 cm apart on the chamber floor. Males were allowed a 5 min acclimation period, after which 15 uL (representing 1 female equivalent) of pooled extracts from either conspecific or heterospecific females were pipetted onto the filter paper. Male behaviors (walking, flying, antennation) were monitored continuously for 10 minutes during which we recorded how long each male spent in the vicinity (within 2 cm) of each filter paper square. Bioassays for diurnal fireflies were similar, except chambers consisted of a 9 cm petri dish, as

these males generally approach females by walking; trials were conducted on 8 *L. atra* males and 9 *E. corrusca* males.

For each species, we compared the duration of time males spent associated with the conspecific and the heterospecific extracts; because these paired data were not normally distributed, we used a non-parametric Wilcoxon signed-rank test (2-tailed) to determine if beetles showed a preference for either stimulus. Because differences of zero between paired stimuli are excluded in this analysis, sample sizes were reduced for some species.

RESULTS

Gas chromatography analysis revealed major differences in CHC profiles among firefly species. In the nocturnal *P. greeni*, no detectable CHC were found in any females (Figure 1A) or males (Figure 1B). Similarly, both sexes of *P. ignitus* as well as all *P. obscurellus* females and most *P. obscurellus* males exhibited no detectable CHC (data not shown; 2 of 8 *P. obscurellus* males showed measurable but low CHC abundance). In contrast, both diurnal firefly species exhibited a much greater abundance and diversity of CHC (Figure 2). *L. atra* showed the highest CHC abundance, with both females (Figure 2A) and males (Figure 2B) showed the same 8 CHC peaks. In *E. corrusca* (Figure 2C and D), overall CHC abundance was lower than in *L. atra*, and again, similar peaks were present in both sexes. Principal component analysis of relative peak areas for these diurnal fireflies (Figure 3) showed that these two species separated mainly along principal component 1, with a fairly wide range of individual variation within species.

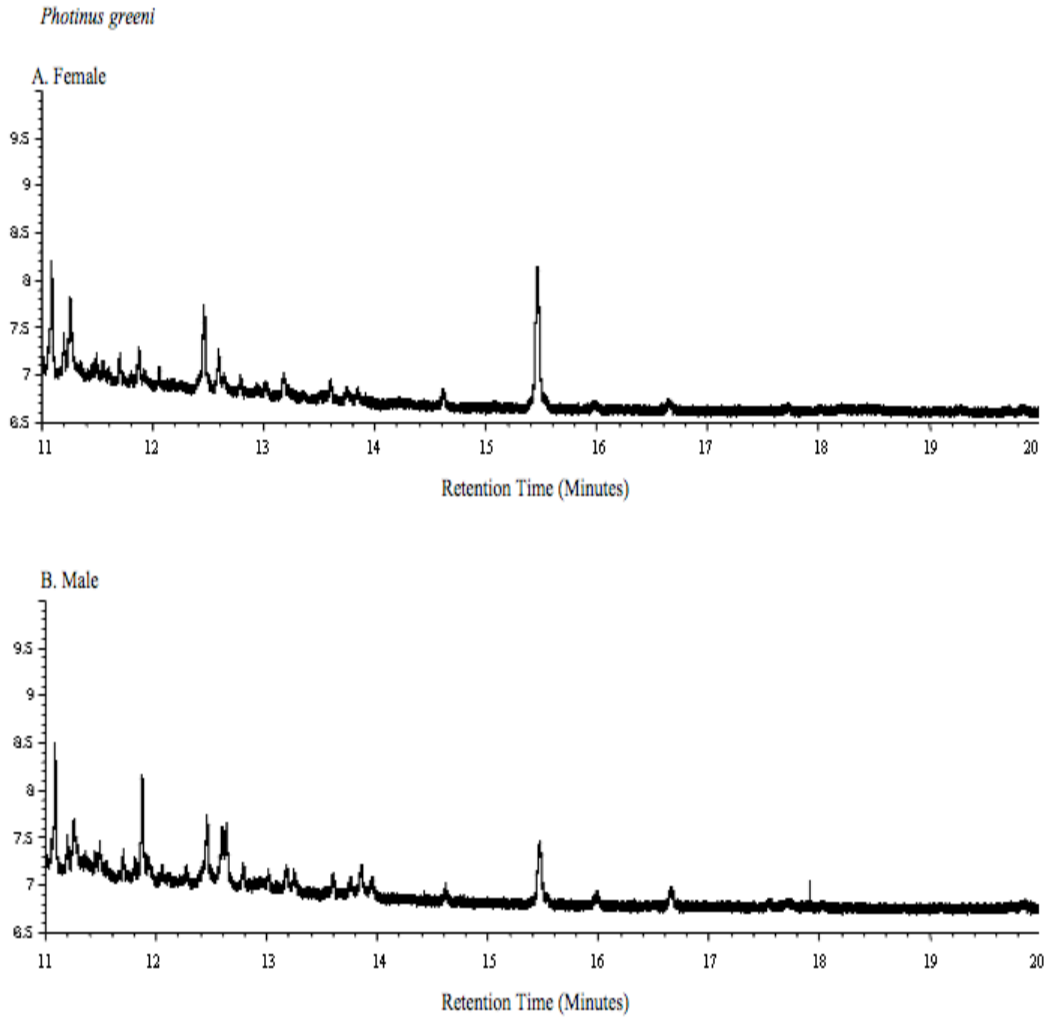

Figure 1. Representative GC profiles of cuticular hydrocarbons from nocturnal fireflies, *Photinus greeni:* A. female, B. male. All peaks seen in both chromatograms were also present in quality control samples.

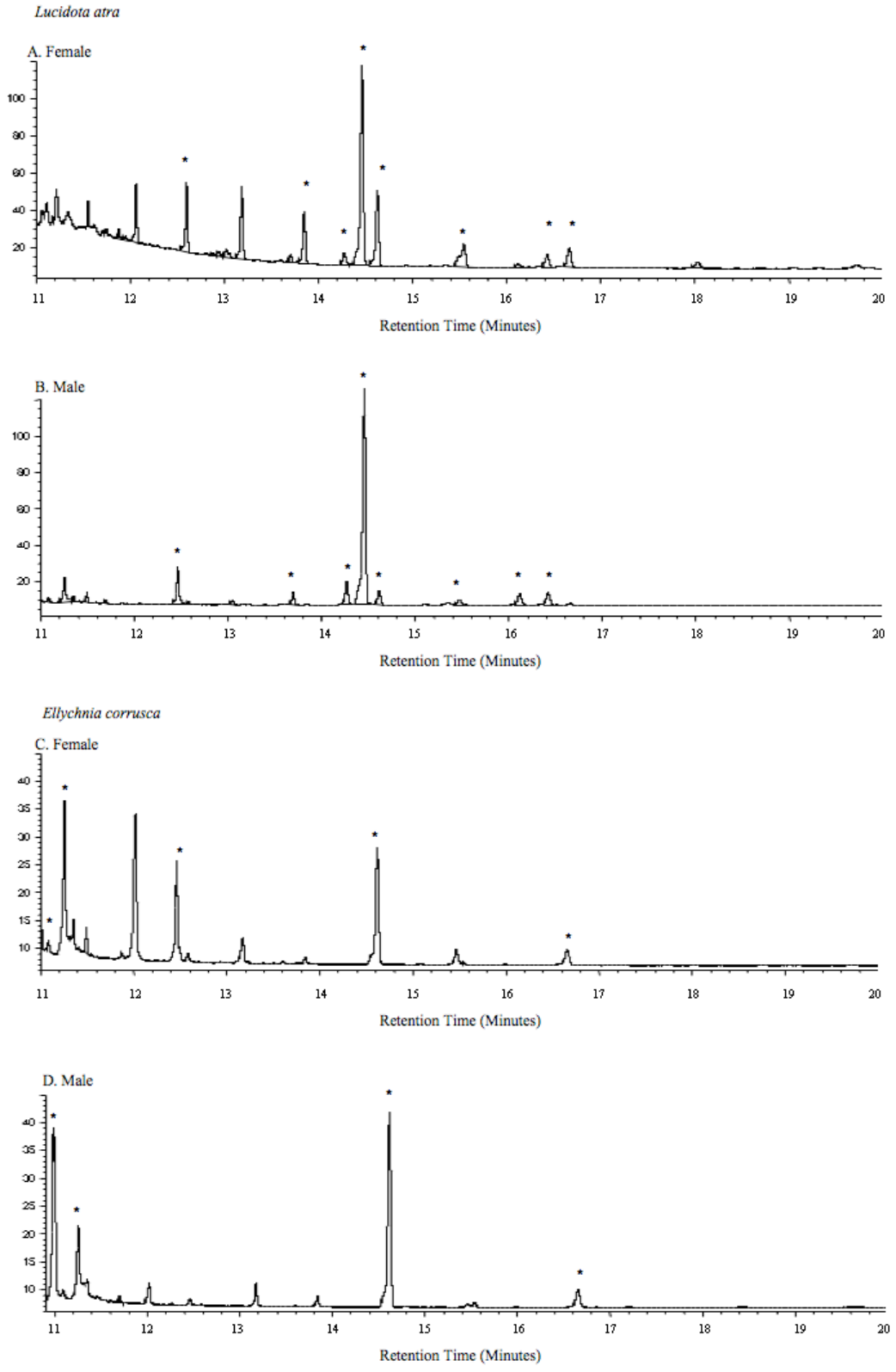

Figure 2. Representative GC profiles of cuticular hydrocarbons from diurnal fireflies (note change in Y-axis scale from Figure 1): *Lucidota atra* - A. female, B. male*; Ellychnia corrusca* – C. female, D. male Peaks with asterisks represent cuticular hydrocarbons based on retention times and absence in quality controls.

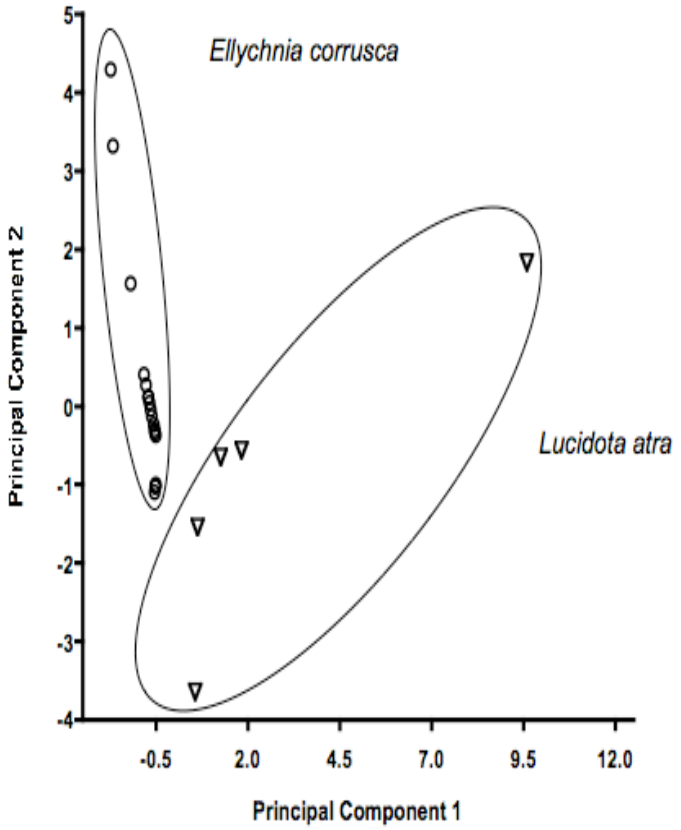

Figure 3. Principal component analysis summarizing species differences and individual variation in CHC relative peak areas for two diurnal firefly species (18 *E. corrusca* individuals, 5 *L. atra* individuals).

In bioassays tests of nocturnal fireflies, there were no significant differences in the time that focal males spent in close proximity to extracts from conspecific vs. heterospecific females for either *P. greeni* (Figure 4A; n = 11 males, Wilcoxon signed ranks test $W_s = 48$, $p = 0.182$) or for *P. obscurellus* (Figure 4B; n = 8, $W_s = 18$, $p = 1.0$). Nocturnal firefly behavior during the bioassays was characterized by periods of inactivity interspersed with brief periods of flight or walking.

