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Chapter 2

EVIDENCE OF NATURAL AND SEXUAL SELECTION SHAPING THE SIZE OF NUPTIAL GIFTS AMONG A SINGLE BUSH-CRICKET GENUS (*POECILIMON*; TETTIGONIIDAE): AN ANALYSIS OF SPERM TRANSFER PATTERNS

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ABSTRACT

During mating, male bush-crickets transfer a complex spermatophore to the female. The spermatophore is comprised of a large nuptial gift which the female consumes while the sperm from the ejaculate-

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containing ampulla are transferred into her. Two main functions of the nuptial gift have been proposed: the ejaculate protection hypothesis and the parental investment hypothesis. The former, founded on sexual selection theory, predicts that the time to consume the gift is no longer than necessary to allow for full ejaculate transfer. The latter maintains that gift nutrients increase the fitness or quantity of offspring and hence the gift is likely to be larger than is necessary for complete sperm transfer. With an aim to better understanding the primary function of nuptial gifts, we examined sperm transfer data from field populations of five *Poecilimon* bush-cricket taxa with varying spermatophore sizes. In the species with the largest spermatophore, the gift was four times larger than necessary to allow for complete sperm transfer and is thus likely to function as paternal investment. Species with medium and small gifts were respectively sufficient and insufficient to allow complete sperm transfer and are likely to represent, to various degrees, ejaculate protection. We also found that species that produce larger spermatophores transfer greater proportions of available sperm than species producing smaller spermatophores, and thus achieve higher paternal assurance.

Keywords: Ejaculate protection, mating effort, paternal investment, spermatophore size, sperm transfer, sperm competition

INTRODUCTION

Nuptial feeding has been observed in several insect taxa (Thornhill and Alcock, 1983; Vahed, 1998). Male bush-crickets (Tettigoniidae) transfer a substantial, and often costly, spermatophore to the females for consumption during mating (Wedell, 1994a, 1994b; Vahed, 2007a). The spermatophore consists of an ampulla that contains the sperm, and a spermatophylax that, in most species, is a large gelatinous mass. The female first eats the spermatophylax and then eats the smaller ampulla along with any remaining sperm and seminal fluid (Bowen et al., 1984). There is debate over the selective pressures that maintain nuptial gift size in bush-crickets (for reviews see Thornhill and Alcock, 1983; Simmons and Parker, 1989; Vahed, 1998; Gwynne, 2001; Vahed, 2007; Gwynne, 2008; McCartney, et al 2008, 2010, 2012). Despite recent discussions concerning the effect of sexual conflict on nuptial gift size (e.g. Vahed, 2007b; Gwynne, 2008; Lehman, 2012) two hypotheses remain central to understanding the role of gift size: the ejaculate protection hypothesis and the parental investment hypothesis.

The ejaculate protection hypothesis argues that the nuptial gift is sexually selected; it increases fertilisation success by diverting the female away from the sperm ampulla while maximum insemination is achieved (Gerhard, 1913; Boldyrev, 1915; Gwynne, 1984; Sakaluk and Eggert, 1996; Vahed and Gilbert, 1996; Simmons, 2001). The parental investment hypothesis proposes that the function of the nuptial gift is derived from its nutritive value and that these nutrients are passed into the donating males' offspring; the gift is thus under natural selection to increase the quality and/or the quantity of the male's offspring (Trivers, 1972; Thornhill, 1976; Gwynne, 1986, 1988a, 1988b, 1990; Reinhold, 1999).

The ejaculate protection and paternal investment hypotheses are not mutually exclusive (Quinn and Sakaluk, 1986) and present research focuses on the relative importance of the two hypotheses in different taxa. It is likely that the spermatophylax evolved through sexual selection for ejaculate protection in bush-crickets (Gwynne, 1986, 1990, 1997, 2001), but there is evidence that both functions can be involved in the maintenance of spermatophylax size in various tettigoniid species (for reviews see Vahed, 1998; Gwynne, 2001; McCartney et al. 2008).

Nuptial gifts that function to protect the ejaculate are predicted to be smaller, less nutritious, and of a size that co-varies with either sperm number and/or ampulla size and should be no larger than necessary to allow for complete insemination (Reinhold and Heller, 1993; Wedell, 1993a, 1994a, 1994b; Heller and Reinhold, 1994; Vahed and Gilbert, 1996). Nuptial gifts that are influenced by paternal investment are likely to be large, nutritious (Wedell, 1994a, 1994b), and take longer to consume than it takes to transfer a full complement of sperm (Wedell, 1994b). While it can be relatively simple to test the prediction of the ejaculate protection hypothesis, at least three further criteria underpin paternal investment in nuptial-gift-bearing species and are needed to distinguish it from the ejaculate protection hypothesis: 1) the degree of last-male mating advantage; 2) the time that it takes for the nutrients of the spermatophylax to directly affect the donating males' offspring; and 3) the relationship between female mating interval and egg laying interval (see Vahed, 1998 and references cited therein).

