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**Spermatophore size variation
in the bush-cricket genus *Poecilimon***

A thesis presented in partial fulfilment

of

the requirements for the degree

of

Doctor of Philosophy

in

Ecology

at

Massey University,

Palmerston North – New Zealand

Jay McCartney

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I dedicate this thesis to the light and strength in my life,

Mary,

and the beauty in my world,

Milla and Nikau

Spermatophore size variation across the bush-cricket genus *Poecilimon*

Abstract

During mating, male bush-crickets transfer a costly nuptial gift to the female to consume while the ejaculate is transferred into her. The nuptial gift functions primarily as ejaculate protection, although in some larger spermatophore-producing species the gift functions additionally as paternal investment. While costly, production of large spermatophores may increase male fitness by providing a way in which males outcompete conspecific male sperm competition and female control over mating. For females, the nuptial gift may provide nutrients that increase her fecundity or allow greater fitness; however, larger gifts may also reduce a female's mating optima. A large variation in spermatophore size exists among bush-crickets; traditionally this is attributed to environmental and physiological differences. However, interspecific size variation may also be due to behaviour or common ancestry. Few studies have documented the evolutionary ecology of spermatophore size variation while accounting for environmental variation and relatedness.

Controlling for body mass, common ancestry, and diet, my thesis is a study of the variations in spermatophore size of the genus *Poecilimon*. I investigate aspects of operational sex ratio, reproductive effort, mating effort, paternal investment, ejaculate protection, sperm competition, mate choice, sexual conflict and reproductive fitness. I

gathered previously unpublished data and extracted data from the literature to make comparative analyses among 33 *Poecilimon* taxa. For specific focal comparisons, I further intensively studied five taxa in the field that vary markedly in spermatophore size.

First, I observed that variation in *Poecilimon* spermatophore size is as wide as that of the entire bush-cricket family (Tettigoniidae), and thus can be viewed as the ideal model system for investigating gift size variations across tettigoniids. Furthermore, using a phylogenetically independent contrast analysis I showed that evolutionary history has been of little importance in preventing changes in spermatophore size. I present evidence that both ejaculate protection and paternal investment are behind the evolution of larger spermatophore investments within *Poecilimon*. However, potential increases in spermatophore size are predicted to be selected against by female opportunities to increase fitness through multiple mating. In contrast, in a small spermatophore-producing species I found female mate choice for young, virgin males that are likely to transfer greater sperm volumes than previously mated males. In this small spermatophore-producing species I found selection for larger spermatophores. Theory predicts further restrictions to nuptial gift production, as a trade-off between alternative reproductive efforts. However, I found increases in paternal assurance enhanced by transferring larger spermatophores may allow for increased selection to advertise expensive gifts; because spermatophore size and investment in mate attraction are coupled, it appears there is no trade-off between these expensive mating efforts. Moreover, I found that spermatophore size within *Poecilimon* is correlated with a risk-shift in pair-formation protocol between taxa

whereby stationary males that call and wait for females to approach are able to produce larger spermatophores than males that approach calling females. Sexual conflict has been predicted to influence spermatophore size variation because dose-dependent manipulations of gift size on female polyandry occur in most insects, yet I found large spermatophore-producing *Poecilimon* taxa to have a larger per mating fitness increase than small spermatophore-producing taxa. Furthermore, I observed no direct cost of spermatophore size on female fitness. In fact, independent of the spermatophore size received per mating, females of different taxa typically receive similar volumes of spermatophore over their lifetime. Spermatophore size variation across *Poecilimon* reflects predictable within-species adjustments that males make to each spermatophore component in response to environmental constraints, ejaculate protection, paternal investment, and female selection as conditional strategies to maximize reproductive fitness.

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This thesis, in no unsure terms, has occupied a large portion of my life. Many factors have played a crucial part over its duration, yet none more so than the people I have inflicted my thesis upon; most have supported, aided and even cajoled me along the way towards its eventual completion.

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While sometimes hard to believe (although there are photos to prove it), I did have a life before my PhD. A handful of people, during that time, had such an influence on me that I was driven to seek something beyond the paper route. I am deeply indebted to my parents, Joe and Deborah McCartney, who supported my fascination with all things living, through exploding fish tanks, insomnia-inducing tree frogs, axolotls in the swimming pool, man-eating spiders, hairy eels in the toilet at 3.00am, and spluttering geckos in the vacuum cleaner. Without their enduring belief in me I'd still be working with pot-heads - chipping foam and laminating kitchen panels. I am also deeply grateful to my sister Cher; for many years I was the horse in our cowboys and Indians routine (I still don't know why I couldn't be an Indian). Without staring so closely at the ground for interminable hours, I would never have been introduced, or become fascinated by, the billions of creatures living beneath my feet. Seriously, Cher was an inspiration. She taught me the value of determination, hard work, self-belief, and a good hair-cut, all traits that helped me make it through the long-haul thesis flight. So, "thank-you Big Sis", from the bottom of my heart. I am also indebted to Kim Teltscher for her support from the early days of my Honours thesis, through to encouraging me to apply for the PhD position in Germany, and beyond, to her massive contribution to field-work in Greece over two years. I will always be grateful to her for this.

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At the risk of sounding like I have spent much of my time watching the All Blacks, this dissertation can be grossly classified as a thesis of two halves; the temporal point connecting these halves was not only when I returned to New Zealand from Germany, but when my objectives changed from the primary goal of data collection and entry and analysis, to the secondary goal of getting it all down in an intelligible form on paper.

The German thesis. I owe great thanks to Dr Klaus-Gerhard Heller who had the most difficult task of mentoring a naïve Kiwi lad in international research. He played a major role in my induction to a foreign land and obtained the original funding from the D.F.G. (many thanks to the Deutsche Forschungsgemeinschaft which supported my PhD). I must also thank him for introducing me to the most interesting research I could have hoped to discover. I also thank Roland and Dagmar Achmann, who taught

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Table of Contents

	Page
Abstract.....	i
Acknowledgements.....	v
Table of Contents	xi
 Chapter 1: Introduction.....	 1
NUPTIAL GIFT FEEDING IN INSECTS	3
OPERATIONAL SEX RATIO	4
MATING EFFORT AND PARENTAL INVESTMENT	5
EJACULATE PROTECTION AND SPERM COMPLETION	7
SEXUAL CONFLICT AND REPRODUCTIVE FITNESS	8
NUPTIAL FEEDING IN BUSH-CRICKETS	10
NUPTIAL GIFT FUNCTION IN BUSH-CRICKETS	11
NUPTIAL GIFT SIZE VARIATION IN BUSH-CRICKETS	13
NUPTIAL FEEDING IN <i>POECILIMON</i>	17
SUMMARY.....	19
A NOTE ON THESIS STRUCTURE AND CO-AUTHOR CONTRIBUTIONS.....	20
CHAPTERS	23
REFERENCES	32
 Chapter 2: Understanding nuptial gift size in bush-crickets: an analysis of the genus <i>Poecilimon</i> (Tettigoniidae: Orthoptera).....	 45
ABSTRACT	47
INTRODUCTION.....	48
METHODS.....	52
Collection.....	53
Determination of male body mass, spermatophore size, and sperm number.....	53
Analysis.....	55
RESULTS	57
Comparisons between <i>Poecilimon</i> and other Tettigoniidae	57
Variation within <i>Poecilimon</i>	63
Intraspecific variation	64
Spermatophore components.....	65
DISCUSSION	69
Spermatophore variation, ejaculate protection and paternal investment	69
Spermatophore size variation within <i>Poecilimon</i>	72
Spermatophore differences between field and laboratory-raised individuals.....	74
CONCLUSIONS	75
REFERENCES	77
APPENDIX 1	85

	Page
Chapter 3: A preliminary analysis of mate choice in a bush-cricket (<i>Poecilimon laevis</i> Tettigoniidae) suggests virginity is more important than body size	87
ABSTRACT	89
INTRODUCTION	90
METHODS	92
RESULTS AND DISCUSSION	94
REFERENCES	98
 Chapter 4: Evidence of natural and sexual selection shaping the size of nuptial gifts among a bush-cricket genus (<i>Poecilimon</i>, Tettigoniidae): an analysis of sperm transfer patterns	 103
ABSTRACT	105
INTRODUCTION	106
MATERIALS AND METHODS	110
Species and sites	110
Spermatophore consumption time, male body mass and spermatophore mass	112
Sperm transfer	113
ANALYSIS	115
Sperm transfer and spermatophore consumption	115
Relative spermatophore mass and proportion of sperm transferred	116
Phylogenetic independent comparisons	116
RESULTS	117
Spermatophore consumption and sperm transfer	117
Relative spermatophore mass and proportion of sperm transferred	120
DISCUSSION	121
REFERENCES	129
 Chapter 5: Lifetime spermatophore investment in natural populations of two closely related bush-cricket species (Orthoptera: Tettigoniidae: <i>Poecilimon</i>).....	 135
SUMMARY	139
INTRODUCTION	140
METHODS	144
Spermatophore size	144
RESULTS	145
Investment pattern	145
Seasonality	147
DISCUSSION	150
Seasonality of investment	150
Spermatophore scaling	151
REFERENCES	155

	Page
Chapter 6: Sex roles in mate attraction and searching: a comparative test using bush-crickets (<i>Poecilimon</i>: Tettigoniidae)	161
ABSTRACT	163
INTRODUCTION	164
METHODS	169
Our study taxon: <i>Poecilimon</i>	169
Male body mass, spermatophore size, and sperm number	169
Analysis	174
RESULTS	175
DISCUSSION	176
REFERENCES	181
 Chapter 7: Is there evidence of a macro-evolutionary trade-off between reproductive investments in mate attraction and nuptial gift size in bush-crickets?	 189
ABSTRACT	191
INTRODUCTION	192
METHODS	196
Male body mass, spermatophore size, and sperm number	196
Syllable and impact number per day and PCF	200
Phylogenetic construction	200
Comparative analyses	201
RESULTS	204
DISCUSSION	206
REFERENCES	212
 Chapter 8: Larger nuptial gifts increase male per-mating fitness across a bush-cricket genus (<i>Poecilimon</i>), but do they “manipulate” females?	 221
ABSTRACT	223
INTRODUCTION	224
METHODS	229
<i>Poecilimon</i>	229
Data collection, body mass and spermatophore mass	229
Population mating frequency	232
Mature female longevity	233
Daily egg batch laying frequency	234
Egg mass and hatching success	235
Male per-mating reproductive fitness	235
Female lifetime reproductive fitness and total lifetime spermatophore material received	236
Analysis	236
Phylogenetic independent comparisons	237

	Page
Chapter 8 (continued)	
RESULTS	238
Body mass and spermatophore mass	238
Population mating frequency	238
Mature female longevity	242
Daily egg batch laying frequency	243
Egg mass and hatching success	244
Male per-mating reproductive fitness (eggs laid, egg mass and number of eggs hatched per mating)	246
Differences between species in female lifetime reproductive fitness	248
Male per-mating reproductive fitness	249
Female lifetime reproductive fitness and total lifetime spermatophore material received	251
DISCUSSION	251
REFERENCES	260
 Chapter 9: Discussion and conclusions	 267
<i>POECILIMON</i> AS A MODEL TAXON	269
CONTROLLING FOR NATURAL VARIATIONS IN A CLOSELY RELATED TAXON	271
MATING EFFORT, PATERNAL INVESTMENT, EJACULATE PROTECTION AND SPERM COMPETITION	274
REPRODUCTIVE EFFORT	278
MATE CHOICE	282
OPERATIONAL SEX RATIO, SEXUAL CONFLICT AND REPRODUCTIVE FITNESS	285
FUTURE RESEARCH	288
CONCLUSIONS	289
REFERENCES	291

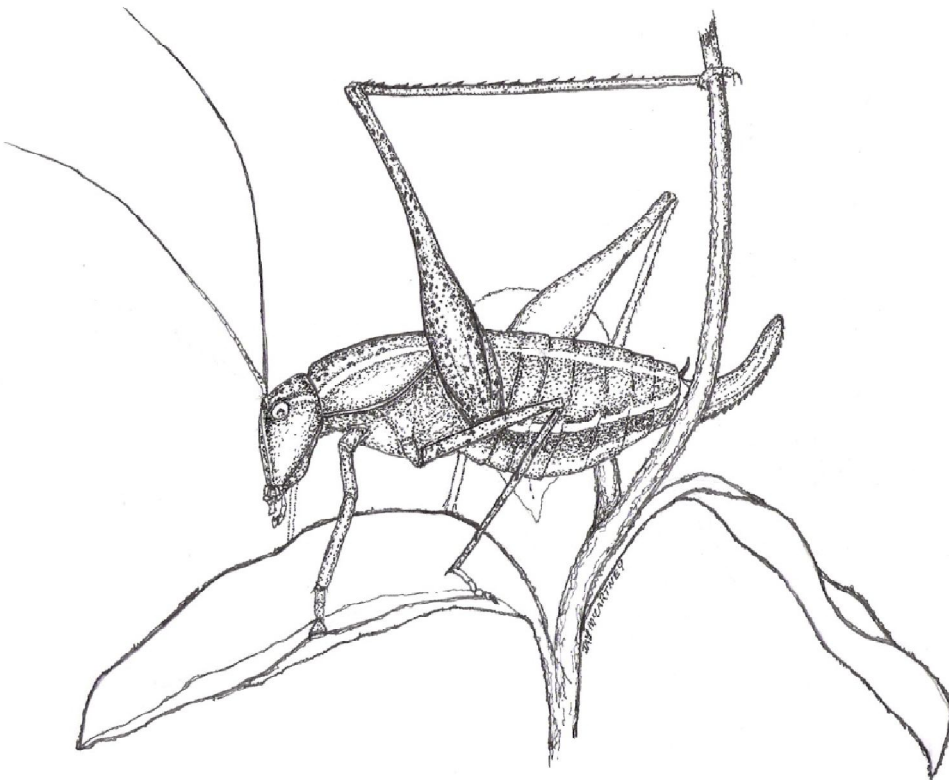
"[Sexual selection] depends, not on a struggle for existence, but on a struggle between the males for possession of the females; the result is not death to the unsuccessful competitors, but few or no offspring."

Darwin (1859), *On the Origin of Species* (p. 103)

Chapter 1

Introduction

Jay McCartney



Darwin (1959) was the first to make a clear distinction between those evolutionary forces that enhance an individual's survival – natural selection – and those that enhance reproductive success by acquiring mates – sexual selection – yet the relative degree to which natural and sexual selection respectively affect mating behaviour is still largely unknown. Few examples delineate the complex interaction between natural and sexual selection more so than the act of nuptial gift transfer in insects. This dissertation, exactly 150 years after the publication of Darwin's principal treatise *On the Origin of Species* (1859), is dedicated to understanding how the forces of natural and sexual selection influence the behavioural evolution of nuptial gift size in bush-crickets.

Nuptial gift feeding in insects

Nuptial gifts are nutrient-containing items that a male transfers to the female during or immediately after courtship or copulation. Despite their potentially high associated costs, nuptial gifts are widespread across invertebrate taxa (for reviews see Thornhill and Alcock 1983; Mann 1984; Andersson 1994; Vahed 1998; Gwynne 2001). These gifts vary, from offerings of prey (Thornhill 1976; Iwasaki 1996; Bockwinkel and Sauer 1994; Brown 1997; Engqvist 2009), to body parts (Gwynne 1997; Fedorka and Mousseau 2002), regurgitated food (Steele 1986), fluid secretions (Dodson et al. 1983; Eggert and Sakaluk 1994; Brown 1997), and spermatophores (Gerhardt 1913; Boldyrev 1915; Vahed and Gilbert 1996; Voigt et al. 2008). In some spiders (e.g., Newman and Elgar 1991; Arnqvist 1992; Forster 1992; Andrade 1996; Wilgers et al. 2009), flies (e.g., Downes

1978), preying mantids (e.g., Lawrence 1992; Kynaton et al. 1994; Barry et al. 2009), and crickets (e.g., Burr et al. 1923), a nuptial gift may even constitute the male himself (for reviews see Thornhill and Alcock 1983; Boggs 1995; Gwynne 1997, 2001; Vahed 1998). The large variation found in both nuptial gift size and form continues to be one of the most interesting and widely debated aspects of animal mating systems.

Over the last 150 years a handful of theories have themselves evolved from Darwin's natural/sexual selection foundation, and become the contemporary basis of the way the evolution of nuptial gift feeding in insect mating systems is understood. These selective pressures form the basis of my thesis: operational sex ratios, reproductive effort, mating effort, parental investment, ejaculate protection, sperm competition, mate choice, sexual conflict and reproductive fitness. The aim of this introduction is to outline these selective pressures and to relate them to my research.

Operational sex ratio

While Darwin and many other naturalists understood that "the female, with the rarest exceptions, is less eager than the male ... she is coy, and may often be seen endeavouring for a long time to escape" (Darwin 1879, p. 245), Bateman (1948, p. 365) formalised the concept in his influential study on the fruitfly *Drosophila melanogaster*: "in unisexual organisms there is nearly always a combination of an indiscriminating eagerness in the males and a discriminating passivity in the females". With his concept that male reproductive success was much more variable than that of the female, Bateman (1948) initiated a new era of interest in sexual selection (Thornhill and Alcock

1983). In terms of energetic expenses, Bateman (1948) explained that male sperm, compared to the female's eggs, are relatively cheap to produce. As a result of anisogamy – the relative difference in size of the male and female gametes – Bateman (1948) concluded that males are limited by the number of females they can acquire whereas females are limited by the number of eggs they can produce. As a result, females become a limiting resource within typical mating systems. Males compete for access to fewer available females and are, resultantly, sexually selected by those females. Thus, the concept of the operational sex ratio – the ratio of sexually available males to sexually available females (Emlin and Oring 1977) – became the foundation for understanding mating systems. This is especially important when understanding the mating system of gift-giving insects: gift-production costs may reduce a male's remating frequency similar to that of the female, resulting in a decrease or shift in selection pressure from the male by reducing the inequality in relative resource demands between the sexes (Gwynne 1991).

Mating effort and parental investment

Building on Darwin (1859; 1871) and Bateman (1948), Trivers (1972) further extended the concepts of sexual selection and relative reproductive investments. Trivers recognised that females are guaranteed to be the genetic parent of their offspring but males are not. This 'genetic certainty', allows females to provide higher levels of parental investment than males (without risk of cuckoldry) and, as a result, they are not always available for subsequent fertilisation (i.e., gravid, brooding, lactating etc).

Trivers (1972) further noted, however, that species do not always exhibit the typical 'ardent' male and 'coy' female behaviour. He argued that "the sex whose typical parental investment is greater than that of the opposite sex will become the limiting resource for that sex", and recognised there was a conceptual need for a common currency that could explain the degree of variation in relative investment that each sex makes toward their offspring.

While the term 'reproductive effort' had been coined a few years earlier to explain the proportion of an organism's total available energy used in reproduction – not just gamete size (Williams, 1966) – Trivers' (1972) 'parental investment', which he defined as "any investment by the parent in an individual offspring that increases the offspring's chance of surviving at the cost of the parent's ability to invest in other offspring", explained why males that contribute more to their offspring than females become the discriminating sex. Due to the relative increase in a male's reproductive investment in gift-giving insects, males and females may not only invest equally in offspring, but males may become a limiting resource for which females compete (Alexander and Borgia 1979; Gwynne 1984). Parental investment, along with 'mating effort' – effort expended in acquiring mates (Low 1978; Alexander and Borgia 1979) – came to respectively represent the two opposing forces of natural and sexual selection known to shape mating systems. The two forms of reproductive effort became fundamental to understanding variations in nuptial gift size.

In terms of parental investment, nuptial gifts in insects are naturally selected to enhance the fitness or quantity of the donating male's offspring, whereas a mating effort function argues that nuptial gifts are sexually selected to attract females, facilitate

mating and/or maximise ejaculate transfer (for reviews see Vahed 1998; Gwynne 2001). While greater parental investments made by gift-giving males provided a convincing argument for the evolution of larger nuptial gifts, there was, at the time, little empirical evidence to support the idea that offspring benefited from the donating male's gift (Boggs and Gilbert 1979; Boggs 1981; Boggs and Watt 1981).

Ejaculate protection and sperm competition

Concurrent to the formulation of ideas on parental investment, another convincing paradigm, that of sperm competition, was popularised by Parker (1970a) and provided persuasive arguments in favour of the mating effort function of the nuptial gift. Arguments for the mating effort function of the gift; that of ejaculate protection, propose that the gift should be consumed over a time proportional to that in which the sperm takes to transfer into the female. This proposition was supported by empirical evidence from a range of insect taxa (reviewed in Vahed 1998; Gwynne 2001).

Despite the fact that the sperm from a single mating are often sufficient in number to fertilise all the eggs of a female (reviewed in Neubaum and Wolfner 1999; Simmons 2001), the majority of female insects, indeed most animals, accept the sperm from more than one male. Female insects commonly have a sperm storage organ, the spermatheca, in which sperm is typically stored for long periods. The storage of sperm gives females greater control over when it is used or the potential to 'choose' the paternity of her offspring (reviewed in Birkhead and Møller 1998; Simmons 2001). Depending on the sequence in which females utilise the stored sperm, whether it be the

first male she mates with, the last, or a 'raffle' between all males, a male's sperm will be selected on the basis that it can supersede the sperm of rival males (Parker 1990). Given high last male sperm precedence, where the last male to mate sires the majority of offspring, which is typically the case in insects, males will also be selected if they can prevent subsequent males from depositing further sperm into the female (Parker 1970a,b,c,d). As sperm competition ultimately arises over the presence of sperm from two or more males, an increase in ejaculate volume offered by males is typically associated with the degree of female polyandry (reviewed in Thornhill and Alcock 1983; Birkhead and Møller 1998; Simmons 2001; Shuster and Wade 2003; Parker and Ball 2005).

In an idea initially proposed by Gerhardt (1913, 1914) and Boldyrev (1915), the nuptial gift can be viewed as a protective device for sperm and ejaculate transfer. Males that can keep the female occupied with a nuptial meal while their ejaculate is transferring can transfer greater sperm numbers than those males that offer no such gifts.

Sexual conflict and reproductive fitness

Sexual conflict is the conflict of evolutionary interests between different sexes of a species (for reviews see Parker 2006; Gwynne 2007). Each sex may have specific reproductive optima, and if these cannot be obtained simultaneously a selective disadvantage will be conferred to the manipulated sex, forming the basis for subsequent inter-sexual competition (Parker 2006). Male bush-crickets, for example, are

typically available for mating more quickly than females; males have a higher potential mating frequency. Mating at an optimum frequency for males will yield a greater reproductive fitness than if, for example, they mated at the lower mating frequency optimal for conspecific females. The reproductive success of an individual therefore is dependent on the reproductive optima of the opposite sex and may, if different, lead to conflict between the sexes while attempting to retain control over their own optimum reproductive strategy (reviewed in Arnqvist and Rowe 2005; Gwynne 2007; Vahed 2007).

While sexual conflict had been implicit as a potential driving force for evolutionary change (Darwin 1871), mating systems were typically viewed as a cooperative affair (for a review see Gwynne 2007). Williams (1966), however, viewed mating as “a battle of the sexes” and sexual conflict to be at the centre of sexual selection theory. By the 1990s, the theory of sexual conflict seemed to occupy much of the literature as a realistic selective pressure influencing variations in mating systems, including those of gift-bearing species (e.g., Arnqvist 1989, 1992, 1997; Simmons 1991; Thornhill and Sauer 1991; Part et al. 1992; Jormalainen et al. 1994; Rowe 1994; Arnqvist and Rowe 1995; Clutton-Brock and Parker 1995; Warner et al. 1995; Chapman and Partridge 1996; Arnqvist et al. 1997; Brown 1997; Chapman 1997; Parker 1998; Watson 1998). Beginning with Parker’s (1979) first theoretical analysis of sexual conflict in evolution, the nuptial gift came to be viewed as a way that a male may overcome female mate choice in an effort to raise his own reproductive fitness (reviewed in Gwynne 2007).

Nuptial feeding in bush-crickets

Due to increased male reproductive investment in gift-giving insects, males and females may come close to investing equally in offspring. These insects therefore provide the ideal model mating system to test the relative influence that natural and sexual selection have on nuptial gift size variation. While nuptial feeding is widespread in insects, one model group, Orthoptera (grasshoppers, crickets, locusts and their allies), has perhaps generated more research than any other group of insects. Orthoptera are particularly conducive to studying mating system variations: they are readily found in most environments, are generally large which makes them easy to manipulate, and are easy to raise under laboratory conditions.

Male bush-crickets transfer a substantial nuptial package, the spermatophore, to the female during mating (for reviews see Gwynne, 1990, 2001; Wedell 1993a, 1994a; Vahed 1998). The spermatophore is comprised of a large gelatinous, protein rich, nuptial gift – the spermatophylax – and a smaller, sperm-filled pouch – the ampulla. During the initial phase of courtship the spermatophore is formed from accessory glands within the thorax of the male (Boldyrev 1915; Gwynne 1997). The male then transfers the ampulla and attached spermatophylax to the female's genital pore. With the ejaculate-filled ampulla and spermatophylax left hanging from the female, she bends under and starts to consume it as sperm transfer into her (Boldyrev 1915). After the consumption of the spermatophylax-gift the female then removes and consumes the ampulla and its remaining contents (Boldyrev 1915).

A large inter-specific variation in nuptial gift size exists within the orthopteran bush-cricket family Tettigoniidae (Gwynne 1983, 1990; Wedell 1993a, 1994a, 1994b; Vahed and Gilbert 1996). Spermatophores can contribute to a net investment of between 2% (*Acripeza reticulata*: Wedell 1993a) and 40% of male body mass (*Epiphigger*, Busnel and Dumortier 1955; *Sterolpleurus stali*, Bateman 1997; *Poecilimon thessalicus*: McCartney et al. 2008). Bush-cricket species are numerous, with over 6,000 described species, and ubiquitous, appearing on all continents except Antarctica (Gwynne 2001).

Nuptial gift function in bush-crickets

The function of the ampulla as an ejaculate-holding transfer vessel is relatively clear in bush-crickets yet the role of the spermatophylax-gift is more complicated. The two main functional explanations for nuptial gifts in insects – mating effort and parental investment – may similarly explain spermatophore size variation in Tettigoniidae. Mating effort in bush-crickets has been attributed to an ejaculate protection function whereby the spermatophylax increases fertilisation success by acting as ejaculate protection (Gerhard 1913; Boldyrev 1915; Low 1978, Gwynne 1984, 2001; Sakaluk and Eggert 1996; Vahed and Gilbert 1996; Simmons 2001). A large gift allows the transfer of the complete ejaculate into the female before she removes and consumes the ampulla and the remaining sperm. The ejaculate protection function assumes that the nuptial gift in bush-crickets is sexually selected, as it increases the male's assurance in sperm competition (Wedell 1991; Reinhold and Heller 1993; Heller and Reinhold 1994;

Gwynne 1997; Reinhold and von Helversen 1997). In this case, the spermatophylax size should co-vary with the number of sperm transferred (Reinhold and Heller 1993; Wedell 1993a, 1994a; Heller and Reinhold 1994). Under the ejaculate protection hypothesis the nuptial gift may also influence the speed at which a female will re-mate, thereby affecting the number of eggs the male fertilises (Gwynne 1986; Wedell and Arak 1989; Simmons and Gwynne 1991; Reinhold and Heller 1993; Wedell 1993a), or by directly influencing egg laying frequency; either hastening the onset of oviposition or the development of eggs (Loher 1981, 1984; Wedell and Arak 1989; Eberhard and Cordero 1995), (for reviews see Vahed 1998; Gwynne 2001). Consequently, female inter-mating interval and the gift size should co-vary.

The paternal investment hypothesis proposes that the function of the orthopteran spermatophylax is derived from its nutritive value. In this hypothesis, natural selection favours large spermatophylaxes since this has a direct positive effect on the eggs or offspring of the donating male, resulting in an increase in the fitness and/or quantity of surviving offspring (Alexander and Borgia 1979; Wickler 1985; Gwynne 1986b, 1988, 1990; Simmons and Parker 1989; Heller et al. 1998, 2000; Reinhold and Heller 1993; Reinhold 1999). Large benefits for females consuming larger gifts are also predicted; large gifts may even supply a female with her total energy requirements (Voigt et al. 2005). Paternal investment is argued to be possible only if the donating male's offspring benefit from his spermatophylax; if the benefits of female gift consumption only occur to offspring sired by subsequent male partners, the nuptial gift is considered 'pseudo-parental investment' (Wickler 1985).

While not mutually exclusive, the paternal investment and ejaculate protection hypotheses highlight the selective pressures that distinguish between the relative influences of natural and sexual selection on the evolutionary maintenance of spermatophore feeding in bush-crickets. Within the past thirty years or so, the adaptive significance of nuptial gifts in Orthoptera, and bush-crickets in particular, have formed the central debate on the selective pressures that maintain nuptial gift size variation in insects (for reviews see Vahed 1998; Gwynne 2001).

Nuptial gift size variation in bush-crickets

The two functional hypotheses explaining nuptial gift variation – paternal investment and ejaculate protection – both predict that larger gifts confer greater benefits to males than smaller gifts. Why, then, is there such a large size variation in bush-crickets? Why do many species produce small gifts when it is clear that large gifts confer large benefits?

A number of reasons have been proposed to account for ‘sub-optimal’ gift sizes and the accompanying variation found in spermatophylax-gift size between orthopteran species. Spermatophores are costly to produce; they incur higher metabolic costs than smaller spermatophores, and may reduce a male’s future reproductive potential (Dewsbury 1982; Simmons 1990, 1995b; Heller and von Helversen 1991; Hayashi 1993). Given sub-optimal environmental conditions, large gifts may be either difficult to produce at efficient rates, or the production process may potentially decrease a male’s overall reproductive fitness.

Environmental constraints are important because they underpin the costs of reproduction (Fischer 1930; Stearns 1989; Partridge and Sibley 1991; Simmons 1993), and as a major determinant of reproductive success, they are an important aspect of sexual selection (Halliday 1987; Simmons 1988, 1993). Diet, for example, has been shown to be a major factor influencing spermatophore size and function (Gwynne 1985; Wedell 1993a, 1994a,b). Environmental stresses created by variations in the availability of food resources, such as water (Reinhold and Heller 1993; Ivy et al. 1999) and protein (Heller et al. 1998; Wedell 1994a), are important influences on spermatophore production (Heller et al. 1998, 2000). Food and nutrient availability may also alter the urgency with which females require spermatophore nutrients, placing further premiums on spermatophore size and quality (Parker and Simmons 1989; Gwynne 1985, 1990; Simmons 1988b; Simmons and Gwynne 1991). While males attempt to retain larger spermatophores by reducing spermatophore quality (Wedell 1994a), mating frequency (Simmons 1988a; Gwynne 1990; Simmons and Bailey 1990; Simmons et al. 1992; Heller and Reinhold 1994; Reinhold and von Helversen 1997, although see Wedell 1993b), and energy allocated to other functions such as calling (Simmons et al. 1992), gift size may be adversely affected by nutrient assimilation due to such factors as food availability (Wedell 1993a) or parasite load (Lehmann and Lehmann 2006).

More recently, theories of sexual conflict have provided convincing arguments for explaining evolutionary change in spermatophylax-gift size among Orthoptera. Sexual conflict may also largely explain why orthopteran nuptial gifts are often smaller than can be predicted solely from their functional explanations. While spermatophylax-

gifts confer considerable benefits to males and females, spermatophylaxes may be used to manipulate the female's mating behaviour to align with a male's own reproductive optima; gift size, in these cases, is likely the product of sexually antagonistic co-evolution (Arnqvist and Rowe 2005; Vahed 2007; Gwynne 2007; Wagner and Basolo 2008). As a result of male manipulation, females are predicted to resist if the optima of each sex cannot be achieved simultaneously. As a result, counter-selection is predicted and retaliatory changes in females may be selected as females try to maintain their own reproductive optima (Sakaluk et al. 2006).