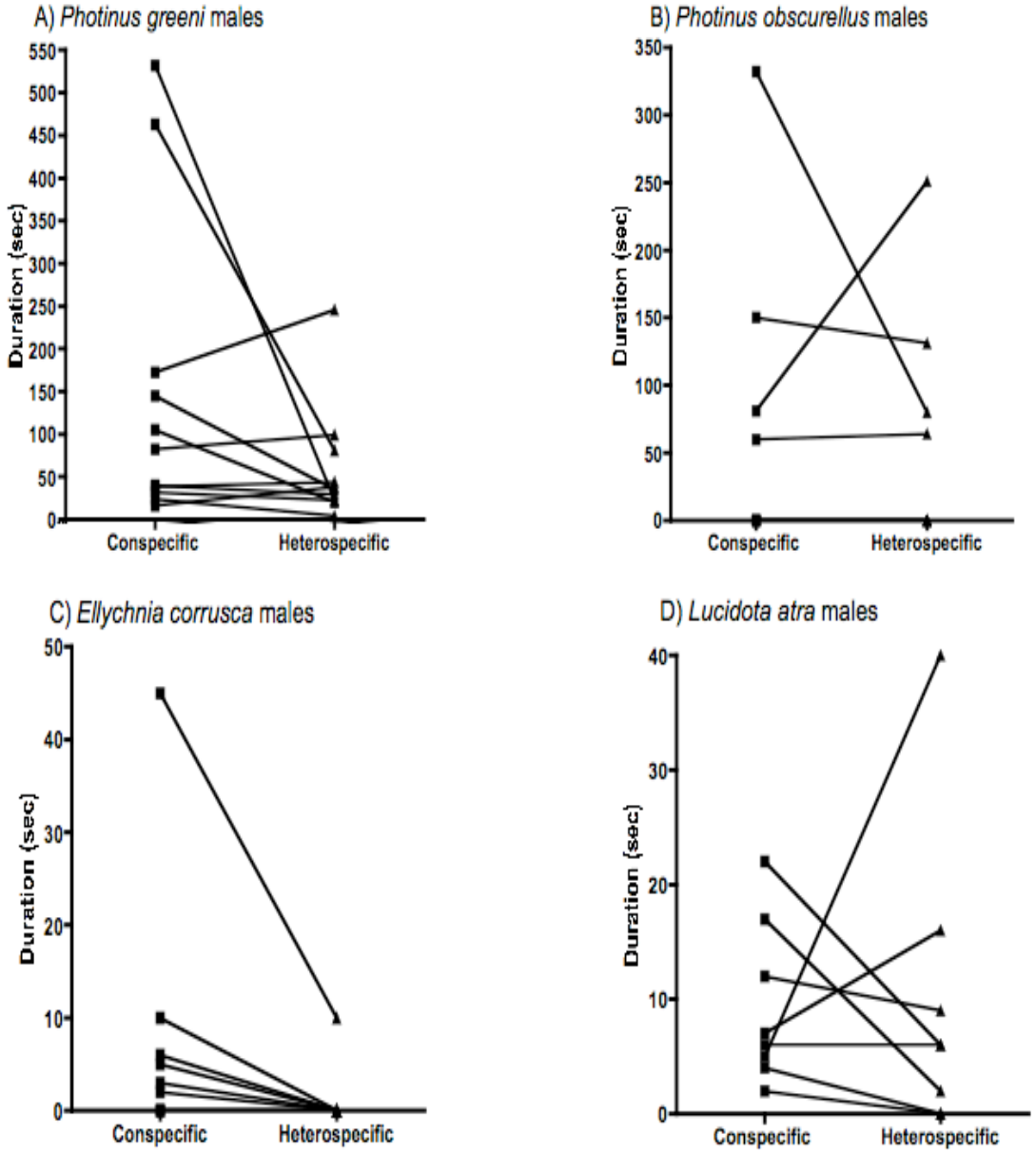

Figure 4. Behavioral bioassay results for male fireflies responding to extracts from conspecific vs. heterospecific females. Duration of time (in seconds) spent in proximity to extracts on filter paper was recorded during 10 min observation periods. Each line represents the behavioral responses of a single male: A. *Photinus greeni* (n = 11 males), B. *Photinus obscurellus* (n = 8 males), C. *Ellychnia corrusca* (n = 9), D. *Lucidota atra* (n = 8).

For diurnal fireflies, *E. corrusca* males spent significantly more time in contact with extracts from conspecific compared to heterospecific females (Figure

4C: n= 6, W_s= 21, p = 0.028), while *L. atra* males showed no significant difference (Figure 4D: n = 7, Ws= 17, p = 0.612). Diurnal fireflies showed a marked increase in walking and antennation when they encountered filter paper containing the extracts

DISCUSSION

A communication role for cuticular hydrocarbons has been well-documented in many social and solitary insect species; contact CHC signals have been shown to function in species, sex and nestmate recognition, as well as in mate choice (Howard 1993; Singer 1998; Ginzel & Hanks 2003; Ginzel *et al*. 2003; Sumana *et al*. 2005; Dani 2006; Barbour *et al*. 2007; Johansson & Jones 2007; Mullen *et al*. 2007). Sex-specific differences in CHC presence and abundance have been found in several coleopterans (Ginzel *et al*. 2003; Barbour *et al*. 2007; Peterson *et al*. 2007). Our is the first study to provide a comprehensive assessment of species and sex differences in CHC profiles across several firefly species using multiple individuals. CHC were abundant in two diurnal fireflies, *E. corrusca* and *L. atra*. However, CHC were not detected in the nocturnal fireflies *P. greeni*, *P. ignitus*, or in *P. obscurellus* females, and CHC were found in very low abundance in a few *P. obscurellus* males. These low CHC levels found in nocturnal fireflies are unlikely to reflect methodological artifacts, as we readily detected CHC in diurnal fireflies using identical procedures. Although new techniques may allow detection of higher molecular weight CHC excluded by the standard GC approach used here (Cvacka et al. 2006), it is possible that lower dessication risk in nocturnal fireflies precludes the need for high CHC abundance as CHC function to prevent

dehydration in many insects (Howard & Blomquist 2005). Our results are consistent with those obtained by Shibue *et al*. (2004), who also found greater CHC diversity and abundance in diurnal compared to nocturnal fireflies, and identified 8 CHC peaks in two *L. atra* females. The present study also revealed major differences in CHC profiles between two sympatric diurnal fireflies, suggesting that these contact signals might play a role in species recognition and pre-mating reproductive isolation in this group.

Results from the behavioral bioassays also provide support for the idea that species recognition by *E. corrusca* males may involve contact chemical signals, as these males preferentially responded to extracts from conspecific females compared to *L. atra* females. Based on the lack of discrimination by *L. atra* males in bioassays, however, contact chemicals appear less important as recognition signals in this other diurnal firefly. *L. atra* females produce volatile pheromonal signals that attract males (Lloyd 1972), although the active compounds have yet to be identified.

Bioassay results for the two nocturnal *Photinus* species, *P. greeni* and *P. obscurellus,* indicated that males did not discriminate between contact chemicals from conspecific vs. heterospecific females. Unfortunately, in the absence of any known chemical attractants for these beetles, positive controls could not be incorporated into the bioassay design. However, evidence supports an interpretation of our bioassay results as indicating *Photinus* males do not use chemical cues for close-range species discrimination. First, these bioassay results are consistent with our gas chromatographic analyses, which showed CHC were

below detection limits in these nocturnal fireflies. Second, it is not likely that the observed lack of discrimination exhibited during behavioral bioassays reflected a lack of motivation to mate, as all bioassays were conducted during the appropriate evening mating period for each species. Additionally, previous studies have used similar extraction and bioassay methods to show behavioral discrimination in many different insects (Ginzel *et al*. 2003; Bryning *et al*. 2005; Barbour *et al*. 2007), suggesting that these methods should also have been sufficient to detect any use of contact chemical signals by fireflies. Finally, it seems unlikely that lack of discrimination by *Photinus* males reflects insufficient statistical power, as a significant preference for conspecific female extracts was demonstrated for *E. corrusca* males using similar sample sizes. Taken together, these findings suggest that contact chemical signals do not play a major role in species recognition for nocturnal *Photinus* fireflies.

In conclusion, cuticular hydrocarbons seem unlikely to play a major role in species or sex recognition in nocturnal firefly species. Current evidence suggests that species, sex and mate recognition in nocturnal fireflies relies primarily on long-range bioluminescent visual signals. However, CHC may be important species recognition signals for at least one diurnal firefly, and may replace or act in conjunction with volatile pheromones shown to be important in other diurnally active fireflies. Future studies of multimodal signaling in fireflies might focus on close-range tactile signals associated with pre-mating behavioral interactions.

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Chapter VII. Correlated evolution of female neoteny and flightlessness with male spermatophore production in fireflies (Coleoptera: Lampyridae)

Abstract

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.

Introduction

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 dramatically 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, 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 Liebherr 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 (Figure 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 (1998) 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.

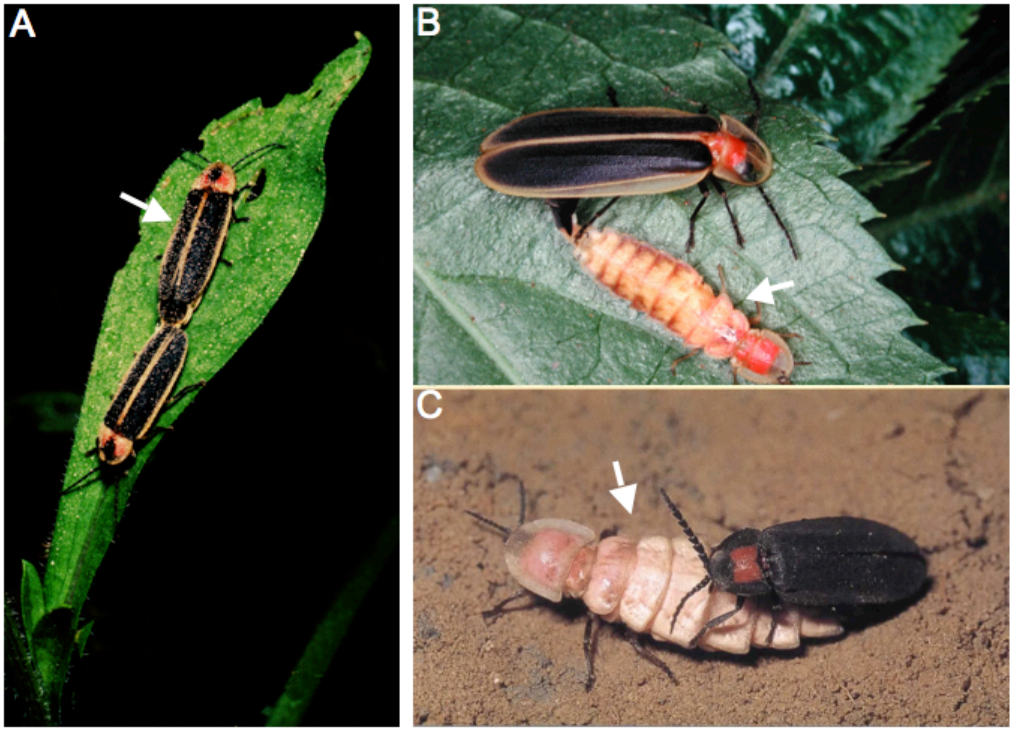

Figure 1. Range of neoteny exhibited by females (indicated by arrows) in various lampyrid fireflies. **A.** *Photinus sp*. (neoteny score of 0). **B.** *Diaphanes sp*. (neoteny score of 2). **C.** *Pyrocoelia analis* (neoteny score of 2).

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 non-gametic) 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 Simmons1989, 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 tradeoffs 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 vs. flightless females. In species where females are capable of flight, female reproductive allocation is assumed to be limited by biomechanical constraints and by tradeoffs 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 towards 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 & 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 while adults of most species have degenerate 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 *P. 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, *personal communication*).

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 multi-state characters, female flightlessness (associated with higher levels of neoteny) and male spermatophore production (presence/absence) as binary characters, and sexual signal mode as a multi-state 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, non-neotenic 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: *Lampyris 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 (*personal communication*).

CHARACTERIZING MALE REPRODUCTIVE TRAITS

Data on male reproductive anatomy and spermatophore production was 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 7 species (*Photinus pyralis*, *Ellychnia corrusca*, *Pyractomena angulata*, *Photuris versicolor*, *Luciola cruciata*, *L. lateralis* and *Phausis 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 (online supplemental material). 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 & 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 2 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 (*Microphotus 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 demonstrated 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 1x phosphate buffered saline using 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). 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) (Figure 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 *Pyropyga nigricans* (neoteny score=1), a species which exhibits substantial inter-population 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 (non-luminescent), 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 (intitiates 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 Mattysen 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 v. 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

(6 states), female neoteny (5 states), and sexual signal mode (4 states) were coded as multi-state characters.

To test for correlated evolution between the two binary traits, we used Pagel's (1994) correlation analysis as implemented in Mesquite v. 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 (4-parameter model) or dependent (8-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 multi-state characters (4-6 states) as continuous traits and tested for trait correlations using independent contrast analysis (Felsenstein 1985, as implemented in Mesquite v. 2.6 and the PDTREE module v. 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 (Pagel 1992: contemporaneous tips and internode segments set equal) and Grafen's (Grafen 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 v.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*, *Stenocladius*, and *Rhagopthalmus* 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 (Figure 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 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 2 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, while the remaining 3 species (*Pleotomus pallens, Lampyris noctiluca,* and *Phosphaenus hemipterus*) had neoteny scores of 2. In all 12 of the lampyrid species lacking male spermatophores, the corresponding females were flightless (Table 1).