The ejaculate protection hypothesis is supported by comparative studies across taxa showing positive correlations between spermatophylax size and ampulla mass or sperm number (Wedell, 1993a; Vahed and Gilbert, 1996; McCartney et al., 2008, 2012), as well as studies within species showing that the size of the nuptial gift or the consumption time of the gift is roughly similar to the time that it takes for the majority of sperm to transfer into the

female (e.g. Wedell and Arak, 1989; Wedell, 1991; Reinhold and Heller, 1993; Heller and Reinhold, 1994; Vahed, 1994; Simmons, 1995a). Evidence of paternal investment has also been observed in some species (Gwynne et al., 1984; Gwynne, 1986, 1988a, 1988b; Simmons, 1990; Wedell, 1994a, 1994b; Simmons et al., 1999; Reinhold, 1999), yet almost all insect species studied thus far, including those with properties of paternal investment, have nuptial gifts (or nuptial gift consumption times) that approximate the size necessary for complete sperm transfer (Heller and Reinhold, 1994; Simmons, 1995a; Simmons and Gwynne, 1991; Vahed, 1994), and are therefore likely to be maintained primarily through sexual selection via the ejaculate protection hypothesis (Vahed, 1998).

Diverse examples of this rule can be found in Mecoptera as prey and salivary masses, Diptera as nuptial prey and regurgitated food, Coleoptera and Zoraptera as cephalic gland secretions, and other Orthoptera, as hind-wing and glandular secretion feeding (Vahed, 1998 and references cited therein).

Possibly the only exception is *Requena verticalis*, initially reported to have a spermatophylax twice as large as necessary to allow for complete sperm transfer of the ampulla (Gwynne et al., 1984; Gwynne, 1986, 1988*b*). However, further research on this species (Simmons, 1995*a*, 1995*b*; Simmons et al., 1999) and different interpretations of what constitutes 'complete' sperm transfer (Vahed, 1994, 1998; Simmons, 1995*a*) suggest that complete sperm transfer may not be achieved until close to, or even after gift consumption (Vahed, 1998).

Additionally, males have a substantial first-male paternity advantage (Gwynne, 1988b; Simmons and Achmann, 2000; Simmons et al., 2007) and variable spermatophylax sizes, perhaps as a result of variability in female availability, re-mating interval (Simmons, 1995b), and sexual status (Simmons et al., 1993). At times, therefore, gift size approximates the size necessary for complete sperm transfer.

In order to better understand the relationship between nuptial gift size and sperm transfer pattern and the selective pressure that most influences its variation, there is perhaps no better model than the bush-cricket genus *Poecilimon* (Tettigoniidae) (McCartney et al. 2008, 2012). This genus contains species with a large diversity in mating behaviours. Comparisons among species within genera can be particularly useful as characters shared by congeners are often held constant and thus control to a large degree for similarities that may be caused by relatedness (Harvey, 1991; Harvey and Pagel, 1991). With around 140 described *Poecilimon* species (Eades and Otte, 2008), the variation in nuptial gift size is unmatched among Orthoptera and

approaches the magnitude of family-wide variation (McCartney et al., 2008), with spermatophore size varying from 6.1% (*Poecilimon laevissimus*) to 37% (*P. thessalicus*) of the relative body mass of the male (McCartney et al., 2008). This clearly represents large variation in male reproductive investment.

Few bush-cricket studies have investigated nuptial gift function from a sperm transfer perspective and, of these most have used laboratory-reared individuals despite concerns about the validity of this approach (see McCartney et al., 2008 for discussion). Even fewer studies still, have considered sperm transfer patterns within field populations (eg. Heller & von Helversen, 1991; Reinhold, 1994; Vahed and Gilbert, 1996). Furthermore, interpretation of data has been complicated by the diversity of taxa involved; variations in sperm transfer may ultimately be linked to taxon differences and not nuptial gift size *per se* (for discussion see Gwynne, 1995; Vahed and Gilbert, 1996; McCartney, 2010).

Our aim here was to better understand the premise that nuptial gift size relates to function. First, in order to assess the match between nuptial gift consumption time and optimum sperm transfer time across closely related species with marked variation in nuptial size, we combined published sperm transfer and nuptial gift consumption time data from two field-observed *Poecilimon* taxa that produce medium and large gifts (Reinhold and Heller, 1993, Heller & Reinhold, 1994), with sperm transfer and gift consumption data from three novel field-observed *Poecilimon* species; two with small gifts and one with very large gifts.

A close match between gift consumption and sperm transfer would be consistent with the sperm protection hypothesis, whereas if complete sperm transfer occurs long before spermatophylax gift consumption is completed, we have grounds to infer a paternal investment function. Secondly, we controlled for body mass and relatedness, and compared spermatophore size between species to the proportion of sperm that has transferred into the female by the time she has consumed the spermatophore.

A significant relationship would indicate that males of *Poecilimon* taxa that produce larger spermatophores have increased confidence of sperm transfer, and thus paternal assurance, compared to taxa producing smaller spermatophores.