Interspecific variation in spermatophylax-gift size in Orthoptera therefore can be explained as a balance between the reproductive benefits that each sex may receive, i.e., increases in reproductive fitness and offspring quality gained through reproductive investments, and the environmental and reproductive constraints that directly influence male spermatophore production (Fischer 1930; Dewsbury 1982; Stearns 1989; Partridge and Sibley 1991; Simmons 1990, 1992, 1993, 1995; Heller and von Helversen 1991; Hayashi 1993). However, the relative strength of these factors in selecting the size of spermatophylax-gifts is largely unknown.

Potentially the most effective way to understand the selective pressures that influence gift size variation is through an in-depth comparative analysis of closely related, field-observed species. Such a group should not only produce a large variation in spermatophylax-gift size but should also share similar life histories, habits and habitats. Comparisons among species within a genus, for example, can be particularly informative for indicating evolutionary trends because many variables that are shared by congeners are held constant (Ridley 1983; Felsenstein 1985; Harvey 1991; Harvey

and Pagel 1991). To date, however, only a few studies have attempted to understand spermatophylax-gift variation across a range of species. However, understanding the interspecific variation in gift size and function in these studies has been difficult; complications occur with interspecific diet variation, different levels of relatedness between taxa, and observing field-populations in their natural environment.

Wedell (1994a), for example, showed that ecological variables such as diet might influence the cost of the spermatophore, affecting its size and quality across 22 varied bush-cricket species. Wedell (1993a, 1994b) also found, across 19 varied species of bush-cricket, that spermatophylax-gifts may be categorized into two main types: those that protect the ejaculate during insemination and have no (or little) nutritional benefit to the female, and those that are large and highly nutritious to the female. Gwynne and Brown (1994), however, observed that in these cases “Variation in nuptial gifts may be due to common ancestry rather than ecology as taxonomy and diet are perfectly confounded.” More recently, Vahed and Gilbert (1996) showed that, while controlling for relatedness across 43 widely related bush-cricket taxa, differences in nuptial gift size correlate with sperm number and ejaculate volume. However, some species were lab reared – a condition known to affect sperm number (e.g., Reinhold 1994) and spermatophylax size (e.g., Heller and von Helversen 1991) – and no specific reference was made to patterns of variation in spermatophore size and their relation to the ecology of the species.

Large differences in spermatophore components have previously been found to occur between laboratory and field-raised species (see, for example, Heller and von Helversen 1991; Heller and Reinhold 1994). Data from either captive or natural

populations on survival and reproduction across the lifespan is rare and has often lead to major assumptions being made and the use of individual fitness traits being used to estimate lifetime reproductive success (Reed and Bryant 2004). Single-species studies have often been used to understand the selective pressures that affect gift variation, yet the reliability of these results is limited when trying to understand evolutionary trends. What has been lacking is an ideal model taxon for which the selective pressures can be compared across species with varying spermatophore sizes in the field.

Nuptial feeding in Poecilimon

Around 140 species of the barbistine bush-cricket genus *Poecilimon* (Phaneropterinae, tribe Barbistini) have been formally described (Eades and Otte 2008). Approximately 65 species are found throughout Europe, most of which are situated in the east Mediterranean (Heller 2004), where they constitute the most species-rich genus of bush-cricket in the region. Over the past 20 or so years a large amount of life history data has been collected on this genus, allowing a relatively resolved understanding of *Poecilimon* morphology, taxonomy, phylogeny and acoustics (Heller 1984, 1988, 1990, 2004, 2006; Heller and Reinhold 1992; Willemse and Heller 1992; Lehmann 1998; Warchalowska-Sliwa et al. 1995, 2000; Ünal 2001, 2003a,b, 2004, 2005; Heller and Lehmann 2004; Heller and Sevgili 2005; Lehmann et al. 2006; Ulrich et al. in press). In addition, a range of mating behaviour data from field-studied *Poecilimon* species have been collected: *P. affinis* (Heller and von Helversen 1991; von Helversen et al. 2000), *P. hoelzeli* (Achmann 1996; Lehmann and Lehmann 2000a,b, 2001; Lehmann and Heller

2001), *P. intermedius* (Lehmann et al. 2007), *P. mariannae* (Lehmann and Heller 1998; Lehmann and Lehmann 2000a, 2006), *P. ornatus* (Heller et al. 1997), *P. thessalicus* (Lehmann et al. 2001; Lehmann and Lehmann 2008a), *P. veluchianus* (Heller and von Helversen 1991; Achmann et al. 1992; Reinhold and Heller 1993; Heller and Reinhold 1994; Reinhold 1996, 1998, 1999; Lehmann and Heller 1998; Reinhold and von Helversen 1997; Lehmann and Lehmann 2000b) and *P. zimmeri* (Lehmann and Lehmann 2008b, 2009); thus providing a broad overview of the variations in mating behaviour found within *Poecilimon*. This makes the genus an ideal model taxa candidate for understanding the selective pressures that influence gift size variation in insects.

Moreover, recent studies indicate that *Poecilimon* species are closely related; they belong to a confirmed monophyletic clade (Ulrich et al. in press), and they seem to share similar feeding habits and ecologies. This, to some degree, helps control for larger phenotypic variations that might otherwise be expected in more distantly related groups. Perhaps most importantly, initial behavioural observations on *Poecilimon*, nearly all of which were conducted on natural populations in the field, show that large interspecific variations exist in mating behaviour and spermatophylax-gift size (Heller and von Helversen 1991; Heller and Reinhold 1994; Vahed and Gilbert 1996; Wedell 1993a; Achmann et al. 1992). Furthermore, recent work on *Poecilimon* has supported aspects of both mating effort (e.g., Heller and Reinhold 1994; Heller and von Helversen 1991; Reinhold 1999; Reinhold and Heller 1993; Reinhold and von Helversen 1997) and parental investment (Reinhold and Heller 1993; Reinhold 1999) as a source for variation of spermatophylax-gift size. There is also evidence to suggest that

spermatophore production in larger spermatophore-producing *Poecilimon* is costly for males, both in direct energetic investment (Voigt et al. 2006, 2008), and in future reproductive potential (Heller and von Helversen 1991; Lehmann and Lehmann 2000a,b, 2006; Lehmann et al. 2001). Furthermore, male *Poecilimon* also invest heavily in mate acquisition (Heller 1992; Heller and von Helversen 1990, 1993; Heller et al. 1997) by initiating mating through emitting expensive acoustic signals (e.g., Heller 1990). The combination of these factors suggest that comparing life history traits (such as mating frequencies, spermatophore consumption time, sperm transfer patterns, mate choices such as body size to virgin partner preferences), to gift size variation across *Poecilimon* species may help elucidate behavioural selection pressures such as mate choice, sexual conflict, operational sex ratios, reproductive effort and fitness.

One further benefit of studying *Poecilimon* may come from the knowledge that the sperm precedence pattern has been established in two species; one with a large spermatophore, *P. veluchianus*, and the other with a medium size spermatophore *P. hoelzeli*, both of which have a high last male sperm precedence pattern (Achmann et al. 1992; Achmann 1996). In summary, a relatively large body of published literature exists concerning the genus *Poecilimon*, and provides an ideal model taxon in which size variation in spermatophylax-gifts can be better related to selection pressures.

Summary

Between-species variation in spermatophore size may be attributed to environmental, physiological and/or behavioural factors. To a large degree environmental and

physiological effects are well studied in bush-crickets, yet the influences that natural and sexual selection have on mating behaviour and their subsequent effect on spermatophore size variation are poorly understood. This thesis is an examination of the behavioural factors.

Within this thesis I bring together published and unpublished field data with novel field observations on *Poecilimon* to answer some of the more elusive theoretical aspects of gift size variation. In particular, I use both comparative and focal species analyses to answer questions relating to the behavioural relationships between spermatophylax-gift size variation and operational sex ratio, reproductive effort, mating effort, parental investment, ejaculate protection, sperm competition, mate choice, sexual conflict, and reproductive fitness.

I bring together these aspects of mating behaviour across *Poecilimon* into a general model, linking natural (fecundity) and sexual (mating success) selection to potential constraints in spermatophore investment, such as mate attraction and sexual conflict. My intention is to provide a better understanding of the relative investments that male and female gift-offering insects make in pair-formation and the effect that this may have on their respective reproductive fitness.

A note on thesis structure and co-author contributions

All chapters of this thesis have been submitted for publication and are in review, in press or published. Each chapter therefore has been presented in the style that each journal requires for submitting. For consistency and ease of reading, however, figures

and tables have been set within document text (c.f. individually placed, separate from their captions, at the end of each document). The introduction and discussion to this thesis have not been submitted for publication and have been formatted in the style required for submitting to the journal *Evolution*.

While this thesis is entirely my own work, co-authors have been included on all papers due to their significant input. For the most part, I organised and conducted field and laboratory work, but it was significantly aided by the help of two people who worked under my supervision and direction, namely, Kim Teltscher who helped for two seasons (eight months), and Louise Penny for one season (four months). Klaus-Gerhard Heller and I worked closely in the field for the first three weeks of two seasons, sharing observations, experiments and ideas. These people provided significant help and have been duly placed as co-authors in the first exploratory *Poecilimon* paper, chapter two. The placement of all further co-authors on chapter two are due to their respective contributions of species data. Murray Potter and Alastair Robertson have been included because of their significant support and direction during the thesis organisation, manuscript preparation, statistical and theoretical discussion.

Klaus-Gerhard further acted to obtain the initial funding for the research and set up the project in Germany. He also provided stimulating discussion throughout the organisation of the project and has contributed a significant amount of species data to chapter two, without which two of the subsequent papers would have suffered. Klaus-Gerhard has therefore been acknowledged as co-author on the majority of submitted papers.

I am the sole author of all chapters in this thesis, with the exception of the introduction and discussion of chapter six wherein Darryl Gwynne had the wisdom and knowledge to expand the hypotheses, initially proposed in my original manuscript, to include theories of resource advertising. Additionally, the introduction and discussion of the chapter on lifetime spermatophore investment for two *Poecilimon* species (chapter five) were co-authored by Arne and Gerlind Lehmann; a topic which they understand intimately.

Lastly, I conducted all analyses contained herein, other than that for chapter five. Analyses for chapter five were conducted by Arne Lehmann the Biological Statistician at the Humbolt University (Berlin).

The remaining section of this introduction is devoted to outlining each chapter, how each chapter relates to previous chapters and to the underlying concepts that have thus far been presented.

Chapters

Chapter Two

Understanding nuptial gift size in bush-crickets: an analysis of the genus *Poecilimon* (Tettigoniidae; Orthoptera). McCartney, J., K.-G. Heller, M. A. Potter, A. W. Robertson, K. Teltscher, G. Lehmann, A. Lehmann, D. v. Helversen, K. Reinhold, and R. Achmann. (2008). Journal of Orthoptera Research 17:231-242.

While a large amount of data had previously been collected on *Poecilimon*, much of this was unpublished. In order to better understand the variations in spermatophore, spermatophylax and ampulla size and sperm number across *Poecilimon*, I needed to bring this unpublished data together with that previously published. Previously, only a handful of published works compared differences across taxa, all of which either include observations from lab-reared individuals or observed species that vary in diet and ecology. To date no studies have attempted to understand spermatophore size variation across closely related field-observed bush-cricket taxa. Here I bring together data on male body mass and spermatophore components (spermatophylax-gift mass, ampulla mass and sperm number) from 62 *Poecilimon* populations (36 taxa). Primarily, this chapter is a comparative exploration of gift size variation and function among *Poecilimon* taxa with respect to gift function. Secondly, however, I compare male body mass and spermatophore component variations across the genus and relate my findings to other bush-cricket comparative studies in order to determine the reliability of using *Poecilimon* as a model taxon for understanding spermatophore size variation.

Keywords: mating effort, natural selection, paternal investment, *Poecilimon*, sexual selection, spermatophore function, spermatophore mass.

Chapter Three

A preliminary analysis of mate choice in a bush-cricket (*Poecilimon laevisissimus*: Tettigoniidae) suggests that virginity is more important than body size. McCartney, J. and K. -G. Heller. (2008). Journal of Orthoptera Research 17:227-230.

Mate choice is an important principle governing selection pressures in mating systems.

Two main factors: body size and mating status, are fundamental to understanding nuptial gift size variation. Female body size is important to males as it determines the level of reproductive fitness in females, especially in gift-giving insects where males may be donating more than just sperm. Similarly, male body size in gift-giving insects is important to females; it typically determines the quantity of investment a male may offer during copulation. Virginity, however, is also important as males that have just mated may transfer smaller gifts and mated females typically contain a full complement of sperm and are therefore, in terms of sperm competition, likely to be riskier to mate with. Here I compare virgin/body mate choice preferences in males and females of a small spermatophore-producing *Poecilimon* species, *P. laevisissimus*, with the aim of determining how mate choice (virgin/body size status) may affect spermatophylax-gift size variation.

Keywords: body mass, mate choice, *Poecilimon laevisissimus*, sperm competition, virgin, nuptial gift, spermatophore.

Chapter Four

Evidence of natural and sexual selection shaping the size of nuptial gifts among a bush-cricket genus (*Poecilimon*; Tettigoniidae, Orthoptera): an analysis of sperm transfer patterns. McCartney, J., M. A. Potter, A. W. Robertson, and K.-G. Heller. (In review.). Biological Journal of the Linnean Society.

The time it takes for female insects, including bush-crickets, to consume the nuptial gift is almost unanimously coupled with the time it takes for the majority of sperm to transfer into the female. There is perhaps only one well-documented example from all insect species, where this does not occur. This implies that paternal investment has little evolutionary influence on size variation in nuptial gifts. Moreover, nuptial gift size, ampulla size and sperm number were thought to be phylogenetically conserved and have a low potential for evolutionary change in closely related groups such as genera. Together these suggest that nuptial gifts are likely, in closely related taxa, to have evolved in a similar fashion with similar functions. In light of this, I compare the sperm transference pattern between five *Poecilimon* species (including two sub-species) which vary markedly in total spermatophylax-gift investment. I then relate this to the time it takes females to consume the nuptial gift. If there is a close match between the time that females take to consume the gift and the time taken for the majority of sperm to transfer into the female of large and small spermatophore-producing species, the ejaculate protection function is supported across *Poecilimon*. If, however, sperm transfer and gift-consumptions times are not coupled in some species (i.e., the majority of the sperm have transferred before the consumption of the gift), there is evidence to suggest that the gift functions additionally as paternal investment. This would show that spermatophore function is variable between species, evolutionary labile and

highly susceptible to selection pressures. Controlling for body mass and phylogeny, I also test the relationship between spermatophore size and the proportion of sperm that has transferred into the female by the average time the female of each species consumes the spermatophore. A greater proportion of sperm transferred in larger spermatophore producing species, would show that the males of these species, in addition to the paternal investment function, obtain greater paternal assurance.

Keywords: mating effort, paternal investment, reproductive fitness, reproductive investment, spermatophore size, sperm competition.

Chapter Five

Lifetime spermatophore investment in natural populations of two closely related bush-cricket species (Orthoptera: Tettigoniidae: *Poecilimon*). McCartney, J., A. W. Lehmann, and G. U. C. Lehmann. (2010). Behaviour 147:285-298.

While it is important to understand larger trends in spermatophore investment across *Poecilimon* taxa, it is equally important to critically evaluate this variation within a single species framework. To date, only a handful of studies have analysed the investment pattern in spermatophore components over repeated matings among bush-crickets in the field and none, to my knowledge, compare closely related taxa that show a large difference in total spermatophore investment. Here I examine, at the species level, how investments in spermatophyllax-gift and sperm number vary over the season in two *Poecilimon* species that differ markedly in spermatophore investment and size. I present observations on two closely related field-observed *Poecilimon* species: *P. thessalicus*, which produces a massive spermatophore of up to 40% of male

body mass, and *P. veluchianus minor*, with a modest spermatophore of around 20%. My aim is to understand, in detail, the variation in spermatophore component size across species and over the entire mating season. Large gifts are typically associated with the transfer of large quantities of sperm, both within and across species. Sperm depletion is therefore predicted by current models of sperm expenditure toward the end of the mating season in species that transfer larger spermatophylax-gifts. As a result, the reproductive success of species that produce gifts of different sizes may not only vary independently over the season but may be influenced to different degrees by paternal investment and ejaculate protection. Understanding temporal variation of gift size over the season between species that invest different amounts in gift production allow us to understand the temporal selective pressures that may drive evolutionary selection of spermatophore size across species.

Keywords: sexual conflict, spermatophore, spermatophylax, sperm competition, sperm number, trade-offs.

Chapter Six

Sex roles in mate attraction and searching: a comparative test using bush-crickets (*Poecilimon*: Tettigoniidae). Biological Journal of the Linnaean Society. McCartney, J., D. T. Gwynne, and K-G. Heller. (In review).

While the main factors typically used to understand spermatophore size variation are in the first four chapters i.e., paternal investment, ejaculate protection, mate choice and seasonal investment, a range of minor hypotheses have received less attention. In this chapter I explore three additional hypotheses involving alternative reproductive efforts in mate attraction and gift size. Large spermatophores are expensive to produce; male

Orthoptera therefore may have evolved the ability to advertise these large resources through calling. Similarly, alternative efforts spent in mate attraction, such as calling, are also expensive and trade-offs are therefore expected between mate attraction and spermatophore investment. Alternatively, because males are typically sexually selected to perform the riskier behaviour in pair formation, spermatophore size may be coupled with a higher 'risky' calling. This 'risky' behaviour may therefore be selected by females. Here I assess much of the life history data on spermatophore component variation presented in Chapter Two and compare it, across 32 *Poecilimon* taxa, to the two pair formation protocols that occur in *Poecilimon*: the orthopteran-typical female search-for-calling male, and the *Poecilimon*-derived male search-for-calling female (in response to the initial male call). Calling is energetically expensive and mate-searching is risky. If larger gifts are associated to either calling and moving, or calling alone in the males, I will either have support for the hypothesis that males advertise larger resources, trade-off spermatophore resources for mate attraction, or alternatively produce greater spermatophore resources due to sexual selection on males by the females for greater nutritional investment. Variations in nuptial gift size and pair forming behaviour within *Poecilimon* allow, for the first time, a critical quantitative examination of resource advertising, risk-shift and trade-off hypotheses in Orthoptera.

Keywords: mate choice, mate searching, nuptial gift, pair formation, resource advertising, risk shift, trade-off.

Chapter Seven

Is there evidence of a macro-evolutionary trade-off between reproductive investments in mate attraction and nuptial gift size in bush-cricket? McCartney, J., and K.-G. Heller. (In review). *Evolution*.

Larger nuptial gifts may be advantageous to both the males that produce them and to females that receive them. Paradoxically, however, many bush-cricket species produce small nuptial gifts. It is likely that such a contradiction is in part due to trade-offs that males must make between nuptial gift investment and alternative reproductive efforts such as mate attraction. In this chapter I control for male body mass and relatedness, and compare investments made between spermatophore component investments (nuptial gift, ampulla and sperm) to three components of mate-attraction (syllable and impact number and peak carrier frequency). Previously, only single species data were available for these comparisons and few conclusions could be drawn. Here, I make these comparisons across 36 *Poecilimon* species. Because larger gifts are expensive to produce, trade-offs are expected to occur between alternative reproductive efforts. This is likely to result in a reduction of potential paternal investment and ejaculate protection afforded by larger spermatophores.

Keywords: ejaculate protection, energetic partitioning, mate attraction, trade-off.

Chapter Eight

Larger nuptial gifts increase male per-mating fitness across a bush-cricket genus (*Poecilimon*), but do they “manipulate” females? McCartney, J. (Submitted manuscript). Proceedings of the Royal Society of London B: Biological Sciences.

The ultimate evolutionary measure of nuptial gift variation, indeed of any reproductive investment, ultimately lies in reproductive output or offspring fitness.

This chapter builds on the findings of all previous chapters. Published data on spermatophore size, together with novel data presented here on field mating frequency, egg laying frequency, egg weight, and hatching success, is used to calculate the reproductive fitness of five field-observed *Poecilimon* species with varying spermatophore sizes. I relate potential reproductive output to the individual investments made by males and females. Males especially stand to gain substantial reproductive benefits through the transference of larger gifts; the assumption therefore is that species that produce larger spermatophylax-gifts have greater reproductive fitness. Males with larger spermatophores, I hypothesise, should either 1) father a greater number of surviving offspring, 2) father better quality offspring, or 3) have greater assurance in paternity than those of species with smaller spermatophores. The females receiving larger gift spermatophylaxes should produce either a fitter or a greater number of offspring. Offspring fitness is measured by egg weight and hatching success; two measures known to correspond to fitness. Offspring number is measured by the number of eggs laid. Because the sperm precedence pattern of two *Poecilimon* species is known to be largely last male, and larger transferred sperm volumes are

typically associated to greater paternity; paternity is calculated as the number of offspring a male is likely to sire per mating.

Keywords: fecundity, fitness, offspring provisioning, paternal investment, reproductive success, sexual conflict.

Chapter Nine

Discussion

In each chapter, the contribution of that chapter to understanding mating systems in spermatophore bearing Poeciliimon is discussed. In this final chapter, I bring these contributions together, unifying the fundamental tenets that have shaped my research; namely, operational sex ratios, reproductive effort, mating effort, parental investment, ejaculate protection, sperm competition, mate choice, sexual conflict and reproductive fitness. Here, I connect the main outcomes of each chapter, showing how each new hypothesis builds on the outcomes of those previously presented.

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Chapter 2

Understanding nuptial gift size in bush-crickets: an analysis of the genus *Poecilimon* (Tettigoniidae: Orthoptera)

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Poecilimon laevis with pronotal mites.

We dedicate this paper to Dagmar von Helversen (1944-2003), who contributed data to this study and devoted many years of her academic career to understanding the nature of *Poecilimon*.
Anonymous (2004) Bibliographie der wissenschaftlichen Publikationen von Dr.
Dr. h.c. Dagmar von Helversen (1944-2003). – Articulata 19: 124-126.

Abstract

During mating, male insects of certain species transfer a costly nuptial gift, a large spermatophore, which is eaten by the female as sperm transfer into her. The spermatophore components (the sperm-free spermatophylax and the sperm-ampulla) vary greatly in size between species, and have a direct influence on male fitness. Studies of the relationship between spermatophore size variation and male fitness have concentrated on associations between evolutionary changes in spermatophylax size and either ampulla size or sperm number. Two main hypotheses have been put forward to explain the function of the spermatophylax: the ejaculate-protection hypothesis and the paternal investment hypothesis. A strong correlation between the spermatophylax and ampulla or sperm number suggests an ejaculate-protection function because it protects the ampulla from being removed prematurely. However, comparative support comes mainly from disparate bush-cricket species (Tettigoniidae) that vary greatly in relatedness and diet. Furthermore, data are often from animals reared under laboratory conditions. Our study describes the significance of size-variation in bush-cricket nuptial gifts, with an analysis from field populations of 33 species within the genus *Poecilimon*. *Poecilimon* share similar diets and the variation in spermatophore size within the genus approximates family-wide variation, so confounding influences from diet and relatedness are, to a certain extent, controlled. Previous support for the ejaculate-protection hypothesis is almost universal, so we expected to find similar results. However, unlike previous studies, there was no relationship between body mass and each of the three spermatophore components

when body mass was accounted for, or between spermatophylax mass and sperm number. We also found only a weak relationship between ampulla mass and sperm number, suggesting that caution is needed when using ampulla size to predict sperm number or sperm number to predict ejaculate size. In support of the ejaculate-protection hypothesis we found a positive relationship between spermatophylax size and ampulla mass. While our results support the ejaculate-protection hypothesis, they are not inconsistent with the paternal investment hypothesis.

Keywords: mating effort, natural selection, paternal investment, *Poecilimon*, sexual selection, spermatophore function, spermatophore mass

Introduction

The degree to which natural and sexual selection respectively affect mating behavior is largely unknown in evolutionary biology, and few examples delineate the problem more clearly than the maintenance of nuptial gift size in Orthoptera. During mating, male bush-crickets (Tettigoniidae) transfer a variable (in size), yet often substantial, spermatophore to the female (for reviews see Gwynne 1990, 2001; Vahed 1998). When transfer is complete the pair uncouple and the female reaches under her abdomen and starts to consume the spermatophore (Boldyrev 1915). As the ejaculate (sperm and seminal fluid) discharges from the ampulla into the female, she consumes the spermatophylax, a large, sperm-free, gelatinous mass. After that, she consumes the

ampulla and remaining ejaculate (Boldyrev 1915, Bowen *et al.* 1984).

Although the function of the ampulla to house the ejaculate is relatively clear, the role the spermatophylax plays in mating is more complicated. Two non mutually exclusive hypotheses have been suggested for spermatophylax size (for reviews see Vahed 1998, Gwynne 2001). First, the ejaculate-protection hypothesis states that the spermatophylax is sexually selected by preventing the female from removing the ampulla prematurely (Gerhardt 1913, 1914; Boldyrev 1915) and therefore directly increasing a male's assurance in sperm competition in a dose-dependent manner (for reviews see Eberhard 1996, Vahed 1998, Gwynne 2001, Simmons 2001, Arnqvist & Rowe 2005). There may be additional benefits under this hypothesis – consumption of a large spermatophylax may reduce the speed at which a female will remate, thereby indirectly increasing the number of offspring and the number of ova that may be fertilised by the male (Gwynne 1986, Wedell & Arak 1989, Simmons & Gwynne 1991, Wedell 1993a, b, Vahed 2007), or may increase the chance of female survival until oviposition (*e.g.*, Voigt *et al.* 2005, 2006). Males that produce relatively large spermatophores are also more likely to transfer more ejaculate and therefore succeed in sperm competition (for a review see Simmons 2001). A large ejaculate may also induce longer intermating refractory periods in females (Heller & Helversen 1991, Heller & Reinhold 1994, Lehmann & Lehmann 2000a, Vahed 2007), allowing males to father a greater share of eggs laid in the next oviposition (Gwynne 1986, Wedell & Arak 1989, Simmons & Gwynne 1991, Wedell 1993a, b). Under this hypothesis, spermatophylax size should covary with the size of the ampulla (Reinhold & Heller 1993, Wedell 1993a, Heller & Reinhold 1994) or the number of sperm.

Alternatively, the paternal investment hypothesis suggests that the spermatophylax is under natural selection to provide a positive nutritional effect on the donating male's progeny (Trivers 1972, Gwynne *et al.* 1984). In this case, spermatophylax size should correspond to a relative increase in fitness and/or quantity of offspring (Trivers 1972; Thornhill 1976; Simmons & Parker 1989; Gwynne 1986, 1988, 1990; Wedell 1991; Reinhold 1999) but is not expected to covary with ampulla size or sperm number (for reviews see Vahed 1998, Gwynne 2001).

Both natural and sexual selection functions of the spermatophore have been observed in tettigoniids, and are reflected in considerable interspecific variation in spermatophore size (Gwynne 1983, Wedell 1993a, Vahed & Gilbert 1996). Spermatophore mass ranges from about 2% of total male body mass (relative mass) (*Acripeza reticulata*, Wedell 1993a; *Anonconotus alpinus*, Vahed 2002) to about 40% (*Ephippiger ephippiger*, Busnel & Dumortier 1955), and sperm numbers range between 38,000 (*Phaneroptera nana*, Vahed & Gilbert 1996) and 37.3 million sperm (*P. thessalicus*, McCartney & Heller this issue, p. 227). With respect to spermatophore function it is clear that size variation has significant fitness implications for each sex and species.

Despite the likely benefits to males, producing large spermatophores is expensive, as they represent a loss in future reproductive potential (Simmons 1988a, 1990, 1995a; Heller & von Helversen 1991; Vahed 2007), the costs of which will vary with factors such as local growing conditions and diet (Halliday 1987, Simmons 1988a, Simmons *et al.* 1993).

The variation found in spermatophore size among species may be, at least partly, a consequence of phylogenetic relatedness (Gwynne 1995, Vahed & Gilbert

1996). Nevertheless, in an analysis of 19 bush-cricket genera, Wedell (1993a, 1994a) showed that interspecific differences in spermatophore size, spermatophylax mass and ampulla mass are largely influenced by diet. Controlling for phylogeny in 43 tettigoniid species, Vahed & Gilbert (1996) found that there was also a large residual variation in sperm number and spermatophore size. Vahed & Gilbert (1996) however, did not control for diet, and used laboratory-reared bush-crickets (Vahed 1994) — a condition that may affect sperm number (*e.g.*, Reinhold 1994) and spermatophylax size (*e.g.*, Heller & von Helversen 1991).

Comparisons among species within a genus can be particularly informative because many variables that are shared by congeners are held constant (Ridley 1983, Felsenstein 1985, Harvey 1991, Harvey & Pagel 1991). The aim of this study was to compare spermatophore and body-mass data from field observations within the diverse bush-cricket genus *Poecilimon*. *Poecilimon* species share a similar diet and morphology, and while we recognise that this genus does not represent the full diversity found in bush-crickets, we show here that variation in spermatophore size approximates family-wide variation, so variations in diet and relatedness are, to a certain extent, controlled for. In this paper, we test the ejaculate-protection and paternal-investment hypotheses in *Poecilimon* by examining the correlations between the spermatophore components: spermatophylax mass, ampulla mass and sperm number.

Methods

Poecilimon Fischer, 1853, (Fig. 8) is a genus of barbistine bush-crickets (Phaneropterinae, Tribe Barbistini) (Orthoptera: Ensifera: Tettigoniidae). There are 128 currently recognized species and subspecies (Otte *et al.* 2005), with about 65 European species, mostly situated in the east Mediterranean (Heller 2004). While the current position of species within the *Poecilimon* clade is under constant review (*e.g.*, Heller 2004, Heller & Lehmann 2004, Heller *et al.* 2004, Heller 2006), the status of *Poecilimon* at the genus level is well supported (Ramme 1933, Bey-Bienko 1954, Heller 1984). Since the description of the genus in 1853 there has been no dispute about the homogeneity of this group (see references in Otte 1997). The nomenclature used here follows that of Otte *et al.* (2005), with additional species *P. gerlindae* (Lehmann *et al.* 2006), *P. ege* (Ünal 2005), and *P. ukrainicus* (Bey-Bienko 1951).

The genus *Poecilimon* is quite uniform in terms of behavior and life-history patterns. Notable exceptions include differences in how females consume the spermatophore, and timing of the active mating phase. Most *Poecilimon* species consume the spermatophylax directly from underneath the abdomen, where it remains attached to the ampulla. However, at least one species, *P. erimanthos*, detaches the spermatophylax from the ampulla before consumption. Most species used are nocturnal. Notable exceptions are *P. erimanthos*, *P. mytilenensis*, and *P. werneri*, which are predominantly active during the day. *P. nobilis*, *P. affinis*, and *P. gracilis* seem to be active both night and day (Heller & von Helversen 1993). All species are semelparous, have obligate diapause and most have a univoltine lifecycle. All the *Poecilimon* species

employed eat flowers and leaves, so are foliovores when ordered into gross feeding categories, such as those given by Wedell (1994a): 1) omnivorous-predaceous, 2) seed eaters, and 3) foliovores.