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
Photuris versicolor	0	Yes	3	Yes	3—Flash	2,4,5
Pleotomus pallens	2	No	3	Yes	2—Glow	2,4,5
Lampyris noctiluca	2	No	2	Yes	2—Glow	2,4,5
Pyrocoelia discicolis	2	No	1	No	1—Pheromone/Glows	1,3,4,5
Pyrocoelia fumosa	2	No	1	No	1—Pheromone/Glows	1,3,4,5
Pyrocoelia m. matsumurai	2	No	2	No	1—Phermone/Glows	1,3,4,5
Diaphanes formosus	2	No	1	No	2—Glow	2,4,5
Pyrocoelia rufa	2	No	1	No	2—Glow	1,3,4,5
Pyrocoelia miyako	2	No	0	No	2—Glow	1,3,4,5
Pyrocoelia atripennis	2	No	1	No	2—Glow	1,3,4,5
Microphotus angustus	3	No	1	No	2—Glow	2,4,5
Ellychnia corrusca	0	Yes	4	Yes	0—Dark	2,4,5
Photinus pyralis	0	Yes	4	Yes	3—Flash	2,4,5
Pyropyga nigricans	1	No	5	Yes	0—Dark	2,4,5
Pyractomena angulata	0	Yes	3	Yes	3—Flash	2,4,5
Lucidota atra	0	Yes	5	Yes	0—Dark	2,3,5
Lucidina biplagiata	0	Yes	3	Yes	0—Dark	1,3,4,5
Lamprohiza splendidula	2	No	2	No	2—Glow	2,4,5
Phosphaneus hemipterus	2	No	3	Yes	0—Dark	2,4,5
Phausis reticulata	3	No	2	No	2—Glow	2,4,5
Cyphonocerus ruficollis	0	Yes	3	Yes	1—Pheromone/Glows	1,3,4,5
Stenocladius shiraki	4	No	1	No	0—Dark	1,3,4,5
Rhagophtalmus ohbai	4	No	1	No	2—Glow	1,3,4,5
Hotaria parvula	1	Yes	3	Yes	3—Flash	1,3,4,5
Luciola yayeyamana	1	Yes	3	Yes	3—Flash	1,3,4,5
Luciola cruciata	0	Yes	3	Yes	3—Flash	2,4,5
Luciola lateralis	0	Yes	3	Yes	3—Flash	2,4,5
Luciola kuroiwae	0	Yes	3	Yes	3—Flash	1,3,4,5
Curtos okinawana	0	Yes	3	Yes	3—Flash	1,3,4,5
Curtos costipennis	0	Yes	3	Yes	3—Flash	1,3,4,5
Pristolycus sagulatus	0	Yes	2	Yes	0—Dark	1,3,4,5
Drilaster axillaris	0	Yes	3	Yes	0—Dark	1,3,4,5

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

RECONSTRUCTING PATTERNS OF TRAIT EVOLUTION

Female neoteny was found to be a derived trait (Figure 2). Ancestral state reconstruction-revealed that the common ancestor had non-neotenic females (Figure 2, proportional likelihood for neoteny score of 0 = 0.99) and thus these females were capable of flight (Figure 3, proportional likelihood = 0.94). From

this non-neotenic ancestor, there were at least 7 independent increases (and no reductions) in female neoteny (Figure 2), associated with at least 5 independent losses of female flight capability (Figure 3).

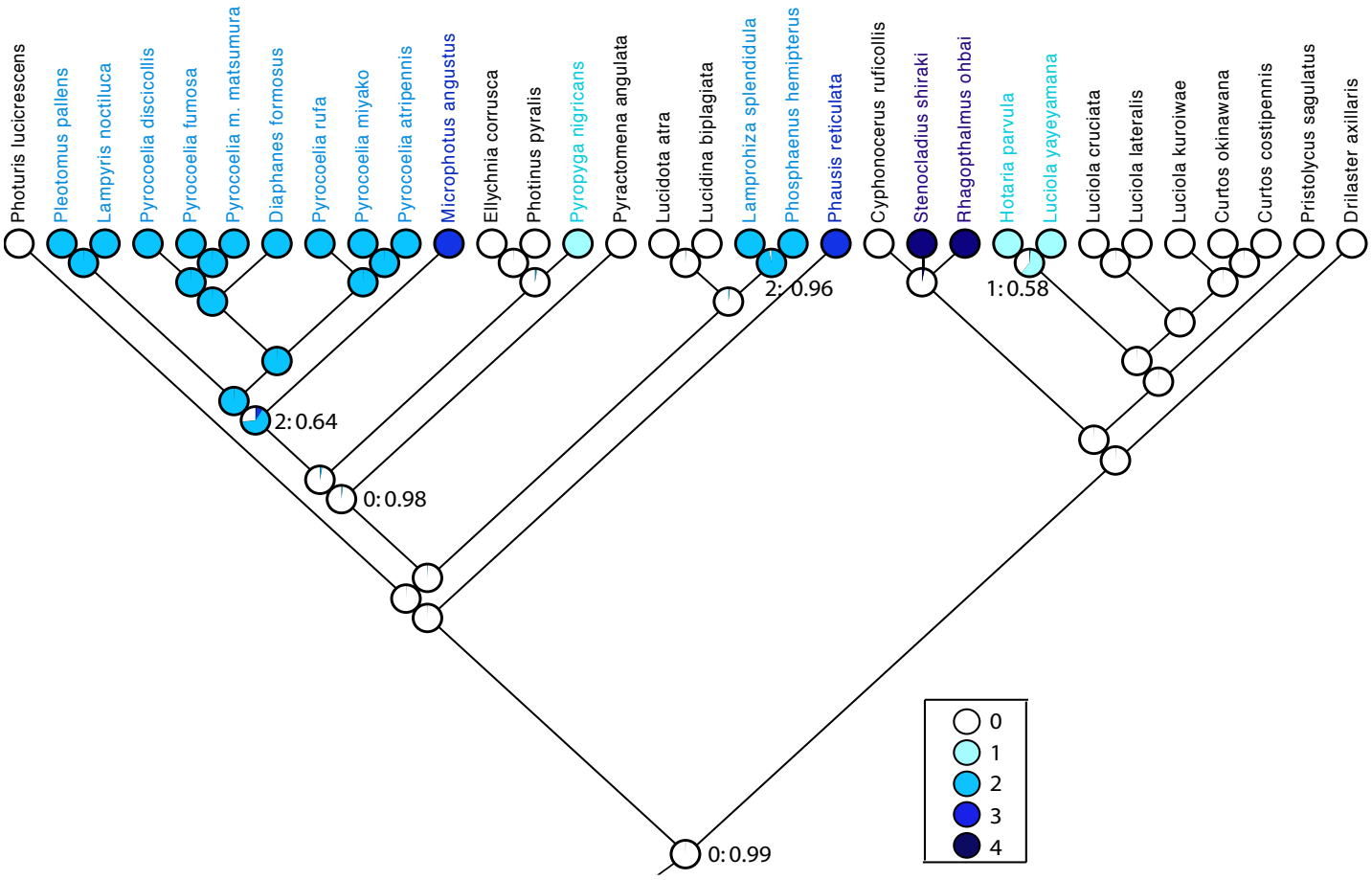

Figure 2. Maximum likelihood reconstruction of female neoteny for 32 firefly species. The pie charts indicate the ML support (proportional likelihood) for the ancestral state at each node, with values given at transition nodes.

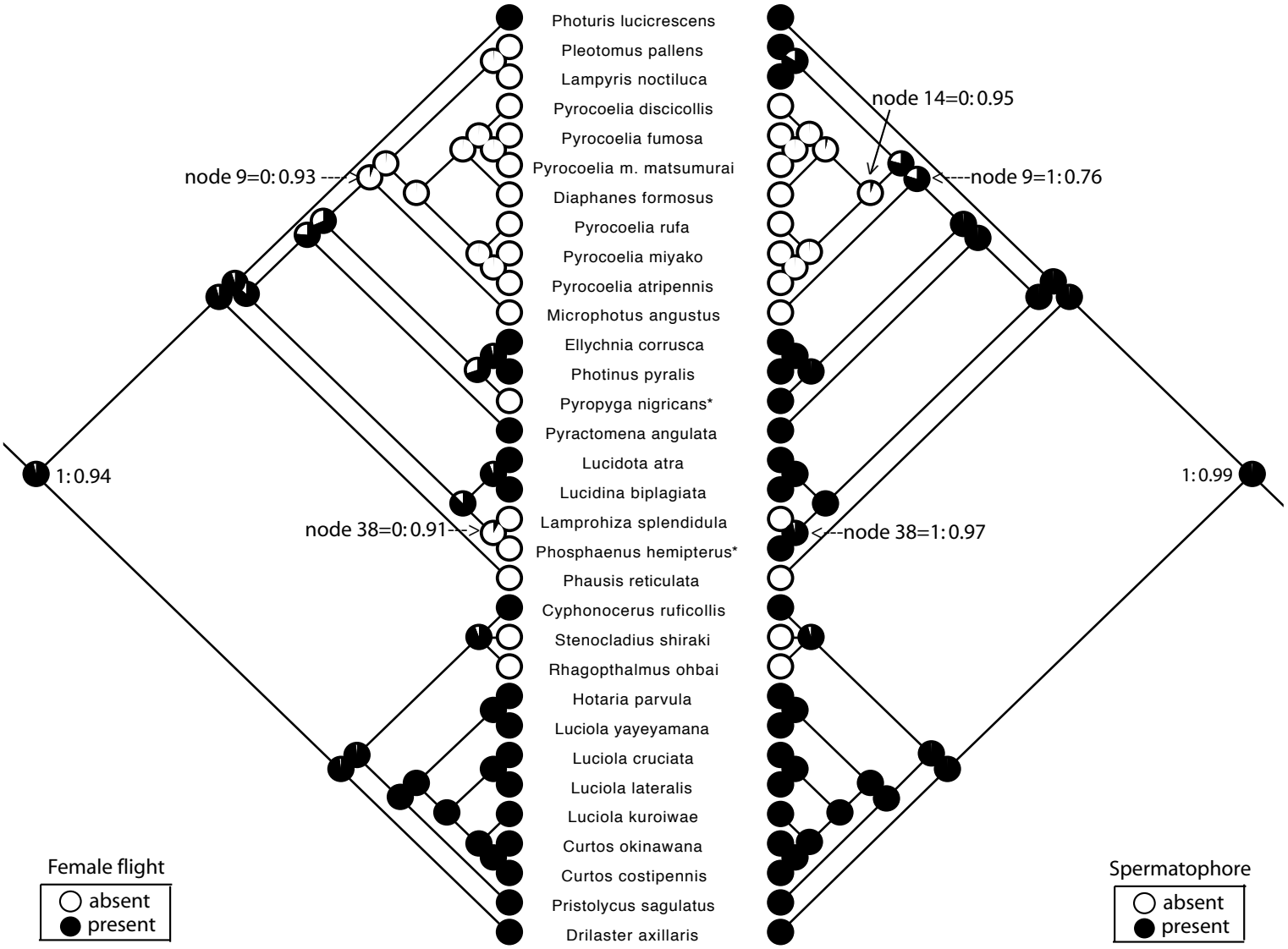

Figure 3. Maximum likelihood reconstructions of female flightlessness (left) and spermatophore production (right). The pie graphs indicate the ML support (proportional likelihood) for the ancestral states at each node, with values given at transition nodes. Numbered nodes with arrows are discussed in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

Maximum likelihood reconstructions revealed that male spermatophore production was the ancestral state (Figure 3, proportional likelihood = 0.99). Loss of spermatophore production occurred subsequently in at least 4 lineages, with no reversions. Ancestral lampyrid males most likely used 3 paired reproductive accessory glands to produce spermatophores (Figure 4, proportional likelihood = 0.98). In our taxon sample the number of male accessory glands independently increased twice (to 4 or 5 pairs), and also decreased 4 or 5 times (to 2 or 1 pairs). In addition, there were 2 independent reversals where male accessory gland number increased from a single pair to either 2 or 3 pairs. In the case of *Pyrocoelia m. matsumurai*, such a reversal led to males having 2 pairs of accessory glands and losing spermatophore production (Table 1, Figures 3, 4). The other reversal occurred in the common ancestor of *Pleotomus pallens* and *Lampyris noctiluca;* males in both extant species produce spermatophores, the former with 3 gland pairs and the latter with 2 gland pairs. Thus, with the exception of *Lampyris noctiluca,* all taxa in which males transfer spermatophores show ≥ 3 pairs of accessory glands (Table 1, Figures 3, 4).

Analysis of this taxon sample indicated that light was not used for sexual communication in the lampyrid ancestor (Figure 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 4 times: 3 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 (*Pyrocoelia discicollis* group), and once from a dark ancestor (*Cyphonocerus ruficollis*; see also Stanger-Hall *et al*. 2007).