MATERIALS AND METHODS

Species and Sites

Poecilimon is a genus of bush-crickets (Phaneropterinae, tribe Barbistini) (Orthoptera: Ensifera: Tettigoniidae), with about 65 European species that are mostly situated in the east Mediterranean (Heller, 2004). Three species, *Poecilimon laevissimus* (Fischer, 1853), *P. erimanthos* Willemse and Heller, 1992, and *P. thessalicus* Brunner von Wattenwyl, 1891, were chosen to represent the genus in this study, as a previous study found that these species had some of the largest differences in relative spermatophore size and sperm number within the genus (McCartney et al., 2008). The spermatophore sizes of *P. laevissimus* and *P. thessalicus* represent the upper and lower limits, with *P. erimanthos* producing a small to medium-sized spermatophore of 7.2% relative mass (McCartney et al., 2008). Sperm number from single matings range between 90,000 and 140,000 - 210,000 for *P. laevissimus* and *P. erimanthos* respectively, and up to about 14,500,000 in *P. thessalicus* (McCartney et al., 2008). Data for two further species, *P. v. minor* and *P. v. veluchianus* were obtained from the literature because these species represent medium to large-size spermatophores and sperm numbers respectively (Reinhold and Heller, 1993, Heller & Reinhold, 1994). All species examined here are nocturnal except *P. erimanthos* which is diurnal and mates during the day.

Any important differences between the methods used on the novel species presented here, *P. laevissimus*, *P. erimanthos* and *P. thessalicus*, and previously published species, *P. v. veluchianus and P. v. minor*, are outlined below. However, see Reinhold and Heller (1993) and Heller and Reinhold (1994) for detailed methods on *P. v. veluchianus* and *P. v. minor*.

Fieldwork on all novel species was carried out during the summers of 1990, 1997 and 1998 on the Peloponnese Peninsula and mainland Greece. *Poecilimon erimanthos* and *P. laevissimus* were observed at Erimanthos Valley (east of the village of Kumani, N. Elia, 37°46'N, 21°47'E.), and *P. thessalicus* at a site inland from Katerini (north-west of the village of Elatochori, 40°19'N, 22°15'E). Both sites were semi-pastoral with forest margins, and population borders were demarcated by roads, forests or cliffs.

Spermatophore Consumption Time, Male Body Mass and Spermatophore Mass

All measurements on spermatophore consumption of novel species were taken from field observations of marked (*P. erimanthos*) or non marked animals (*P. laevissimus* and *P. thessalicus*) throughout their mating season. Captured animals were paired in containers or hanging mesh cages in the field. Male and female *P. laevissimus* were captured as sub-adults and allowed to mature for around seven days before pairing to allow for full development of the accessory glands (males) and full receptivity (Heller and Helversen, 1991; see Reinhold & Heller, 1993; McCartney et al., 2008 for discussion on cage and laboratory effects in *Poecilimon*). To minimize disturbance of females, observations of spermatophore consumption progress were made at intervals rather than continuously. *Poecilimon laevissimus* and *P. thessalicus* were only used in observations after witnessing the onset of spermatophore consumption, whereas we estimated onset for *P. erimanthos* as half of the interval between the first observation of a female without a spermatophore, and again with a spermatophore (females observed about every hour). Spermatophore consumption times of all species were also estimated as half of the interval between the observation of the female last seen with a spermatophore, and subsequently without a spermatophore.

Spermatophore consumption time and male body mass were measured in the 1997 and 1998 breeding seasons and pooled for *P. thessalicus* (data did not differ significantly between years; spermatophore consumption time, $t_{14}= -0.561$; $p = 0.584$; male body mass $t_{66} = -1.501$; $p = 0.138$). Spermatophore masses for *P. thessalicus* are reported from 1998. Measurements of spermatophore consumption time, male body mass and spermatophore mass for *P. laevissimus* are reported from 1997. Spermatophore consumption times were recorded for *P. erimanthos* in 1990 and the male body mass and spermatophore mass were recorded in 1997.

Sperm Transfer

Poecilimon thessalicus and *P. laevissimus* were observed in 1998 whereas *P. erimanthos* was observed in 1997 and 1998. *Poecilimon erimanthos* (in 1997) and *P. thessalicus* (in 1998) were observed at the locations where they were collected. In 1998, we collected approximately 50 sub-adult *P.*

laevissimus and *P. erimanthos* east of the village of Kumani, N. Elia and took them to Central Greece, where we made further caged observations.