Collection.—Previously published and unpublished data were compiled from a range of sources for 33 species (36 taxa, 62 independent observations) of *Poecilimon* to supplement the data we collected ourselves. All were found in Greece, Turkey, Italy, Slovenia or the Ukraine (see Appendix 1 for the location of each population). The data for several species were obtained from the paper by Vahed & Gilbert (1996). Although these authors did not present relative spermatophore, spermatophylax and ampulla mass, we calculated these percentages directly from the table in their paper (see below for calculations of relative mass). The sources for all novel data included here are appended to Table 1; the locations where they were observed are listed in Appendix 1. For 11 species, two (or more) independent measurements from different populations or different years were included (designated by Roman numerals), and two species were sampled at the subspecific level: *P. veluchianus veluchianus*, *P. veluchianus minor*, and *P. jonicus jonicus*, *P. jonicus superbus*, *P. jonicus tessellatus*. In all, 62 taxa-site-year combinations were collated from 36 taxa (Table 1, Appendix 1).

Determination of male body mass, spermatophore size, and sperm number.—We separated field-caught juveniles (ex-field larvae) and field-caught adults (EL and F respectively, Table 1) into cages of each sex. Field-caught juveniles were separated until at least seven days after their imaginal moult, in order to ensure sexual maturity (Heller & Reinhold 1994). Field-caught adults were separated for at least three days prior to pairing, in order to ensure full receptivity (Heller & von Helversen 1991,

Lehmann & Lehmann 2000b). Two exceptions to this were *P. thessalicus* I and *P. v. minor* III (taken from independent mating experiments) where individuals were paired immediately after they were collected. Some data were used from individuals that were reared in the laboratory (for example, *P. elegans*, *P. gracilis*, Table 1). While their treatment and the experimental procedures were otherwise the same as those in the field, they are not included in final interspecific analyses.

For mating, pairs were typically placed in 500-ml containers and observed every 15 min or less until the female bore a spermatophore, which we then carefully removed with forceps for weighing. All weights were measured to the nearest 1 mg. In some cases, the measurements were made in the field from wild matings. Where possible, the spermatophore, spermatophylax and ampulla masses were measured immediately after mating. When this was not possible (for example, *P. laevissimus* IV), male weight loss and female weight gain (with the spermatophore attached) before and after mating were compared (Reinhold & Helversen 1997). If the difference between the male weight loss and female weight gain was larger than 20%, that datum was excluded (following the procedure of Heller & Reinhold 1994). On occasion, either the spermatophylax or the ampulla mass was not measured; in these cases the missing component was calculated as the difference between the full spermatophore mass and the mass from the known component.

Relative spermatophore mass was calculated as the percentage of male body mass for each individual, and then the mean for all individuals taken to calculate a species average. On occasion, the spermatophore mass and male body mass were taken from different males, so the average spermatophore mass was divided by the average

male mass to give relative spermatophore mass.

After weighing, the ampulla was cut from the spermatophylax, added to a known quantity of water (between 1 and 5 ml depending on the organ size), and sliced with a scalpel. We further mixed the solution by passing it repeatedly through a syringe until the sperm had been suspended in the water and fully homogenised. A subsample was taken and the sperm counted on a field haemocytometer (Swift: Neubauer improved). Normally three subsamples were taken and the solution remixed before taking each new subsample. If there was a large variation between subsamples or the sperm was not evenly distributed over the slide, the solution was remixed and further subsamples taken. Sperm from a known volume (50 μ l - 200 μ l) were counted and multiplied by the appropriate dilution factor to give the total number of sperm for the entire ampulla. For *P. mariannae* a Coulter counter was used (for details of the method see Lehmann & Festing 1998). Relative sperm number was calculated as the number of sperm per mg of mean male body mass and expressed as sperm number $\times 10^3$.

Analysis.—Using data from multiple populations or seasons means that some species are over-represented and may inflate the contribution of those taxa in the analyses. However, full data sets with multiple species may give a better understanding of how the environment affects spermatophore size. Therefore, we restricted our use of the full data set to descriptive comparisons, and only performed analyses on reduced data sets that included only one of each taxa. Priority for removal was first given to observation location (*i.e.*, field observations were preferred over lab observations) and then to sample size (Table 1). Unless otherwise stated, statistics with

multiple observations removed are presented in text and figures.

P. mytilenensis is unusual as it has a greatly enlarged ampulla and a large variation in sperm number (between 6.3 and 15.8 million sperm, Heller *et al.* 2004). Data for the current paper were from laboratory-reared individuals for this species, although observations from the field show that this variation in size approximates that found in its natural environment. Our intention in this paper was to compare among field-observed animals, avoiding any confounds imposed by lab-reared species. However, in terms of taxonomy, *P. mytilenensis* is quite typical for *Poecilimon* and large variations in spermatophore components are likely to represent realistic variations within the genus. Preliminary analysis that included data from *P. mytilenensis* also indicated that its impact on our understanding of mating systems within *Poecilimon* required further exploration. We therefore duplicated all analyses a second time, with the inclusion of *P. mytilenensis*, in order to directly compare this with variations found in the rest of the genus.

To normalize the data, all variables were \log_{10} transformed prior to analysis unless otherwise stated. Two types of analysis were performed. First, the correlation coefficients between male body mass and each of spermatophore mass, spermatophylax mass, ampulla mass, and sperm number were calculated. Second, the overall effect of male body mass (MBM) was estimated for each parameter using least-squares regressions and the residuals for each population examined, to reveal cases where male investments were over or under expectation based on the overall allometric relationships. All data were analysed using SAS 9.1.3.

Results

Comparisons between Poecilimon and other Tettigoniidae.—The wide range in each spermatophore component within the genus *Poecilimon* approximates that occurring among the Tettigoniidae as a whole (Fig. 1. *Poecilimon* dataset not reduced). However, the smallest relative spermatophore size in *Poecilimon* is around 6.1% (*P. laevissimus* IV, Table 1), while some other tettigoniids have spermatophores that are even smaller than this: *Mecopoda elongata* and *Meconema thalassinum*, for example, have spermatophores that are barely 1% of male body mass, with little or no spermatophylax. *Poecilimon* have relatively large spermatophores (always >5% relative mass) and nearly always have a larger spermatophylax than an ampulla. *Poecilimon mytilenensis* (Fig. 1), however, is an exception with an unusually large ampulla (14.7 % relative mass) and a relatively small spermatophylax (8.2 % relative mass; see Heller *et al.* 2004 for details). The upper limits of spermatophylax size are similar between *Poecilimon* and tettigoniids in general, with *P. thessalicus*, *P. ornatus* and *P. pergamicus*, for example, and *Steropleurus stali*, producing spermatophylaces that represent between 25% to 28% of male body mass (Fig. 1).

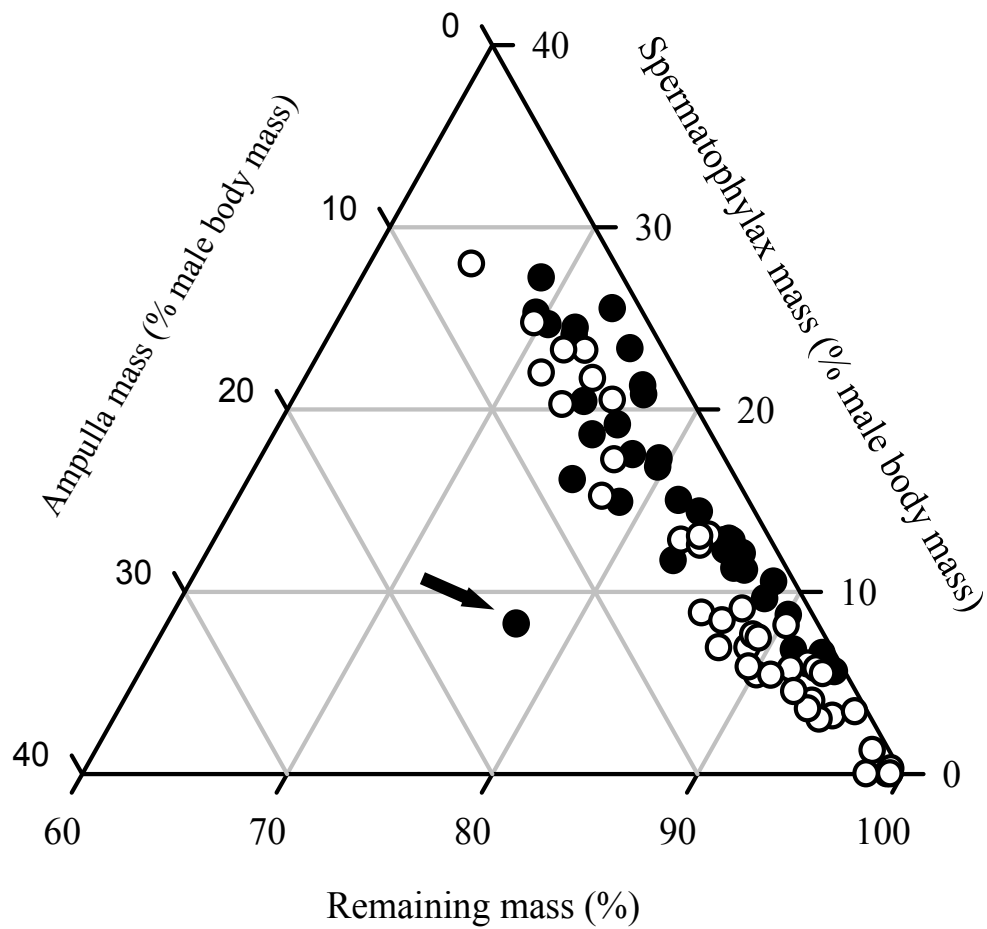


Fig. 1 Male body, spermatophylax and ampulla mass as proportions of combined mass in 29 *Poecilimon* species (closed circles, 31 taxa; n=37) and 40 other Tettigoniid species (open circles, see Vahed & Gilbert 1996 for details), showing that variation in *Poecilimon* approximates family wide variation. The solid arrow points to *P. mytilenensis*, a species that has a remarkably large ampulla (Heller et al. 2004).

Table 1. Mean male body mass and sperm number with relative and actual mean spermatophore, spermatophylax, ampulla masses and sperm number of 33 *Poecilimon* sp. (36 taxa, 62 independent observations), (n= number of individuals). Each species is listed with the describer and with reference to the collectors or source of publication (see key for reference). Some species have more than one independent observation and are distinguished by roman numerals. Status of observations, i.e. field observations (F), ex- larvae specimens (EL) that were field obtained but allowed to mature in large cages in the location of the natural population and purely lab-reared (L) individuals are listed. Relative sperm number (rel#) = $\times 10^3$ sperm/mg⁻¹ of male body mass. Dashes (-) indicate a lack of gathered information and, on occasion, data has been published more than once so we refer to original publications.

Species/source/collector	male body mass			spermatophore mass (mg) relative spermatophore mass (%)				spermatophylax mass				ampulla mass (mg) relative ampulla mass (%)				sperm number			
	Mg	loc	n	Mg	rel %	Loc	n	mg	rel %	loc	n	Mg	rel %	loc	n	$\times 10^6$	rel #	loc	n
<i>P. aegaeus</i> Werner, 1932 ^a	849	EL	10	272	31.4	EL	7	236	27.2	EL	7	34	4.0	EL	7	-	-	-	-
<i>P. affinis</i> I (Frivaldsky, 1867) ^b	1440	F	168	209	15	F	15	-	-	-	-	-	-	-	-	-	-	-	-
<i>P. affinis</i> II (Frivaldsky, 1867) ^c	1572	F	5	230	14.6	F	5	-	-	-	-	-	-	-	-	-	-	-	-
<i>P. affinis</i> III (Frivaldsky, 1867) ^d	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	21.6	-	L	3
<i>P. affinis</i> IV (Frivaldsky, 1867) ^e	1328	F	4	201	15.1	F	4	170	12.8	EL	4	31	2.3	F	3	4.4	3.3	F	3
<i>P. amissus</i> Brunner von Wattenwyl, 1878 ^f	410	EL	8	68	20.5	EL	1	48	11.7	EL	1	20	5.3	EL	1	-	-	-	-
<i>P. anatolicus</i> Ramme, 1933 ^g	694	EL	2	149	22.4	EL	2	-	-	-	-	-	-	-	-	-	-	-	-
<i>P. brunneri</i> (Frivaldsky, 1867) ^h	320	F	9	62	20.7	F	1	48	15.0	F	1	14	3.4	F	1	-	-	-	-
<i>P. deplanatus</i> Brunner von Wattenwyl, 1891 ⁱ	449	F	15	41	9.2	F	7	55	12.3	F	2	9	2.0	F	4	-	-	-	-
<i>P. ege</i> Ünal, 2005 ^f	568	F	4	168	28.7	F	3	140	24.7	F	3	28	4.9	F	3	11.1	19.5	F	3
<i>P. elegans</i> (Brunner von Wattenwyl, 1878) ^j	272	L	3	56	20.4	L	3	47	17.3	L	3	9	3.2	L	3	1.6	5.9	L	3
<i>P. erimanthos</i> I Willemse & Heller, 1992 ^k	650	F	25	47	7.2	F	11	43	6.6	F	13	4	0.6	F	11	0.9	1.4	F	19
<i>P. erimanthos</i> II Willemse & Heller, 1992 ^l	583	F	5	80	13.8	EL	8	-	-	-	-	-	-	-	-	1.2	2.1	F	4
<i>P. gerlindae</i> Lehmann Willemse & Heller, 2006 ^f	552	F	9	154	29.7	F	9	135	24.5	F	9	19	3.7	F	9	2.4	4.3	F	9
<i>P. gracilis</i> (Fieber, 1853) ^d	530	F	6	102	16.7	EL	6	-	-	-	-	-	-	-	-	3.1	5.8	L	3
<i>P. hamatus</i> I Brunner von Wattenwyl, 1878 ^f	517	F	5	121	22.3	F	4	110	21.3	F	4	11	2.1	F	4	0.2	0.4	F	4
<i>P. hamatus</i> II Brunner von Wattenwyl, 1878 ^f	466	F	12	67	14.5	F	5	58	12.4	F	3	9	2.0	F	3	-	-	-	-
<i>P. hoelzeli</i> I Harz, 1966 ^f	2960	F	3	442	14.6	F	1	381	12.9	F	1	61	2.0	F	1	-	-	-	-
<i>P. hoelzeli</i> II Harz, 1966 ^d	2250	F	>10	387	17.2	F	8	-	-	-	-	-	-	-	-	13.4	6.0	F	3
<i>P. ikariensis</i> Willemse, 1982 ^m	473	F	5	71	14.5	F	4	56	11.8	F	4	15	3.2	F	4	0.2	0.4	F	4
<i>P. jonicus jonicus</i> I (Kollar, 1853 in Fieber) ^f	352	F	6	52	14.9	F	6	45	12.8	F	5	7	1.9	F	5	0.4	1.1	F	6

Species/source/collector	male body mass			spermatophore mass (mg) relative spermatophore mass (%)				spermatophylax mass				ampulla mass (mg) relative ampulla mass (%)				sperm number			
	Mg	loc	n	Mg	rel %	Loc	n	mg	rel %	loc	n	Mg	rel %	loc	n	x 10 ⁶	rel #	loc	n
<i>P. jonicus jonicus</i> II (Kollar, 1853 in Fieber) ^e	324	F	4	28	8.6	F	4	22	6.8	F	4	6	1.9	F	3	0.2	0.6	F	3
<i>P. jonicus superbus</i> (Fischer, 1853) ^f	306	F	2	57	18.6	F	2	-	-	-	-	-	-	-	-	0.2	0.7	F	1
<i>P. jonicus tessellatus</i> (Fischer, 1853) ⁿ	721	EL	3	83	11.6	EL	3	69	9.6	EL	3	13	1.9	EL	3	-	-	-	-
<i>P. laevissimus</i> I (Fischer, 1853) ^f	759	EL	1	66	8.7	EL	1	-	-	-	-	-	-	-	-	-	-	-	-
<i>P. laevissimus</i> II (Fischer, 1853) ^f	731	EL	5	85	10.8	EL	3	77	10.5	EL	3	8	1.0	EL	3	1.0	1.14	EL	3
<i>P. laevissimus</i> III (Fischer, 1853) ⁿ	744	EL	4	73	9.9	EL	4	65	8.7	EL	4	9	1.2	EL	4	-	-	-	-
<i>P. laevissimus</i> IV (Fischer, 1853) ^o	781	F	50	48	6.1	F	9	44	5.6	F	7	4	0.5	F	7	0.7	0.9	F	7
<i>P. macedonicus</i> Ramme, 1926 ^d	302	F	12	65	21.8	F	5	-	-	-	-	-	-	-	-	2.0	6.6	F	4
<i>P. mariannae</i> Heller, 1988 ^p	583	EL	21	133	22.8	EL	21	109	18.6	F	21	34	5.8	EL	21	2.4	4.1	EL	21
<i>P. marmaraensis</i> Naskrecki, 1991 ^h	490	EL	8	104	21.2	EL	7	73	14.9	EL	7	31	6.3	EL	7	-	-	-	-
<i>P. mytilenensis</i> Werner, 1932 ^{q, f}	822	F	4	227	29.3	F	6	114	8.2	F	4	113	14.7	F	5	10.4	12.7	L	3
<i>P. nobilis</i> (Brunner von Wattenwyl, 1878) ^f	1405	F	6	194	13.9	F	6	158	11.3	F	6	36	2.6	F	9	6.6	4.7	F	13
<i>P. obesus</i> (Brunner von Wattenwyl, 1878) ^f	1869	F	5	247	13.4	F	5	209	11.2	F	4	38	2.1	F	4	4.0	2.1	F	10
<i>P. ornatus</i> I (Schmidt, 1849) ^r	2552	F	9	310	11.8	F	7	275	25.5	F	7	35	1.4	F	7	-	-	-	-
<i>P. ornatus</i> II (Schmidt, 1849) ^f	2957	EL	8	268	9.2	EL	14	-	-	-	-	-	-	-	-	-	-	-	-
<i>P. pergamicus</i> Brunner von Wattenwyl, 1891 ^f	174	F	5	53	30.4	F	1	44	25.3	F	1	9	5.2	F	1	2.8	16.1	F	1
<i>P. sanctipauli</i> I Brunner von Wattenwyl, 1878 ^f	1234	EL	4	308	25	EL	1	-	-	-	-	-	-	-	-	-	-	-	-
<i>P. sanctipauli</i> II Brunner von Wattenwyl, 1878 ^f	1355	F	1	337	24.9	F	1	316	23.3	EL	2	21	1.6	F	1	2.6	1.9	F	1
<i>P. schmidtii</i> (Fieber, 1853) ^e	525	F	8	73	13.9	F	6	63	12.1	F	6	9	1.7	F	6	0.9	1.7	F	2
<i>P. thessalicus</i> I Brunner von Wattenwyl, 1891 ^s	442	F	48	102	23	F	8	92	20.8	F	8	10	2.2	F	8	3.9	8.8	F	4
<i>P. thessalicus</i> II Brunner von Wattenwyl, 1891 ^s	507	F	5	146	29	F	5	122	24.1	F	5	20	3.9	F	5	-	-	-	-
<i>P. thessalicus</i> III Brunner von Wattenwyl, 1891 ^t	464	F	20	112	24	F	20	89	19.2	F	20	30	4.3	F	20	14.0	30.2	F	20
<i>P. thessalicus</i> IV Brunner von Wattenwyl, 1891 ^d	610	F	3	224	36.7	F	2	-	-	-	-	-	-	-	-	16.5	27.0	F	2
<i>P. turcicus</i> Karabag, 1950 ^f	632	EL	3	152	24.1	EL	2	102	16.1	EL	2	50	8.0	EL	2	6.4	10.1	EL	2
<i>P. ukrainicus</i> Bev-Bienko, 1951 ^f	274	EL	12	60	21.9	F	7	48	17.5	F	7	12	4.4	F	7	0.4	1.5	F	4
<i>P. unispinosus</i> Brunner von Wattenwyl, 1878 ^f	404	F	2	82	20.3	F	2	68	16.8	F	2	14	3.5	F	2	0.9	2.2	F	2
<i>P. v. minor</i> I Heller & Reinhold, 1993 ^f	439	F	19	87	20	F	19	-	-	-	-	-	-	-	-	-	-	-	-

Species/source/collector	male body mass			spermatophore mass (mg) relative spermatophore mass (%)				spermatophylax mass				ampulla mass (mg) relative ampulla mass (%)				sperm number			
	Mg	loc	n	Mg	rel %	Loc	n	mg	rel %	loc	n	Mg	rel %	loc	n	x 10 ⁶	rel #	loc	n
<i>P. v. minor</i> II Heller & Reinhold, 1993 ^a	400	F	83	74	19.1	F	271	-	-	-	-	-	-	-	-	-	-	-	-
<i>P. v. minor</i> III Heller & Reinhold, 1993 ^t	327	F	70	56	17.1	F	19	47	14.4	F	19	9	2.7	F	19	3.4	10.4	F	19
<i>P. v. minor</i> IV Heller & Reinhold, 1993 ^v	367	L	15	-	-	-	-	-	-	-	-	-	-	-	-	7.6	20.7	L	18
<i>P. v. minor</i> V Heller & Reinhold, 1994 ^v	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	7.5	-	F	43
<i>P. v. veluchianus</i> I Ramme, 1933 ^f	821	F	10	212	26.1	F	10	-	-	-	-	-	-	-	-	-	-	-	-
<i>P. v. veluchianus</i> II Ramme, 1933 ^c	661	F	13	150	22.7	F	13	-	-	-	-	-	-	-	-	-	-	-	-
<i>P. v. veluchianus</i> III Ramme, 1933 ^b	660	F	107	162	26.4	F	10	-	-	-	-	-	-	-	-	-	-	-	-
<i>P. v. veluchianus</i> IV Ramme, 1933 ^v	625	L	29	-	-	-	-	-	-	-	-	-	-	-	-	6.8	10.9	L	36
<i>P. v. veluchianus</i> V Ramme, 1934 ^v	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	10.5	-	F	-
<i>P. v. veluchianus</i> VI Ramme, 1933 ^w	-	-	-	-	25.4	L	64	-	-	-	-	-	25.4	L	-	6.3	-	L	34
<i>P. v. veluchianus</i> VII Ramme, 1933 ^e	710	F	1	182	25.6	F	1	145	20.4	F	1	37	5.3	F	1	10.4	14.6	F	50
<i>P. werneri</i> Ramme, 1933 ^f	318	EL	5	47	14.6	EL	5	39	12.3	EL	3	8	2.5	EL	3	0.2	0.6	EL	2
<i>P. zimneri</i> I Ramme, 1933 ^l	711	F	7	150	21.1	F	7	-	-	-	-	-	-	-	-	28.4	39.9	F	5
<i>P. zimneri</i> II Ramme, 1933 ^x	818	EL	91	146	17.8	EL	91	-	-	-	-	-	-	-	-	-	-	-	-

Key:

^a Lehmann, A. & Lehmann, G. (unpub.)

^b Heller & von Helversen (1991)

^c Heller et al. (1998)

^d Reinhold, K. (unpub.)

^e Vahed & Gilbert (1996)

^f Heller, K.-G. (unpub.)

^g von Helversen, D. & Heller, K.-G. (unpub.)

^h Braun, H. (unpub.)

ⁱ Heller, K.-G., Heller, M. & Volleth, M. (unpub.)

^j Ingrisch, S. (unpub.)

^k McCartney, J. & Heller, K.-G. (unpub.)

^l Reinhold, K. & Heller, K.-G. (unpub.)

^m Heller, K.-G. & Volleth, M. (unpub.)

ⁿ Lehmann, G. & Lehmann, A. (unpub.)

^o McCartney, J. Telscher, K.L. & Heller, K.-G. (unpub.)

^p Lehmann & Lehmann (2000a)

^q Heller et al. (2004)

^r Achmann, R. (unpub.)

^s McCartney, J., & Telscher, K.L. (unpub.)

^t McCartney, J., Telscher, K.L., Penny, L. (unpub.)

^u Heller & Reinhold (1994)

^v Reinhold (1994)

^w Reinhold & von Helversen (1997)

^x Lehmann & Lehmann (2007 and unpublished manuscript)

There is also a very large range in sperm number within *Poecilimon*, which could not be accounted for simply by body size ($y = 1.11x - 2.73$, $F_{1,26} = 7.706$, $p = 0.011$, $r^2 = 0.22$; Fig. 2). In most tettigoniids sperm number follows body size quite closely ($y = 1.12x - 3.11$, $F_{1,29} = 60.45$, $p < 0.001$, $r^2 = 0.68$), but in *Poecilimon*, sperm number ranged between about 200,000 sperm per spermatophore (*P. hamatus*, *P. ikariensis*, *P. jonicus* and *P. wernerii*) to about 28 million (*P. zimмери*), although *P. thessalicus* can reach 37.3 million sperm (McCartney & Heller unpub. data). Within other tettigoniids, sperm number ranges between 38,000 for *Phaneroptera nana*, to about 10 million for *Pycnogaster inermis*. Many species of *Poecilimon* had far more sperm than would be expected for their body size, based on the overall pattern within the tettigoniids (e.g., *P. thessalicus*, *P. zimмери* and *P. ege*, Table 1), though there are also a few species with unusually low sperm counts for their size (e.g., *P. jonicus* and *P. wernerii*).

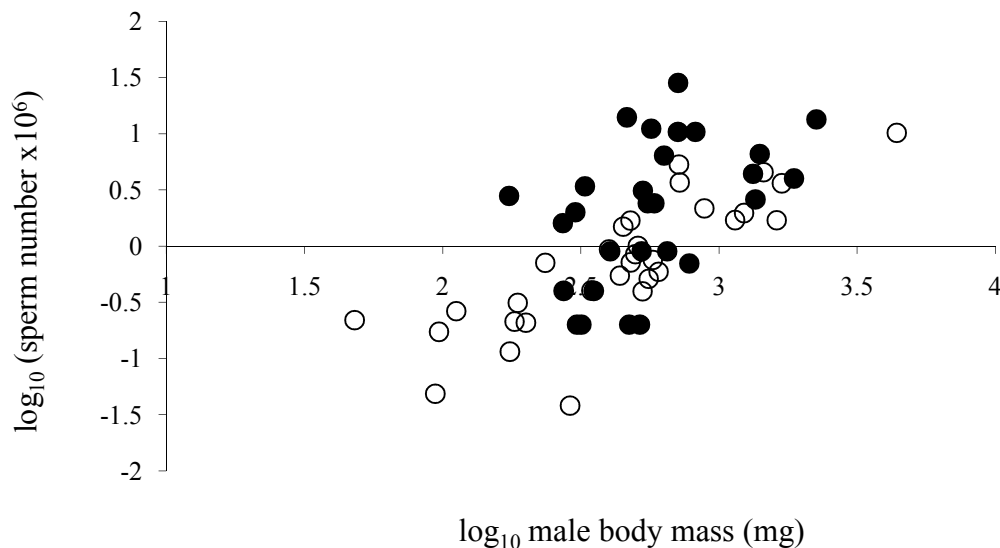


Fig. 2 Male body mass and sperm number within Orthoptera (open circles, Vahed & Gilbert (1996) and *Poecilimon* (closed circles). Male body mass explains little of the variation in sperm number within *Poecilimon*. In contrast, 68% of the sperm number is explained by male body mass in other Orthoptera.

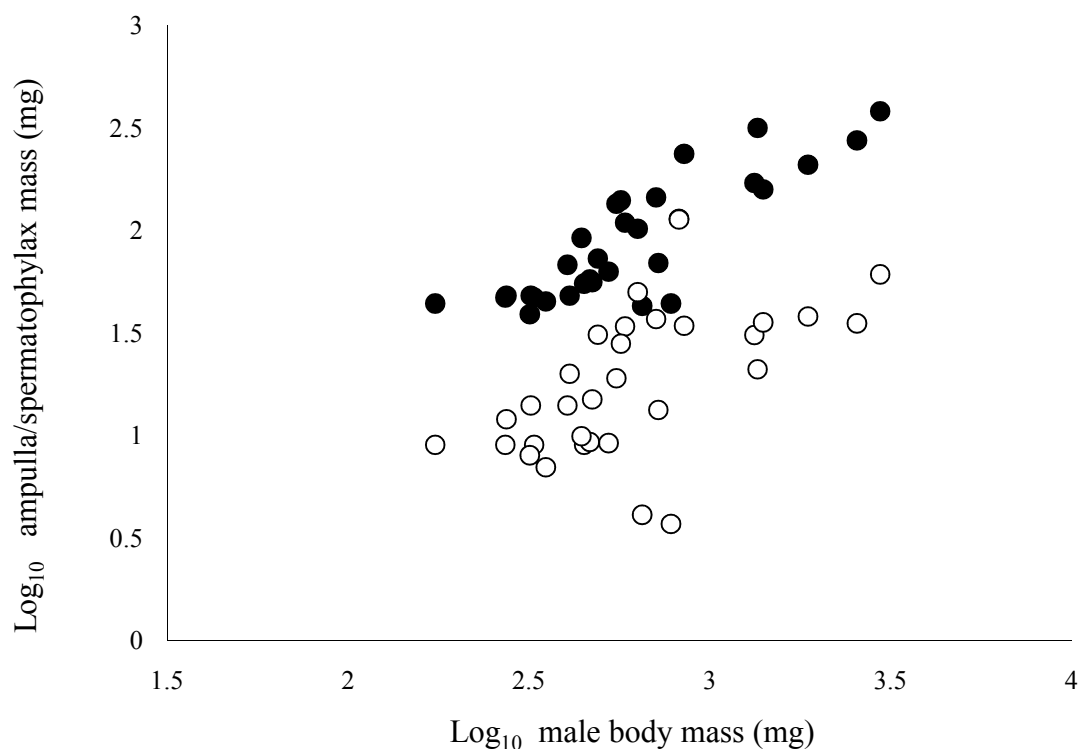


Fig. 3 Spermatophore components are largely dictated by male body mass: the relationships between male body mass and both spermatophylax mass (closed circles) and ampulla mass (open circles) in 31 *Poecilimon* taxa.

Variation within Poecilimon.—Within *Poecilimon* there is a large range in both body mass and spermatophore size. *P. hoelzeli*, for example, is more than fifteen times the weight of *P. pergamicus* (Table 1) and produces an accordingly large spermatophore of up to 454 mg, compared to 18.1 mg in *P. pergamicus*. Within the genus, spermatophore mass is closely correlated with male body mass ($y = 0.7545x + 1.24$, $F_{1,35} = 59.255$, $p = 0.000$, $r^2 = 0.64$, Table 2). Similarly, male body mass is closely correlated with spermatophylax mass ($y = 0.86x - 0.44$, $F_{1,30} = 72.20$, $p < 0.001$, $r^2 = 0.71$), and is significantly correlated with ampulla mass ($y = 0.67x - 0.60$, $F_{1,30} = 12.91$, $p = 0.001$, $r^2 = 0.31$; Fig. 3).