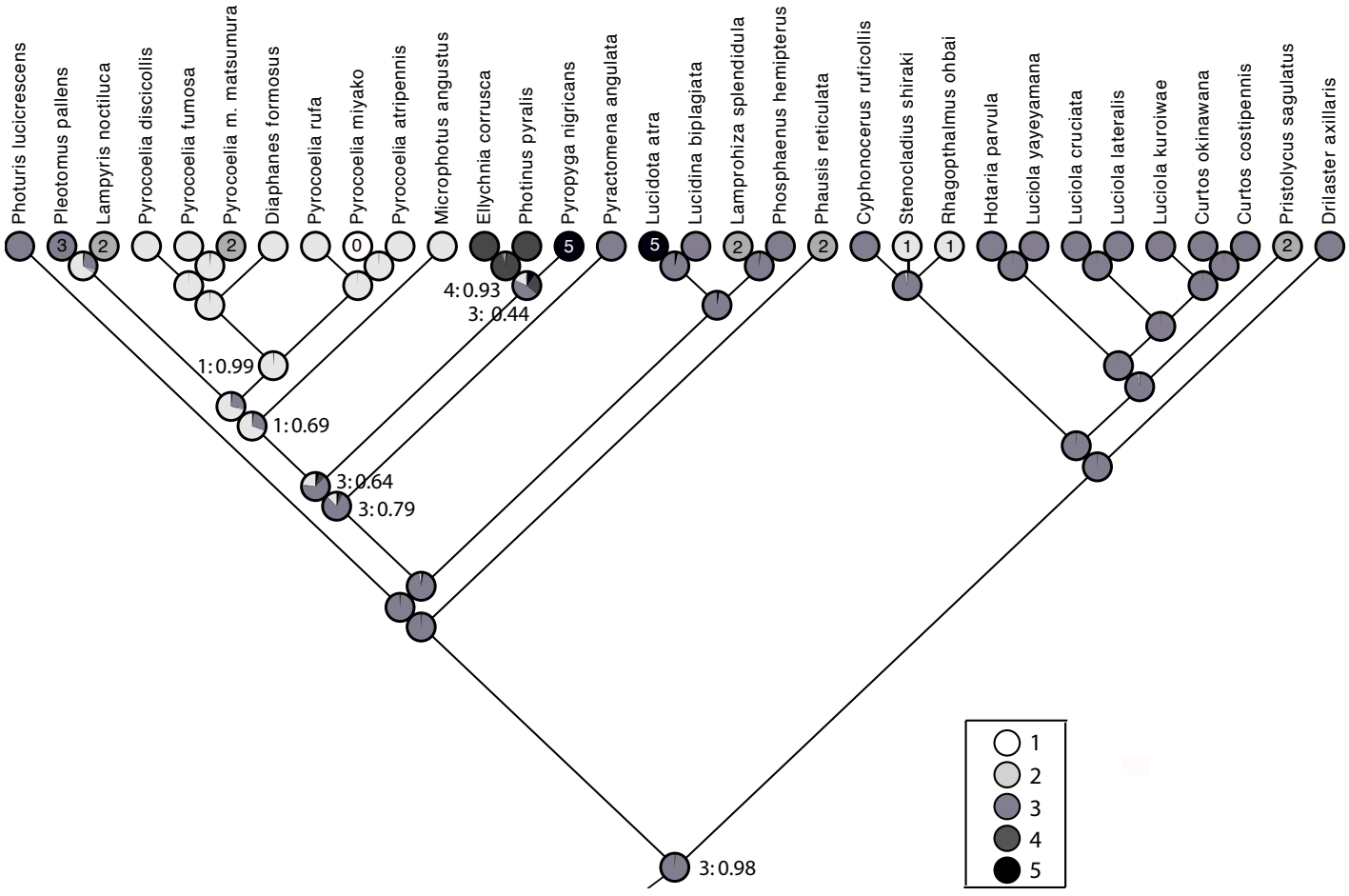

Figure 4. Maximum likelihood reconstruction of the number of male accessory glands for 32 firefly species. The pie graphs indicate the ML support (proportional likelihood) for the ancestral state at each node, with values given at transition nodes.

CORRELATED TRAIT EVOLUTION

Ancestral state reconstruction for female flight ability (proportional likelihood = 0.94) and spermatophore production (proportional likelihood = 0.99) showed remarkable congruence (Figure 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 (Figure 2) and male accessory gland number (Figure 4, r = - 0.6146, $F(1,29) = 18.21$, $P < 0.001$).

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 Figure 3) also represents a shift to glowing females from flashed light signals (Figure 5, proportional likelihood = 0.94). Similarly coinciding with the loss of female flight, *Rhagopthalmus, Phausis* and *Lamprohiza* each independently evolved strong female glows as sexual signals; in these cases such glows evolved from dark ancestors. Interestingly, while 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 Matthysen 2005; De Cock 2009). Bioluminescent signals are also absent in flightless *Pyropyga* and *Stenocladius* females. *Cyphonocerus* is the only taxon in our analysis that retained non-neotenic females with full flight ability and weak glows.

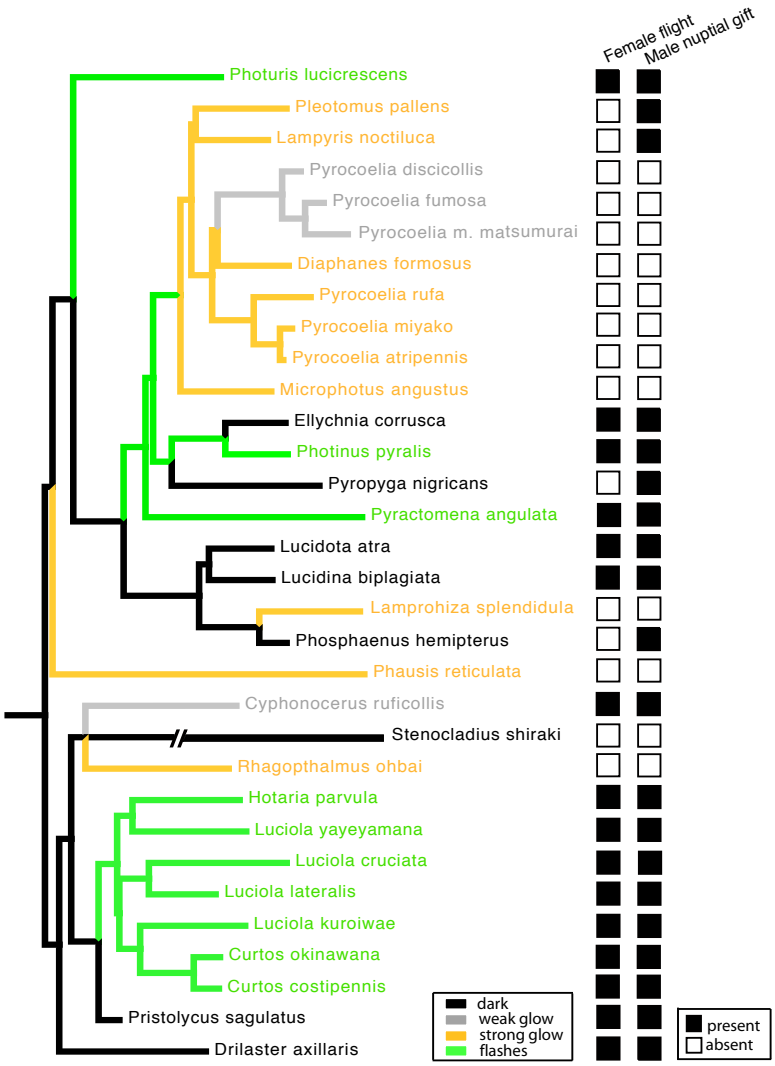

Figure 5. Phylogenetic relationships (from Stanger-Hall et al. 2007) among 32 firefly species indicating signaling mode used for sexual communication, and character matrix for female flight ability and male spermatophores.

RECONSTRUCTING HISTORICAL SEQUENCES OF TRAIT EVOLUTION

The evolution of female neoteny with a concomitant loss of flight ability occurred at least 5 times in our taxon sample, as did the loss of male spermatophores (Figures 2, 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 3 instances. For example, in the lineage leading to *Microphotus*, *Diaphanes*, and *Pyrocoelia* fireflies, the change from non-neotenic females to neoteny level 2 occurred somewhere between nodes 8 and 9 (Figure 2: node 9, proportional likelihood of neoteny level 2 = 0.64); because flight is based on neoteny level, flight was also lost 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 (Figure 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 *Lamprohiza splendidula* and *Phosphaenus hemipterus* (Figure 2, node 38 in Fig. 3: proportional likelihood of female flightlessness = 0.091). This was followed in *Lampyris splendidula* by a reduction in male accessory glands (Figure 4) and spermatophore loss (Figure 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 (Figure 3). Finally, *P. reticulata, S. shirakii, and R. ohbai* all show increased female neoteny, associated loss of flight, and absence of spermatophore production (Figure 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.

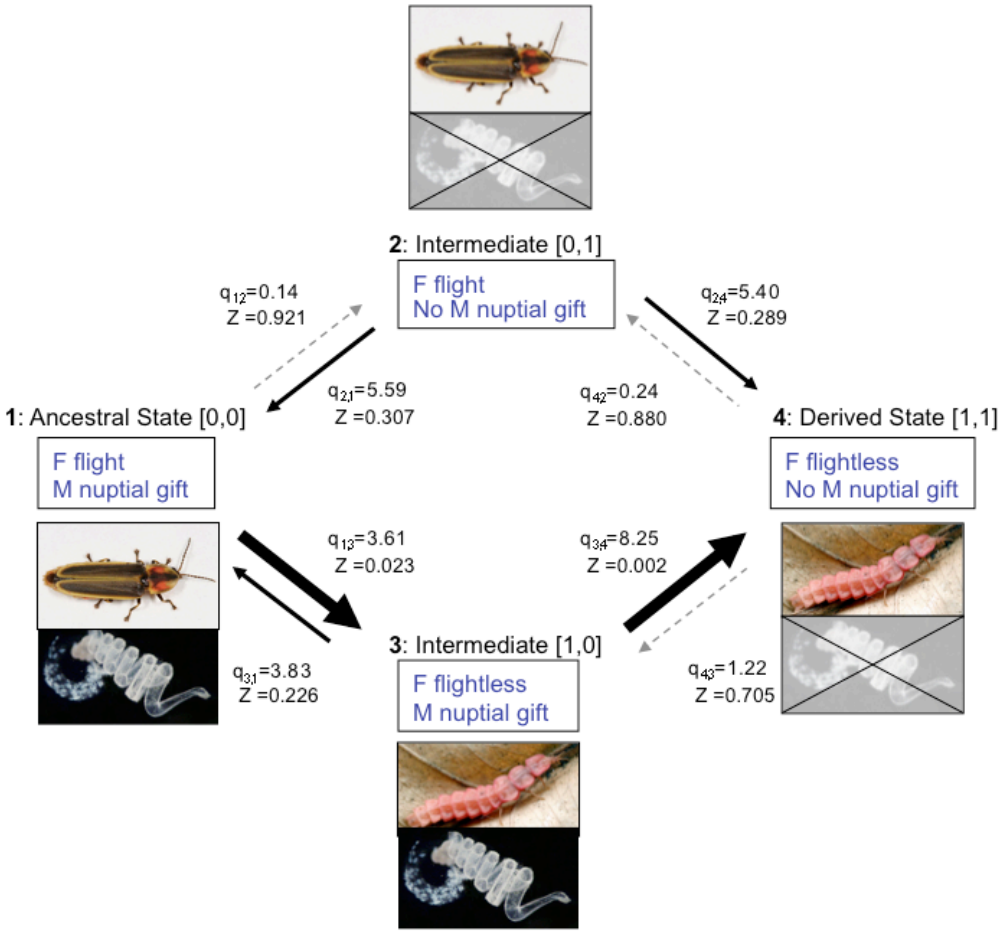

Figure 6. Possible evolutionary transitions between ancestral and derived states for female flight and male spermatophores. For each transition we report both the q value (mean transition parameter between these 2 states) and the Z value (proportion of iterations that were assigned zero), each based 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).

This conclusion is supported by transitional probability analysis (Figure 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 (Figure 6, $Z > 0.90$). Finally, once both flight and spermatophores were lost, reversals to either intermediate were unlikely (Figure 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. While there was no overall association between female neoteny 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 non-neotenic 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 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 Fairborn 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, 1988; 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 *Lampyris 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 non-neotenic 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 vs. 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, *Ellychnia corrusca* and *Photinus 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 towards 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 intra-sexual 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 (South, unpublished data; Fu, *personal communication*).