All bush-crickets taken from the field were sub-adults and were stored separately by sex and species, then allowed to mature for at least seven days. We allowed mating of 20 to 30 virgin pairs of each species. Mated females were allocated randomly to predetermined spermatophore attachment times that were set at intervals relative to the spermatophore consumption time in order to determine the rate of sperm transfer. For each species, the duration of the first sperm transfer trial was set to equal the average spermatophore consumption time for that species (see Table 1). All mated females, except some *P. erimanthos* in 1997, were assigned randomly to a pre-determined transfer time for examination. For *P. laevissimus* and *P. thessalicus* we tested sperm transfer times at appropriately equal periods either side of the average spermatophore consumption time, and repeated this until we had adequately covered the full period from no transfer until (near) full transfer (*P thessalicus* = 120, 240, 480, 780, 1020, 1260 min intervals, *P. laevissimus* = 60, 120, 180, 240 min. intervals). The spermatophores of *P. erimanthos* in 1997 were removed at various intervals between 30-80 min., with two distinct modes of 35 min and 75 min This meant the mean number of sperm that had transfer in six observations between 30-35 min., and six observations between 45-80 min. were pooled into two groups at 35 min. and 80 min. and the mean sperm transfer value was used for each. The spermatophores of *Poecilimon erimanthos* in 1998 were removed at 1 min., 120 min., and 240 min and combined with the data of 1997 (35 min. and 80 min.).

Immediately after mating, each female was placed head-first into a large scintillation tube to prevent her from bending to remove the ampulla. We then stored the females in a cool, shaded area and males were returned to cages. After the assigned period, each female was removed from her vial and the spermatophore removed by grasping the ampulla at its base with dissecting forceps and pulling it carefully from her genital pore and the spermatheca was excised. The female was killed and the spermatheca and the ampulla were stored in separate vials with a known volume of water for sperm counting (1-5 ml depending on the structure's size). If sperm ampullae became semi-detached or sperm had drained outside the female these data were not used in the analysis.

Each ampulla and spermatheca was macerated with a scalpel and mixed by passing it repeatedly through a syringe until the sperm had been suspended in the water and the sample homogenised. A sub-sample was placed on a haemocytometer slide (Swift: Neubauer improved). Sperm from a minimum

volume of 50 µl (or up to 200 µl) were counted and multiplied by the appropriate dilution factor to give the total number of sperm per spermatheca. Five sub-samples were taken and the solution was remixed before each new sub-sample was taken. From total sperm (ampulla and spermatheca) we derived the percentage of sperm within each mating transferred from the spermatophore into the spermatheca.

ANALYSIS

Sperm Transfer and Spermatophore Consumption

In order to compare the match/mismatch of complete sperm transfer and spermatophore consumption of all novel species, average spermatophore consumption times of all species were overlaid on a time-course chart of sperm transfer. In an attempt to compare the sperm transfer profiles of the three species presented here we spent considerable effort fitting regression models to sperm transfer patterns, and were not convinced that they could either reliably resolve the shape of sperm transfer curves, or validly explain the behaviour of sperm transferring into the female. Ultimately, no model we used could clarify the sperm transfer relationship between different species (see discussion). However, in all species examined, the modal sperm transfer time was apparent as the time when the largest change in sperm number was observed between observation intervals; in P. thessalicus this was followed by a clear plateau in the number of sperm transferred. Standard error is given in all cases.

In each species there were mating attempts resulting in no sperm transferring. These data were not included in analyses but are discussed further. Data were analysed using SAS 9.1. The analyses of *P. v veluchianus* and *P. v. minor* were as recorded in Reinhold and Heller (1993) and Heller and Reinhold (1994).

Relative Spermatophore Mass and Proportion of Sperm Transferred

Regression analyses on relative spermatophore mass against the proportion of sperm that had transferred into the female were first performed across taxa. All proportion data were arcsine (square root) transformed and

tested for normality. In conjunction with this regression analysis, corresponding regression analyses were also performed on transformed proportion data with phylogenetic independent contrasts in order to control for relatedness (Felsenstein, 1985). While this method is typically preferred over standard linear regression analyses across species, sample sizes are reduced further using contrasts (n-1) and so have less power.

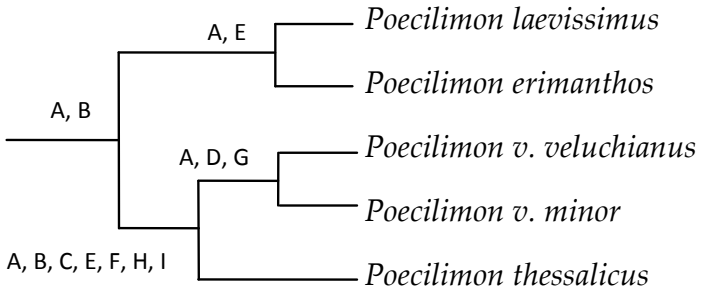

Figure 1. Cladogram representing the phylogenetic relationships between the five *Poecilimon* taxa used in this study. Letters at nodes indicate that subsequent daughter branches are based on information derived from the literature. References cited: A: Ulrich et al. 2010, B Heller 1984, C. Warchalowska-Sliwa et al. 2000, D. based on species geographic location, E. Willemse & Heller 1992, F. Heller 2006, G. Heller & Reinhold 1992, H. Heller 1990, I. Lehmann 1998.