Table 2. Regressions between male body mass (MBM), spermatophore mass, spermatophylax mass, ampulla mass and sperm number between *Poecilimon* 33 species (36 taxa, n=62). *=significant

Hypotheses	F-statistic	p value	r ² -value	df
MBM/spermatophore mass	59.255	<0.001*	0.64	1,35
MBM/spermatophylax mass	72.195	<0.001*	0.71	1,29
MBM/ampulla mass	12.908	0.001*	0.31	1,29
MBM/sperm number	7.406	0.011*	0.22	1,26
MBM/relative spermatophore mass	2.7855	0.104	0.08	1,34
MBM/relative spermatophylax mass	0.0586	0.810	0.00	1,29
MBM/relative ampulla mass	1.4749	0.234	0.05	1,29
MBM/relative sperm number	0.1736	0.680	0.01	1,26
Spermatophylax mass/ampulla mass	16.256	<0.001*	0.36	1,30
“ without <i>P. mytilenensis</i>	23.789	<0.001*	0.46	1,29
Spermatophylax mass/sperm number	1.4827	0.200	0.06	1,22
“ without <i>P. mytilenensis</i>	1.7638	0.200	0.08	1,21
Ampulla mass/sperm number	15.705	<0.001*	0.43	1,22
“ without <i>P. mytilenensis</i>	9.4264	0.006*	0.32	1,21

Intraspecific variation.—In four taxa, *P. erimanthos*, *P. hamatus*, *P. j. jonicus*, and *P. laevissimus*, spermatophore size varied two-fold within populations among seasons, while the numbers of sperm per spermatophore remained relatively constant (Table 1). Most of this variation is attributable to spermatophylax mass rather than ampulla mass, apart from *P. laevissimus*, where the ampulla mass (actual and relative) also varied two-fold among seasons. *P. affinis* showed only a small range in relative spermatophore size (13 to 15%) among years and populations in field conditions, but there was a remarkable difference in sperm number between field and laboratory-reared individuals (4.4 million sperm and 21.6 million sperm respectively). In *P. thessalicus*, while body size varied between 442 and 610 mg over four seasons, spermatophore mass varied from 102 mg (23% relative mass) to 224 mg (36.7% relative

mass), and sperm number showed a four-fold range from 3.9×10^6 to 16.5×10^6 sperm over the same period. The two subspecies of *P. veluchianus* have been sampled repeatedly from both laboratory and field-reared bush-crickets. In *P. v. veluchianus* spermatophore size varied a little from 150 mg to 212 mg (23% to 26% relative mass), but sperm number varied from 6.3 million sperm in laboratory-reared bush-crickets (Reinhold & von Helversen 1997) to 10.5 million sperm in the field (Reinhold 1994). Similarly, the relative spermatophore mass of *P. v. minor* varied from 17% - 20% of body mass but sperm number ranged from 3.4 million to 7.6 million.

Spermatophore components.—The previous sections demonstrate that there is a tendency for relative spermatophore size to increase with an increase in body size. However, there is considerable variation among the species in spermatophore investment that is independent of this general pattern. No significant relationship was found between male body mass and relative spermatophylax mass ($y = -0.0004x + 16.22$, $F_{1,29} = 0.06$, $p = 0.81$, $r^2 = 0.002$), relative ampulla mass ($y = -0.0009x + 4.35$, $F_{1,29} = 1.48$, $p = 0.23$, $r^2 = 0.048$), and relative number of sperm ($\times 10^3$ per 1mg of male body mass, $y = -0.0192x + 86.71$, $F_{1,26} = 0.17$, $p = 0.68$, $r^2 = 0.007$). Allowing for body size reveals that some species invest relatively much more heavily in some spermatophore components than other species (Fig. 4, Table 1). Spermatophore components show considerable variation with some small males producing large spermatophylaxes, ampullae or sperm numbers, and some large males producing small spermatophylaxes, ampullae or sperm numbers.

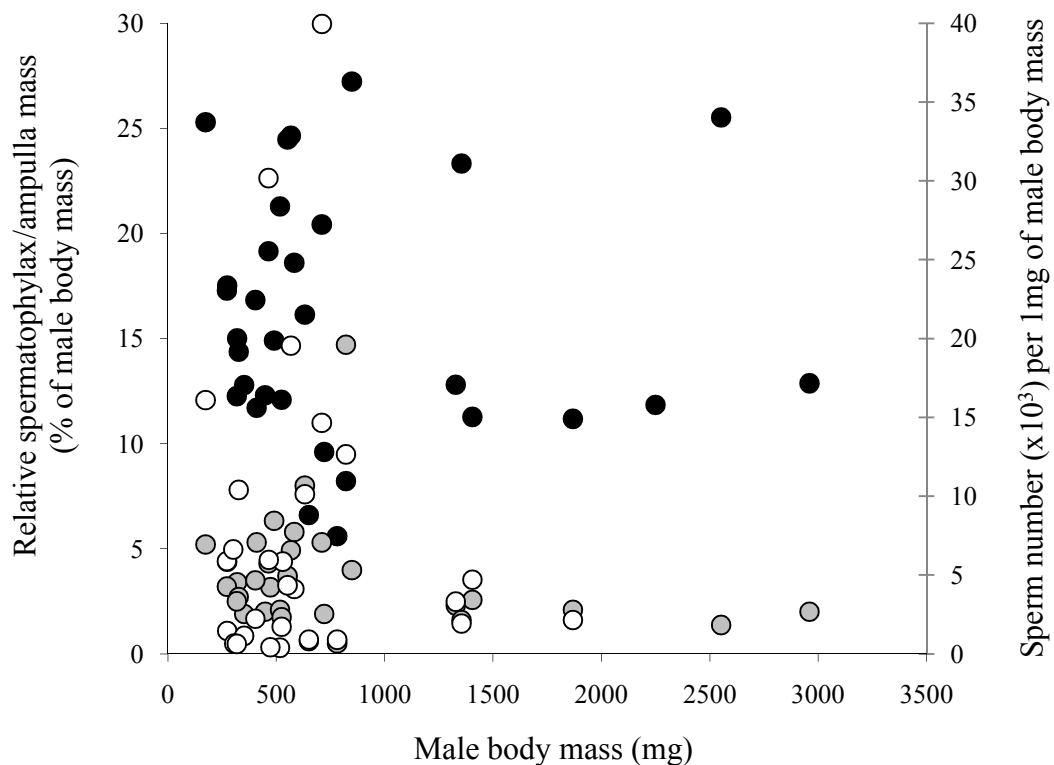


Fig. 4 A large variation in the relative investment to spermatophore components: no relationships between male body mass and relative spermatophylax mass (black circles), relative ampulla mass (grey circles) and relative sperm number (open circles).

We found a significant correlation between the residuals of spermatophylax and ampulla mass ($y = 1.1116x$, $F_{1,30} = 23.79$, $p < 0.001$, $r^2 = 0.46$), although a substantial portion of the variance in residuals did not co-vary (Fig. 5). Including data from *P. mytilenensis* predictably decreased the relationship further ($y = 1.10x + 0.023$, $F_{1,31} = 16.26$, $p < 0.001$, $r^2 = 0.36$; Table 2). Surprisingly, residual spermatophylax mass did not correlate with residual sperm number across *Poecilimon* species ($y = 0.38x$, $F_{1,21} = 1.76$, $p = 0.2$, $r^2 = 0.08$; Fig. 6), and was largely unaffected by the inclusion of *P. mytilenensis*, $y = 0.39x$, $F_{1,22} = 1.48$, $p = 0.2$, $r^2 = 0.06$; Table 2).

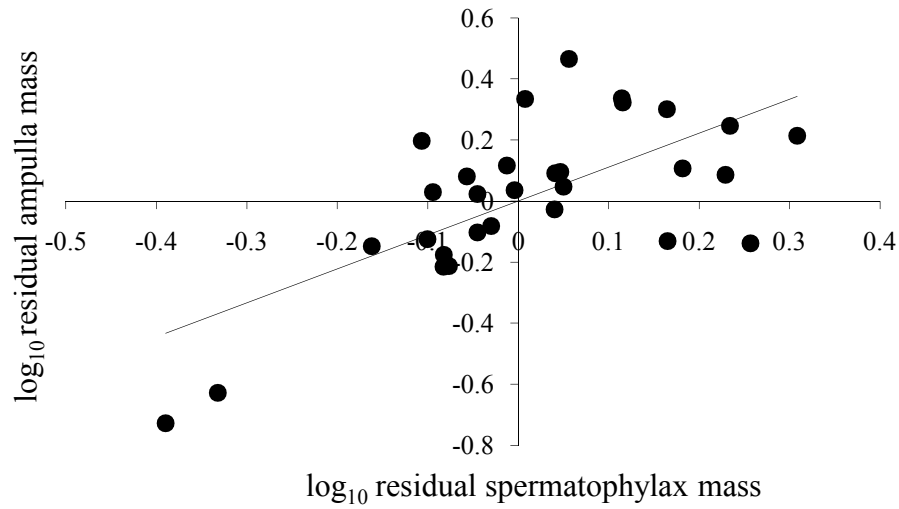


Fig. 5 Positive relationship between residual ampulla mass and residual spermatophylax mass across 31 *Poeciliimon* species.

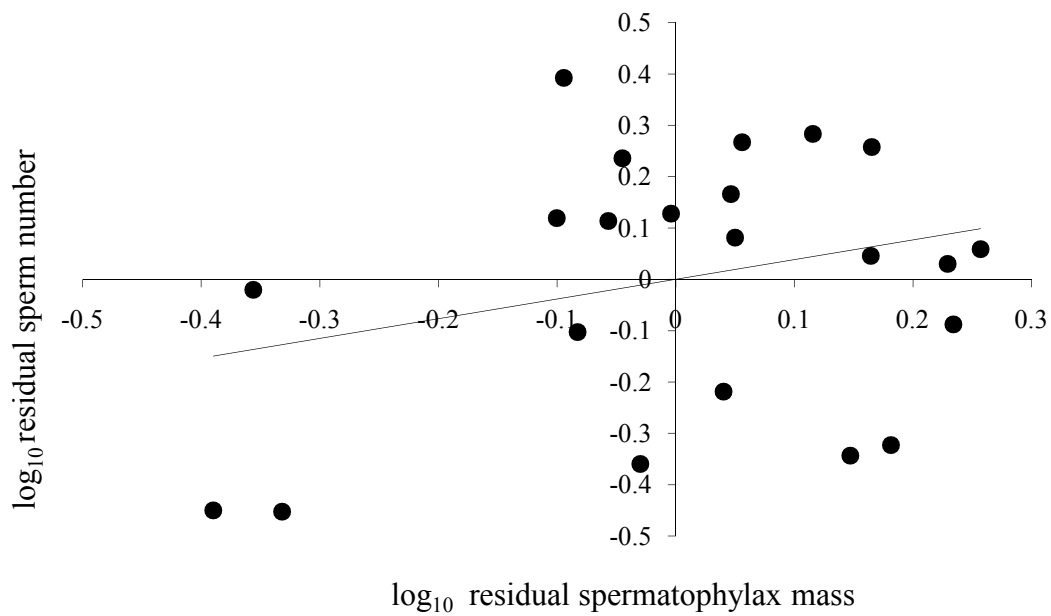


Fig. 6 No relationship exists between residual sperm number and residual spermatophylax mass across 22 species of *Poeciliimon*.

A significant correlation was found between the residuals of ampulla mass and sperm number ($y = 0.4817x - 0.0032$, $F_{1,21} = 9.426$, $p = 0.006$, $r^2 = 0.32$; Fig. 7, Table 2), although a substantial portion of the variance in residuals, about 68%, could not be explained by the model. Including *P. mytilenensis* in this model strengthened this association so that 57% of the variation could not be accounted for ($y = 0.54x$, $F_{1,22} = 15.71$, $p < 0.000$, $r^2 = 0.43$).

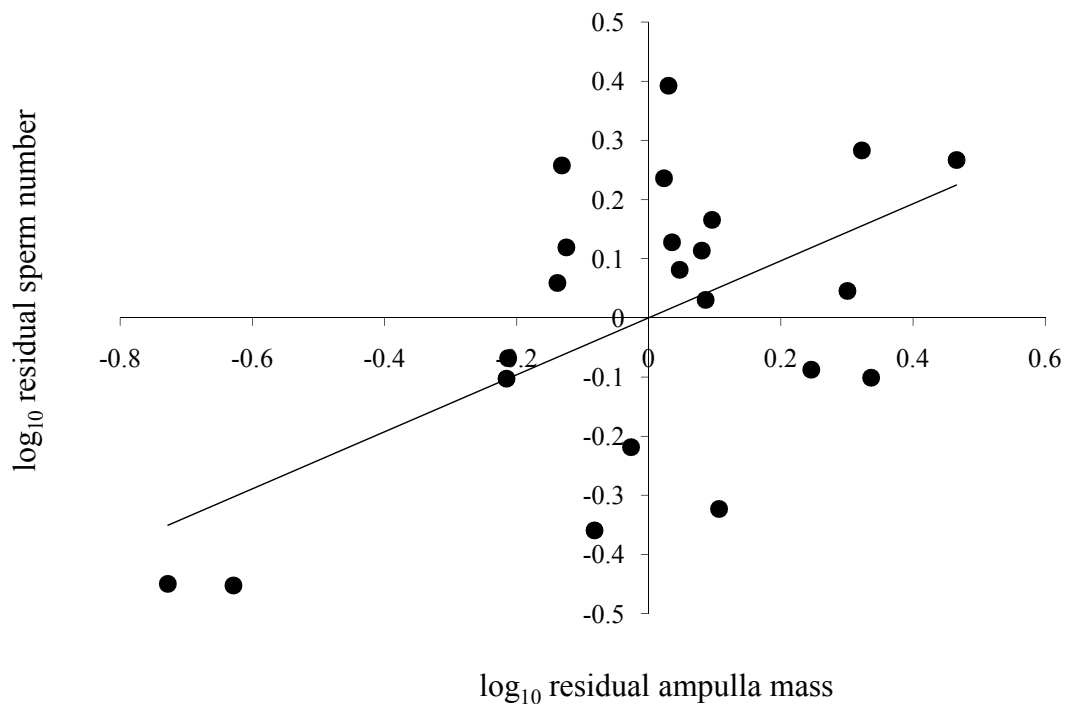


Fig. 7 The relationship between residual sperm number and residual ampulla size across 22 *Poecilimon* species.

Discussion

Spermatophore variation, ejaculate protection and paternal investment.—The positive correlation we found between residual spermatophylax mass and residual ampulla mass is consistent with other research supporting the ejaculate protection hypothesis (Reinhold & Heller 1993; Wedell 1993a, 1994b; Heller & Reinhold 1994; Vahed & Gilbert 1996). Vahed & Gilbert (1996) also found a strong correlation between residual spermatophylax mass and residual ampulla mass within 43 species from nine subfamilies of mostly European bush-crickets. Similarly, Wedell (1993a, 1994b) found a positive correlation between spermatophylax mass and ampulla mass in 19 genera of mostly Australian bush-crickets. While the correlation found between these components within *Poecilimon* was moderate, the relationship was strengthened by the removal of *P. mytilenensis* — a species known to have an inordinately large ampulla, but a modestly sized spermatophylax (Heller *et al.* 2004).

While our findings are consistent with the ejaculate protection hypothesis, they are not inconsistent with the paternal investment hypothesis. The spermatophylax of *P. veluchianus*, for example, is approximately the size required to allow for an optimum amount of sperm to enter into the female (Reinhold & Heller 1993, Heller & Reinhold 1994), although the spermatophore of the last male to mate will have a positive effect on the dry weight of his own offspring (Reinhold 1999). The paternal investment hypothesis assumes selection acts on the spermatophylax through a direct nutritional benefit to the offspring (Trivers 1972, Gwynne *et al.* 1984). Yet compared to the spermatophylax, the ejaculate may be produced relatively inexpensively (*e.g.*, Bateman 1948, Trivers 1972, but see Dewsbury 1982, Reinhold & Helversen 1997; Wedell *et al.*

2002 provide a review) and is critical to male fertilization success. Ampullae size (ejaculate volume) may still modulate spermatophylax size through influences of ejaculate protection, while the primary factors influencing spermatophylax size itself are paternal investment. Males that primarily invest heavily in spermatophylaxes and as a result, provide a significant nutrient investment to their offspring, may also produce greater than normal quantities of sperm in order to 'hedge their bets' and maintain paternity shares in the face of sperm competition (Reinhold & von Helversen 1997, Lehmann & Lehmann 2000b). The ejaculate and/or spermatophylax mass may also have flow-on effects in females by influencing female intermating refractory period (Heller & Helversen 1991, Heller & Reinhold 1994, Lehmann & Lehmann 2000b, Vahed 2007), female lifespan (Brown 1997), the timing of oviposition (Wedell & Arak 1989), and the share of eggs that are laid with the donating males' nutritional investment (Simmons 1990, Vahed 2003).

Under the ejaculate-protection hypothesis, the cost of extra sperm or ejaculate fluid is assumed to be negligible in comparison to the gain in paternity (Simmons 1995b). Evidence showing sperm to be less costly than the production of the spermatophylax has been observed in *P. mariannae*: parasitized males lose their ability to replenish their spermatophylax, but not their sperm (Lehmann & Lehmann 2000b). Similarly, Reinhold & von Helversen (1997) found that spermatophore replenishment, rather than sperm number, limits intermating interval in male *P. veluchianus*.

In contrast to predictions of the ejaculate-protection hypothesis, we did not observe a relationship between sperm number and spermatophylax size in *Poecilimon*. This runs counter to findings from other studies where a positive relationship existed

across taxa (e.g., Wedell 1994b, Vahed & Gilbert 1996). Sperm number has also been found to be independent of spermatophylax mass in an Australian bush-cricket, *R. verticalis* (Simmons et al. 1993), and in *P. veluchianus*, (Reinhold & von Helversen 1997). Reinhold & von Helversen (1997) further predicted that this lack of relationship may represent a general trend in bush-crickets. However, sperm number and spermatophylax mass are adjusted in concert in parasitized *P. mariannae* (Lehmann & Lehmann 2000b), so the situation appears to be more complicated in *Poecilimon*.

While our results confirm the prediction of Reinhold & von Helversen (1997), the validity of the ejaculate-protection hypothesis relies more specifically on the relationship between spermatophore consumption time and sperm discharge time, rather than covariance of spermatophylax mass and sperm number (see for example Reinhold & Heller 1993, McCartney & Heller submitted ms.). An association between spermatophylax consumption time and sperm drainage has been observed in all bush-cricket studies thus far: *R. verticalis* (Gwynne 1984a, 1986, 1997, but see Simmons 1995a, Vahed 1998 for different interpretations), *Decticus verrucivorus* (Wedell & Arak, 1989), *Kawanaphila nartee* (Simmons & Gwynne, 1991), and *Leptophyes laticauda* (Vahed 1994), as well as *Poecilimon hoelzeli* (Achmann 1996), and two subspecies of *Poecilimon veluchianus* (Reinhold & Heller 1993, Heller & Reinhold 1994). However, the spermatophore consumption time and sperm discharge do not correspond in two further *Poecilimon* species (*P. laevissimus* and *P. thessalicus*, McCartney & Heller submitted ms.). This, combined with our detection of a large intraspecific variation in spermatophylax mass and sperm numbers between individuals, populations and years (e.g., *P. thessalicus* and *P. veluchianus*, Table 1) is likely to explain the lack of association we found within the

genus.

Under the ejaculate-protection hypothesis, the spermatophylax may be viewed as a sperm-protection device, allowing the transfer of a maximum number of sperm, and being primarily influenced by sperm competition. However, chemicals in the ejaculate itself can increase male fitness by functioning to increase onset of egg-laying, increase total number of eggs laid and to prolong the female intermating period (*e.g.*, Reinhold & von Helversen 1997; Vahed 1998; 2003, 2006, 2007; Arnqvist & Rowe 2005). Our study indicates that discharge of the ejaculate may be more important in terms of spermatophylax function than the discharge of sperm *per se*. While we found a significant association, ampulla mass only explained a small amount of variation in sperm number within *Poecilimon*.

Only one other comparative study seems to have measured the association between ampulla mass and sperm number among bush-cricket species and no relationship was found (Vahed 2006). This, in combination with our finding that ampulla mass, but not sperm number, correlates with spermatophylax mass, indicates that the spermatophylax, in terms of mating effort, has an ejaculate-protection function, but not a primary sperm-drainage function in *Poecilimon*. Our results lead us to believe that sperm number itself should not be used as an assessment of the ejaculate protection function, nor should ejaculate volume (ampulla size) be used to assess sperm protection or competition (*e.g.*, Wedell 1993a, Wedell 1997) when making interspecific comparisons.

Spermatophore size variation within Poecilimon.—Spermatophore size within the genus *Poecilimon* approximates that found within the entire family *Tettigoniidae* (*c.f.*

Wedell 1993a, Vahed & Gilbert 1996, Wedell 1997, Vahed 2007), indicating that variation in spermatophore size is unlikely due to relatedness or diet alone. This large variation between species is likely to reflect within-species adjustments that male bush-crickets make to specific spermatophore components as a conditional strategy — apparently in order to maximise reproductive output (*e.g.*, *P. affinis*, *P. erimanthos*, *P. hamatus*, *P. jonicus*, *P. laevissimus*, *P. thessalicus*, *P. veluchianus*). We found that all spermatophore components in *Poecilimon* scale approximately with male body mass, but large variations are apparent in relative investment when body mass is taken into consideration.

Preferential investment in spermatophore components suggests that variations in environment and available energy or nutrients are directed to whichever spermatophore component is more effective at increasing reproductive fitness (see for example Voigt *et al.* 2005 and references cited therein). Examples of this have been found in a variety of bush-crickets. Male *Requena verticalis*, for example, increase the number of sperm when mating with older females, or when exposed to a high female sex ratio, effectively increasing their chances of paternity, given the likely increase in sperm competition (Simmons *et al.* 1993, Simmons 1995a). Similarly, *R. verticalis* males disproportionately adjust the ampulla mass over the spermatophylax mass in relation to their remating frequency (Simmons 1995b) or mating potential (Simmons 1995c). Males of another species, *Decticus verrucivorus*, adjust the size of the offered spermatophore depending on whether or not a mate is virgin (Wedell 1992).

Considerable variation in the size of *Poecilimon* spermatophore components was found between and within populations (*e.g.*, *P. erimanthos*, *P. hamatus*, *P. jonicus*, *P.*

laevissimus, *P. thessalicus*, *P. veluchianus*). The foundation for this variation is likely the availability of environmental resources (e.g., Hubbell & Johnson 1987, Gwynne & Simmons 1990, Adamo & Hoy 1994) yet, while related, more proximal causes associated with life histories and mating behavior, including population density, operational sex ratio, and sexual size dimorphism, influence the relative pay-offs in spermatophore production (e.g., Gwynne 1981, 1984a, b; Gwynne & Simmons 1990; Heller & von Helversen 1991; Allen 1995; Bateman 1997). There is little published information on intraspecific variation in spermatophore component size among bush-cricket populations, and evidence presented here suggests that further research on *Poecilimon* is needed to help clarify how environmental factors affect male investment in spermatophore components.

Spermatophore differences between field and laboratory-raised individuals.— Importantly, we found large differences between laboratory-reared individuals and those from the field. For example, *P. v. minor* males reared in the laboratory had a larger body mass and over twice as many sperm per spermatophore, compared to those in the field. The converse was true for *P. v. veluchianus*, which had a larger number of sperm in individuals collected in the field. A large range in ampulla mass was also seen in this subspecies (5.3 to 25.4 mg) and previous studies show that spermatophore consumption time also varies greatly between conditions (Reinhold & Heller 1993). Similarly, *P. affinis* differs considerably in sperm number in laboratory and field observations, with nearly five times more sperm in laboratory-reared individuals; however it is difficult to assess whether this reflects environment differences or bias due to small sample size. Laboratory-reared animals it seems, often

show extreme variations in spermatophore component size. This may provide important information in some circumstances; however, given the highly variable nature of spermatophore production, we recommend caution when interpreting spermatophore function using laboratory-reared animals, small sample sizes, or means from short-term observations.

Conclusions

Detailed analyses of spermatophore size with respect to phylogeny and diet will be important to developing a more complete understanding of the evolutionary significance of variation in spermatophore size. Spermatophore component size in *Poecilimon* appears to be evolutionarily labile and a general lack of association within *Poecilimon* between relative spermatophore-component size and male body mass, reflects differences related to mating strategy. This, combined with a lack of association between spermatophore component size, indicates that effective ejaculate transfer, not sperm drainage *per se*, is a significant influence in the evolution of spermatophore size. Mating effort and paternal investment are not mutually exclusive and further analysis within *Poecilimon* on the direct association between the amount of sperm that drains into the female and its relationship to spermatophore-consumption time is needed for a full understanding of the relative influences of ejaculate protection and paternal investment on spermatophore size. Given the significance of sperm competition in evolutionary biology, studies within and between closely related species in natural

populations are necessary to improve knowledge of the processes that influence the evolution of nuptial feeding in insects.

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Fig. 8. *Poecilimon veluchianus minor* with attached spermatophore. From a population at Makrakomi, mainland Greece, near the village of Tsouka, 1998. Photo by J. McCartney. See Plate III.

Appendix 1. Table showing the location where each *Poecilimon* species was observed. (The site locations for each species taken from the literature are listed at the bottom of Table 1).

<i>P. aegaeus</i> , GREECE: Island of Andros in the Cyclades, (37°83' N, 24°93'E), 29 iv 1996
<i>P. affinis</i> III, GREECE: Near the village Pisodherion, Florina, (40°46'N, 21°16'E) (date unknown)
<i>P. amissus</i> , GREECE: Island of Lesbos. Mytilini, near Vrissa (39°02'N, 26°11'E), 23 v 1993
<i>P. anatolicus</i> , GREECE: Drama, Kato Vrontdou north-east of Serrai (41°16'N, 23°44'E), 1 vi 1983
<i>P. brunneri</i> , GREECE: Evros, 1 km east of Peplos (before the Turkish border) (40°57'N, 26°17'E), 1-31 v 1996
<i>P. deplanatus</i> , GREECE: Island of Karpathos, near Lefkos (35°35'N, 27°4'E), 15-20 v 2005
<i>P. elegans</i> , ITALY: Istrien, near Triest (45°39'N, 13°46'E), 1-31 viii 1992
<i>P. erimanthos</i> I, GREECE: Peloponnes, N. Elia, Erimanthos valley, east of the Koumani village (37°48'N, 21°47'E), 1997
<i>P. erimanthos</i> II, GREECE: Peloponnes, N. Elia, Erimanthos valley, east of the Koumani village (37°48'N, 21°47'E), vi 1990
<i>P. gracilis</i> , GREECE: Near the village Pisodherion, North Florina, (40°46'N, 21°16'E) (date unknown)
<i>P. hamatus</i> I, GREECE: Island of Samos; (37°44'N, 26°46'E), 1998
<i>P. hamatus</i> II, GREECE: Island of Rhodes; (36°11'N, 28°03'E), 2005
<i>P. hoelzeli</i> I, GREECE: Karditsa, between Loutropigi and Mesochori (39°05'N, 22°03'E), 19 v 1989
<i>P. hoelzeli</i> II, GREECE: Karditsa, near Makrirahi, (39°06'N, 22°07'E), vi 1990
<i>P. ikariensis</i> , GREECE: Aegean Islands, N. Samos, Ikaria: 3 km northwest Ag. Kyrikos (37°37'N, 26°16'E), 22 v 1998
<i>P. jonicus jonicus</i> I, GREECE: Thesprotia, Kallithea, 25 km east of Igoumenitsa (39°33'N, 20°27'E), 4 vi 1992
<i>P. jonicus superbus</i> , ITALY: L'Aquila, Gran Sasso: 10 km west of Fonte Cerreto (42°27'N, 13°25'E), 1300 m, 1-3 ix 1996
<i>P. jonicus tessellatus</i> , GREECE: Peloponnes: N Ano Diakoptó, Haikos gorge (37°83'N, 22°93'E), 27 iv 1996
<i>P. laevissimus</i> I, GREECE: Evvoia, Mistras (38°31'N, 23°50'E), 1983
<i>P. laevissimus</i> II, GREECE: Ilia Peloponnes, Erimanthos -Tal 6 km east of Koumanis (37°48'N, 21°47'E), 24 v 1992 and GREECE: Aitolia-Akarnania, Astakos (38°32'N, 21°4'E), 25 v 1992
<i>P. laevissimus</i> III, GREECE: Peloponnes: Ithómi near the ancient Messenian ruins (37°15'N, 21° 94'E), and near a monastery in the Mistras of Lakonía (22°36'E, 36°07'N), 5-6 v 1996
<i>P. laevissimus</i> IV, GREECE: Peloponnes, N. Elia, Erimanthos valley, east of the Koumani village (37°48'N, 21°47'E), 1997
<i>P. macedonicus</i> , GREECE: Mt. Chortiatis east of Thessaloniki above the town of Panorama (1990) (40°34'N, 23°06'E), 1990
<i>P. marmaraensis</i> TURKEY: Kirklareli, 10 km west of Lüleburgaz (intersection after Saricaali) (41°25'N, 27°15'E), 1-31 v 1996
<i>P. nobilis</i> , GREECE: Peloponnes, N. Elia, Erimanthos valley, east of the Koumani village (37°48'N, 21°47'E), v/vi 1992

Appendix 1 continued.

- P. ornatus* I, ITALY: Medeazza; northern Italy (45°47'N, 13°36'E), 1996
- P. ornatus* II, SLOVENIA: Loibl-Pass (46°26'N, 14°15'E), 1995
- P. pergamicus*, GREECE: Island of Lesbos. Mytilini, Moria (Aqueduct) (39°07'N, 26°30'E), 28 v 1993
- P. gerlindae*, GREECE: Domokos, N. Fthiotis (39°06'N, 22°18'E), 8-17 vi 1992
- P. sanctipauli* I, GREECE: Island of Rhodos (28°03'E, 36°11'N), 31 v 1996
- P. sanctipauli* II, GREECE: Island of Samos (37°44'N, 26°46'E), 31 v 1996
- P. ege*, GREECE: Island of Samos (different localities) (37°44'N, 26°46'E), 31 v 1996
- P. thessalicus* I, GREECE: Pieria, north west of the village of Elatochori (40°19'N, 22°15'E), 1997
- P. thessalicus* II, GREECE: Pieria, north west of the village of Elatochori (40°19'N, 22°15'E), 1997
- P. thessalicus* III, GREECE: Pieria, north west of the village of Elatochori (40°19'N, 22°15'E), 1998
- P. thessalicus* IV, GREECE: Mt.Ossa, north east of Thessaloniki (40°49'N, 23°08'E), 1990
- P. turcicus*, GREECE: Island of Lesbos; Mytilini, near Larissos (Kolpos Geras), (39°07'N, 26°26'E), 28 v 1993
- P. ukrainicus*, UKRAINE: Kiev and Cherkaska Oblast, Kanev Forest Reserve, and surrounding area (49°44'N, 31°30'E), 18-23 vi 1996
- P. unispinosus*, GREECE: Island of Chios (different localities) (38°22'N, 26°08'E), v 1995
- P. v. minor* I, GREECE: Nomos Fthiotis, Makrakomi, near the village of Tsouka (38°57'N, 22°05'E), 1995
- P. v. minor* III, GREECE: Nomos Fthiotis, Makrakomi, near the village of Tsouka (38°57'N, 22°05'E), 1998
- P. v. veluchianus* I, GREECE: Nomos Fthiotis, 3 km north of the village of Vitoli, near the village of Makrakomi (38°58'N, 22°01'E), 1995
- P. wernerii*, GREECE: Near the city of Astakos, in the area of Aitolia-Akarnania (38°32'N, 21°4'E), 25 v 1992
- P. zimmeri* I, GREECE: Fokis, near the town of Kalascopi, South of Mt Oiti (38°42'N, 22°19'E), 900 m, v 1990
- P. zimmeri* II, GREECE: Near the Delphi ancient temple in the area of Fokis (38°28'N, 22°29'E), 2002
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Chapter 3

A preliminary analysis of mate choice in a bush-cricket (*Poecilimon laevisissimus*: Tettigoniidae) suggests virginity is more important than body size

J. McCartney and K-G. Heller



Two marked *Poecilimon laevisissimus* mating.