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 neotonic 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*. 2004; Boggs 2009). For example, neotenic *Photinus 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 4 extant taxa (*Pleotomus pallens*, *Lampyris noctiluca*, *Pyropyga*

nigricans, and *Phosphaenus 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 lampyrid 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 lampyrid *Pyropyga 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 lampyrid 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 lampyrids 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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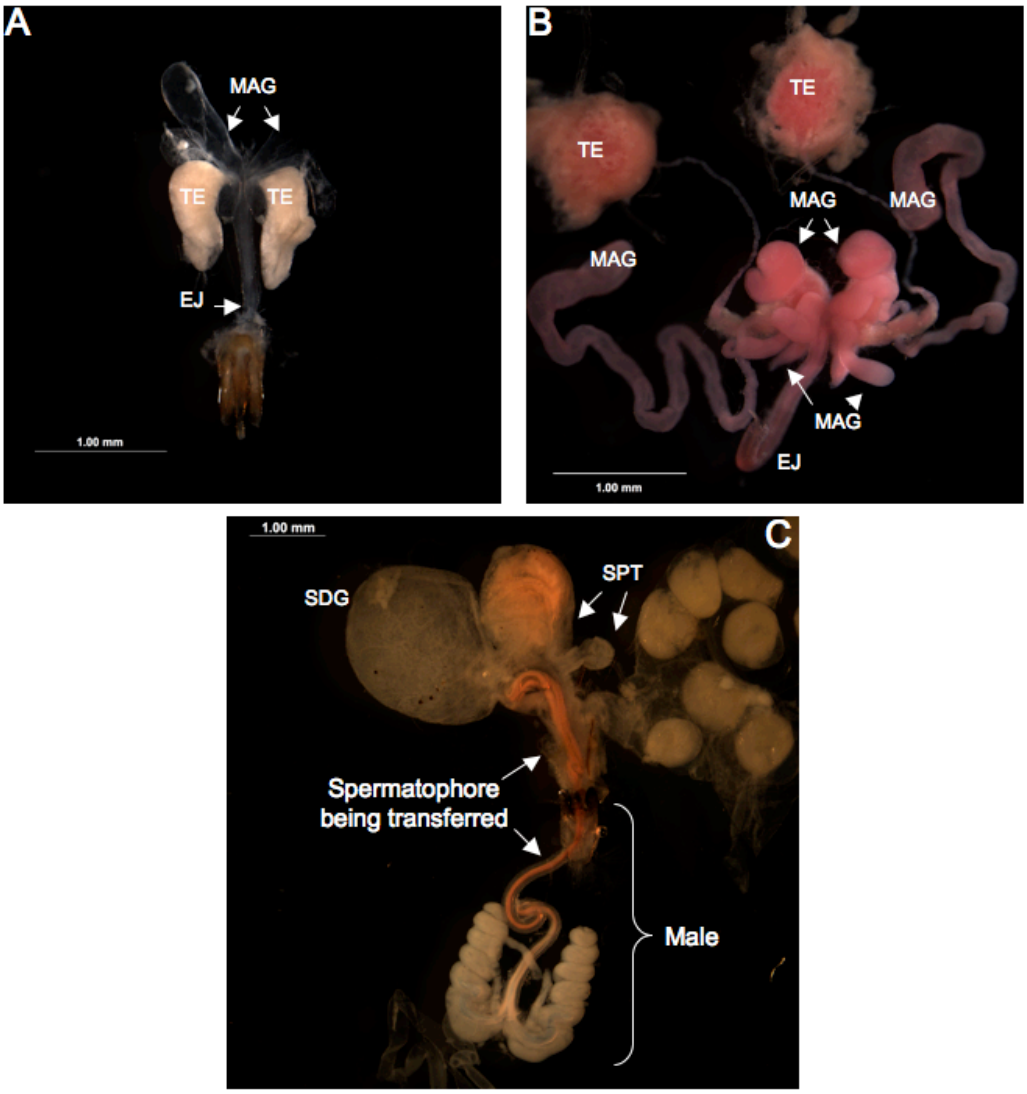

Supplementary Figure 1. Reproductive systems in three lampyrid firefly species, showing evidence used to assess spermatophore production. **A**. *Lamprohiza splendidula* male, showing testes and only a single pair of accessory glands. **B.** *Luciola lateralis* male, with three paired accessory glands. **C.** Male and female reproductive tracts of *Photinus*, showing a spermatophore (stained pink with rhodamine B) in the process of being transferred from the male (lower) to the female (upper). Male reproductive system shows four paired accessory glands. Female shows structures for sperm storage and spermatophore digestion. **Labeled structures:** MAG – male accessory gland, TE – testes, EJ – ejaculatory duct, SDG – spermatophore digesting gland, SPT – spermatheca.

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Chapter VIII. Identification of predicted seminal fluid proteins in *Tribolium castaneum*

Abstract

In several insect species, seminal fluid proteins (SFPs) have been demonstrated to be key regulators of male and female fitness through their ability to alter female physiology and behavior. *Tribolium castaneum* is an economically important pest species and a model system for sexual selection research, but little is known about SFPs in this insect. To create a foundation for the study of *T. castaneum* SFPs, we used mass spectrometry to identify putative SFPs by comparing proteins detected in the male reproductive glands with those found in the reproductive tracts of virgin and mated females. Thirteen putative SFPs were identified through this approach. We also used RT-PCR to examine expression levels across different tissue types. We found strongly male-biased expression in 13 genes, 9 of which were expressed only in male accessory gland tissue. This represents the first proteomic-based method of identifying putative SFPs in any coleopteran species, and is the first study in this species to identify putative SFPs that are likely transferred to the female. This work could lead to functional analyses of the role of SFPs in sexual selection, sexual conflict and potential control of a pest species.

Introduction:

Although the primary function of copulation is gamete transfer, male ejaculates contain numerous other substances produced by secretory tissues in the reproductive tract (Chen, 1984; Gillott, 1996, 2003; Leopold, 1976; Poiani, 2006; Simmons, 2001). Recent work in arthropods has identified many specific non-sperm components of male ejaculates (reviewed in Gillott, 2003; Poiani, 2006; Wolfner, 2007; Avila *et al*. 2011). These substances not only assist in storing and provisioning sperm (Poiani, 2006), but also have diverse physiological and behavioral effects on females. In insects, seminal fluid proteins (SFPs) have been implicated as the principal seminal component responsible for inducing many of these post-mating responses (Gillott, 2003; Wolfner *et al*., 2005; Wolfner, 2007; Avila *et al*., 2011).

The most extensive characterization of SFPs has been accomplished in *Drosophila melanogaster* (reviewed in Wolfner, 2002, 2007; Ravi Ram and Wolfner, 2007), although some characterization of SFPs has occurred in other species of Diptera (Tephritids: Davies & Chapman 2006; Mosquitoes: Sirot *et al*.

2008; Dottorini *et al*. 2007; Rogers *et al*. 2009), honeybees (Collins *et al*. 2006; Baer *et al*. 2009), crickets (Braswell *et al*. 2006; Andres *et al*. 2008) and bedbugs (Reinhardt *et al*. 2009). The *D. melanogaster* studies demonstrate that SFPs influence many processes in females, including stimulating oviposition and egg production, sperm utilization and storage, female re-mating rates and even female life span (reviewed in Eberhard and Cordero, 2003; Gillott, 2003; Chapman and Davies, 2004; Ram and Wolfner, 2007; Wolfner, 2007; Chapman, 2008). SFPs have also been implicated as being crucial mediators of sperm competition (Harshman and Prout, 1994; Prout and Clark, 2000; Wigby *et al*., 2009) thereby influencing fertilization success among rival males. Thus, the complex cocktail contained in male ejaculates and transferred to females during mating is likely to have profound fitness implications for both sexes, influencing evolutionary processes ranging from sexual conflict to reproductive isolation in addition to aspects of a species' mating system, such as the degree of multiple mating and sperm competition. SFPs are among the most rapidly-evolving proteins (Clark *et al*., 1995, 2006, Swanson *et al*., 2001; Swanson and Vacquier, 2002; Clark and Swanson, 2005; Andres *et al*., 2006; Panhuis *et al*., 2006), with attempts to identify homologs of *D. melanogaster* SFPs outside of *Drosophila* being met with limited success, most likely due to these rapid rates of evolution. Thus, it is necessary to identify SFPs for each species of interest, however, this has been accomplished for relatively few taxa. For example, SFPs have not been positively identified in any coleopteran, a highly successful insect order that includes several agriculturally important pests. Indeed, the dearth of information about these proteins in such key taxonomic groups currently limits our understanding of their functional significance.

Tribolium castaneum (Coleoptera:Tenebrionidae) is an excellent model system in which to investigate SFPs. Adults are highly promiscuous and utilize a variety of mechanisms to bias paternity (Fedina and Lewis, 2008), rendering it an appropriate species for exploring the role of these proteins in influencing the consequences of multiple mating and sexual conflict. Previous work on *Tribolium* has revealed that last male sperm precedence varies widely, and several peri- and post-copulatory behaviors influence paternity (Bernasconi *et al*., 2006; Fedina and Lewis, 2006, 2007, 2008). *Tribolium*'s ease of culture, short generation time, sequenced genome and efficacy of genetic manipulation make it an ideal model organism (Richards *et al*., 2008; Brown *et al*., 2009). Finally, *Tribolium* are a worldwide stored

product pest species, and understanding how SFPs influence reproduction could potentially open future avenues for pest control.

Several studies in *Tribolium* and related beetles suggest that substances manufactured by male beetles and transferred to the female could be important in determining paternity success (Fedina and Lewis, 2008). *Tribolium* males have two pairs of accessory glands (AGs), the mesadenia (ME) and ectadenia (EC) glands. Both glands are involved in producing a spermatophore that is transferred to the female (Figure 1). Several previous studies have concluded that these AGs produce not only different proteins, but also large carbohydrate-containing macromolecules (Sevener *et al*., 1992; Novaczewski and Grimnes, 1996). Additionally, 112 genes that are highly expressed in *T. castaneum* AG have been identified with custom microarrays, and 14 of those genes show biased expression in AG (Parthasarathy *et al*., 2009). Furthermore, RNAi knockdown of genes involved in juvenile hormone synthesis decreased expression of these AG genes, lowered sperm production, and lessened male mating vigor. Females mated to those knockdown males produced fewer eggs and progeny than females mated to control males (Parthasarathy *et al*., 2009). Taken together, these results suggest that SFPs might play a role in explaining variance in male reproductive performance and fitness in *T. castaneum*.

Although studies of gene expression in male reproductive glands are useful for identifying potential SFPs, recent studies have shown that not all SFPs show enriched expression in the male reproductive glands (Findlay *et al*. 2008, 2009). Proteomic analyses have allowed the direct identification of proteins produced in the male accessory glands and transferred to females during mating (Findlay *et al*. 2008, 2009). Here, we adopt a proteomic approach for identifying SFPs in *Tribolium*. We use mass spectrometry (MS) to identify putative SFPs by comparing proteins detected in the male reproductive glands with those found in the reproductive tracts of virgin and mated females. This MS based approach has proven successful in identifying putative SFPs in other taxa (e.g. Collins *et al*. 2006; Sirot *et al*. 2008; Reinhardt *et al*. 2009). Using RT-PCR, we then determined the presence/absence of expression of the genes coding for these identified proteins in female and male tissues. This represents the first characterization of putative SFPs in *Tribolium* using direct identification of proteins through mass spectrometry.

Experimental Procedures:

Identification of seminal fluid proteins using mass spectrometry

The reproductive tract of *T. castaneum* males is dominated by a pair of lobed testes and two pairs of male accessory glands (Figure 1A). The testes are connected to the ejaculatory duct through the vas deferentia, which dilate into the slightly wider seminal vesicles as they approach the ejaculatory duct. The two pairs of male AGs are distinguishable by their morphology; the ectadenia glands (EC) are smaller and rod-shaped, while the mesadenia glands (ME) are longer and more tubular. The seminal vesicles and two pairs of AGs terminate in the ejaculatory duct. The two types of AGs together produce a spermatophore, which is transferred to the female bursa copulatrix during mating (Figure 1B).