Phylogenetic Independent Comparisons

A cladogram of the species used in this study was constructed using the literature on the phylogeny for these *Poecilimon* taxa (see Figure 1 for references) and the computer package PDAP (Maddison & Maddison, 2006) (Figure 1). The proportion of sperm and relative spermatophylax data were added to the tree in order to calculate phylogenetically-independent contrasts. In all cases, branch lengths were set to 1. The contrasts were then standardised by dividing them by the variance (square root of the sum of the branch length, Felsenstein (1985)). Generalised linear models were then used to regress the standardised independent contrasts of relative spermatophore size against the standardised independent contrasts of the proportion of transferred sperm response variable. All inferential regressions involving phylogenetically-independent contrasts were forced through the origin (Garland et al. 1992).

RESULTS

Spermatophore Consumption and Sperm Transfer

The spermatophores (spermatophylax and ampulla) were consumed in 101 ± 10.7 min (range 30-165 min., n = 14) for *P. laevissimus* (Table 1), a period too short to allow more than a small portion of the sperm to transfer into the female (Figure 2). Only about 15% of available sperm had transferred during any of the observations made prior to the last observations at 240 min (2.4 times longer than the mean spermatophore consumption period). The four observations at this longest interval revealed that a large amount of sperm still remaining in the ampulla and therefore the spermatophylax appears to be much smaller than is necessary to ensure complete sperm transfer.

Spermatophores in *P. erimanthos* (1990) were consumed in 84 ± 3.5 min (range 55-130 min, n=39, Table 1). This corresponds with a peak in sperm transfer, and more than 50% of sperm had transferred to the spermatheca by this time (Figure 2). After the time usually required for spermatophore consumption, sperm transfer seemed to slow down reaching about 75% of the total transfer after 240 min. Thirty five minutes after mating no sperm had been transferred (n=9) yet after this time all except one female (that was discarded because she was found with no sperm after 4 hours) contained over 50% (n=18) of the available sperm. So a fast transfer process occurs in this species and takes between 35 and 60 min, and is complete just before the spermatophore is normally consumed, indicating that the spermatophore may be of about the correct size for optimum sperm transfer (about 60% of total sperm). Pooled data for *P. thessalicus* from both years gave a spermatophore consumption time of 15.7 h (943 ± 47.6 min, n=16) (Table 1). The sperm transfer pattern of *P. thessalicus* differs from that in the two previous species, in that peak transfer occurred between 13-25% of mean spermatophore consumption time (240 min., Figure 2), and 93% of sperm had transferred by the end of spermatophore consumption. There was a clear plateau in sperm transfer in *P. thessalicus* at around 90-95% of total available sperm and therefore females were inseminated nearly four times more quickly than required for spermatophylax consumption. Even the fastest spermatophore consumption, of about 710 min, would have allowed around 93% of the sperm to transfer by the time one third of the spermatophore was consumed. Five out of 26 matings (19.2%) did not release any sperm into the female after transfer onset (one female at each of 240, 780, and 1260 min and two females at 480

min) and, since all other pairings resulted in close to, or above, 90% sperm transfer, these were not included in calculations of the means or standard errors. Interestingly, the average number of sperm in the ampullae that failed to transfer any sperm was only 8.3 million (n=5, S.E.=2.9 million, range = 2.3-17.8 million), significantly fewer than the 22.6 million (n=22, S.E.=21 million, range = 0.05-37.3) in spermatophores that did transfer (Mann-Whitney rank analysis U=27, $P < 0.007$).

Table 1. Male body mass, spermatophore consumption time (min) and absolute and relative spermatophore mass in three species of *Poecilimon* studied here (mean ± S.E. (range: n); upper three rows) and two sub species taken from Reinhold & Heller (1993), (lower two rows)

Species	Spermatophore consumption time (min) (range: n)	Spermatophore mass (mg)	Male body mass (mg)	Relative spermatophore size
P. erimanthos	84 ± 3.5 (55-135: 39)	47 ± 3 (n=11)	640 ± 4 (n=25)	7.2% (n=11)*
P. laevissimus	101 ± 10.7 (30-165:14)	47 ± 6 (n=9)	781 ± 13 (n=50)	6.1% (n=9)*
P. thessalicus	943 ± 47.6 (710-1380: 16)	112 ± 8 (n=28)	440 ± 7 (n=68)	33% ± 2..34% (n=17)
P. veluchianus veluchianus	570	162	640	24.9%
P. v. minor	200	74	365	19.1%

*No S.E. available because relative spermatophore mass was taken from dividing the average of pooled spermatophore mass from the average of pooled male body mass.

Relative Spermatophore Mass and Proportion of Sperm Transferred

No significant relationship was found between spermatophore size and the proportion of sperm that had transferred into the female by the spermatophore consumption time ($F_{1,4}$ = 7.69, p = 0.069, r^2 = 0.72). While this was not strengthened while controlling for relatedness ($F_{1,3}$ = 3.06, p = 0.179), a strong relationship is apparent (Figure 3). An increase in sample size is likely to produce a significant effect; males of larger spermatophore-producing *Poecilimon* taxa are likely to transfer a greater proportion of sperm than species producing smaller spermatophores.