Abstract

Insects are predicted to prefer larger partners for a number of reasons relating to fitness. In species where males provide an expensive nuptial gift, male and female preferences for a larger partner are likely to be more pronounced. In nuptial-feeding insects however, models of sperm competition and female choice predict that males and females should also prefer virgin partners. Here we test the relative importance of size vs virginity in a Greek bush-cricket, *Poecilimon laevisissimus* in which males offer nuptial gifts during mating. While only a small number of replicates could be implemented, and there is a clear need for further analysis, we found that all males and females preferred to mate with virgins, despite the fact that nearly 90% of the virgins were smaller in size than the nonvirgins offered. In terms of mate choice, virginity therefore appears more important than body size in *P. laevisissimus*.

Keywords: body mass, mate choice, *Poecilimon laevisissimus*, sperm competition, virgin, nuptial gift, spermatophore

Introduction

With respect to mating success, body size is arguably the most prevalent measure of fitness documented in the literature. Males are predicted to prefer larger females because they are generally more fecund (*e.g.* Gwynne 1981, 1984, 1985; Thornhill & Alcock 1983; Simmons & Bailey 1990; Honek 1993; Vincent & Lailvaux 2006), and females are predicted to prefer large males, as body size is associated with several direct and indirect measures of fitness (for a review see Wedell & Ritchie 2004). Such measures include: disease and parasite load (Simmons 1994, Lehmann & Lehmann 2000a, for a review see Zuk & Stoehr 2004), performance during intrasexual competition (Thornhill & Alcock 1983, Simmons 1988), male vigor (*e.g.*, Reid & Roitberg 1995), sperm vigor (*e.g.*, Reinhardt & Siva-Jothy 2005) and good genes (*e.g.*, Beck & Powell 2000, Wedell & Ritchie 2004).

In many insects, males donate a nuptial gift during mating which is costly and typically positively associated with a male's body size (Heller & Reinhold 1994, Vahed & Gilbert 1996, Wedell 1997, McCartney *et al.* 2008). Bush-cricket males, for instance, produce an often expensive nuptial gift (*e.g.*, Dewsbury 1982, Heller & Helversen 1991, Vahed 2007) in the form of a gelatinous spermatophylax which functions in a dose-dependent manner, to optimize male fertilization success, and in some cases, offspring fitness (for reviews see Vahed 1998, Gwynne 2001). Sexual conflict resulting in mate choice is predicted to occur when male and female investment optima differ, such as, for example, when males invest heavily in spermatophores and have a high confidence of paternity (Knowlton & Greenwell 1984, Parker 1984). As a result, males are

predicted to discriminate against females if they pose a threat of cuckoldry and females are predicted to detect males with greater investment ability and discriminate accordingly (Dewsbury 1982, Simmons *et al.* 1993, Gwynne 2001). Mate choice in spermatophore producing bush-cricket is therefore likely to be strong because of the benefits that females potentially receive in the form of fertilisation and nutrients, and the expected returns that males may receive, in the form of greater fecundity, from mating with a larger female.

On the other hand, the cost of spermatophore production for males and the subsequent benefits that this may have for females should also result in selection for a preference by both sexes for virgin partners. Males may be expected to seek virgins in order to avoid sperm competition (*e.g.*, Simmons & Achmann 2000, Simmons 2001) or to take advantage of young females that produce eggs at a greater rate or of better quality than older females (*e.g.*, Rutowski 1982). Females may be predicted to prefer virgin males as they are normally younger and less affected by factors negatively affecting spermatophore size, such as parasites and disease (for reviews see, Lehmann & Lehmann 2000b, Zuk & Stoehr 2002). Younger males may also produce higher quality sperm (*e.g.*, Reinhardt & Siva-Jothy 2005) and, at least in the case of fruit flies, higher quality offspring (Price & Hansen 1998).

Despite the likely benefits of mating with virgins and larger partners, there is surprisingly little documentation showing a direct preference for either in bush-cricket. *Requena verticalis*, for example, showed an initial preference for virgins over nonvirgins, although this advantage was lost when nonvirgins had oviposited (Lynham *et al.* 1992). Later it was found that males prefer youth, not virgins per se, and

ultimately larger females were preferred over virgins (Simmons *et al.* 1993, Simmons *et al.* 1994).

Moreover, the vast majority of work seems to have been done on male preferences for females (for a review see Jennions & Petrie 1997) and there seems to be no documentation on the preference of females for virgin males over body mass in bush-crickets. In *Poecilimon*, larger males produce larger spermatophores (*e.g.*, McCartney *et al.* 2008) and larger females are likely to hold more eggs, but virgin males are likely to contain larger reserves of spermatophore material and virgin females can offer high assurance of paternity to males. Here we present a preliminary analysis of mate preference shown by males and females in a spermatophore-bearing bush-cricket, *Poecilimon laevissimus*, for small virgin or larger nonvirgin partners.

Methods

P. laevissimus (Figs 1,2) is a Greek bush-cricket (Tettigoniidae) of medium size; male body mass ≈ 781 mg ($n=50$, McCartney *et al.* 2008), female body mass ≈ 848 mg ($n = 50$, McCartney & Heller unpub. data), semelparous, and has a univoltine life-cycle with an obligate diapause. The spermatophore ranges in size from 6 to 11% of male body mass in this species (McCartney *et al.* 2008), and consists of a large proteinaceous spermatophylax, which protects the ejaculate contained in the associated ampulla from premature removal as it passes into the female.

The experiment was conducted in a natural population in north Ilia, Erimanthos Valley, east of the village Koumani (lat 37° 48'N, long 21° 47'E),

Peloponnese, Greece, during June and July, 1997. Age was kept approximately constant by ensuring that all bush-crickets used in the experiment were taken on a single day as subadults and all pairings occurred within a single day. Furthermore, adult populations of *P. laevis* only survive for a few weeks (McCartney submitted manuscript) and therefore should all have been similar in age. Males and females were kept separately in field cages until they had reached adulthood, and had sufficient time to attain reproductive maturity. Fresh leaves and flowers, taken from the site of the local population, and water, were supplied *ad libitum*. All individuals were then numbered with an indelible pen on their pronota. The nonvirgin mating partners were taken from the field population on the same day and otherwise handled in a similar fashion as the virgin mating partners. The only difference was that they had mated once or twice previously and been allowed at least five days recovery to ensure they were fully receptive.

The experiments were conducted in mesh cages of approximately 35 × 20 × 20 cm. Each subject was placed in a separate cage with a smaller virgin and a larger nonvirgin of the opposite sex. Four male and five female bush-crickets (nine replicates) were placed with two partners that were matched for mass (to the nearest 1 mg), so that the virgins weighed less than the nonvirgins. In one additional male test, the virgin female was larger than the mated female. When a mating took place, the pairs were observed until the pair had uncoupled. Two of the ten replicates, one male and one female, resulted in no mating. Following mating, the spermatophore was carefully removed from the female with watchmaker's forceps and weighed. Each individual was then weighed and the spermatophore mass from the resulting mating added to the male's current weight.

Results and discussion

In all successful matings, over both male and female treatments, virgins were chosen over nonvirgins, despite their smaller size. Virgin males in the female-choice treatment were on average 17% (mean weight = 0.59 g, range = 0.52 – 0.66g, n = 4) smaller than nonvirgins (mean weight = 0.71 g, range = 0.59 – 0.80 g, n = 4). Virgin females (mean weight = 0.93 g, range 0.84 – 1.1 g, n=3) in the male-choice treatment were on average 9% smaller than the nonvirgins (mean weight = 1.027 g, range = 0.87 – 1.219 g, n=3). In the additional test where the virgin female (weight = 0.873 g) was larger than the nonvirgin female (weight = 0.652), the virgin was also chosen.

There is evidence that in other bush-crickets, males discriminate in favor of virgin females, yet further analysis reveals that it is normally a preference for youth, not virgins *per se*, and ultimately body mass is likely preferred over virginity (e.g., *Requena verticalis*, Lynham *et al.* 1992, Simmons *et al.* 1993, Simmons *et al.* 1994). A strong preference for young females (and virgins) is understandable in *R. verticalis* because the first male to mate has a high confidence of paternity and subsequent mating males are thus likely to have their nutrient investment cuckolded (Gwynne 1988).

In the Botswana armoured ground cricket, *Acanthopplus discoidalis* (Tettigoniidae), males prefer females with a lower mass and reject nonvirgins more often. This, however, was also interpreted as a preference for younger females, not virgins *per se*, because virgins were significantly younger than the nonvirgins tested (Bateman & Ferguson 2004). While it may be preferable for males to detect virgins, more proximate cues used to detect virginity may not exist in these species and youth

may serve as the best proxy. *A. discoidalis* seemed to use cues, such as small size, to detect youth (Bateman & Ferguson 2004), so the apparent preference in *P. laevisissimus* males for virgin females may actually be a preference for small females, rather than the larger, nonvirgin females or virgins per se. However, our single female that was larger than the nonvirgin female was also chosen, consistent with virgin status, and not size, being more important: this needs to be investigated in future studies.

Studies with butterflies, fruit flies, weevils, grasshoppers (see Simmons *et al.* 1994 and references cited therein), and at least two bush-crickets (Wedell 1992, Wedell 1998) all show that males distinguish virgin from nonvirgin partners, apparently because of their greater fecundity and associated increased certainty of paternity; there is no evidence at this stage to indicate that this may be otherwise in *P. laevisissimus*.

Female *P. laevisissimus* actively selected virgin males despite their smaller size. Female *Ephippiger ephippiger* (Tettigoniidae) mate with younger males, as youth in this species indicates mating history and males with fewer matings produce larger, more nutritious spermatophores with more sperm (Wedell & Ritchie 2004). Spermatophore size in *Poecilimon* is closely related to body size (McCartney *et al.* 2008), so it seems unlikely that females select smaller, albeit virgin, males if they require nutritional investment from males. Sperm number however, is not related to body size in *Poecilimon*, and compared to other *Poecilimon* species, *P. laevisissimus*' spermatophylax mass is lower in relation to the sperm number (McCartney *et al.* 2008). So, female *P. laevisissimus* may select virgin males in order to receive greater sperm loads. Other data from *P. laevisissimus* suggest that females rarely contain more than 50-75% of the sperm that males offer in one spermatophore, further suggesting preferences for multiple

mating and a higher sperm load (McCartney & Heller in prep).

Intrasexual competition may be an important factor influencing mate choice in *P. laevisissimus*. Virgin female *Poecilimon* may respond phonotactically to male calls faster than nonvirgins, and virgin males may produce calls or pheromones that are more attractive to females. Furthermore, males may directly interfere with copulating pairs in order to dislodge the copulating male (pers. obs.). The choice made in our treatments therefore may not be a result of mate choice *per se*, but instead of the virgin from each trial showing more concupiscence than the nonvirgin and so ultimately winning access. However, female *P. laevisissimus* respond acoustically to male calls, and males respond to this by moving toward the female (Heller & Helversen 1986). *P. laevisissimus* are therefore more likely to interpret mating status from the mating call and, as with other bush-crickets (e.g., *E. ephippiger*, Wedell & Ritchie 2004), discriminate accordingly. Furthermore, no males in the female choice experiment were observed ‘wrestling’ for access to females, so it is likely that the females similarly used sound or pheromone cues to discriminate.

While the influence of intrasexual interactions on *P. laevisissimus* mate-choice needs further investigation, both sexes of *P. laevisissimus* showed a consistent preference for smaller virgins and there is no direct evidence to suggest any interaction between the virgin and nonvirgin individuals during any of the trials.

It is important to state that given the sample size, the evidence presented here is compelling yet not definitive. Further study is needed for all combinations of virginity, size and age in both sexes before we can state with certainty that *P. laevisissimus* prefer virgin over large partners. However, our evidence is important in that there is little

indication in the literature showing a preference in spermatophore-bearing bush-crickets toward virgin partners over larger partners.

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Fig. 1. *Poecilimon laevisissimus* mating pair. Erimanthos Valley, Peloponnese, Greece, 1997. Photo by J. McCartney. See Plate III.



Fig. 2. *Poecilimon laevisissimus* bearing engorged eutrombiid mites. Erimanthos Valley, Peloponnese, Greece, 1997. Photo by J. McCartney. See Plate III.

Chapter 4

Evidence of natural and sexual selection shaping the size of nuptial gifts among a bush-cricket genus (*Poecilimon*; Tettigoniidae): an analysis of sperm transfer patterns

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Poecilimon erimanthos and *Poecilimon laevisimus* cohabitating.

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-containing ampulla are transferred into her. Two main functions of the nuptial gift have been proposed; the ejaculate protection hypothesis has evolved in a sexual selection context and predicts that the time to consume the gift is no longer than necessary to allow for full ejaculate transfer. The parental investment hypothesis 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 examine 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 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 various degrees of ejaculate protection. Controlling for body mass and relatedness, we also found that species producing larger spermatophores apparently transfer a greater proportion of available sperm than species producing smaller spermatophores, and thus result in a 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). She starts with the nuptial gift, or spermatophylax, a large gelatinous mass in many species, and then eats the smaller ejaculate-containing ampulla along with any remaining sperm and seminal fluid (Bowen *et al.*, 1984). There is current 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). Despite recent discussions concerning the effect of sexual conflict on nuptial gift size (e.g. Vahed, 2007b, Gwynne, 2008), two hypotheses remain central to understanding the role of gift size. 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 the function of the nuptial gift is derived from its nutritive value and that these nutrients are passed into the donating males' offspring. Thus the gift is under natural selection to increase the quality and/or 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), thus present research focuses on the relative importance of the two hypotheses in different taxa. It is likely that the evolutionary origin of the spermatophylax has arisen through sexual selection on ejaculate protection in bush-crickets (Gwynne, 1986, 1990, 1997, 2001), yet evidence for both functions have been suggested for the maintenance of spermatophylax size in various tettigoniid species (for reviews see Vahed, 1998; Gwynne, 2001).

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), 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, 1988b). However, further research on this species (Simmons, 1995a, 1995b; Simmons *et al.*, 1999) and different interpretations of what constitutes 'complete' sperm drainage (Vahed, 1994, 1998; Simmons, 1995a) suggest that complete sperm transfer may be close to or after gift consumption (Vahed, 1998). Additionally, males have a large 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, remating interval (Simmons, 1995b), and sexual status (Simmons *et al.*, 1993). At times, therefore, gift size approximates the size necessary for 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). 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, thus controlling, 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 family-wide variation (McCartney *et al.*, 2008). Initial observations among *Poecilimon* show spermatophore size varies between 6.1% (*Poecilimon laevissimus*) and 37% of male body mass (relative mass), (*P. thessalicus*; McCartney *et al.*, 2008), thus representing a large variation in male reproductive investment.

To date few studies have considered sperm transfer in bush-crickets to understand nuptial gift function. From these, and despite concerns over the validity of understanding mating behaviour from lab reared individuals, fewer still have considered sperm transfer patterns from field populations. Furthermore, taxa used to understand sperm transfer patterns come from a range of taxa; variations found in sperm transfer may ultimately be connected to species differences and not nuptial gift size.

Our aim here is to better understand the premise that nuptial gift size relates to function. Published data from two field-observed taxa with medium and large gifts (Reinhold and Heller, 1993, Heller & Reinhold, 1994) were combined with novel data from three field-observed *Poecilimon* taxa; two with small gifts and one with very large gifts, to understand this premise. We do this by first assessing the match between nuptial gift consumption time and optimum sperm transfer time among these five taxa that vary markedly in nuptial gift size. A close match would be consistent with the sperm protection hypothesis. If, on the other hand, complete sperm transfer occurs long before spermatophylax gift consumption is completed, we have grounds to infer a paternal investment function. Secondly, we control for body mass and relatedness, and compare 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 show that males of *Poecilimon* taxa that produce larger spermatophores have increased confidence of sperm transfer, and thus paternal assurance, compared to taxa producing small 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 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 200,000 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 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. Peloponnese), 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, normally within hours of their capture. Male and female *P. laevissimus* were captured as sub-adults and allowed to mature for seven days (but not much longer) 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 normally made at intervals rather than continuously. *Poecilimon laevissimus* and *P. thessalicus* were observed 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 completion 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 mass for *P. thessalicus* are

reported from 1998. Measurements of 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 location 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 (see above for details). 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 drained 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 was fully homogenised. A sub-sample was placed on a haemocytometer slide (Swift: Neubauer improved). Sperm from a minimum volume of 50 μ l (or up to 200 μ l) were counted and multiplied by the appropriate dilution factor to give the total

number of sperm per spermatheca. Normally, five sub-samples were taken and the solution was remixed before each new sub-sample was taken. From total sperm (ampulla + 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 with the sperm transfer pattern, 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.

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 sperm transferred 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).

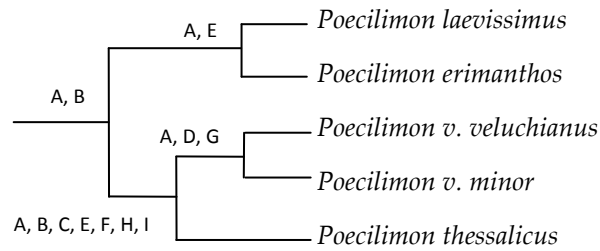


Figure 1. Cladogram representing the phylogenetic relationships between the five *Poecilimon* taxa used in this study. Letters at nodes indicate that subsequent daughter branches are based on information derived from the literature. References cited: A: Ulrich et al. in press, B Heller 1984, C. Warchalowska-Sliwa *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.

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 drain into the female (Figure 2). Only around 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 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.

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

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

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 minutes, 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).

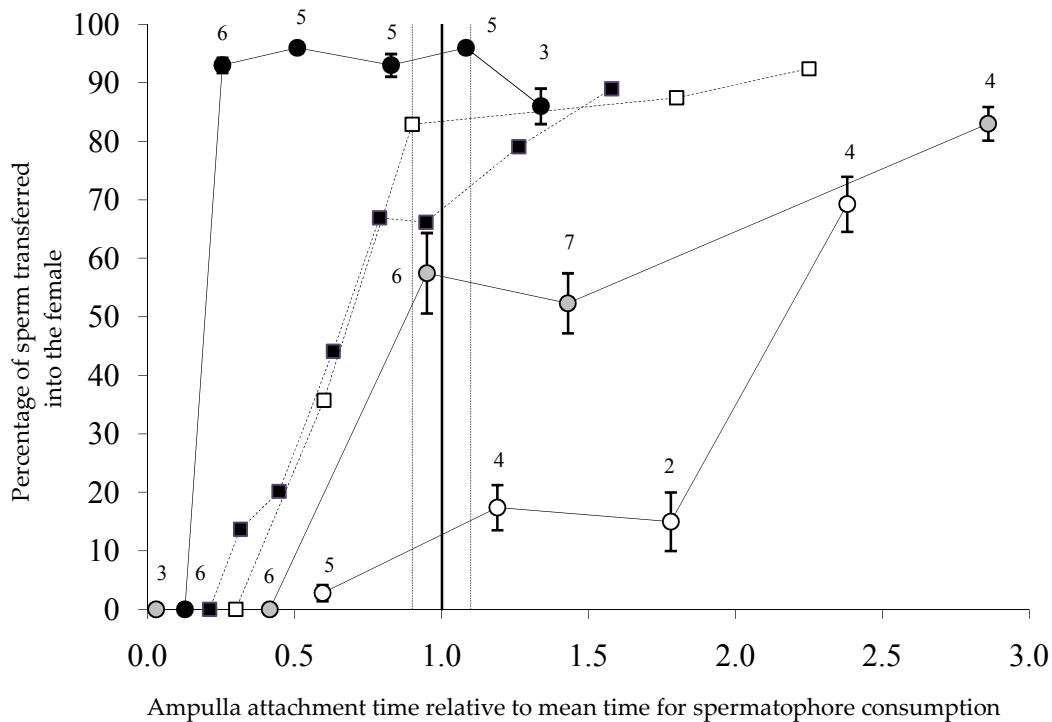


Figure 2. Percentage of sperm transferred after copulation from the male ampulla to the female spermatheca (\pm S.E.) plotted relative to the mean spermatophore consumption time, (numbers above points = n). Novel species are represented by unbroken lines: *P. laevissimus* (open circles), *P. erimanthos* (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).

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 drained 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 ($n=5$, $SE=2.9$ million, range = 2.3-17.8 million), significantly fewer than the 22.6 million ($n=22$, $SE=21$ million, range = 0.05-37.3) in spermatophores that did drain (Mann-Whitney rank analysis $U=27$, $P < 0.007$).

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.

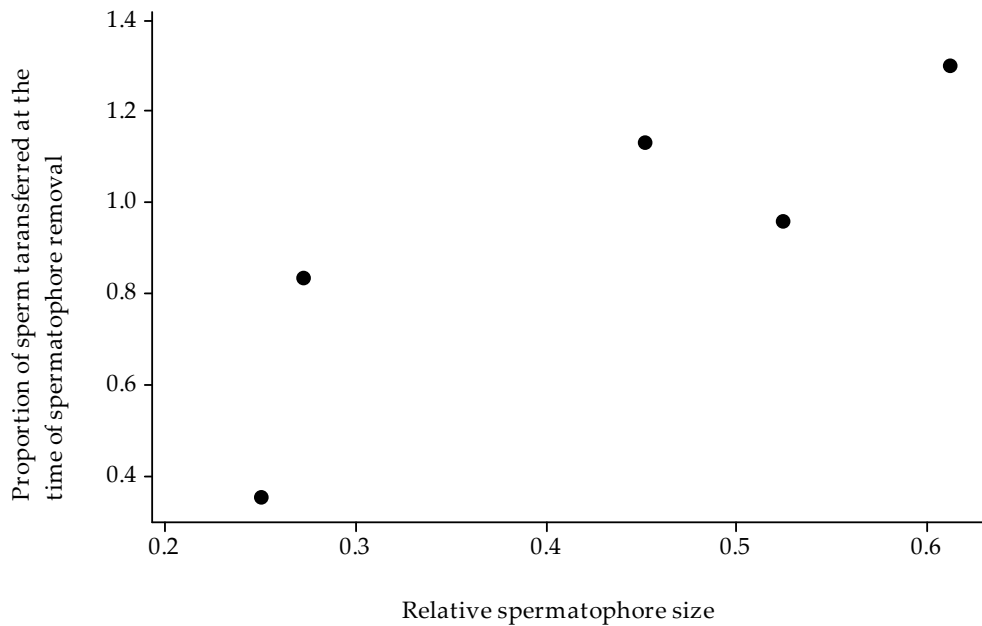


Figure 3. Spermatophore size compared to the proportion of sperm that have transferred into the female at the time of spermatophore consumption among five *Poecilimon* taxa. NB. Data are arcsine (square root) transformed.

Discussion

The percentage of sperm that had transferred into the female by the time it took 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.

Poecilimon laevis 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 a large nuptial gift 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 drains 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 are likely to be considerably 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 large 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 submitted manusc.). 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* however is likely to exclude the possibility of paternal investment; both species remate, on average, mate every 1-2 days and lay eggs every two days (McCartney submitted manusc.). 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, McCartney submitted manusc) and lay eggs every 1-2 days (McCartney submitted manusc.), 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 males in the two species that produce smaller spermatophores don't 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 (5 from 27; 18.5%). Spermatophores are expensive to produce (Dewsbury, 1982; Drummond, 1984; Simmons, 1990, 1995a; Heller and von Helversen, 1991; Vahed, 2007b), 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. laevisissimus*, *P. erimanthos* and a previously studied species, *P. hoelzeli* (R. Achmann *pers comm.*). Importantly, total sperm numbers in ampullae that did not drain were much less (by 63%) than the total number of sperm in ampulla that did drain. 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. laevisissimus*, $S=0.261$, $P=0.61$). 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, in fact, 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. laevisissimus* make significant nutritional paternal investments in their offspring. 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. 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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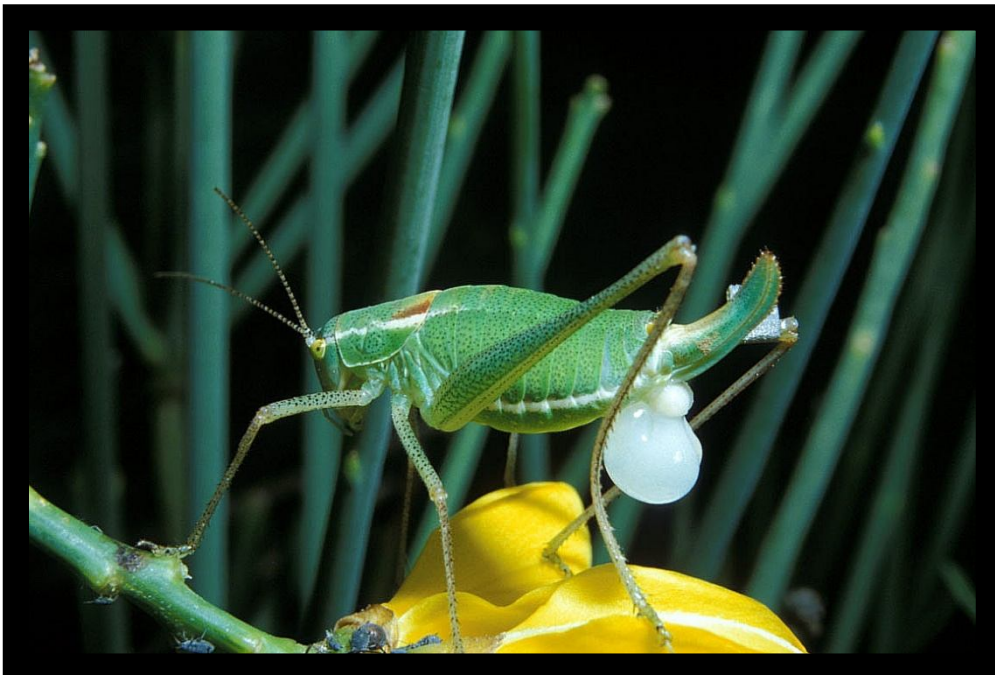
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Chapter 5

Lifetime spermatophore investment in natural populations of two closely related bush-cricket species (Orthoptera: Tettigoniidae: *Poecilimon*)

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A marked female *Poecilimon veluchianus minor*.



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Summary

Male bush-crickets transfer a substantial spermatophore to females during copulation. The spermatophore comprises a large spermatophylax and a sperm-containing ampulla which is consumed by the female while the ejaculate transfers into her. Spermatophores are costly to produce and investment trade-offs are expected to occur between the ejaculate, sperm and spermatophylax. While models of ejaculate allocation predict strategic allocation between current and potential reproductive rates, no comparative studies to date have analysed interspecific spermatophore component variation between bush-cricket species over the entire mating season. Here we compare field data from two bush-cricket species (*Poecilimon*) that differ in spermatophore investment. While *Poecilimon thessalicus* invests heavily in spermatophore production and was variable in body mass and spermatophylax size over the season, they consistently produced similar-sized ampullae and transferred constant numbers of sperm. In contrast, the ampulla size of the less investing species, *P. veluchianus minor*, varied considerably over the season, yet the body mass, spermatophylax mass and sperm number remained constant. Differences between the two species reflect within-species adjustments that male bush-crickets make to specific spermatophore components as conditional strategies in order to maximize reproductive output.

Keywords: spermatophore, spermatophylax, sperm competition, sperm number, trade-offs.

Introduction

Mating and fertilization often involve conflicts of interest and intersexual coevolutionary arms races that potentially lead to substantial fitness costs for both sexes (Arnqvist & Rowe, 2005; Lessells, 2006; Wedell et al., 2006). In some arthropods the male manufactures a nuptial gift that is eaten by the female during ejaculate transfer (Vahed, 1998a). In bush-crickets, the nuptial gift, or spermatophylax, is a large, gelatinous offering which is attached to the ejaculate-containing ampulla. Transfer of the large ejaculate is protected by the spermatophylax, which is eaten by the female while the sperm and ejaculate are transferred from the ampulla (for reviews see Vahed, 1998a; Gwynne, 2001). While these gifts confer considerable benefits to males and females, their production involves substantial fitness costs to the males and are likely the products of sexually antagonistic co-evolution (Arnqvist & Rowe, 2005; Vahed, 2007a; Gwynne, 2008; Wagner & Basolo, 2008).

Females are expected to prefer heavier males which provide more direct benefits to females in the form of larger, high-quality spermatophylax meals (Gwynne, 1982; Arnqvist & Rowe, 2005; Lehmann & Lehmann, 2008a; McCartney & Heller, 2008). As in other arthropods (Arnqvist & Nilsson, 2000), bush-cricket studies report positive effects of spermatophylax consumption on female reproductive output via increased fecundity or offspring survival (for a review see Gwynne, 2001). There is evidence for spermatophylaxes as an important source of nutrition (e.g., Bowen et al., 1984; Wedell, 1993b; Voigt et al., 2006); indeed, females may metabolise spermatophylax gift nutrients within hours of consumption (Voigt et al., 2008). Theoretically, a bush-cricket

female may obtain all her food by mating, as a single nuptial gift meets her energy requirements for 1 to 2 days (Voigt et al., 2005). Spermatophore-consuming females are also likely to be exposed to lower predation risk due to reduced foraging activity (e.g., Heller, 1992).

While larger nuptial gifts may provide benefits to females, they may alternatively be viewed as a means by which males can overcome the resistance of the female to accepting larger ejaculates (Vahed, 1998b, 2007a). Substances in the ejaculate manipulate female mating behaviour in a dose-dependent manner; longer spermatophore attachment times and larger volumes of sperm and ejaculate transferred increase the period during which the female remains unreceptive to further mating (for reviews, see Gwynne, 1997, 2001). This increase in male fertilisation success results in a lower lifetime degree of polyandry among bush-crickets (Vahed, 2006). Sexual selection should, therefore, strongly act on male bush-crickets to maintain or increase spermatophore investment.

However, substantial costs incurred by males may limit such investment. Males with relatively large nuptial gifts and ejaculates have long sexual refractory periods while they replenish the spermatophore glands (Vahed, 2007b). Previously mated males transfer smaller spermatophores than virgin males (Simmons & Bailey, 1990; Wedell, 1993b; McCartney & Heller, 2008), and spermatophore size typically increases with male age (Wedell & Ritchie, 2004; Lehmann & Lehmann, 2009) and mating interval (Simmons, 1990, 1993, 1995; Heller & Helversen, 1991; Reinhold & Helversen, 1997; Lehmann & Lehmann, 2000). Models of ejaculate expenditure (Parker & Ball, 2005) and nuptial gift allocation (Kondoh, 2001) assume that there will be a trade-off in

males between resources spent on current reproduction and lifetime mating rate. Accordingly, trade-off theory predicts that males should strategically allocate sperm and ejaculates with respect to mating status (Wedell et al., 2002; Parker & Ball, 2005; Williams et al., 2005; Cameron et al., 2007).

In the Australian bush-cricket *Requena verticalis*, differences in spermatophore mass or spermatophylax weight were observed in subsequent matings when males were fed a low nutrient diet (Gwynne, 1990; Simmons, 1993). In the large carnivorous species *Decticus verrucivorus*, where investment in the spermatophore is relatively low, there was no change in spermatophore weight over eight consecutive matings (Wedell, 1993b). In the relatively high spermatophore-investing *Ephippiger ephippiger*, the results were more differentiated. Males transferred spermatophores with similar weight in both the spermatophylax and the ampulla regardless of mating history, yet sperm number and nitrogen content was significantly reduced on a male's fourth mating, indicating that male mating history influences a male's investment over the mating season (Wedell & Ritchie, 2004). While a large variation in spermatophore component investment appears to occur among bush-crickets, the few studies that have analyzed investment patterns over repeated matings are typically laboratory based and deal with single species: comparisons between species sampled *in situ* provide additional insights into the factors that influence investment.