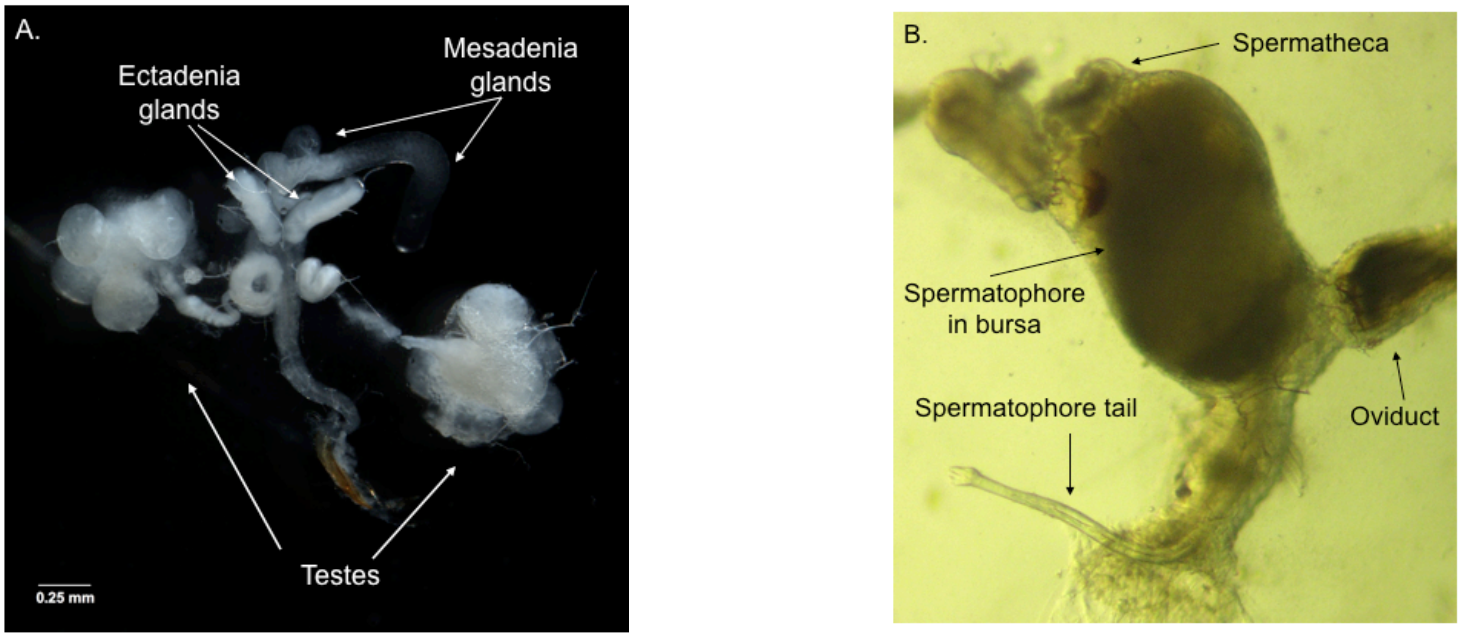

Figure 1. Reproductive anatomy of male (A) and female (B) *Tribolium castaneum*. Proteins were separately collected from mesedenia and ectadenia glands. Bursal tissue was collected from both virgin and mated females.

Putative *Tribolium* SFPs were directly identified using mass spectrometry. For this analysis, proteins were collected and pooled from several different tissue types: virgin male reproductive AGs (EC and ME separately), the bursa copulatrix from mated females containing a newly-transferred spermatophore (Figure 1B), and the bursa copulatrix of virgin females. Additionally, male body tissue lacking AGs was collected. For each tissue type, at least 2 independent biological replicates representing tissue pooled from 30 individuals were collected. Mated females were obtained by placing a virgin male

and female into a mating arena. Within 30 sec of the cessation of copulation, females were removed and the entire female reproductive tract was dissected in autoclaved, Dulbecco's PBS (Sigma) with protease inhibitors (Roche). This rapid removal of the female reproductive tract following mating reduces the possibility of our sample containing proteins that the female produces in response to the presence of male-derived proteins. The bursa copulatrix containing the spermatophore was then separated from the remainder of the tissue. Similar dissections were done to remove the bursa from virgin females and AGs from virgin males. By including both the mated and virgin female bursa copulatrices in our analysis, we were able to distinguish probable male-derived proteins from proteins normally present in the female bursa copulatrix. For the samples of the male and female bodies without the above tissue types, as much of the remainder of the tissue was removed from the abdomen, thorax and the head as possible. Exoskeleton fragments were removed from the samples. Following dissection, tissues were placed in 40 µL 10% Dulbecco's PBS with protease inhibitors. Samples were centrifuged at 11,000×*g* for 30 min at 4 °C, and supernatant was removed and placed in new tubes. Pellets were re-suspended in 20 µL 10% DPBS with protease inhibitors and 20 µL 2× SDS sample buffer (125 mM Tris-HCl pH 6.8, 20% glycerol, 4% SDS, 10% ß-mercaptoethanol, 0.001% Bromophenol blue) was added to the supernatant and pellet samples. Proteins were separated on a one-dimensional 5–15% gradient SDS-polyacrylamide gel and stained with SimplyBlue™ SafeStain (Invitrogen) for visualization.

Bands were selected for mass spectrometry-based identification if they were present in the AGs (we only selected proteins from the ME sample, as they more closely matched the mated female sample) and mated female bursa samples but light or absent in the virgin female bursa (indicated with boxes in Figure 2).

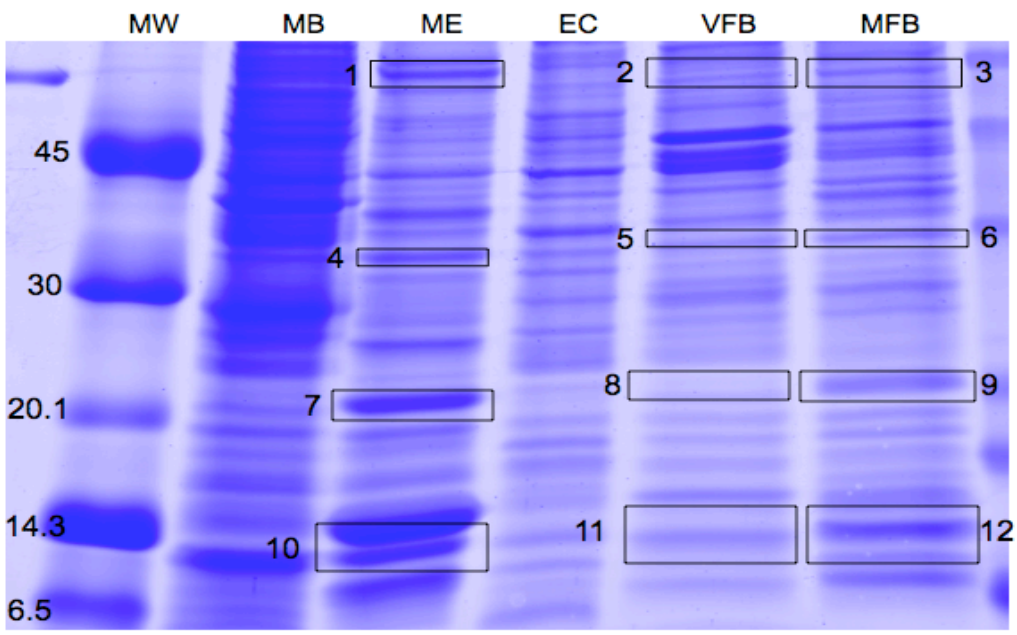

Figure 2. Coomassie-stained one-dimensional protein gel of tissue samples from *Tribolium castaneum* male reproductive accessory glands and the bursa copulatrix of virgin and mated females. Numbered boxes indicate protein bands selected for analysis that are present in male accessory glands and mated females but lighter or absent in virgin females. MB = male body, ME = mesadenia glands, EC = ectadenia glands, VFB = virgin female bursa, MFB = mated female bursa. Proteins were separated on a one-dimensional 5-15% gradient SDS-polyacrylamide gel and visualized with SimplyBlue™ SafeStain

This increased the likelihood that our samples contained male-derived proteins that were transferred to females. In-gel digestion, tryptic peptide extractions and Nano LC-MS/MS were conducted by the Cornell University Life Sciences Core Proteomics and Mass Spectrometry facility following previously published methodology (Morris *et al*., 2007). The data emerging from the mass spectrometry were submitted for database comparison using ProteinPilot software (Applied Biosystems) against the *Tribolium* predicted peptide fasta database (www.beetlebase.org), with identifications being based on a ProtScore >1.3 and representing > 95% statistical significance. We also tested for predicted secretion signal sequences using SignalP 3.0 (www.cbs.dtu.dk/services/SignalP/) and for predicted protein domains with SMART (http://smart.embl-heidelberg.de/) and InterProScan (http://www.ebi.ac.uk/Tools/InterProScan/).

Predicted protein sizes were calculated with the protein molecular weight calculator at the Sequence Manipulation Suite (http://www.bioinformatics.org/sms/prot_mw.html).

We compared the amino acid sequence of the identified putative SFPs to translated *D. melanogaster*, *Ae. aegypti*, *An. gambiae*, *A. mellifera* transcripts using tblastn against predicted *D.*

melanogaster, (http://flybase.bio.indiana.edu/) *Ae. aegypti* (http://aaegypti.vectorbase.org/index.php) *An. gambiae* (http://agambiae.vectorbase.org/index.php) and *A. mellifera* (http://hymenopteragenome.org/beebase/) gene databases. The amino acid sequences of the top hits were then compared back to the Beetlebase3_NCBI_DB database on the Beetlebase website (http://beetlebase.org/) using tblastn to determine whether the original *D. melanogaster*, *Ae. aegypti*, *An. gambiae* or *A. mellifera* transcript was the top hit. *T. castaneum* genes were considered homologs if the two were reciprocal best BLAST hits with e-values $<1x10^{-3}$ and identities ≥ 30%.

Patterns of gene expression

The expression patterns of the putative SFP genes identified through mass spectrometry were investigated using qualitative reverse transcription PCR (RT-PCR). Total RNA was extracted separately from male ectadenia and mesadenia glands (pooled from 40 males), male whole bodies without the accessory glands, and female bodies (pooled from 30 individuals of each sex). All *T. castaneum* individuals were virgin and approximately 2 weeks old. Dissections were done as described above. Pooled tissue samples were kept on ice in Trizol (Invitrogen) until dissections for each tissue type were completed. Tissues were then ground in Trizol, and RNA was extracted following manufacturer's instructions. Extracted RNA was treated with RNAse free DNase (Promega) to remove any DNA contamination. For each tissue type, 1 ug of RNA was used to synthesize the first strand cDNA with Superscript II Reverse Transcriptase (Invitrogen). TCRPS6, a proteins that is part of the small ribosomal subunit, was used to standardize the amount of cDNA used to determine the presence/absence of seminal fluid protein transcripts in each tissue type and also act as a control. PCR was conducted with first strand cDNA using Platinum PCR Supermix (Invitrogen) and gene-specific primers. PCR cycles were as follows: 1 cycle at 94°C 5 min; 33 cycles of 94°C 30 s, 62 °C 30 s, 72°C 30 s; followed by 5 min at 72°C. For two genes, TC006088 and TC010066, primers failed to amplify gene products using this PCR program. Therefore, the PCR program used for those genes were: 1 cycle at 94°C 5 min; 16 cycles of 94°C 30 s, 66 °C 30 s, 72°C 1 min; 20 cycles of 94°C 30 s, 62 °C 30 s, 72°C 1 min; followed by 5 min at 72°C. TC006088 and TC010066 PCR product was sequenced to ensure proper product amplification. PCR was conducted on at least two independent biological replicates of each tissue type. The results from each gene specific RT-PCR from all tissues tested were visualized on the same gel to

determine the presence of a transcript, but relative expression was not quantified. Gel imaging was done using Quantity One (Bio-Rad) software.

Results and Discussion:

Identification and expression patterns of putative SFPs

We conducted mass spectrometry on a subset of bands of proteins separated through 1D gel electrophoresis that showed patterns suggestive of SFPs (i.e., presence in male reproductive glands and reproductive tracts of mated females, but not in reproductive tracts of virgin females; Figure 2). Through mass spectrometry of proteins from male mesadenia glands (ME; Figure 1) and virgin and mated female reproductive tracts, we identified 14 distinct proteins that were detected in the ME and the mated female, but not in the virgin female reproductive tracts (Table 1). Of the proteins identified via mass spectrometry, 12 are newly-identified putative reproductive proteins and two, TC005744 and TC010066, were previously identified as expressed in the AGs (Parthasarathy *et al*., 2009). These proteins represent only a subset of the possible reproductive proteins that are produced by both male and female *T. castaneum*. The expression patterns of genes encoding 13 of the 14 proteins we identified via mass spectrometry were highly male-biased, with undetectable transcript levels in female tissue (Figure 3). Nine of the genes produced transcripts that were detectable only within male AGs, with one of those genes, TC015849, expressed only in the ectadenia glands (EC). Given that proteins from the mesadenia glands (ME) only were submitted for MS identification, this result is surprising and warrants future investigation. It is possible that the protein product could be synthesized in the ME and transferred to the EC, or that the presence of expression in the ME could be detected by more sensitive means, such as qPCR. The remaining 4 genes were found not only in both types of AGs, but also in male body tissue samples (which lacked AGs). Thus, two lines of evidence suggest that the 13 proteins with male-specific gene expression represent putative SFPs. 1) These proteins were identified from both male AGs and the bursa copulatrices of mated females but were not detected from the bursa copulatrices of virgin females. 2) RT-PCR did not reveal detectable expression levels in females. The remaining protein, TC010066, was also identified previously as an AG gene (Parthasarathy *et al*. 2009) but does not have male specific expression.