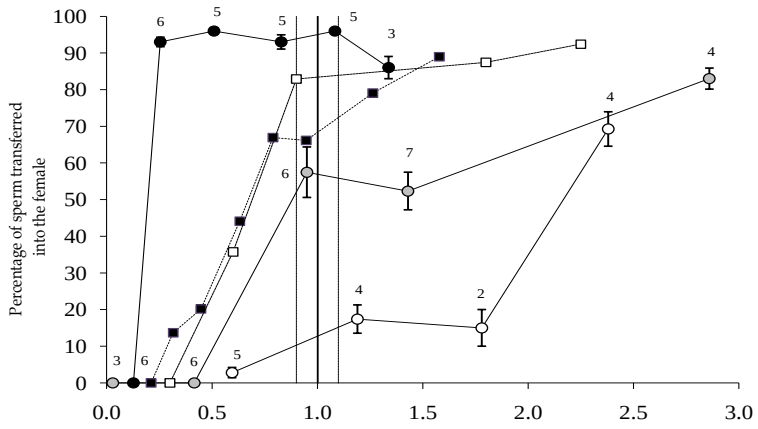

Figure 2. Percentage of sperm transferred after copulation from the male ampulla to the female spermatheca (± S.E.) plotted relative to the mean spermatophore consumption time, (numbers above points = n). Novel species are represented by unbroken lines: *P. laevissimus* (open circles), *P. erimanthos* (grey circles), and *P. thessalicus* (black circles). Broken lines represent *P. v. veluchianus* (closed squares) and *P. v. minor* (open squares) calculated from the published data (S.E and n not presented; for details see Heller and Reinhold (1994)). Dashed vertical lines show one SD in consumption time for *P. thessalicus* (the species with the largest SD).

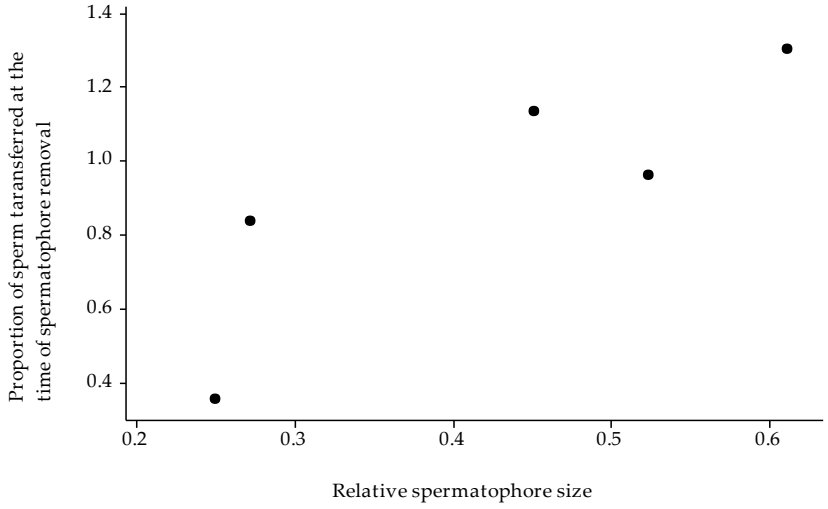

Figure 3. Spermatophore size compared to the proportion of sperm that have transferred into the female at the time of spermatophore consumption among five *Poecilimon* taxa. NB. Data are arcsine (square root) transformed.

DISCUSSION

The percentage of sperm that had transferred into the female by the time it took her to consume and remove the spermatophore differed markedly between the five species. The match/mismatch between complete sperm transfer and spermatophore consumption time found across our species correspond to our predictions that nuptial gifts of different sizes are affected by ejaculate protection and paternal investment to different degrees. Large nuptial gifts in *Poecilimon* are apparently either of correct size or larger than necessary to allow for a full transfer of sperm, whereas small nuptial gifts seem to be less than capable of protecting the ejaculate and allowing the complete complement of sperm to be transferred. Sexual selection for larger spermatophores in *Poecilimon* is likely to increase male confidence in sperm transfer (McCartney et al., 2008, 2010) and correspond to greater level of courtship related female mating investment (McCartney et al., 2012).

Poecilimon laevissimus and *P. erimanthos* have small spermatophylaces which seem to be either smaller than necessary for sperm transfer, or have consumption times marginally correspondent with the time it takes for sperm to transfer into the female; thus they are likely to function primarily as ejaculate protection. *Poecilimon thessalicus*, on the other hand, has one of the largest spermatophores reported (McCartney et al., 2008), and a nuptial gift almost four times larger than necessary for complete sperm transfer. It is therefore likely to function as both ejaculate protection and paternal investment.