In order to better understand the relationship between nuptial gift size and the selective pressures that influence seasonal variation, there is perhaps no better model than the bush-cricket genus *Poecilimon* (Tettigoniidae). With around 140 described species (Eades & Otte, 2008) the variation in spermatophore size within *Poecilimon*

approximates that of the entire family (McCartney et al., 2008). Two species, *Poecilimon thessalicus* and *P. veluchianus minor*, represent the genus particularly well; both species are medium sized, flightless bush-crickets with similar ecologies and feeding habits, yet there is a large difference between the relative investments that each species make toward spermatophore production (Lehmann A.W., 1998; Lehmann & Lehmann, 2008b; McCartney et al. 2008). The spermatophore size of *P. thessalicus* represent the upper limit for bush-crickets (up to 37% of male body mass), whereas *P. v. minor* produces a medium-sized spermatophore of around 17–20% of male body mass (McCartney et al., 2008). Furthermore, associations between ejaculate transfer and nuptial gift consumption in these two species are relatively well studied: *P. thessalicus* produces a nuptial gift that is almost four times larger than necessary for complete ejaculate transfer (McCartney et al., data not shown). *Poecilimon v. minor*, on the other hand, produces a nuptial gift that is similar to the predicted size required to achieve optimal sperm transfer (Heller & Reinhold, 1994; McCartney et al., data not shown).

Our aim is to better understand investment patterns through time as they are expected to differ between species with different relative investment in spermatophore production per mating; high investing species, such as *P. thessalicus*, may be resource depleted much earlier than species with a lower per mating investment, such as *P. v. minor*. Specifically, we examine strategic male investment into the spermatophylax, ampulla and sperm of each species with respect to male mating season. To our knowledge this is the first comparative study to analyse investment patterns from the spermatophore components of males collected throughout reproductive maturity in natural populations.

Methods

Fieldwork was carried out during the summer of 1998 in mainland Greece in semi-pastoral habitats with forest margins. *P. thessalicus* were observed once every 5–7 days on five occasions between June and July 1998, at a site inland from Katerini (north-west of the village of Elatochori 40°19'N, 22°15'E). *P. v. minor* were observed once every 3–4 days on four occasions between May and June 1998 at a site near Makrakomi above the Village of Tsouka (38°57'N, 22°05'E). Both species are semelparous and have a univoltine lifecycle.

Spermatophore size

Throughout the entire adult life of each species, approximately ten field-caught males were weighed on electric scales (± 1 mg) and then randomly paired with ten field-caught females. Pairs were placed in 500-ml plastic insect chambers which typically resulted in at least five successful matings; however, only three matings occurred in *P. thessalicus* for the last two mating intervals and only four matings occurred for *P. v. veluchianus* in the first mating interval. Pairs were observed constantly until a successful mating had occurred and then we immediately removed the spermatophore. The complete spermatophore was weighed fresh (± 1 mg), followed by a separate weighing of both components; the spermatophylax and ampulla. All males and females were subsequently placed in large field cages for later experiments.

Each ampulla was macerated with a scalpel and mixed by passing it repeatedly through a syringe until the sperm had been suspended in the water and fully homogenized. 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. Normally, five sub-samples were taken and the solution was remixed before taking each new sub-sample.

The statistical analysis was performed using OpenStat Version 3 (Miller, 2008) and WinSTAT 2001.1 (Fitch, 2001). Reproductive investment is frequently coupled with male body mass, so we follow the recommendations of Darlington & Smulders (2001) and García-Berthou (2001), by entering male body mass as a covariate in the analysis of variance.

Results

Investment pattern

Males of the two bush-cricket species provided their mates with a spermatophore representing on average 24% of male body weight in *P. thessalicus* and 16% in *P. v. minor*. These are medium to large spermatophores compared to bush-crickets in general and to the genus *Poecilimon* in particular. Spermatophore and component details of each species are summarized in Table 1. Males from the *P. thessalicus* population were typically larger and heavier, and their spermatophores were typically heavier and contained more sperm than *P. v. minor* males ($t = 5.62\text{--}7.63$, $p < 0.001$ for all comparisons).

Table 1. Male body mass and reproductive investment of male *Poecilimon thessalicus* (N = 20, N = 18 for sperm number) and *P. v. minor* (N = 19) pooled over the mating season (\pm SE).

	<i>P. thessalicus</i>	<i>P. v. minor</i>
Male body mass (mg)	464.25 \pm 50.38	347.68 \pm 44.68
Wet spermatophore mass (mg)	111.50 \pm 33.42	56.00 \pm 15.93
% spermatophore vs. body mass	23.75 \pm 5.82	16.06 \pm 3.87
Wet spermatophylax mass (mg)	91.45 \pm 28.03	47.05 \pm 13.64
% spermatophylax vs. body mass	19.47 \pm 4.89	13.51 \pm 3.40
Wet ampulla mass (mg)	20.15 \pm 7.32	8.95 \pm 4.80
% ampulla vs. body mass	4.32 \pm 1.45	2.56 \pm 1.24
Sperm number ($\times 10^6$)	13.97 \pm 6.69	3.42 \pm 2.08

Male body mass was highly correlated with spermatophore mass in both species (Table 2). However, of the spermatophore components, the spermatophylax had a strong correlation with male body mass in both species (Table 2), whereas the relationship between male body mass and ampulla was relatively weak. Males of *P. thessalicus* were not only heavier than males of *P. v. minor*, but also, after correction for male body mass, invested relatively more into their spermatophylax than males of *P. v. minor* (ANCOVA: $F_{1,38} = 46.21$, $p < 0.001$). Spermatophylax investment shows a steeper increase with male body mass suggesting more pronounced selection on spermatophylax size in *P. thessalicus* (*P. thessalicus*: $y = 0.39x - 88.89$, $R^2 = 0.49$, $N = 20$, *P. v. minor*: $y = 0.15x - 6.32$, $R^2 = 0.25$, $N = 19$).

Table 2. Correlation analysis of reproductive parameters of male *Poecilimon thessalicus* ($N = 20$, $N = 18$ for sperm number) and *P. v. minor* ($N = 19$) pooled over the mating season.

	Spermatophylax (mg)	Ampulla (mg)	Sperm number ($\times 10^6$)	Male body mass (mg)
<i>P. thessalicus</i>				
Spermatophore (mg)	(0.96)	(0.60)	0.21	0.49
Spermatophylax (mg)		0.40	0.19	0.49
Ampulla (mg)			0.22	0.20
Sperm number ($\times 10^6$)				0.10
<i>P. v. minor</i>				
Spermatophore (mg)	(0.92)	(0.35)	0.25	0.28
Spermatophylax (mg)		0.12	0.14	0.25
Ampulla (mg)			0.36	0.11
Sperm number ($\times 10^6$)				0.23

The correlation of spermatophylax and ampulla mass with spermatophore mass is given in parentheses only for convenience, as both are partly autocorrelations.

In both species sperm number was highly correlated with ampulla mass but less so with spermatophylax mass (Table 2). Controlling for ampulla mass revealed no significant differences in relative sperm number between the species (ANCOVA: $F_{1,36} = 0.001$, $p = 0.98$), whereas ampulla mass was highly correlated with sperm number (ANCOVA: $F_{1,36} = 8.49$, $p = 0.006$).

Seasonality

Field-caught males differed seasonally in body mass over the mating period in *P. thessalicus* (ANOVA: $F_{4,19} = 3.84$, $p = 0.024$), but not in *P. v. minor* (ANOVA: $F_{3,18} = 0.64$, $p = 0.60$). However, given the large influence of body mass on spermatophore investments, we tested for the influence of the season by using body mass as a covariate. Spermatophylax size in *P. thessalicus* changed over the mating season (ANCOVA: $F_{4,19} = 7.72$, $p = 0.002$), increasing at the beginning, peaking in week 3 and 4 and decreasing at the end of season (ANCOVA post-hoc pair-wise comparison: $F_{4,19} >$

76.17, $p < 0.001$; $\{1 * -2 * -3 - 4 * -5\}$). In *P. v. minor* the transferred spermatophylax was similar in size over the season (ANCOVA: $F_{3,18} = 0.28$, $p = 0.84$) (Figure 1).

Ampulla weight remained relatively constant between weekly samples in *P. thessalicus* ($F_{4,19} = 0.099$, $p = 0.76$). In contrast, *P. v. minor* ampulla weight varied significantly over the season ($F_{3,18} = 4.53$, $p = 0.02$), with a strong increase in the last week (ANCOVA post-hoc pair-wise comparison: $F_{3,18} > 48.29$, $p < 0.001$; $\{1 - 2 - 3 * -4\}$) (Figure 2).

Sperm number did not differ significantly between weeks in either *P. thessalicus* (ANCOVA: $F_{4,17} = 1.01$, $p = 0.44$), or *P. v. minor* (ANCOVA: $F_{3,18} = 1.79$, $p = 0.20$). Sperm number was significantly correlated with ampulla mass in both species, however the effect was not significant in either species once ampulla mass was used as a covariate: *P. thessalicus* (ANCOVA: $F_{4,17} = 0.83$, $p = 0.40$); *P. v. minor* (ANCOVA: $F_{3,18} = 0.28$, $p = 0.84$).

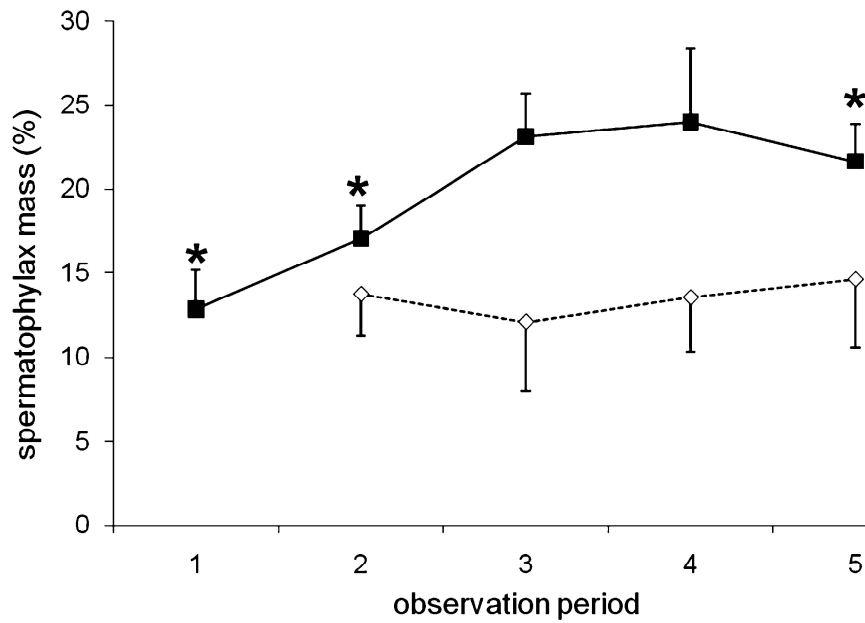


Figure 1. Relative spermatophylax mass standardized for male body mass in *P. thessalicus* (closed squares) and *P. v. minor* (open diamonds) over the mating season. Means marked with an asterisk differ significantly from all other week means.

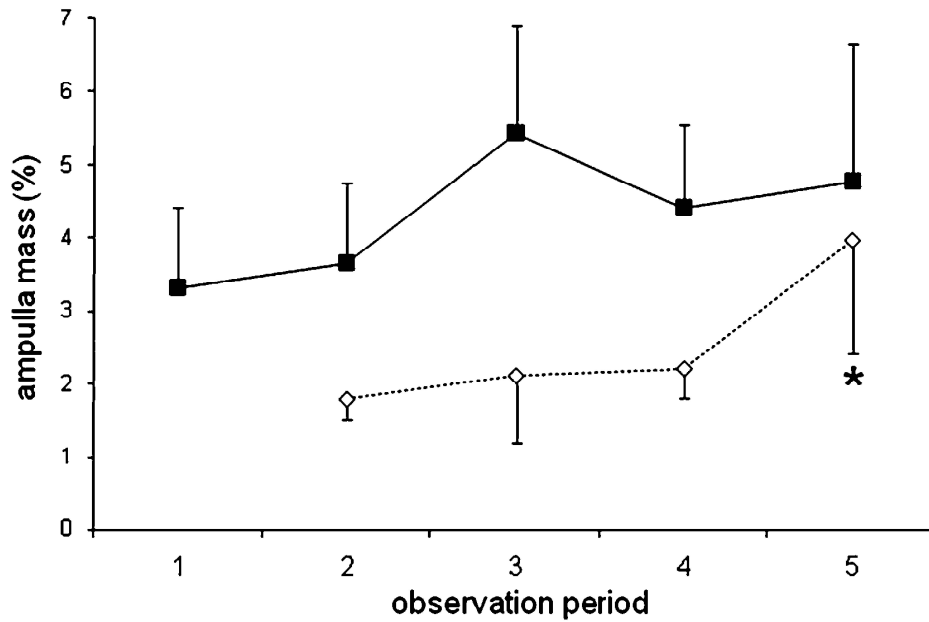


Figure 2. Relative ampulla mass standardized for male body mass in *P. thessalicus* (closed squares) and *P. v. minor* (open diamonds) over the mating season. Means marked with an asterisk differ significantly from all other week means.

Discussion

Seasonality of investment

Field-observed male *P. thessalicus* invest more in relative and absolute spermatophore size than *P. v. minor* males, yet both species have surprisingly uniform spermatophore sizes over the season. In agreement with this, spermatophylax weight was stable throughout the mating season in the less-investing *P. v. minor*. Spermatophylax size of the heavily investing *P. thessalicus*, however, seems more labile; showing remarkable variation. Sperm number was independent of season even after correcting for ampulla weight. While we had expected to find *P. thessalicus* males to be sperm depleted at the end of the season, given the large volumes compared to other members of the genus and family (McCartney et al., 2008), this was not the case. There was, however, a remarkable increase in ampulla weight without a corresponding increase in sperm number in *P. v. minor* at the end of the season; strengthening the idea that ampullae are not merely sperm containers, but size-independent under sexual selection.

Transferred spermatophylaxes were similar in size over the mating season in *P. v. minor*. This may be explained by the fact that spermatophylax size is closely coupled with sperm transfer in this species (Reinhold & Heller, 1993; Heller & Reinhold, 1994). A male benefits if he transfers a spermatophylax large enough to protect the ejaculate. Additional investment in spermatophylax size may reduce a male's capability for future matings and may select for regular modest investments (Simmons, 1995). Greater variation in spermatophore production over the mating season was found in the spermatophylax of *P. thessalicus*. The increase at the beginning of the season is

comparable to the adjustment of spermatophylaxes with increasing virgin age after eclosion in male *P. zimmeri* (Lehmann & Lehmann, 2009), another member of the *P. propinquus*-group (Lehmann, 1998). Spermatophylax size subsequently increases in relation to the male's age at first mating. These age-related differences in spermatophylax may reflect adaptive plasticity in male effort spent in current mating, holding back resources for future matings (Simmons, 1995). Such an adjustment is commonly seen as strategic investment in insects (Wedell & Cook, 1999; Engqvist & Sauer, 2001, 2002), although it may have little effect on sperm transfer in this species because the spermatophylax is much larger than necessary to ensure a full sperm transfer (McCartney et al., submitted); males could effectively decrease nuptial gift size by at least one half without compromising sperm transfer.

Spermatophore scaling

Our data show that sexual selection for a large spermatophylax might be a driving force for species-specific male body mass. Spermatophore mass scales with male body mass in both *P. thessalicus* and *P. v. minor*. The spermatophylax, as the largest component and an autocorrelate of the spermatophore, also predictably scales with male body mass in both species. This is consistent with comparative studies of both *Poecilimon* (McCartney et al., 2008) and bush-cricket species in general (Wedell, 1993a; Vahed & Gilbert, 1996; Vahed, 2006, 2007b). Similarly, comparative studies have also found that ampulla mass scales with body mass across bush-cricket species (Vahed & Gilbert, 1996; Vahed, 2006, 2007b) including within *Poecilimon* (McCartney et al., 2008; with phylogenetic control, McCartney & Heller, submitted manuscript). This correlation is found within many bush-cricket species (Wedell, 1993b; Wedell & Ritchie, 2004;

Lehmann & Lehmann, 2008), although not all (Gao & Kang, 2006a,b). The amount of variation in ampulla mass explained by male body mass is, however, relatively small. Species with ampullae that represent a higher relative proportion of male body mass show more pronounced scaling. Our results fit well into this pattern: there is a strong scaling effect between ampulla mass and male body mass in the relatively large ampulla-offering *P. thessalicus*, but only a moderate relationship in the relatively less-investing *P. v. minor*. The selection gradient within species seems to be related to the basic investment pattern; the ampulla mass of species investing comparatively less is not as influenced by male body mass and may, therefore, vary more than the ampullae of more heavily-investing species.

Spermatophylaces should be at least large enough to enable the transfer of the majority of sperm into the female (for reviews, see Vahed, 1998; Gwynne, 2001). One implication, which is strongly supported in the literature, is that spermatophylax and ampulla mass should co-vary (Vahed & Gilbert, 1996; Vahed, 2006, 2007b; Wedell, 1993a, 1994). While this correlation across 31 *Poecilimon* species was moderate (McCartney et al., 2008), in our study the components were only size-coupled in the heavier *P. thessalicus*. With respect to ejaculate protection, this raises the question: to what extent does nuptial gift function differ between species? This is hard to determine without understanding the function of the different spermatophore components. At least the selection pressure seems to follow a species-specific pattern, which strongly acts on body mass and spermatophylaces in concert, but not to such a large extent between ampulla mass and body mass. Despite comparative studies previously finding strong correlations across species between sperm number and spermatophylax size

(Vahed & Gilbert, 1996; Vahed, 2006), in terms of mating effort, spermatophylaxes have a primary ejaculate-protection function; sperm transfer time, per se, does not necessarily define spermatophylax size. Sperm number is independent of spermatophylax weight within other species (Simmons et al., 1993; Reinhold & Helversen, 1997; Gao & Kang, 2006a,b) and, at least in *Poecilimon*, sperm number does not correlate with spermatophylax size across 23 species (McCartney et al., 2008), which is reflected in a lack of correlation within the two species we observed here.

Ampulla mass and sperm number sometimes co-vary within species (Simmons & Kvarnemo, 1997; Lehmann & Lehmann, 2000), but not in all species (Simmons et al., 1993; Gao & Kang, 2006a,b). The sperm number and ampullae of both *P. thessalicus* and *P. v. minor* are highly correlated, which reflects the strong relationships previously found across 23 *Poecilimon* species (McCartney et al., 2008). The main reason for a covariance might be the fact that sperm form a major fraction of the ampulla content along with seminal substances. However, little is known about the ability of the ejaculate to manipulate female mating behaviour in bush-crickets (Heller et al., 1998, 2000) and ejaculate volume may vary independently from sperm number under strong sexual selection.

Given that both species studied here are closely related, such differences show nuptial gifts are evolutionarily labile and that sexual selection can quickly act on shaping mating systems. This large variation between the two species likely reflects within-species adjustments that male bush-crickets make to specific spermatophore components as a conditional strategy, apparently in order to maximize reproductive output. Preferential investment in spermatophore components after controlling for

body mass might reflect differences in life histories: available energy or nutrients are directed to whichever spermatophore component is more effective at increasing reproductive fitness (Voigt et al., 2005).

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Chapter 6

Sex roles in mate attraction and searching: a comparative test using bush-crickets (*Poecilimon*: Tettigoniidae)

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Poecilimon laevis being attacked by a Greek lycosid.

Abstract

Signalling by males is argued to have evolved to advertise resources important to females. However, in the absence of male resources, sexual selection on males is suggested to lead to signalling as a more risky (but profitable) way of obtaining mates than searching. The resource-advertising hypothesis may explain why long-distance male songs evolved in nuptial gift-bearing Orthoptera, such as Tettigoniidae where the food gift is a spermatophylax attached to the ejaculate. Species in the diverse genus *Poecilimon* exhibit one of two pair-formation protocols: the orthopteran-typical female-search-for-calling male, and also male-search-for-calling female (in response to the initial male call). Using field observations we test the resource-advertising hypothesis by comparing variations in pair-forming systems to variation in nuptial gift size among 32 *Poecilimon* taxa. As predicted, taxa with female-search produce significantly larger nuptial gifts than those with male-search. Our data provide the first support of the hypothesis that long-distance signalling by males evolves when males offer a substantial resource to females. We also found that male-search species produce smaller ejaculates and fewer sperm which may represent a trade-off with effort invested in mate-seeking movements, thus providing an alternative explanation for the association of small gifts with male search.

Keywords: Mate searching, nuptial gift, pair formation, resource advertising, risk shift, trade-off.

Introduction

Darwin (1871) asked why males "should invariably have acquired the habit of approaching the females, instead of being approached by them". His answer was that sexual selection on competing males (i.e. their high potential reproductive rate: Kokko & Wong, 2007) leads them to search for any distance cue indicating the presence of a sexually receptive female.

While males typically search for calling females in most mating systems that involve pheromones (e.g. many Lepidoptera), a number of animal groups show a distinct departure from Darwin's "male search" pattern (e.g. Thornhill, 1979; Alexander & Borgia, 1979; Alexander *et al.*, 1997). In most acoustically calling insects (Orthoptera and cicadid homopterans) and anurans, females typically search for the calling male, although there can be variation in which sex searches within species (Kokko & Wong, 2007) such as silent male searching for mates at high population densities (Cade & Cade, 1992). Notable exceptions to these patterns are mecopterans (scorpionflies) where females search for males that emit attracting pheromones (Byers & Thornhill, 1983).

What factors explain this variation in which sex searches? Males with their high potential reproductive rates are expected to search for females but Thornhill (1979) and Alexander & Borgia (1979) (see also Alexander *et al.*, 1997) argued that in taxa such as ensiferan Orthoptera (crickets and allies) female searching evolves if males advertise resources important to females (i.e. that enhance female reproductive fitness) such as nuptial gifts (Gwynne, 1997, 2001) or burrows (Gwynne, 1995a). Another

hypothesized influence on whether a sex signals or searches relates to the risk of each aspect of pair formation which is expected to be higher in the sex with the higher potential reproductive rate (i.e. subject to greater sexual selection). Under this risky-calling hypothesis males are expected to assume the signalling role (Thornhill, 1979; Alexander *et al.*, 1997). Acoustic signals are known to attract more natural enemies than chemical signals (Zuk & Kolluru, 1998) so in most cases lengthy bouts of calling by male anurans and cicadas is probably riskier than female searching (examples: bats attracted to calling male frogs (Tuttle & Ryan, 1981) and sarcophagid fly parasitoids attracted to cicada song (Soper *et al.*, 1976)). In contrast, pheromone calls appear to be less risky which might explain why females typically produce long-distance pheromone signals (e.g. Lepidoptera) (Alexander *et al.*, 1997). Scorpionfly (Mecoptera) mating systems support these ideas because it is the male that produces the long distance calling pheromone and also provide carrion or prey as nuptial gifts to females (Byers & Thornhill, 1983); thus resource advertisement may have selected for male calling and female searching in these insects (Thornhill, 1979).

Recent models of sexual differences in searching (Kokko & Wong, 2007) predict that the common male-search-for-female pattern evolves when there is sperm competition (i.e. multiple mating by females). Their models predict female searching only when both sperm competition is absent and when search costs are lower for females than males. Given these results Kokko and Wong (2007) dismiss the intuitive notion that opportunities for males to increase reproductive rates selects for male searching (thus avoiding high costs for missed opportunities to mate). However their results do not explain why females search for signaling males in so many acoustic

animals. Both high levels of female multiple mating (Simmons, 2001) and male resource provisioning (Gwynne, 1995a) are typical of mating in ensiferan Orthoptera with calling males. In contrast to scorpionflies, it is unclear whether resource-advertising (Alexander & Borgia, 1979; Thornhill, 1979) and/or risky-calling has selected for female searching and male calling (forewing stridulation) in most orthopteran species. The fact that an important resource – nuptial gifts of glandular secretions – appear to have evolved in the major groups of ensiferan Orthoptera before male calling (Gwynne, 1995a; also supported by the phylogeny of DeSutter-Grandcolas, 2003) provides some support for the attraction to resources hypothesis.

Studies of behavioural variation within species have also revealed that the value of glandular gifts can affect calling, searching and risk-taking. Male bush crickets (Tettigoniidae) attach a spermatophylax gift to an ejaculate-containing ampulla (Gerhardt, 1913; Boldyrev, 1915; Gwynne, 2001). When this gift is of high value, an excess of sexually-receptive females typically compete for males and may even increase the relative risk of female searching versus male calling (e.g. Gwynne & Dodson, 1983) because males greatly reduce calling (e.g. Gwynne, 1985).

Tettigoniidae are an ideal group to investigate the association between gift value and pair formation patterns because there is extensive interspecies variation not only in nuptial gift size (from 2-40% of male body mass: Wedell, 1993a, 1994a; Vahed & Gilbert, 1996; McCartney *et al.*, 2008) but also in the variation in signalling and searching. Although pairs form in most tettigoniids when the female searches for the calling male, female acoustical ‘answers’ to short male calls - with subsequent male searching - have evolved multiple times in this family (Naskrecki, 2000). Variation in

these characteristics occurs within the subfamily Phaneropterinae and especially within one large genus *Poecilimon* (Heller, 1990). Female acoustical response to an initial male call followed by male searching appears to be the ancestral state for this subfamily given the widespread occurrence of female stridulatory apparatus (Naskrecki, 2000). However, in some *Poecilimon* species females do not answer males but instead show secondary evolutionary origins of the typical ensiferan pairing protocol of female searching for the calling male (Heller, 1990, 1992; Heller & Helversen, 1991, 1993; Heller *et al.*, 2006). The wings of these females are either vestigial or are lost altogether (Heller, 1990). The presence of both male and female searching within the same genus allows a controlled examination of hypotheses concerning sex specific pressures that select for signalling and searching. The evidence to date suggests that all sexual *Poecilimon* species are polyandrous and show extensive sperm competition (Achmann *et al.*, 1992; Achmann, 1996) thus female searching resulting from a lack of sperm competition (along with higher search costs for males) (Kokko & Wong, 2007) does not explain female searching in many *Poecilimon* species.

The resource-advertising hypothesis predicts that nuptial gift (spermatophylax) size should be larger in species where females search for calling males and this is the case for two *Poecilimon* species: Heller and Helversen (1991) reported that gift size (as a percentage of male mass) for the female searches (male calls) *P. veluchianus* is almost twice that of the male searches *P. affinis*. Furthermore, results of a field study of the two species were not consistent with the assume risk hypothesis, as pair formation was far less risky for males in the female-searches *P. veluchianus* than in the male-searches *P. affinis* (Heller, 1992): survivorship of the sexes was similar in the former and

significantly higher for females in the latter. Heller (1992) was also able to relate mortality risk directly to increased movement. Thus searching appears to be more costly than calling in these two *Poecilon*.

Despite the significance of understanding the diversity of mate attraction and searching strategies in animal pairing, and the formulation of hypotheses proposed to explain this diversity (Thornhill, 1979; Alexander & Borgia, 1979), including those generated by mathematical models (Hammerstein & Parker, 1987; Ide & Kondo, 2001; Kokko & Wong, 2007), there have been no comparative empirical tests. Here we provide a test using field observations for 32 *Poecilon* taxa. We predict that nuptial gifts are larger in those species where the male is stationary and calls (female searches) compared to those with male searching (female calls).

Allocation of resources is also likely to necessitate trade-offs between reproductive activities such as mate searching and investment in gifts. There is some evidence that the calls of male tettigoniids affect their ability to invest in nuptial gifts (Bailey *et al.*, 1993; Del Castillo & Gwynne, 1997; McCartney & Heller, submitted manuscript). Nevertheless, no comparative studies have observed the effect that reproductive efforts, such as mate attraction, may have on nuptial gift size in natural populations. Thus, we also consider a third hypothesis for the association of large gift size and female search protocol, i.e. that the lack of effort put into searching movements by males allows them to invest in larger nuptial gifts. This may either be a direct result of the energy that males conserve by being stationary, or as an indirect result of the added time that stationary males have to feed and build on energy reserves for further gift investment. Larger gifts may enhance male fitness by

increasing paternity (ejaculate transfer), and/or increasing the quality and/or quantity of the male's offspring (Halliday, 1987; Simmons *et al.*, 1992; Simmons, 1993; Lehmann & Lehmann, 2008). The potentially costly (e.g. Vahed, 2007) tettigoniid spermatophylax gift protects the ampulla from being prematurely eaten by the female before the transfer of sperm and other ejaculate components from the ampulla has been completed (for reviews see Vahed, 1998; Gwynne, 2001). Thus we measure not only the gift portion of male reproductive effort but also ejaculate volume and sperm number.

Methods

Our study taxon: Poecilimon

Spermatophore size ranges between 4 and nearly 40% of male body mass across this genus, representing the upper and lower limits for spermatophore size known for all tettigoniid species (McCartney *et al.*, 2008). Within *Poecilimon* species the gift is known to both protect the ejaculate, thus enhancing paternity, and increase offspring fitness (Reinhold & Heller, 1993; Heller & Reinhold, 1994; for a review see McCartney *et al.*, 2008) and males are known to invest heavily in both spermatophore production (Heller & Helversen, 1991; Lehmann & Lehmann, 2006; Vahed, 2007; Voigt *et al.*, 2008) and mate acquisition (Heller, 1992; Heller & Helversen, 1990; Heller *et al.*, 1997).

Male body mass, spermatophore size, and sperm number

All data, except those concerning which sex searches (Table 1), were extracted from McCartney *et al.* (2008). We included data from 32 field-observed *Poecilimon* taxa (29 species). Only species that were observed in the field and had measurements of male

body mass and at least one spermatophore component were used. Typically only one observation was made for each taxon in McCartney *et al.* (2008), yet, in cases where more than one observation was made, priority for inclusion was first given to species data that included the most spermatophore component measurements (e.g. McCartney *et al.*, 2008; *P. veluchianus*), then sample size (e.g. McCartney *et al.*, 2008; *P. laevissimus*). Data concerning which sex searches as determined by whether the female has sound producing wings (see Introduction), were novel and directly observed in the field.

All data were obtained from populations in Greece, Turkey, Italy, Slovenia or the Ukraine. Field-caught individuals were separated into cages defined by status (adults/sub-adults) and sex. Juveniles were separated until at least seven days after their imaginal moult in order to ensure sexual maturity (Heller & Reinhold, 1994). Adults were separated for at least five days prior to pairing in order to ensure full receptivity (Heller & Helversen, 1991). For mating, pairs were typically placed in 500 ml containers and observed either continuously or at short intervals (e.g. five minutes) until the female carried a spermatophore. The spermatophore was subsequently removed carefully with forceps and measured at least to the nearest 1 mg. The ampulla was then dissected from the spermatophylax and both were weighed. On occasion, either the spermatophylax or the ampulla mass could not be measured; in these cases the missing datum was calculated as the difference between the full spermatophore mass and the mass from the known component.