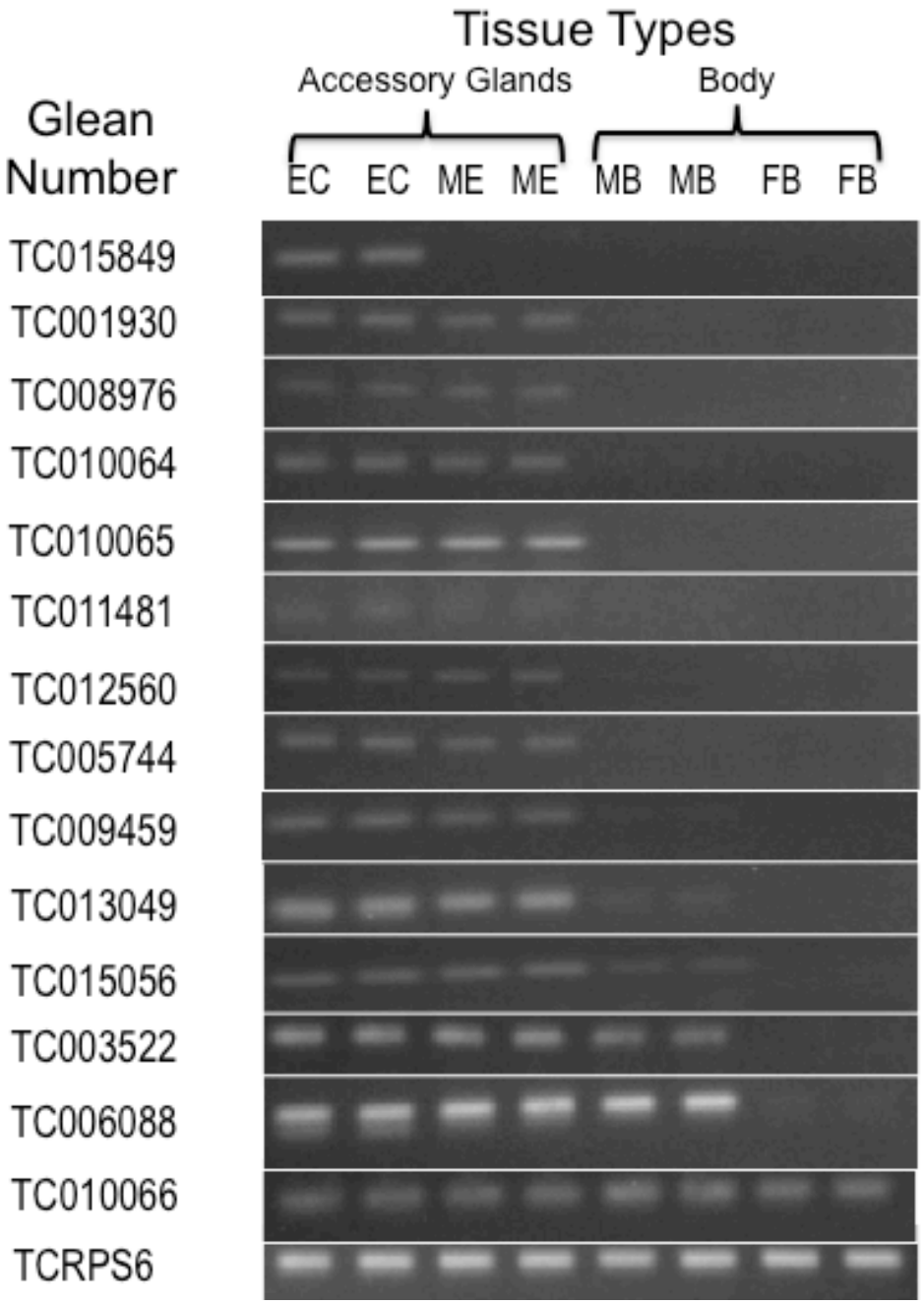

Figure 3. Qualitative RT-PCR to determine the presence of 14 putative seminal fluid protein gene transcripts in different tissue types of Tribolium castaneum. EC = ectadenia gland, ME = mesadenia gland, MB = male body, FB = female body. The bottom row shows expression of TCRPS6, a protein that is part of the small ribosomal subunit. This was used to standardize the amounts of template cDNA used. This figure represents a composite of gels, as the products of the RT-PCR from each gene were visualized on separate gels and then assembled for this figure.

Table 1. Putative *Tribolium castaneum* seminal fluid proteins identified by mass spectrometry with their predicted protein class and homologues in other insects

Glean number	Predicted size (kDa)	Band number from Fig. 2	Tissue of expression	Predicted protein class	*Aedes aegypti* homologue	*Anopheles gambiae* homologue	*Drosophila melanogaster* homologue	*Apis mellifera* homologue
TC001930	20.75	10, 12	EC, ME	No homology				
TC003522	11.64	10,12	EC, ME, MB	No homology				
TC005744	41.86	4,6,7,9	EC, ME	Serine protease inhibitor			CG8137 Serine-type endopeptidase inhibitor Known SFP	GB16472-RA Serine protease inhibitor
TC008976	31.89	10,12	EC, ME	Serine protease inhibitor	AAEL009795 Papilin glycoprotein	AGAP009766 Known SFP	CG33103 Papilin glycoprotein	GB16153-RA Papilin glycoprotein
TC009459	11.28	10,12	EC, ME	Pheromone/odorant binding protein				
TC010065	12.10	10,12	EC, ME	No homology				
TC010064	12.15	10,12	EC, ME	Pheromone/odorant binding protein				
TC011481	22.60	1,4,6,7,12	EC, ME	No homology				
TC012560	10.66	10,12	EC, ME	Inosine triphosphate pyrophosphatase	AAEL000200			
TC013049	33.81	4,6,9	EC, ME, MB	Senescence marker protein	AAEL000757 Fat body protein	AGAP007794	CG7390 Senescence marker protein	GB18633-RA Senescence marker protein
TC015056	13.74	10,12	EC, ME, MB	No homology	AAEL006131	AGAP001708	CG31326 Serine-type endopeptidase	
TC015849	41.89	1,3	EC	Prophenoloxidase	AAEL014544 Prophenoloxidase	AGAP002825 Prophenoloxidase	CG8193 Monophenol monooxygenase activity	GB18313-RA Prophenoloxidase
TC006088	11.18	10,12	EC, ME, MB	Nucleoside triphosphate hydrolase				
TC010066	13.05	10,12	EC, ME, MB, FB	Pheromone/odorant binding protein	AAEL002617 Odorant binding protein			GB18363-RA Odorant binding protein

Tissue types: EC, ectadenia gland; ME, mesadenia gland; MB, male body; FB, female body.

Homology and characterization of putative seminal fluid proteins

Seven of the 14 proteins we identified had homologs in either *D. melanogaster*, *Aedes aegypti*, *Anopheles gambiae* or *Apis mellifera* (Table 1), and 2 of these homologs are known or predicted SFPs in these species. The predicted protein classes among our putative SFPs are consistent with protein classes that have been identified in the SFPs of other organisms (Mueller *et al*., 2004; Braswell *et al*., 2006; Collins *et al*. 2006; Davies and Chapman, 2006; Dottorini *et al*. 2007; Ravi Ram and Wolfner, 2007; Sirot *et al*., 2008; Walters and Harrision, 2008; Andres *et al*., 2008; Baer *et al*. 2009; Reinhardt *et al*. 2009; Rogers *et al*. 2009). The suite of proteins identified here contain several proteolysis regulators and insect pheromone/odorant-binding proteins. Additionally, several of the proteins that were identified had no conserved protein domains or homologs among any of the comparison species, and some of the proteins identified here are novel putative SFP classes. As many SFP sequences are thought to be rapidly evolving (Clark *et al*., 1995, 2006; Swanson *et al*., 2001; Swanson and Vacquier, 2002; Clark and Swanson, 2005; Andres *et al*., 2006), this is not surprising.

Proteolysis-regulating SFPs have been implicated as key modulators of reproductive biology in both males and females of many species. In *D. melanogaster*, these proteins are thought to influence the protein cascade that regulates post-copulatory processes in females such as ovulation and sperm storage (Ravi Ram *et al*. 2006) as well as defense against microbial infections (Khush and Lemaitre 2000, Mueller *et al*. 2007). *D. melanogaster,* SFPs in this class have also been suggested to play roles in mating plug formation (Lung and Wolfner 2001), hormone cleavage and prohormone protection (Monsma *et al*. 1990, Mueller *et al*. 2004, Wolfner 2009). In mammals, proteolysis-regulating SFPs influence the breakdown of semenogelin and affect sperm motility (Kise *et al*. 1996, Robert *et al*. 1997, Malm *et al*. 2000). Two of the putative *T. castaneum* SFPs reported here, TC005744 and TC008976, are predicted to be proteolysis regulators (Table 1). Interestingly, TC005744 is a predicted protease inhibitor with serpin domains that has a homolog among the known *D. melanogaster* SFPs (Ravi Ram *et al*. 2005; Findlay *et al*. 2008). That *Drosophila* SFP, CG8137, has predicted hydrophobic domains similar to those found in serpin class members which constitute a large component of mammalian seminal fluid. These serpins are capable of binding hormones and are crucial for male fertility in mammals (Uhrin *et al*. 2000). Given the known conservation of the general roles of reproductive proteins, it is possible that the

T. castaneum putative proteolysis-regulating SFPs identified here share functions with those observed in other taxa.

Mating has the potential to introduce a variety of pathogens into the female reproductive tract that could negatively affect the reproductive success of both sexes. In *D. melanogaster*, several SFPs have been found to have either direct antimicrobial activity and may protect gametes and zygotes or may modulate a female's ability to fight infection (Samakovlis *et al*., 1991; Lung *et al*., 2001; Mueller *et al*., 2007, Wolfner 2009). Putative SFPs in both *Ae. aegypti* and *An. gambiae* proteins fall in protein classes suggesting that may also play a role in immune response (Rogers *et al*. 2008; Sirot *et al*. 2008.) Here we identify a putative SFP in *Tribolium*, TC015849, which is possibly involved in an immune response. This putative SFP is a predicted prophenoloxidase, a class of proteins that have been demonstrated to be important components of innate immune response within arthropods. The activation of prophenoloxidase leads to melanin synthesis and the stimulation of the Toll signaling pathway, which in turn induces the assembly of antimicrobial proteins (Kanost and Gorman 2008; Kan *et al*. 2008, Ferrandon *et al*. 2007; Buchon *et al*. 2009; El Chamy *et al*. 2008; Roh *et al*. 2009;). The homologs of TC015849 in *Ae. aegypti*, *An. gambiae* and *A. mellifera* are also predicted to have prophenoloxidase domains (Baer et al. 2009; Lawson *et al*. 2009), while the *D. melanogaster* homolog, CG8193, is predicted to have monophenol monoxygenase activity and be involved in defense response (Asano and Takebuchi 2009). However, none of those homologs are putative or known SFPs. Prophenoloxidase activity is typically associated with hemocytes (Cerenius and Soderhall 2004), and given that hemocytes would be likely found in male and female body samples, it is surprising that TC015849 transcripts were not detected in those tissues if expression is indeed limited to hemocytes. Furthermore, we did not detect TC015849 proteins in virgin female reproductive tracts using mass spectrometry. Given that mating represents an opportunity for the introduction of foreign materials into the female reproductive tract and that prophenoloxidase is responsible for initiating melanin deposition around such foreign objects, it is conceivable that prophenoloxidase could be transferred within the male ejaculate to serve a defensive function within the female. The lack of expression at levels detectable in our study of TC015849 in any other tissue besides male AGs lends credence to this prediction, but certainly requires further investigation before any definitive conclusions can be drawn. Additionally, it is worth noting that

such defensive strategies are likely to be coupled with other, more immediate forms of protections, such as lysis of bacterial cells (Otti *et al*. 2009).

Several of the possible *Tribolium* SFPs fall into the classification of being predicted pheromone/odorant binding proteins (Table 1). These are small, soluble proteins that bind semiochemicals such as pheromones and odor molecules and deliver those molecules to olfactory receptors (see Pelosi *et al*. 2006 for review), supporting a role in insect molecular recognition. Odorant binding proteins are a class of putative SFPs identified in *D. melanogaster* and *Ae. aegypti* (Findlay *et al*. 2008; Sirot *et al*. 2008). The functional significance of these proteins in reproduction has yet to be identified, but recent work suggests that odorant molecules play a chemoattractant role for sperm (Takuda *et al*. 2004). Others (Takemori and Yamamoto 2009) have suggested that the odorant binding proteins in *D. melanogaster* have organ-specific signaling roles in reproduction.