It may be assumed that *Poecilimon* species with a similarly large spermatophore size as *P. thessalicus* will also have similarly long consumption times. This, however, does not appear to be the case in either of the subspecies of *P. veluchianus* which also have large nuptial gifts but comparatively quick consumption times (Table 1, Figure 2). While *P. thessalicus* and *P. veluchianus* indeed have larger gifts, the difference does not seem to lie in the speed at which the sperm transfers into the female, but rather with the extended period over which female *P. thessalicus* consume nuptial gifts. This point is important when understanding a key assumption of the paternal investment hypothesis; males must invest in their own offspring. Lengthened consumption time increases ejaculate transfer which delays the speed at which a female re-mates and increases the time in which nutrients of the donating male's gift can be incorporated in his offspring. Reasons for an extended feeding time in *P. thessalicus* are thus far unknown, however the study population was at relatively high altitude (ca. 1,100 m a.s.l.), with night time

temperature often at around 10-15°C, compared to *P. v. veluchianus* and *P. v. minor* (330 m a.s.l.; Reinhold and Heller, 1993) and *P. erimanthos* and *P. laevissimus* (around 600 m a.s.l.) where night temperatures are typically 20°C or above (unpubl. data). The metabolism of *P. thessalicus* at these temperatures is likely to be lower than that of the other species, resulting in consumption duration and digestion times of nuptials gift being significantly slower. However, temperature differences are unlikely to have affected our results because spermatophores are costly to produce and are evolutionary labile (McCartney et al., 2008); males would be expected to allocate fewer resources to gift production – to a size more appropriate to ejaculate protection – if there were no fitness benefits to having a proportionately large spermatophylax gift. A further explanation for the slow gift consumption time of *P. thessalicus* may be related to possible bitter substances in the spermatophylax of *P. thessalicus*. These may affect the speed at which females are able to consume the nuptial gift (as suggested by Heller et al., 1998) but further work is needed in order to verify the substances, their palatability, and the effect they have on females.

While there is a match between nuptial gift consumption and sperm transfer times in *P. v. veluchianus* and *P. veluchianus minor* these species have nuptial gift sizes that further correspond to our predictions that larger gifts are influenced by paternal investment. *Poecilimon v. veluchianus* has a substantial last male mating advantage (Achmann et al., 1992) and nutrients from the nuptial gift of the donating male are likely invested into his own offspring (McCartney 2010). Evidence of paternal investment in this species comes from a correlation of nuptial gift size on the dry mass of the donating males' offspring, and a greater lifespan of starved offspring (immediately after eclosion) fathered by males with large spermatophores (Reinhold, 1999).

Typically it takes 3-4 days for the nutrients in nuptial gifts to be incorporated in egg batches in the female (Bowen et al., 1984; Gwynne and Brown, 1994; Wedell, 1993b; Simmons and Gwynne, 1993; Reinhold, 1999, although see Voigt et al., 2006, 2008). While sperm precedence patterns have only been analysed in two *Poecilimon* species (*P. veluchianus*; Achmann et al., 1992 and *Poecilimon hoelzeli*; Achmann, 1996), both show a last male mating advantage. The combination of these factors in both *P. erimanthos* and *P. laevissimus* is, however, likely to exclude the possibility of paternal investment; both species remate, on average, every 1-2 days and lay eggs every two days (McCartney 2010). It is therefore unlikely that there is sufficient time for gift nutrients to be incorporated into the donating male's offspring. In contrast, field observations from *P. thessalicus* suggest that

females may have extended inter-mating refractory periods of about 7-8 days (and up to 19 days) and lay eggs every 1-2 days (McCartney, 2010), so males are likely to have their nutrients incorporated into the majority of eggs before females remate.

Transfer of a full ejaculate is necessary to ensure optimum fertilisation for males especially in polyandrous species (Smith, 1984). It is difficult therefore to understand why, in the two species that produce smaller spermatophores, males do not protect their ejaculate with larger nuptial gifts. In *P. erimanthos* and *P. laevissimus* females consumed 48% and 87% respectively of the sperm that males produced. While *P. laevissimus* seemed to remove and eat the spermatophore nearly eight times faster than expected for maximum sperm transfer, sperm from *P. erimanthos* transferred into the female at a rate that arguably approximated spermatophore consumption time, but still resulted in a waste of a large portion of sperm. Similarly, 9% of spermatophores are estimated to be prematurely consumed in *P. v. veluchianus* (Reinhold and Heller, 1993). It is likely that there is conflict between the sexes over optimum sperm number and resulting spermatophore attachment duration. Premature removal of the ampulla may constitute a form of post-copulatory female discrimination (Sakaluk and Eggert, 1996), but it is unlikely that such a high number of matings observed here resulted in removal discrimination. Sperm loading, the adjustment of copulation duration and ejaculate size according to the risk of sperm competition (Parker et al., 1990), has been observed in some other insects species (see for example Dickenson, 1986; Garcia-Gonzalez and Gomendio, 2004) including bush-crickets *Uromenus rugosicollis* (Vahed, 1997), and may well be a feature in some *Poecilimon*. Males may produce an optimum number of sperm ideal for sperm competition but in *P. laevissimus*, females may "have the edge" over this conflict by being able to consistently consume and remove the nuptial gift and sperm ampulla before the sperm is fully transferred (reviewed in Vahed, 2007b; Gwynne, 2008). This assertion of a conflict between the sexes is further corroborated by evidence in *P. laevissimus* where the pairs struggle for some time as the females appear to try and escape the clasp of the male's cerci, and may additionally represent a form of female discrimination that leads to 'fit' males transferring more sperm overall (Eberhard, 1996).