In order to count sperm, the ampulla was sliced with a scalpel within a known quantity of water (between 1 and 5 ml). When the ampulla was fully broken down we further mixed the solution by repeatedly passing it through a syringe until the sperm

had fully homogenised. We then took a sub-sample and the sperm were counted on a field haemocytometer (Swift: Neubauer improved). Normally three sub-samples were taken and the solution was remixed each time. If there was a large variation between sub-samples or the sperm was not evenly distributed over the slide, the solution was remixed and further sub-samples were taken. Sperm from a known volume (50 μ l - 200 μ l) were counted and multiplied by the appropriate dilution factor to give an estimate of the total of number of sperm in the ampulla. For *P. mariannae* sperm counting was completed on a Coulter counter (for details of the method see Lehmann & Festing, 1998). Relative sperm number was calculated as the number of sperm per mg of mean male body mass and expressed as sperm number $\times 10^6 \text{ mg}^{-1}$.

Table 1. Male body mass and spermatophore, spermatophylax and ampulla weight and relative percentage of male body mass (rel %), sperm number and relative sperm number ($\times 10^3 \text{ mg}^{-1}$) and female acoustic response of 32 *Poecilimon* taxa.

Species	Female acoustic response	Male body mass		Spermatophore mass			Spermatophylax mass			Ampulla mass			Sperm number		
		mg	n	Mg	rel %	n	mg	rel %	n	mg	rel %	n	$\times 10^6$	$\times 10^3 \text{ mg}^{-1}$	n
<i>P. affinis</i>	Y	1328	4	201	15.1	4	170.3	12.8	4	30.9	2.3	3	4.4	3.3	3
<i>P. brunneri</i>	Y	320	9	62	20.7	1	48.0	15.0	1	14.0	3.4	1	-	-	-
<i>P. deplanatus</i>	Y	449	15	41	9.2	7	55.0	12.3	2	9.0	2.0	4	-	-	-
<i>P. erimanthos</i>	Y	650	25	47	7.2	11	42.8	6.6	13	4.1	0.6	11	0.9	1.4	19
<i>P. hamatus</i>	Y	517	5	121	22.3	4	110.0	21.3	4	11.0	2.1	4	0.2	0.4	4
<i>P. hoelzeli</i>	Y	2250	>10	387	17.2	8	381.0	12.9	1	61.0	2.0	1	13.4	6.0	3
<i>P. ikariensis</i>	Y	473	5	71	14.5	4	56.0	11.8	4	15.0	3.2	4	0.2	0.4	4
<i>P. jonicus jonicus</i>	Y	352	6	52	14.9	6	45.0	12.8	5	7.0	1.9	5	0.4	1.1	6
<i>P. j. superbus</i>	Y	306	2	57	18.6	2	-	-	-	-	-	-	0.2	0.7	4
<i>P. j. tessellatus</i>	Y	721	3	83	11.6	3	69.3	9.6	3	13.3	1.9	3	-	-	-
<i>P. laevissimus</i>	Y	781	50	48	6.1	9	44.0	5.6	7	3.7	0.5	7	0.7	0.9	7
<i>P. macedonicus</i>	Y	302	12	65	21.8	5	-	-	-	-	-	-	2.0	6.6	4
<i>P. nobilis</i>	Y	1405	6	194	13.9	6	158.4	11.3	6	35.6	2.6	9	6.6	4.7	13
<i>P. obesus</i>	Y	1869	5	247	13.4	5	209.0	11.2	4	38.0	2.1	4	4.0	2.1	10
<i>P. ornatus</i>	Y	2552	9	310	11.8	7	274.6	25.5	7	35.2	1.4	7	-	-	-
<i>P. sanctipauli</i>	Y	1355	1	337	24.9	1	316.0	23.3	2	21.0	1.6	1	2.6	1.9	1
<i>P. schmidtii</i>	Y	525	8	73	13.9	6	63.0	12.1	6	9.2	1.7	6	0.9	1.7	2
<i>P. ukrainicus</i>	Y	274	12	60	21.9	7	48.0	17.5	7	12.0	4.4	7	0.4	1.5	4
<i>P. unispinosus</i>	Y	404	2	82	20.3	2	68.0	16.8	2	14.0	3.5	2	0.9	2.2	2

Species	Female acoustic response	Male body mass		Spermatophore mass			Spermatophylax mass			Ampulla mass			Sperm number		
		mg	n	Mg	rel %	n	mg	rel %	n	mg	rel %	n	x 10 ⁶	x 10 ³ mg ⁻¹	n
<i>P. werneri</i>	Y	318	5	47	14.6	5	39.0	12.3	3	8.0	2.5	3	0.2	0.6	2
<i>P. aegaeus</i>	N	849	10	272	31.4	7	236.1	27.2	7	34.3	4.0	7	-	-	-
<i>P. amissus</i>	N	410	8	68	20.5	1	48.0	11.7	1	20.0	5.3	1	-	-	-
<i>P. ege</i>	N	568	4	168	28.7	3	140.0	24.7	3	28.0	4.9	3	11.1	19.5	3
<i>P. gerlindae</i>	N	552	9	154	29.7	9	135.0	24.5	9	19.0	3.7	9	2.4	4.3	9
<i>P. mariannae</i>	N	583	21	133	22.8	21	109.0	18.6	21	34.0	5.8	21	2.4	4.1	21
<i>P. marmaraensis</i>	N	490	8	104	21.2	7	73.0	14.9	7	31.0	6.3	7	-	-	-
<i>P. pergamicus</i>	N	174	5	53	30.4	1	44.0	25.3	1	9.0	5.2	1	2.8	16.1	1
<i>P. thessalicus</i>	N	464	20	112	24.0	20	89.0	19.2	20	30.0	4.3	20	14.0	30.2	20
<i>P. turcicus</i>	N	632	3	152	24.1	2	102.0	16.1	2	50.0	8.0	2	6.4	10.1	2
<i>P. veluchianus minor</i>	N	327	70	56	17.1	19	47.0	14.4	19	9.0	2.7	19	3.4	10.4	19
<i>P. v. veluchianus</i>	N	710	1	182	25.6	1	145.0	20.4	1	37.0	5.3	1	10.4	14.6	50
<i>P. zimmeri</i>	N	818	91	146	17.8	91	-	-	-	-	-	-	28.4	39.9	5

Analysis

By comparing species within a single genus we control, to a certain extent, for similarities that may be caused by relatedness (Harvey, 1991; Harvey & Pagel, 1991). Ideally, phylogenetic signal should be accounted for in order to fully understand the relationship between pair-forming behaviour and spermatophore size variation across species (Harvey & Pagel, 1991; Gwynne, 1995b; Vahed & Gilbert, 1996). While a gain in female searching has apparently occurred independently in *Poecilimon* at least three times (*P. propinquus* group, *P. pergamicus* group, and the *P. heroicus* group: Heller, 1990, 1992; Heller & Helversen, 1993; Heller *et al.*, 2006; Heller, unpubl. data), insufficient data currently exist on the phylogeny and spermatophore size of these groups for a comparative analysis using phylogenetically independent contrasts of *Poecilimon* taxa that differ in searching behaviour. However, while accounting for ancestry and body mass among a smaller group (23) of *Poecilimon* species for which phylogenetic relationships are known, a recent study (McCartney & Heller, submitted manuscript) found a significant relationship between spermatophylax mass and ampulla mass. Thus, there is a strong indication that spermatophore component sizes are not confounded by ancestry and are evolutionarily labile and responsive to selection. Furthermore, the variation in spermatophore size within *Poecilimon* approximates variation within the Tettigoniidae as a whole (McCartney *et al.*, 2008). Given this degree of variation of species within a single genus, evolutionary history appears to have had little influence in preventing change (Harvey & Pagel, 1991).

A well-documented relationship exists between male body mass and the spermatophore components in *Poecilimon* (McCartney *et al.*, 2008; McCartney *et al.*, in

press) and must be accounted for in analyses to obtain data on the relative allocation to the nuptial gift. Thus, to give relative component mass, all morphological data were first \log_{10} transformed, then residuals calculated for male body mass versus spermatophore mass, spermatophylax mass, ampulla mass, and sperm number. Following Ruxton (2006), we calculated the central tendency of the species using an unequal variance, one-way *t*-test on the residuals.

Results

In support of the resource-advertising hypothesis, spermatophylaxes (nuptial gifts) in the female-search (for calling male) group were proportionally larger (mean 20% of male body mass) than those in which there is male searching (13% of male body mass) (Table 2).

In support of the alternative hypothesis that males trade-off energy utilised in mate attraction for spermatophore production, although not inconsistent with the resource- advertising hypothesis, stationary males of *Poecilimon* taxa in which females search produce significantly larger spermatophores in proportion to their body mass (24% of male body mass: stationary male spermatophore mass, mean 0.114, SE 0.025) than searching males (16% of male body mass, mean -0.07, SE 0.035). Stationary male species also produce significantly larger ampullae in proportion to their body mass (5% of male body mass: mean 0.171, SE 0.029, and seven-fold more sperm (mean proportional sperm number 16.6×10^5 , mean 0.177, SE 0.055 than those in which males

search (mean proportional ampulla size 2% of male body mass; mean -0.10, SE 0.054: mean proportional sperm number 2.4×10^5 : mean -0.10, SE 0.055), (Table 2)).

Table 2. Difference in body mass residuals of spermatophore, spermatophylax and ampulla size and sperm number between groups of males that respond phonotactically to females and females that respond phonotactically to males in 32 *Poecilimon* taxa.

	Searching males			Stationary males			<i>t</i> -obs	<i>d.f.</i>	<i>p</i> -value
	<i>n</i>	\bar{x}	SE	<i>n</i>	\bar{x}	SE			
Spermatophore mass	20	-0.07	0.035	12	0.114	0.025	4.25	1,29	<0.001
Spermatophylax mass	18	-0.07	0.034	11	0.111	0.029	3.98	1,26	<0.001
Ampulla mass	18	-0.10	0.054	11	0.171	0.032	4.39	1,25	<0.001
Sperm number	16	-0.10	0.055	9	0.177	0.055	3.45	1,20	0.001

NB. *d.f.* for one-way T-test are based on unequal variance not group size (Ruxton, 2006)

Discussion

Compared to groups of singing animals such as anurans, cicadas and other acoustic Orthoptera, there is substantial variation within the genus *Poecilimon* in which sex does most mate searching. Our results show that *Poecilimon* species where the female searches for a calling male have proportionally larger spermatophores than species in which the male searches. The main nuptial gift portion of the spermatophore, the spermatophylax, is also significantly larger in the former group. This is the first comparative evidence in support of the hypothesis that long-distance signalling by males evolves when males offer a substantial resource to females (Thornhill, 1979; Alexander & Borgia, 1979). Large gifts are likely to be of higher value than simply their size alone: comparative studies of tettigoniid species in general showed that larger

spermatophylaces are higher in protein and are associated with greater fecundity in females (Wedell, 1994b). The alternative risky-calling hypothesis is that sexual selection has led to the more risky calling role of males in pair formation. However, field studies of sex differences in mortality in two *Poecilimon* species are consistent with the hypothesis that continuous calling by the male is not more risky than searching (Heller, 1992).

What about other hypotheses for our finding an association between sedentary male calling and larger gift size? One possibility is that sexual selection within choruses of competing male callers (Alexander, 1975) led to the evolution of larger gifts as a way to attract more mates. This does not appear to be a general explanation for the evolution of a spermatophylax gift within ensiferan orthopterans because phylogenetic analyses of this suborder (Gwynne, 1995a; Desutter-Grandcolas, 2003) support the hypothesis that the origin of this gift, widespread in several related families in the suborder Ensifera, preceded the origin of male calling in tettigoniids and haglids (Gwynne, 1995a). Moreover, there are a large number of tettigoniid species with both female search (for a sedentary calling male) and relatively small spermatophylax gifts (Gwynne, 2001). This is inconsistent with the suggestion that a reversion to the standard ensiferan female search strategy in *Poecilimon* taxa would have selected for large gifts.

A further possibility, one consistent with our main hypothesis that male calling advertises the large gift, is that within *Poecilimon* the secondary origin of female searching may have led to an increase in gift size through sexual selection, via female choice for material benefits (Gwynne, 2001). This is likely if proteinaceous food in the

environment was scarce (see Gwynne, 1993), which seems to be the case for some *Poecilimon* species where densities and food availability vary between years and between and within populations (McCartney *et al.*, 2008). Once male calling evolved, female mate choice and male resource advertising, coupled with increased sexual selection for a larger spermatophylax to protect the transfer of a larger ejaculate from premature consumption by the female thus enhancing paternity, collectively selected for greater gift size. A protection role for the gift has been supported for tettigoniids (both focal and comparative studies: Gwynne, 1986a; Wedell & Arak, 1989; Simmons & Gwynne, 1991; Reinhold & Heller, 1993; Wedell, 1993a; Heller & Reinhold, 1994; Vahed & Gilbert, 1996) including *Poecilimon* (Heller & Reinhold, 1994; Reinhold & Heller, 1993; McCartney *et al.*, 2008; McCartney *et al.*, 2010). Indeed, our results show that *Poecilimon* species with searching females have seven-fold more sperm than those with searching males. This compares to a 2.5x greater ampulla size but only a 25% larger gift size. Protection of the ejaculate in *Poecilimon* would explain the strong relationship between gift size, sperm ampulla size and number of sperm, thus supporting the contention that larger spermatophylax gifts result in greater paternity assurance (Wedell, 1991, 1994a, b) by transferring more sperm (or refractory-inducing substances: Wedell, 1993a; Vahed, 2006).

Either paternity advantages or increased male fitness via additional gift nutrients invested into their own offspring (Wickler, 1985; Gwynne, 1986 a, b) predict reproductive investment into larger gifts. Therefore, while not mutually exclusive with the resource-advertising hypothesis, another explanation for our findings is that the association of small gifts with male-search pair formation is a consequence of the

energetic cost of searching, i.e. a trade-off. The spermatophylax gift appears to be the most energetically costly component of the spermatophore. For example, in *Poecilimon* male mating frequency is restricted by the production of larger gifts (e.g. Reinhold & Helversen, 1997), and gift size is reduced when males are infected with parasites (Lehmann & Lehmann, 2000). Furthermore, the higher proportions of costly proteins and nutrients in large spermatophores, (Wedell, 1994b; Heller *et al.*, 1998, 2000; Voigt *et al.*, 2006) indicate that the spermatophylax size is likely to be compromised by searching effort.

Ultimately, the resource-advertising hypothesis can be separated from sexual-selection on calling males and trade-off hypotheses in that resource-advertising predicts that large spermatophylax gift size in *Poecilimon* species originated before male calling, which appears to be the case with the origin of male calling in the tettigoniid-haglid clade of orthopterans (Gwynne, 1995a; Desutter-Grandcolas, 2003). In contrast, increased sexual selection leading to spermatophylax elaboration, or search effort being channelled into a similar elaboration, predict that male calling evolved first. An examination of transition states of pair-formation protocols within *Poecilimon* awaits additional data from other species and the development of a full phylogeny for this speciose genus.

Indeed our analysis would be improved with phylogenetic information to control for ancestry rather than an analysis of taxa within a single genus. However, comparing closely related species that are ecologically similar, as we have done here, provides us with an approximation of the extent to which a character has been influenced by relatedness (Harvey & Pagel, 1991).

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Chapter 7

Is there evidence of a macro-evolutionary trade-off between reproductive investments in mate attraction and nuptial gift size in bush-crickets?

Jay McCartney and Klaus-Gerhard Heller



Male *Poecilimon laevissimus* calling on a fern.

Abstract

During mating, male bush-crickets attract females with intense acoustical calls and transfer costly nuptial gifts. This gift functions primarily in ejaculate protection although its nutrients may also be invested into the donating male's offspring. While selection favours larger, more expensive gifts, the energy required for production must also be traded-off against alternative reproductive efforts such as mate attraction. Thus, any potential reproductive advantages conferred through larger gifts may be compromised. Controlling for phylogeny across 37 closely related, field-observed *Poecilimon* taxa, we first examine the ejaculate protection hypothesis by testing the relationship between nuptial gift size and ejaculate size. Secondly, we assess whether males partition energy between nuptial gifts and three measures of mate attraction (syllable number, impact number and peak carrier frequency). In support of ejaculate protection, we found a positive relationship between nuptial gift and ejaculate size. Surprisingly, nuptial gift size was positively correlated with syllable and impact number but negatively correlated with peak carrier frequency. While meaningful energetic relationships between carrier frequency and gift size are difficult to interpret, we show that species investing more in gifts also invest more in mate acquisition; there seems to be little support for a trade-off between gift size and calling effort.

Keywords: Ejaculate protection, energetic partitioning, mate attraction, trade-off, *Poecilimon*

Introduction

During mating, male bush-crickets transfer a substantial spermatophore to the female. The nuptial gift, or spermatophylax, constitutes the bulk of the spermatophore and is attached to the smaller ejaculate-containing ampulla (Gerhardt 1913; Boldyrev 1915). The nuptial gift is consumed by the female and protects the ejaculate ampulla from being prematurely removed while the sperm and ejaculate transfer into her spermatheca (for reviews see Vahed 1998; Gwynne 2001). Large variation in nuptial gift size exists between species (Wedell 1993a, 1994a; Vahed and Gilbert 1996; McCartney et al. 2008) and two main hypotheses have been proposed to explain this variation. Under the ejaculate protection hypothesis, nuptial gifts are sexually selected and function to optimise ejaculate transfer. The gift size or consumption time is predicted to correspond to sperm number, ejaculate volume (typically measured as ampulla mass), or optimum ejaculate transfer time (Sakaluk 1984; Wedell and Arak 1989; Reinhold and Heller 1993; Wedell 1993a, 1994a, b; Heller and Reinhold 1994; Vahed and Gilbert 1996; McCartney et al. 2008; McCartney et al. 2010). Under the paternal investment hypothesis large nuptial gifts are naturally selected as they contain a significant stock of nutrients (Wedell 1994a, b; Heller et al. 1998, 2000; Voigt et al. 2006, 2008), water (Reinhold and Heller 1993; Ivy et al. 1999), and other substances, which pass into the donating male's offspring (Gwynne 1988; Reinhold and Heller 1993; Reinhold 1999; Heller et al. 1998, 2000). This may result in a nuptial gift size that is larger than necessary to allow for a full complement of sperm to transfer into the female (e.g., Gwynne 1986a; McCartney et al. submitted manusc.).

While large spermatophores are costly to produce, there is strong selection on gifts to be large as they facilitate the transfer of larger ejaculates and induce longer non-receptive refractory mating periods in females (e.g., Vahed 2006; for reviews see Vahed 1998; Gwynne 2001). This gives greater fertilisation success to the donating male because the female typically lays eggs fertilised by the last male during this non-receptive period (Parker 1970; Gwynne 1986b; Wedell and Arak 1989; Simmons and Gwynne 1991; Achmann et al. 1992; Wedell 1993a; McCartney submitted manuscript). Larger nuptial gifts also enhance oviposition onset and rate (Wedell and Arak 1989) or enhance females directly by supporting female survival (Wickler 1994; Voigt et al. 2005, 2006, 2008). Furthermore, environmental nutrient availability may alter the urgency with which females require spermatophylax nutrients and thus place a further premium on nuptial gift size and quality (Parker and Simmons 1989; Gwynne 1985, 1990; Simmons 1988a; Simmons and Gwynne 1991). The spermatophylax therefore may act in a dose-dependent fashion to maximise ejaculate transfer and, if paternity assurance is high, nutrient investment into the female and offspring. As such, nuptial gifts are under strong selection from sperm competition and paternal investment to be large (for reviews see Vahed 1998; Gwynne 2001).

Activities associated with reproduction can be energetically demanding (e.g., Simmons et al. 1992; Simmons 1993). Resources employed in reproduction are often limited and trade-offs between activities are likely to be made in order to optimise a net gain in reproductive fitness (Halliday 1987; Simmons et al. 1992; Lehmann and Lehmann 2008; McCartney et al. 2010). Due to the high production costs of the nuptial gift, it has been suggested that males attempt to conserve gift size by reducing nuptial

gift quality (Wedell 1994a), mating frequency (Simmons 1988b; Gwynne 1990; Simmons and Bailey 1990; Simmons et al. 1992; Heller and Reinhold 1994; Reinhold and Helversen 1997; although see Wedell 1993b), and energy allocated to calling (Simmons et al. 1992; McCartney et al. submitted manuscript.). There is evidence that nuptial gift size is constrained by the male's available energy reserves in crickets (Simmons et al. 1992; Lehmann and Lehmann 2000a, 2000b; Cueva del Castillo and Gwynne 1997; Vahed 2007). The majority of research to date has focussed on the consequences of variation in nuptial gift size on paternity; the effects that alternative reproductive efforts have on nuptial gift size have largely been ignored. Significant efforts made in mate attraction may affect a male's ability to invest in nuptial gift size (Simmons et al. 1992); any advantages that large spermatophores confer over sperm competition and paternal investment may therefore be lost (Simmons et al. 1992). The trade-off between gift size and attraction effort is likely to be pronounced in the bush-cricket genus *Poecilimon*, where males use high-energy calls to attract females (Heller 1984, 1990, 2006) but also donate large, expensive nuptial gifts for the female to consume (McCartney et al. 2008).

Around 140 taxa of the bush-cricket genus *Poecilimon* have been formally described (Eades and Otte 2008). In the genus, spermatophores range between 4% and nearly 40% of male body mass which represents the upper and lower extremes of spermatophore size found in bush-crickets (Tettigoniidae) (Vahed and Gilbert 1996; McCartney et al. 2008). Evidence of both functions of the nuptial gift have been observed within *Poecilimon* (Reinhold and Heller 1993; Heller and Reinhold 1994; Reinhold 1999; for a review see McCartney et al. 2008) and Spermatophore production

can thus be very costly for some males, both in direct energetic investment (Voigt et al. 2006, 2008) and in future reproductive potential (Heller and Helversen 1991; Lehmann and Lehmann 2000a, b, 2006; Lehmann et al. 2001). Furthermore, male *Poecilimon* also invest heavily in mate acquisition (Heller 1992; Heller and Helversen 1990, 1993; Heller et al. 1997) by initiating mating through emitting expensive acoustic signals (e.g., Heller 1990).

Three main measures of calling cost are typically considered when understanding orthopteran signalling (e.g. Prestwich and Walker 1981, Prestwich and O'Sullivan 2005): (1) total calling bout duration, (2) wing stroke rate and (3) length of the stridulatory file. Based on these, we use syllable number per day, impact number per day and peak carrier frequency, to estimate energetic investment in mate attraction across 37 *Poecilimon* taxa. Our first measure of calling cost; number of syllables (the number of stridulatory movements) per day, is a combined measurement of calling bout duration and wing stroke rate. Each syllable is composed of a series of sound impulses which are produced by discrete tooth impacts from the file each time the wing is closed. Under otherwise similar conditions, the total number of these impulses may also affect calling cost. Thus, our second measure of calling cost is the impact number per day (= syllable number per day x file tooth number). The third measure used here, peak carrier frequency (PCF), may also be costly, but this is only assumed from narrow-band resonant-singing tettigoniid species where the carrier frequency is related to the wing movement (Montealegre-Z 2008). While measurements of PCF have previously been used to assess overall calling expenditure in resonant-singing Orthoptera (e.g., Cueva del Castillo and Gwynne 2007; Montealegre-Z 2006, 2009),

there are currently no mechanisms that can reliably explain variations in energy expenditure with PCF across broadband (non-resonant singing) species such as *Poecilimon* (Montealegre-Z 2008). Higher carrier frequencies may not only be produced independent of body size but may not require more energy to produce. However, in at least two bush-crickets: *R. verticalis* (Bailey et al. 1993) and *Kawanaphila nartee* (Gwynne and Bailey 1988), higher frequencies are preferred by females; therefore, in the hope of better understanding the relationship, if any, between carrier frequency and spermatophore size, we included a test of this relationship among *Poecilimon* taxa.

We control for phylogeny and body mass in field populations of 30 *Poecilimon* species (37 taxa) to first test nuptial gift function, i.e., if evolutionary changes in nuptial gift size correspond with evolutionary changes in ampulla size or sperm number. We then analyse the effect of syllable number per day, impact number per day, and peak carrier frequency on spermatophore size and test whether males that invest more in mate attraction invest less energy in spermatophore production.

Methods

Male body mass, spermatophore size, and sperm number

For the most part, body mass and spermatophore mass data, including spermatophylax and ampulla mass and sperm number were selected from McCartney et al. (2008). From this dataset we chose to include only 33 observations of the 62 contained there so that there was just one set of observations from each taxa (Table 1). The data were selected using the following prioritisation: 1) field observations rather than laboratory observations); 2) observations where there was the highest number of

variables available (specifically including, but not limited to, male body mass and at least one further spermatophore component (i.e., spermatophylax mass, ampulla mass or sperm number), (e.g., *P. v. veluchianus*); 3) largest sample size available. One species, *P. mytilenensis*, has an extraordinarily large ampulla which could significantly bias the analysis (Heller et al. 2004; McCartney et al. 2008) and was therefore removed. Body mass, spermatophore mass and PCF data from four additional species were added (*P. anatolicus* [Northern Greece, 20 vi 1985], *P. ebneri* [Northern Greece, 1989 and 1990], *P. elegans* [Italy, Mte. Gargano, 22 vi.1992] and *P. thoracicus* [Northern Greece, 24 vi 1986]) all collected by Dagmar von Helversen (unpublished records), making a total of 37 species available for analysis. Data on syllable number, impact number and PCF were either novel and presented here, or are from Heller (1988), Willemse and Heller (1992), Heller and von Helversen (1993), Heller et al. (2006), or Lehmann et al. (2006) (Table 1).

Table 1. Male body mass, the masses of spermatophore, spermatophylax and ampulla (expressed as raw weights and as percentages of male body mass - rel %), sperm number and sperm per mg of body mass, syllables per day, impact number per day and peak carrier frequency of 37 *Poecilimon* taxa.

	Male body mass		Spermatophore mass			Spermatophylax mass			Ampulla mass			Sperm number				Impact number		PCF
Species	mg	n	mg	rel %	n	mg	rel %	n	mg	rel %	n	x 10 ⁶	x 10 ³ mg ⁻¹	N	Syl/d	teeth	/d x10 ³	(kHz)
<i>P. aegaeus</i>	849.0	10	272.0	31.4	7	236.1	27.2	7	34.3	4.0	7	-	-	-	-	-	-	-
<i>P. affinis</i>	1328.0	4	201.0	15.1	4	170.3	12.8	4	30.9	2.3	3	4.4	3.3	3	1649	225	371	22
<i>P. amissus</i>	410.0	8	68.0	20.5	1	48.0	11.7	1	20.0	5.3	1	-	-	-	-	-	-	-
<i>P. anatolicus</i>	602.0	1	129.0	21.4	1	-	-	-	-	-	-	-	-	-	-	-	-	26
<i>P. brunneri</i>	320.0	9	62.0	20.7	1	48.0	15.0	1	14.0	3.4	1	-	-	-	-	-	-	45
<i>P. deplanatus</i>	449.0	15	41.0	9.2	7	55.0	12.3	2	9.0	2.0	4	-	-	-	-	-	-	34
<i>P. ebneri</i>	362.0	21	77.0	21.3	8	-	-	-	-	-	-	-	-	-	-	-	-	27
<i>P. ege</i>	568.0	4	168.0	28.7	3	140.0	24.7	3	28.0	4.9	3	11.1	19.5	3	-	-	-	-
<i>P. elegans</i>	332.0	4	80.5	24.2	4	-	-	-	-	-	-	-	-	-	-	-	-	41
<i>P. erimanthos</i>	650.0	25	47.0	7.2	11	42.8	6.6	13	4.1	0.6	11	0.9	1.4	19	7201	100	720	40
<i>P. gerlindae</i>	552.0	9	154.0	29.7	9	135.0	24.5	9	19.0	3.7	9	2.4	4.3	9	61018	37	2258	25
<i>P. gracilis</i>	530.0	6	102.0	16.7	6	-	-	-	-	-	-	-	-	-	4626	148	685	33
<i>P. hamatus</i>	517.0	5	121.0	22.3	4	110.0	21.3	4	11.0	2.1	4	0.2	0.4	4	-	-	-	32
<i>P. hoelzeli</i>	2250.0	10	387.0	17.2	8	381.0	12.9	1	61.0	2.0	1	13.4	6.0	3	2378	173	535	15
<i>P. ikariensis</i>	473.0	5	71.0	14.5	4	56.0	11.8	4	15.0	3.2	4	0.2	0.4	4	-	-	-	32
<i>P. j. jonicus</i>	352.0	6	52.0	14.9	6	45.0	12.8	5	7.0	1.9	5	0.4	1.1	6	-	-	-	40
<i>P. j. superbus</i>	306.0	2	57.0	18.6	2	-	-	-	-	-	-	0.2	0.7	4	-	-	-	37
<i>P. j. tessellatus</i>	721.0	3	83.0	11.6	3	69.3	9.6	3	13.3	1.9	3	-	-	-	-	-	-	-
<i>P. laevisimus</i>	781.0	50	48.0	6.1	9	44.0	5.6	7	3.7	0.5	7	0.7	0.9	7	1882	90	169	36
<i>P. macedonicus</i>	302.0	12	65.0	21.8	5	-	-	-	-	-	-	2.0	6.6	4	-	-	-	57
<i>P. mariannae</i>	583.0	21	133.0	22.8	21	109.0	18.6	21	34.0	5.8	21	2.4	4.1	21	91880	60	5513	28
<i>P. marmaraensis</i>	490.0	8	104.0	21.2	7	73.0	14.9	7	31.0	6.3	7	-	-	-	-	-	-	30
<i>P. nobilis</i>	1405.0	6	194.0	13.9	6	158.4	11.3	6	35.6	2.6	9	6.6	4.7	13	2207	117	258	24
<i>P. obesus</i>	1869.0	5	247.0	13.4	5	209.0	11.2	4	38.0	2.1	4	4.0	2.1	10	-	-	-	17

	Male body mass		Spermatophore mass			Spermatophylax mass			Ampulla mass			Sperm number				Impact number		PCF
Species	mg	n	mg	rel %	n	mg	rel %	n	mg	rel %	n	x 10 ⁶	x 10 ³ mg ⁻¹	N	Syl/d	teeth	/d x10 ³	(kHz)
<i>P. ornatus</i>	2552.0	9	310.0	11.8	7	274.6	25.5	7	35.2	1.4	7	-	-	-	-	-	-	20
<i>P. pergamicus</i>	174.0	5	53.0	30.4	1	44.0	25.3	1	9.0	5.2	1	2.8	16.1	1	-	-	-	35
<i>P. sanctipauli</i>	1355.0	1	337.0	24.9	1	316.0	23.3	2	21.0	1.6	1	2.6	1.9	1	-	-	-	15
<i>P. schmidtii</i>	525.0	8	73.0	13.9	6	63.0	12.1	6	9.2	1.7	6	0.9	1.7	2	-	-	-	23
<i>P. thessalicus</i>	464.0	20	112.0	24.0	20	89.0	19.2	20	30.0	4.3	20	14.0	30.2	20	-	-	-	30
<i>P. thoracicus</i>	502.0	4	107.4	21.4	4	-	-	-	-	-	-	-	-	-	-	-	-	33
<i>P. turcicus</i>	632.0	3	152.0	24.1	2	102.0	16.1	2	50.0	8.0	2	6.4	10.1	2	-	-	-	27
<i>P. ukrainicus</i>	274.0	12	60.0	21.9	7	48.0	17.5	7	12.0	4.4	7	0.4	1.5	4	-	-	-	37
<i>P. unispinosus</i>	404.0	2	82.0	20.3	2	68.0	16.8	2	14.0	3.5	2	0.9	2.2	2	-	-	-	31
<i>P. v. minor</i>	327.0	70	56.0	17.1	19	47.0	14.4	19	9.0	2.7	19	3.4	10.4	19	-	-	-	27
<i>P. v. veluchianus</i>	710.0	1	182.0	25.6	1	145.0	20.4	1	37.0	5.3	1	10.4	14.6	50	9954	67	667	25
<i>P. wernerii</i>	318.0	5	47.0	14.6	5	39.0	12.3	3	8.0	2.5	3	0.2	0.6	2	20045	87	1744	36
<i>P. zimmeri</i>	818.0	91	146.0	17.8	91	-	-	-	-	-	-	28.4	39.9	5	8210	45	369	25

Syllable and impact number per day and PCF

Data for the number of syllables per day were taken from Heller and von Helversen (1993) (*P. propinquus* of that paper is now named *P. gerlindae*). Impact number per day was calculated by multiplying syllable number per day with the number of teeth on the stridulatory file. This number is an overestimate of the impact number but we assume that a similar percentage of the file is used in all species. These teeth numbers were either taken from Heller (1988) or from Lehmann et al. (2006; *P. gerlindae*). Both the published (Heller 1988) and novel peak carrier frequencies presented here are from species recorded in the laboratory using a Racal store 4D tape recorder with Brüel and Kjaer 4133 and 4135 microphones (frequency response flat up to 40 resp. 70 kHz). The male songs were then digitised on a computer. Sound analysis was conducted using the program Amadeus (Apple). Digitised recordings of some species are available at the taxonomic database Systax (<http://www.biologie.uni-ulm.de/systax>).