Three of the other classes of proteins identified here represent novel types of putative SFPs, but little or nothing is known about how these classes of proteins might function in reproduction. TC013049 is predicted to be a senescence marker protein, and its homolog in *D. melanogaster*, CG7390, and *A. mellifera*, GB18633, are known to be senescence marker proteins (Tweedie *et al*. 2009). This protein has been implicated in mammalian cells to have a role in Ca^{2+} ion homeostasis and signaling (Inoue *et al*. 1999, Son *et al*. 2008). Ca^{2+} has been demonstrated to play a crucial role in mammalian sperm function where it regulates such activities such as capacitation, chemotaxis and acrosome reaction (Publicover *et al*. 2007; Costello *et al*. 2007). TC012560 is predicted to be an inosine triphosphate pyrophosphatase, a protein that hydrolyzes nucleoside triphosphates into monophosphates and is typically referred to as a house-cleaning enzyme (Galperin 2006). Finally, TC006088 is predicted to be a nucleoside triphosphate hydrolase, an enzyme that can degrade nucleotides into simpler forms. The potential role of these proteins in reproductive processes remains to be investigated.

Five of the putative *Tribolium* SFPs did not fall into any predicted protein class. Four of these proteins had no homologs in any of the four species (*Ae. aegypti*, *An. gambiae*, *D. melanogaster and A. mellifera)* investigated; one of the five proteins had homologs in all three Dipteran species, but functional information is available for only one of those homologs, CG31326 in *D. melanogaster*, which is a serine-type endopeptidase that is upregulated in response to bacterial infection (Maia *et al*. 2007).

We have no functional information on the remaining four proteins in this group. However, all of these proteins remain of interest because they show male-biased expression, with TC010065 expression detected exclusively in the male accessory glands.

Conclusions

The primary goal of this research was to identify a subset of the SFPs that are transferred to female *T. castaneum* in the male ejaculate. We have successfully identified several novel putative SFPs in *T. castaneum* by utilizing a combination of proteomics and gene expression approaches. This represents the first proteomic-based method of identifying SFPs in any coleopteran species, and is the first to identify putative SFPs that are likely transferred to the female in the male–derived spermatophore. Fourteen putative SFPs were identified by mass spectrometry and 13 of those 14 were shown to be male-specific, 9 showing expression limited to the two pairs of male reproductive AGs. Two of the 14 identified genes were demonstrated to have homologs among the respective known and putative SFPs of *D. melanogaster* and *An. gambiae*. The predicted protein classes of many of these putative SFPs are similar to those SFPs reported from a variety of other insects. However, several of the identified putative SFPs did not have conserved protein domains, or identifiable homologs in BLAST searches, indicating that these proteins may be rapidly evolving.

T. castaneum represents an extremely amenable model system for addressing questions about sexual selection and sexual conflict due to the successful implementation of a systematic, integrative approach towards uniting episodes of sexual selection (Fedina and Lewis 2008). By knocking down individual SFPs (e.g., using RNA interference) in such a system, researchers can assess the role and importance of ejaculate proteins in mediating mating success, fertilization success, sperm competition and potentially even reproductive isolation (Walters and Harrison, 2008; Findlay and Swanson 2010). Therefore, exploring the male reproductive proteins identified in this and another recent investigation (Parthasarathy *et al*. 2009) may not only provide insight into the possible mechanisms of sexual selection, but also help control the reproductive output of this economically important agricultural pest.

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Chapter IX: Final Synthesis

Competition between members of the same sex and mechanisms of choice before, during and after mating have been an extremely potent force driving the evolution of a large variety of sexual traits. Historically, empirical studies of sexual selection have focused on just one potential selective episode, in effect examining possible mechanisms of selection in relative isolation. This can result in a distorted perspective of the importance of a given trait or episode to the total fitness of an individual. As outlined in Chapter V, empirical studies have indicated that sequential episodes of sexual selection can be both reinforcing or opposing, but few have combined these episodes into one measurement that expresses the totality of sexual selection. Therefore, the goal of this research was to come to an understanding of how male nuptial gifts operate in sequential episodes of sexual selection. This research provides insights into not only fitness ramifications, but nuptial gift taxonomy and correlated evolution between the sexes. In this final chapter, I review and synthesize these topics.

Photinus nuptial gifts as targets of selection across multiple episodes of selection:

As nuptial gifts are distinctly male traits, the adaptive significance of gift giving has historically been framed in the context of two hypotheses that are not mutually exclusive: mating effort and parental investment. In Chapter II we present an alternative perspective on the adaptive significance of nuptial gifts by considering them as targets of selection before, during and after mating. Here I would like to consider genital nuptial gifts in *Photinus* fireflies from this targets

of selection perspective, as the insights gleaned from the chapters in this dissertation present an opportunity to apply this model to fireflies.

Currently, we have no evidence for the functioning of nuptial gifts in the long range mate attraction of female fireflies, as this appears to be the domain of the species specific flash phrases employed by male *Photinus* fireflies (reviewed in Lewis & Cratsley 2008). However, Chapter V presents evidence that genital nuptial gift size is influencing the choice of female *P. greeni* fireflies to accept mating attempts after the potential mating pair makes first contact (episode 2). As the decision to accept the mating attempts of a male inevitably leads to spermatophore (and associated sperm) transfer in most *Photinus* species, the influence of nuptial gift size on mating success thus also indirectly affects a male's ability to inseminate a female (episode 3). *Photinus* male nuptial gifts also influence postcopulatory selection episodes. Chapter V demonstrates an effect of gift size on *P. greeni* male paternity share (episode 6), as second mating males transferring a relatively larger spermatophore enjoy a significant advantage in P_2 relative to males transferring smaller spermatophores. Previous work in *Photinus* fireflies has demonstrated an effect of number of gifts on fecundity (episode 7); *P. ignitus* females receiving more spermatophores had a significant increase in female reproductive output (Rooney & Lewis 2002). Results from Chapter IV reveal a possible synergistic effect of increased female lifespan due to the receipt of a larger spermatophore with this increase in fecundity. *P. obscurellus* females that received a single large spermatophore relative to females receiving a single small spermatophore experienced a 2 d increase in life span. This increase in

longevity could lead to increased opportunities for females to mate and receive additional spermatophores, resulting in a higher lifetime fecundity (episode 7).

Thus, applying this episodes of selection framework to *Photinus* fireflies clearly indicates an effect of nuptial gifts across multiple episodes of sexual selection. Nuptial gifts are an important component of not only successfully securing copulations, but in also securing fertilizations and increasing the fecundity of mated females. However, pieces of the sexual selection puzzle in *Photinus* fireflies remain to be put into place, and some previous results help to fill in some of these spaces.

A previous investigation into the nature of postcopulatory selection in *P. greeni* fireflies documented a negative relationship between male courtship attractiveness and paternity share (Demary & Lewis 2007). One possible explanation of this result was that females could initially utilize longer distance measures of male attractiveness (e.g. flash signals) to evaluate males, but then switch to close contact cues once this male made initial contact. Thus, paternity might be negatively correlated with measures of precopulatory courtship, but could be positively correlated with some type of pericopulatory signal. Two of these possible signals, cuticular hydrocarbons (Chapter VI) and male pericopulatory behaviors (Chapter V) were evaluated in this dissertation. In Chapter VI we present evidence that titers of cuticular hydrocarbons in nocturnal *Photinus* fireflies are below detectable levels, so these compounds are unlikely to be a usable signal. In Chapter V, we report that a variety of male pericopulatory behaviors exist, but they are correlated with neither mating or paternity success.

Additionally, as these behaviors do not change between a males' first and second mating, these behaviors are unlikely to be correlated with nuptial gift size. Taken together, these results indicate that a potential trade-off exists between male's producing an attractive courtship signal vs investment into genital nuptial gifts. Additionally, our results suggest that another signal modality is potentially being utilized by female fireflies to evaluate male quality, and this signal could be correlated with genital nuptial gift size.

The nature of sexual selection in *Photinus* fireflies highlights the necessity of integrating investigative efforts across selection episodes, as copulation does not necessarily result in fertilization. The results presented in this dissertation indicate that females likely make an initial choice based on male flash, but another modality is being used to make a pericopulatory assessment of males. Therefore, mating success depends on a combination of these two episodes. Some degree of postcopulatory selection functions following this initial mate acceptance, but the relative role of cryptic female choice vs. sperm competition in determining the paternity of offspring in multiply mating females remains unknown. Whatever the mechanism, it appears that some degree of concordance exists across multiple selection episodes as males producing relatively larger nuptial gifts experience benefits in both mating and paternity success.

Classification and definitions of nuptial gifts:

In addition to the targets of selection framework, we present several novel insights into the nature of nuptial gifts in Chapter II. The first is an expansion of a seemingly arbitrary distinction about exactly what constitutes a nuptial gift and a

suggestion for the classification of these types of gifts. We believe that this reflects the realities of how selective pressures could affect these gifts. For example, selection can likely directly shape the character of endogenous nuptial gifts in a different manner from exogenous gifts. While the selection of exogenous gifts could certainly be shaped by evolution, the composition of gifts such as arthropod prey would be subject to a host of selective regimes not related to the gift giver.

Secondly, this chapter makes radical changes to the definition of nuptial gifts. We make little distinction between ejaculates that are packaged (e.g. spermatophores) and unpackaged (in seminal fluid). Unpackaged ejaculates have typically not been considered to be nuptial gifts, but two lines of evidence in this dissertation argue against this distinction. The results of the meta-analysis in Chapter III demonstrate that these different categories of ejaculates have similar effects on female fecundity: receiving relatively greater quantities of both results in an increase in female fecundity. The magnitude of effect on female longevity also did not differ between these two categories. In Chapter VIII we present the results of our proteomic and mass spectrometry identification of seminal fluid proteins in *Tribolium castaneum*. This chapter is part of a growing body of evidence that demonstrably indicates that the seminal fluid proteome of unpackaged and packaged ejaculates is composed of similar protein classes, lending credence to the idea that each is actually a type of nuptial gift. Additionally, our broadening of the nuptial gift definition to include transdermal nuptial gifts marks a major shift in deciding exactly what constitutes a nuptial gift.

By removing any prejudices about the source, target, size or shape of nuptial gifts and instead considering the impacts on fitness, a robust, testable framework emerges that also acknowledges the potential lability of selective pressures that could have affected gift origin and maintenance.

Chapter VIII also highlights the importance of identifying the specific components of nuptial gifts in a wide variety of species, as these molecules are likely to be important in proximate mechanisms across multiple selection episodes that will determine total sexual selection and could also be important mediators of reproductive isolation. While several of the classes of seminal fluid protein identified in *T. castaneum* are consistent with those found in other species, others demonstrated no homology with known protein classes. Typically, such results are believed to stem from the strong selection and subsequent rapid evolution of reproductive proteins. Therefore, an understanding of the evolutionary history of SFPs and a true understanding of how they might vary according to life history traits (type of mating system, habitat, etc.) can only be achieved by broadening the array of species studied. However, seminal fluid proteins remain only one potential class of molecule in nuptial gifts. Other classes, such as free amino acids, have been demonstrated to be important nuptial gift components that can affect female sexual behavior (Gordon et al. 2012). Large scale screens of specific nuptial gift components across a diverse array of taxa will undoubtedly reveal valuable insights into the dynamics of sexual selection.

Patterns of nuptial gift evolution

To achieve optimal fitness, an organism must make a variety of resource allocation decisions with tradeoffs often being an inevitable outcome. While impactful to all insects, the nature of these tradeoffs can be especially important in capital breeders, with their reliance on larval acquired nutrients. Nuptial gifts, with their ability to supplement adult nutritive reserves, have long been recognized as a potential link between the energy budgets of the sexes (Boggs 1990, 1995). Cross taxonomic studies in nuptial gift giving Lepidoptera have demonstrated an association between mating systems and proportional sex specific allocation of male derived nutrients (e.g. Karlsson 1995), as females in polyandrous species allocate less of their own nutrients to reproduction relative to monandrous species. Additionally, the importance of male derived nutrients is expected to vary according to parameters that could influence sex specific allocation to nutrient budgets, such as degree of female mobility. If females can allocate more to reproduction because they do not need to allocate their own nutrients to movement such as flight, selection upon male nuptial gift production should be relaxed.

In Chapter VII we demonstrate in a worldwide sampling of firefly species that the ancestral state for the common ancestor of those species was one in which males were capable of producing nuptial gifts and females were capable of flight. Therefore, extant species in which the female cannot fly and the male cannot produce a nuptial gift are derived states. We show that a loss female flight predated the loss of male nuptial gift production in the same taxa, but that these evolutionary patterns were signicantly linked. As female flight ability was

reduced (and by extension their energy allocation to movement), males of the same species experienced reduction in the number of reproductive accessory glands and loss of their ability to produce a nuptial gift. This is the first phylogenetic analysis that clearly links changes in female mobility as a potential causative factor in male nuptial gift production and provides phylogenetic support for nutritional ecology hypotheses of nuptial gifts being critical components of female nutrient budgets, at least in certain taxa. Another important element of this chapter is the significant evolutionary linkage between not only male nuptial gift production and female mobility, but also the link with sexual signaling mode. These connections between seemingly disparate life history traits indicates that potential selection on one sex can have ripple effects on a correlated suite of life history characters in both sexes, indicating the importance of a consideration of additional characters when evaluating the impact of evolutionary processes.

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