In a different form, large quantities of sperm and spermatophore material are also wasted in *P. thessalicus.* We found that a large proportion of males did not transfer any sperm (18.5%; n=27). Spermatophores are expensive to produce (Dewsbury, 1982; Drummond, 1984; Simmons, 1990, 1995a; Heller and von Helversen, 1991; Vahed, 2007b; Lehmann, 2012), so those that fail to

initiate represent a considerable waste in time and energy to *P. thessalicus* males. It may be that constriction of the females in scintillation tubes affected the onset of sperm transfer in *P. thessalicus*, although this is unlikely as onset was not affected in *P. laevissimus, P. erimanthos* and a previously studied species, *P. hoelzeli* (R. Achmann *pers. comm*.). Importantly, total sperm numbers in ampullae that did not transfer were much less (by 63%) than the total number of sperm in ampulla that did transfer. While these data suggest that the mechanical initiation of sperm transfer may be dependent on the internal pressure or volume of sperm or ejaculate, mechanisms behind the sperm transfer process are poorly understood in bush-crickets. Future studies would benefit by further assessment of sperm transfer initiation during this critical onset period (Achmann et al., 1992; Reinhold and Heller, 1993; Simmons and Achmann, 2000; Simmons, 2001).

Ultimately, no model we used for analysis could clarify the sperm transfer relationship between different species. Vahed (1994), however, previously fitted models using data from Gwynne et al. (1984) and Gwynne (1986) and showed that there was no difference between the sperm transfer curves for *Leptophyes punctatissima* and *Requena verticalis*, two species with varied sperm transfer profiles. Vahed (1994) suggested that the variation found within the sperm transfer among individuals of each species may be too large to easily detect a difference among species, although ultimately concluding that the function of the spermatophylax in *R. verticalis* is likely the same as that for *L. punctatissima*; to protect the ejaculate. As a comparison, we adopted the model used by Vahed (1994) and similarly found no difference between the sperm transfer curves of the two most different *Poecilimon* species (i.e. *P. thessalicus* and *P. laevissimus*, S=0.261, P=0.61). We therefore suggest that the variation found within the sperm transfer among individuals of each species is too large to detect a difference between species and that the curves are unlikely to be considered the same.

It is important to keep in mind that the function of the nuptial gift is influenced by substances in the ampulla, other than sperm, that are transferred during mating (McCartney et al. 2008; McCartney, et al. 2010). Some of these substances are known to influence female intermating refractory period (Heller & Helversen, 1991; Heller & Reinhold, 1994; Lehmann and Lehmann, 2000b; Vahed, 2007b), the timing of oviposition (Arnqvist and Rowe, 2005; Vahed, 2007b), and the share of eggs that are laid with the donating male's nutritional investment (Simmons 1990; Vahed, 2003). Indeed, the positive relationship we found between spermatophore size and the proportion of sperm transferred may tie closely to the total volume of ejaculate substances transferred. If these

substances affect fertilisation success or the incorporation of nutrients into offspring, the size or function of the nuptial gift may instead vary in accordance with these and be an important factor governing gift size (Vahed, 2003, 2007a; McCartney et al., 2008).

It is unlikely that male *P. erimanthos* or *P. laevissimus* make significant paternal investments in their offspring in terms of nutrients. While paternal investment has been directly observed in *P. v. veluchianus* (Reinhold 1999), the disparity in time between complete sperm transfer and spermatophore consumption in *P. thessalicus* is also best explained by paternal investment. Larger spermatophores apparently increase male confidence in sperm transfer – and perhaps total ejaculate transfer – and are likely to ensure a greater level of paternal assurance. Furthermore, a recent study has shown that females of *Poecilimon* species that have males that invest more in spermatophore production will compete for access to males, invest relatively more in mating effort, and take greater risks in finding mates than species with smaller spermatophores (McCartney et al. 2012).

In terms of ejaculate protection and paternal investment, we present evidence that both sexual selection and natural selection influence spermatophore size within the single bush-cricket genus *Poecilimon.* However, irrespective of function, it is clear that sperm is wasted in all species presented here, and a better understanding is needed of the cost of sperm production as well as the mechanisms which affect sperm transfer if we are to fully understand the relationship between nuptial gift size, paternal investment, and ejaculate protection. Future studies would do well to assess how other substances in the ejaculate may control female re-mating, ova production and oviposition rate, and how the transference of these substances relate to gift consumption time.

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