Phylogenetic construction

Previously published and unpublished data were compiled for 34 species of *Poecilimon* (37 taxa) to place the species into a phylogenetic tree (Figure 1). The basal nodes of the cladogram are taken mainly from Ulrich et al. (in press), Heller (1984, 1990), and Warchalowska-Sliwa et al. (2000) as these papers present the most extensive coverage of *Poecilimon* phylogeny (see Figure 1 caption for a full list of references used). However, where appropriate, recent analyses were preferred to older analyses when there were more characters analysed or where the analyses had used DNA data (Figure 1). DNA-sequence data and/or morphological character analyses were used in

preference to cyto-taxonomic analyses using C-banding patterns, chiasma frequency and chromosome character morphology, because the latter tend to change quickly (Warchalowska-Sliwa et al. 2000).

Comparative analyses

The relationships between the contrasts in male body mass, spermatophore component (spermatophylax mass, ampulla mass and sperm number), and mate attraction (syllable and impact number and PCF) were analysed using the comparative method of non-direction phylogenetically independent contrasts (PICs; Felsenstein 1985). All morphological data were normalised using \log_{10} transformations prior to analysis.

In order to account for male body mass, correlation coefficients between independent contrasts of male body mass and seven male reproductive investment parameters - spermatophore size, spermatophylax mass, ampulla mass, sperm number, syllable and impact number and PCF - were calculated using least-squares regressions. All analyses revealed a significant relationship except those between male body mass and syllable and impact number (Table 2). These results indicated that subsequent analyses involving the five male reproductive investment parameters; spermatophylax mass, ampulla mass, sperm number and PCF needed to account for body mass. Subsequently, all paired comparisons between spermatophore components (spermatophylax mass, ampulla mass and sperm number) and PCF were investigated using the phylogenetic residuals taken from the regression of that component over male body mass (Table 2). Spermatophore mass clearly autocorrelates with its components (spermatophylax mass, ampulla mass and sperm number) and because the gift itself – the spermatophylax – along with the ampulla are the important

components in question, spermatophore mass was not used in subsequent analyses with other spermatophore or mate attraction parameters.

For phylogenetic independent contrast analyses the data set of each paired comparison was cropped to account for missing data from either component under analysis. From these data a tree was re-constructed using the computer package PDAP of Mesquite Software (Maddison and Maddison, 2006) following the relationships of the standard phylogenetic hypothesis presented here. \log_{10} transformed data were added to the pruned 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 branch length, Felsenstein 1985). Analyses of variance were then used to regress the standardised PICs of each reduced data (except those for syllable number and impact number) set against male body mass to obtain independent residual contrasts. These were then regressed against the independent residual contrasts of the second trait being tested to test the fit of the slope. All inferential regressions involving PICs were forced through the origin (Garland et al. 1992). All regressions were performed using Minitab 15.

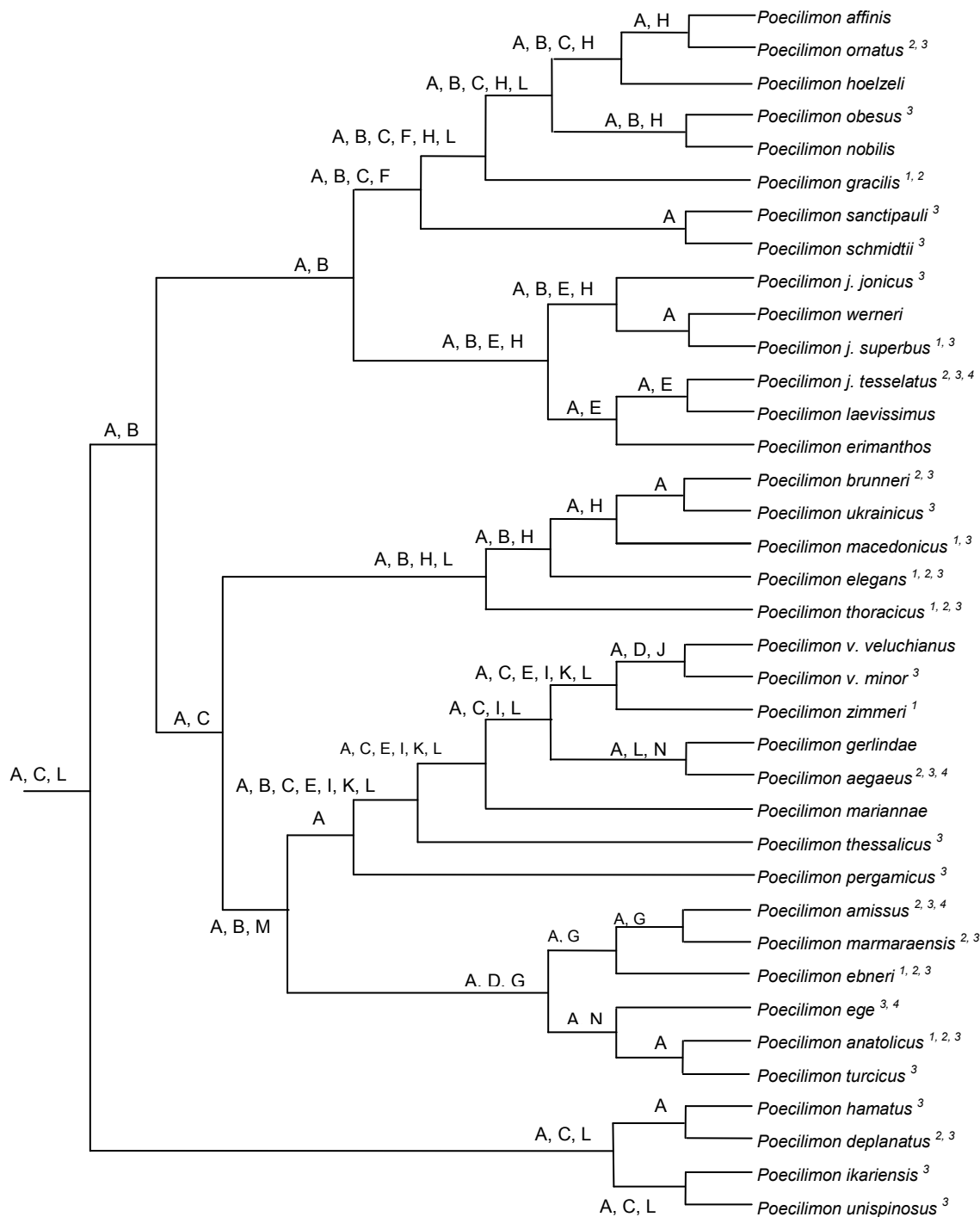


Figure 1. Cladogram representing the hypothesized phylogenetic relationship between 37 *Poecilimon* taxa. Letters at nodes indicate that subsequent branches are based on information derived from the literature. References cited: A: Ulrich et al. in press, B Heller 1984, C. Warchalowska-Sliwa et al. 2000, D. based on species geographic location, E. Willemse & Heller 1992, F. Heller & Sevgili 2005, G. Heller & Lehmann 2004, H. Heller 1988, I. Heller 2006, J. Heller & Reinhold 1992, K. Heller 1990, L. Lehmann 1998, M. Lehmann et al. 2006, N. Ünal 2005. NB. ¹ species without spermatophylax or ampulla data, ² Species without sperm number data, ³ species with syllable /day, teeth and impact number, ⁴ species without peak carrier frequency data.

Results

Positive correlations were found between contrasts of male body mass and contrasts of the spermatophore and its components (spermatophylax mass, ampulla mass and sperm number) (Table 2). Contrasts in male body mass were not significantly correlated with contrasts in syllable or impact number but were negatively associated with contrasts in PCF (Table 2). After removing the effects of male body mass and phylogenetic signal, the hypothesis of an ejaculate protection function of the nuptial gift was supported since the residual contrasts of spermatophylax mass and ampulla mass were correlated significantly with residual sperm number. Residual contrasts of spermatophylax mass and ampulla mass were significantly correlated to residual contrasts of syllable and impact number, and negatively correlated to residual contrasts of PCF.

Residual contrasts of syllable number and impact number both had strong positive relationships with residual contrasts in nuptial gift size suggesting no energetic partitioning between nuptial gift investment and investment in mate attraction. To the contrary, our results suggest that males that invest greater amounts in nuptial gift size complement this reproductive investment with increased investment in calling rates. Interestingly, we also found a significant negative relationship between peak carrier frequency and gift size.

Further strong positive relationships were also found between ampulla mass and male mate calling (syllable number and impact number), indicating a corresponding increase in ampulla volume investment and greater investment in mate

attraction – yet there was no corresponding relationship between residual contrasts in sperm number and residual contrasts in syllable number or impact number.

The only association we observed with sperm number was a moderate negative relationship with PCF, which was also negatively associated with ampulla mass. We also observed a significant negative relationship between residual contrasts in sperm number and residual contrasts in PCF. However, keeping in mind that it is currently difficult to relate energetic principles of carrier frequency production to body size between species, we found little, if any evidence to suggest there is a trade-off between reproductive investments within *Poecilimon*.

Table 2. Phylogenetically independent standardised contrasts of male body mass and spermatophore components (spermatophylax, ampulla, sperm number) and mate calling (syllable number, impact number and PCF) and residual contrasts in spermatophore components (adjusted for body size) with each other and with PCF, syllable number, and impact number. Significant contrasts are shown in bold.

Relationship	Hypotheses	F-statistic	p value	r ² -value	df
1	Male body mass/spermatophore mass	28.42	<0.001	0.45	35
2	Male body mass/spermatophylax mass	57.67	<0.001	0.68	27
3	Male body mass/ampulla mass	17.22	<0.001	0.40	27
4	Male body mass/sperm number	10.44	0.004	0.32	23
5	Male body mass/syllable number	3.59	0.095	0.31	9
6	Male body mass/impact number	2.73	0.137	0.26	9
7	Male body mass/PCF	-17.26	<0.001	-0.37	31
8	Spermatophylax mass/ampulla mass	24.63	<0.001	0.49	27
9	Spermatophylax mass/sperm number	8.98	0.007	0.32	20
10	Spermatophylax mass/syllable number	40.15	0.001	0.87	7
11	Spermatophylax mass/impact number	15.72	0.007	0.72	7
12	Spermatophylax mass/PCF	-20.93	<0.001	-0.49	23
13	Ampulla mass/sperm number	25.04	<0.001	0.57	20
14	Ampulla mass/syllable number	16.75	0.006	0.74	7
15	Ampulla mass/impact number	8.99	0.024	0.60	7
16	Ampulla mass/PCF	-16.47	<0.001	-0.43	23
17	Sperm number/syllable number	1.58	0.249	0.19	8
18	Sperm number/impact number	0.57	0.474	0.08	8
19	Sperm number/PCF	-10.79	0.004	-0.34	22

Discussion

In support of the ejaculate protection hypothesis, a significant positive relationship was observed between nuptial gift and ampulla mass. *Poecilimon* nuptial gifts are sexually selected to enhance sperm transfer and have potential, when large, to provision the donating male's offspring (Wedell 1994a). However, little if any evidence was found to support the prediction that energy used for mate attraction negatively impacted nuptial gift size and its efficacy in ejaculate transfer or offspring provisioning. In fact, syllable number and impact number were positively associated with gift size: i.e. males that spent greater amounts of energy attracting mates actually produced relatively larger gifts, while the least likely mate attraction parameter predicted to affect spermatophore production; that between peak carrier frequency and gift size, resulted in a strong significant negative correlation. In terms of energetic partitioning, however, a meaningful relationship between gift size and PCF is currently inexplicable. As a result, partitioning of energy required for alternative reproductive efforts does not appear to occur across *Poecilimon*. Contrary to our predictions, a positive macro-evolutionary trend across *Poecilimon* is evident: males that invest more in mate attraction apparently retain sufficient residual energy to invest optimally in nuptial gift and ampulla size and are resultantly able to invest unimpeded in ejaculate protection.

A positive association between nuptial gift and ampulla size is considered evidence of ejaculate protection across a range of bush-cricket taxa (e.g., Wedell 1993a, 1994b; Vahed and Gilbert 1996) including *Poecilimon* (McCartney et al. 2008, 2010). While our results demonstrate that investments in ejaculate are coupled with

investments in mate attraction, the precise effect that corresponding changes in mate attraction and gift size have on the ability of the spermatophylax gift to function as paternal investment is not easy to predict. Larger nuptial gifts containing higher proportions of protein are predicted to have a paternal investment function (Wedell 1993a, 1994b). *Poecilimon* males produce larger spermatophores on average than males in other bush-cricket clades (McCartney et al. 2008) and there is both direct and indirect evidence of paternal investment in this genus (Reinhold and Heller 1993; Reinhold 1999; McCartney et al. 2008; McCartney et al. submitted manusc.). Furthermore, larger nuptial gifts increase the latency period before the female's next mating (Vahed 2006, 2007) and the quantity of eggs fertilised by the donating male (Wedell 1994b; McCartney submitted manusc.). Because males which invest most in mate attraction have larger gifts, it is likely that these males gain the added benefits of paternal investment, transferring larger, better quality gifts that raise female nutrient reserves (Voigt et al. 2005, 2006, 2008) and provision a higher number of offspring from the donating male (Wedell 1994b). At least two *Poecilimon* species have been found with high last-male sperm precedence (*P. veluchianus*; Achmann et al. 1992 and *Poecilimon hoelzeli*; Achmann 1996); it is therefore likely that males with larger gifts benefit from the coupled association between increases in mate attraction and gift size. Combined with the support we have for an ejaculate protection function, these results support the disruptive selection ideas put forward by Wedell (1994b) who proposed that there are two types of gift: those that are large and high quality, serving to protect ejaculate transfer as well as provision offspring, and those that are small, less nutritious and serve solely to protect sperm transfer. Our results indicate that the former are

‘significant resources’ that are accordingly advertised and may help ultimately discriminate between those gifts that are solely sexually selected and those that are additionally influenced by paternal investment (McCartney et al. 2008; McCartney et al. submitted manuscript). Given this, we conclude with caution, that larger gifts of *Poecilimon* likely influence offspring survival over and above any function in ejaculate protection.

With respect to energetic partitioning, the positive relationships that syllable and impact number have with nuptial gift and ampulla size are quite clear. The number of syllables produced is considered as a reliable indicator of energy consumption. The negative relationships we observed between peak carrier frequency and nuptial gift and ampulla size and sperm number imply that males with greater investments in carrier frequency have less energy to invest in spermatophore production. This conclusion, however, remains tentative for at least two reasons: peak carrier frequency is largely determined by the resonating properties of the mirror (such as length of the mirror frame; Sales and Pye 1974), and is therefore not predictably responsive to the energetic movements required to produce sound, i.e., increasing the rate of calling does not change the carrier frequency. Second, carrier frequency may vary significantly between species independently of body size; *Poecilimon tschorochensis*, for example, has unusually large wings and produces an unusually low carrier frequency for this genus (Heller 2006). Small animals are typically limited to producing high carrier frequencies unless specific physiological mechanism have evolved, such as the physiological “spring” mechanism found in some tropical narrow-band Ensifera (e.g., Montealegre-Z et al. 2006; Montealegre-Z 2009). The energy

relationship between body mass and carrier frequency is complex, and, at this stage, it is premature to predict with certainty the relative impact that carrier frequency may have on energy reserves and the resulting consequences that it may have on spermatophore components between species. This relationship therefore cannot currently be quantified in a meaningful way and is perhaps more likely the result of a third untested variable.

Given the positive association between mate attraction and nuptial gift investment in *Poecilimon* males, and the fact that trade-offs are expected among costly components of reproductive investment (Simmons et al. 1992; Stearns 1992; Bailey 1993), how are males able to invest effectively in both larger gifts and mate attraction? The notion of a trade-off requires males to be resource-limited. Results showing partitioning or decreased investments in gift production are typically conducted under controlled laboratory conditions on males that are fed low-quality diets (e.g., Gwynne 1990; Simmons et al. 1992). Results from field observed males, such as those presented here, indicate that species producing large gifts may not necessarily be resource limited (McCartney et al. 2010) – at least for the majority of the mating season – and that nuptial gifts are in fact more responsive to sexual selection pressures exerted by local females (McCartney et al. submitted manusc.). Further support for a lack in resource limitation may also be seen in the fact that while energy is required to produce the higher syllable numbers, *Poecilimon*, in general, produce relatively low syllable rates. Conocephaline bush-crickets, for example, produce syllable numbers one or two magnitudes higher than *Poecilimon* species and typically produce relatively smaller nuptial gifts (Walker 1983; Heller 1993). Given this, *Poecilimon* males may be more

likely to 'advertise' greater resources – larger gifts – at a relatively low cost (McCartney et al. submitted manuscript.). Operational sex ratios in large gift-giving bush-cricket species often fall closer to unity (Gwynne 1991; Heller 1992) due to the lengthened time-out that males require to regenerate spermatophores reserves (Heller and von Helversen 1991; Vahed 2007; McCartney submitted manuscript.). Given the concomitant increase in female inter-mating period due to larger gifts, greater paternal insurance may be secured by the gift-donating male than may be obtained by offering smaller gifts.

In this context, the assertion that males emitting lower carrier frequencies also produce relatively larger spermatophylax gifts is not in conflict with our findings that males investing in larger nuptial gifts also invest higher amounts of energy in mate attraction. Due to less attenuation of the male call, low carrier frequencies travel greater distances and likely attract a greater number of better quality mates from greater distances. Males producing lower frequencies therefore may take further risks in attracting predators over greater distances (Heller 1992). Furthermore, *Poecilimon* males that produce relatively larger gifts apparently wait for females to approach, whereas the males of species with smaller nuptial gifts approach acoustically-responding females and incur greater movement costs (Heller 1992; McCartney et al. submitted manuscript.). It seems likely that males with large gifts may also advertise significant resources with lower carrier frequencies and that there is no trade-off in energy between carrier frequency and gift size, only a risk-shift between attracting predators in male pair-forming phonotaxis to attracting predators in mate calling.

Our data reveal that greater syllable number and impact number increase along with greater investments in nuptial gift size. While a predictable relationship between the energy invested in carrier frequency and gift size is difficult to interpret, increases in gift size are predicted under the advertising resources hypothesis (Alexander 1979; Thornhill 1979; McCartney et al. submitted manuscript) and males apparently benefit from greater ejaculate transfer and paternal investment. An ultimate test of this set of interactions would lie in establishing whether males that do invest more in mate attraction and gift size do indeed have greater reproductive fitness than the males of species that invest relatively less in both reproductive efforts. While we can confirm that a positive macro-evolutionary relationship exists between nuptial gift size and mate attraction, a better understanding of the functions of carrier frequencies and the limitations to produce them across different bush-cricket taxa is needed before we can fully understand their connection to nuptial gift function.

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Chapter 8

Larger nuptial gifts increase male per-mating fitness across a bush-cricket genus (*Poecilimon*), but do they “manipulate” females?

Jay McCartney



Female *Poecilimon laevissimus*; ready for conflict.

Abstract

The evolutionary measure of any reproductive investment ultimately lies in reproductive fitness. Male bush-crickets transfer a substantial spermatophore-gift to females during mating. Sexual conflict theory predicts that larger spermatophores are expected to increase male fitness while decreasing female fitness. Despite recent literature predicting sexual conflict in gift-giving insects, these sex-coupled responses have not yet been empirically tested in relation to spermatophore size variation across species. Here, in order to understand the fitness effects of spermatophore size variation, I analyse the relationships between spermatophore size, female longevity and offspring fitness (egg mass, number and hatching success), across five closely related, field-observed, *Poecilimon* bush-crickets that vary markedly in spermatophore investment. Controlling for body size, polyandry and relatedness, males of taxa that produce relatively large spermatophores fertilised more eggs, of a greater overall mass, and, in particular, obtained relatively more hatching per-mating than taxa producing smaller gifts. However, no relationship between relative spermatophore size and female longevity, relative lifetime production of egg number, egg mass or hatching success was found across females. I conclude that while males of large spermatophore-bearing taxa receive greater fitness, conflicts of interest are unlikely because overall female fitness remains unaffected.

Keywords: Hatching success, Egg number, Egg size, Fitness, *Poecilimon*, Sexual conflict.

Introduction

Conflicts of interest are predicted in species where males and females invest differentially in reproduction (reviewed in Arnqvist & Rowe 2005; Gwynne 2008; Vahed 2007a). Males from a diverse range of invertebrate taxa offer material gifts to the female during mating (Thornhill & Alcock 1983; Vahed 1998, 2007a), and these can range from non nutritious seed fluff or silk gifts, to expensive material donations that offer large direct benefits (reviewed in Gwynne 2008; Vahed 1998, 2007a). Nuptial gifts in bush-crickets (Tettigoniidae) are costly to produce; ranging from 2% to 40% of male body mass, they are perhaps more variable in size than in any other insect taxon (Vahed 1998; McCartney *et al.* 2008), and thus best characterise the complex relationship between gift investment and sex-specific offspring fitness.

Male bush-crickets transfer a substantial spermatophore to females during mating (Wedell 1994a,b; Gwynne 2001; Vahed 2007b). The spermatophore is comprised of a large spermatophylax (nuptial gift) which is consumed by the female while the ejaculate from the other component, the smaller sperm-containing ampulla, transfers into her (Gerhardt 1913; Boldyrev 1915). Previously protected by the spermatophylax gift, the ampulla is subsequently consumed by the female, along with any remaining sperm and ejaculate (reviewed in Bowen *et al.* 1984; Gwynne 2001). Two non-mutually exclusive hypotheses have been proposed to explain nuptial gift function: the nuptial gift may be selected to protect ejaculate transfer, or selected as paternal investment if nutrients are passed from the gift into the donating male's offspring. Most evidence suggests that gifts primarily protect the ejaculate during transfer (Wedell 1993a; Vahed

& Gilbert 1996; reviewed in Vahed 1998; Gwynne 2001; McCartney *et al.* 2008), although gifts in some species may additionally act to increase offspring fitness (e.g., Wedell 1994a,b; Reinhold 1999; reviewed in Vahed 1998; Gwynne 2001; McCartney *et al.* 2008).

While environmental selection pressures and competition, coupled with the costs associated with manufacturing larger spermatophores, have traditionally been used to account for spermatophore size variation (reviewed in Gwynne 2001), males stand to gain substantial reproductive benefits through the transfer of larger spermatophores. Both focal and comparative studies show that larger spermatophylax gifts and/or their corresponding larger ejaculate volumes (Wedell 1993a; Vahed & Gilbert 1996; Vahed 2006; McCartney *et al.* 2008; McCartney *et al.* 2010) are positively correlated with female mating interval (Wedell 1993a; Heller & Reinhold 1994; Sakaluk *et al.* 2006; Vahed 2006; Lehmann & Lehmann 2000a,b). This increases the time that females spend laying eggs sired by the donating male, as well as increasing fertilisation success and enhancing female egg production (reviewed in Vahed 1998; Gwynne 2001). Larger gifts may also confer greater direct benefits to females; females are expected to prefer large males which provide larger, high-quality gifts (Wedell 1994b; Gwynne 1982; Arnqvist & Rowe 2005; Lehmann & Lehmann 2008; McCartney & Heller 2008; McCartney *et al.* 2008). Theoretically, females could receive their entire lifetime nutrient requirements from spermatophore meals (Voigt *et al.* 2005).

Given the clear volume of evidence showing larger gifts to be beneficial, why then do some species produce small spermatophores? Recently, attention has turned to the sexual conflict that may arise over the sex-specific costs of spermatophore

components (reviewed in Chapman *et al.* 2003; Arnqvist & Rowe 2005; Sakaluk *et al.* 2006; Wedell *et al.* 2006; Vahed 2007a; Gwynne 2008). Larger ejaculate volumes may induce a female's mating rate to drop below her optimal level to the detriment of her fitness (reviewed in Arnqvist & Nilsson 2000; Arnqvist & Rowe 2005; Gwynne 2008; Vahed 2007a,b). Polyandry (an increase in mating partners) in gift-giving insects may increase direct material and indirect genetic benefits in females. In a meta-analysis, Arnqvist and Nilsson (2000) observed species with nuptial feeding, and found polyandry positively influenced female longevity and egg production. Furthermore, ejaculates from large spermatophores may reduce female lifespan (e.g., Wedell *et al.* 2008). Increases in genetic benefits from multiple matings may include the acquisition of genetically superior males and/or sperm, which can lead to an increase in egg number or egg and embryo viability (reviewed in Ridley 1988; Tregenza & Wedell 1998; Arnqvist & Nilsson 2000; Jennions & Petrie 2000; Simmons 2001, 2005; Zeh & Zeh 2003; Garcia Gonzales & Simmons 2005; Hosken & Snook 2005; Gwynne 2008; Vahed 2007a).

In practice, however, it is difficult to tease apart the effects these mechanisms – paternal investment, ejaculate protection and sexual conflict – have on spermatophore size, and it remains unclear as to how variations in male spermatophore investment influence male and female fitness (Gwynne 2008; Vahed 2007).

Two important questions concerning the governing principles of spermatophore size variation and its influence over sex-specific reproductive fitness remain unanswered: 1) Do males of species that produce larger gifts and ejaculates gain greater fitness, as a result of their larger investment, than males of smaller

spermatophore-producing species? 2) Do large male spermatophore investments negatively influence the quality or quantity of the offspring that females produce? Only a detriment to female fitness can implicate sexual conflict (Gwynne 2008).

The genus *Poecilimon* is an ideal model taxon in which to study this relationship for a number of reasons: evidence for both paternal investment and ejaculate protection have been observed in *Poecilimon* (Reinhold & Heller 1993; Heller & Reinhold 1994; Reinhold 1999; McCartney *et al.* 2008; McCartney *et al.* 2010; McCartney *et al.* submitted manusc.), and the sperm utilisation patterns of each species observed in this study have previously been considered (Achmann *et al.* 1992; Reinhold & Heller 1993; Heller & Reinhold 1994; Achmann 1996; McCartney *et al.* 2008; McCartney *et al.* 2010; McCartney & Heller submitted manusc.). Furthermore, two *Poecilimon* species, *P. v. veluchianus* (Achmann *et al.* 1992) and *P. hoelzeli* (Achmann 1996), show last male precedence patterns.

To address the two questions above, I use observations from field populations of five closely related *Poecilimon* bush-cricket taxa. I use the complete spermatophore mass as the measure of male investment rather than its components independently (spermatophylax-gift and the ejaculate-ampulla). The sizes of both components are highly correlated within and among *Poecilimon* species (Reinhold & Heller 1993; Heller & Reinhold 1994; McCartney *et al.* 2008; McCartney *et al.* 2010). In practice it is often difficult, and, in a sexual conflict framework, perhaps meaningless, to separate the effects of each; the combined effects of both components potentially work in concert to influence female remating behaviour and variations in sex-specific offspring fitness.

To address the first question I control for body mass and relatedness to test whether male per-mating investment translates directly into a higher quantity or quality of offspring. I expect the males of larger spermatophore-producing species to sire a greater quantity and/or a better quality of offspring (measured here as the number of eggs laid per mating, egg mass per mating and the number of eggs hatching per mating). To address the second question, I first compare the field mating frequency with spermatophore size in order to determine whether male spermatophore size does indeed influence female mating latency. I then compare interspecific variations in spermatophore size with female longevity (reproductive lifespan duration), and total female lifetime reproductive success (total number of eggs laid, total egg mass, and total number of eggs hatched).

While these questions have recently received attention (reviewed in Vahed 1998, 2006, 2007a,b; Arnqvist & Nilsson 2000; Gwynne 2001, 2008), this is the first comparative test of the relative effects of spermatophore size on male and female fitness among gift-bearing species. These findings are discussed with reference to sperm precedence and spermatophore size variation.

Methods

Poecilimon

The genus *Poecilimon* Fischer-Waldheim (1853) is a barbistine bush-cricket (sf: Phaneropterinae, tribe barbistini) belonging to the family Tettigoniidae (Orthoptera: Ensifera). Around 140 species have been described (Eades & Otte 2008) with at least 72 European species mostly situated in the east Mediterranean (Otte 1997). Five *Poecilimon* taxa: *P. laevissimus*, *P. erimanthos*, *P. veluchianus* minor, *P. veluchianus veluchianus*, and *P. thessalicus*, were deemed appropriate to quantify lifetime reproductive success in *Poecilimon* because they represent the full range of spermatophore size variation found within Tettigoniidae (McCartney *et al.* 2008). Field populations were used in order to avoid artifacts caused by artificial rearing (Maklakov *et al.* 2005; Simmons *et al.* 2007; McCartney *et al.* 2008). The species used are quite similar in terms of behaviour and life history patterns with the exception of *P. erimanthos* where the female detaches the spermatophylax from the ampulla with her mouth before consuming it (most other *Poecilimon* consume it piecemeal directly while the ampulla is in place in the genital pore). *Poecilimon erimanthos* is also predominantly active during the day; all other *Poecilimon* observed here are primarily nocturnal. All species have a univoltine lifecycle and obligate diapause.

Data collection, body mass and spermatophore mass

Data for male body mass and spermatophore mass (relative and absolute) were taken from field studied species presented in McCartney *et al.* (2008). The standard errors for

these data (not presented in McCartney *et al.* (2008)), were calculated and presented here along with the original data (Table 1).

Data on female body mass, egg mass, field-mating frequency, egg laying frequency, numbers of eggs/batches laid and hatching success were obtained concurrently and from the same natural populations as the data presented in McCartney *et al.* (2008). All data (except female body mass for *P. v. veluchianus* and *P. erimanthos*) were collected from Greece during the summer months of May, June and July of 1997 (*P. laevissimus*, *P. erimanthos* and *P. thessalicus*) and 1998 (*P. thessalicus*, *P. v. veluchianus* and *P. v. minor*) (see McCartney *et al.* (2008). Female body mass for *P. v. veluchianus* and *P. erimanthos*, were collected from the same populations but from previous years (1989 and 1990 respectively). Relative and absolute spermatophore mass for *P. thessalicus* (taken in 1997) was also added here (see McCartney *et al.* 2010) using the same protocol as those described for males in McCartney *et al.* (2008).

Table 1. Female and male body masses, and spermatophore mass, of five different *Poecilimon* taxa.

Body and spermatophore mass	<i>P. laevissimus</i>			<i>P. erimanthos</i>			<i>P. thessalicus</i>			<i>P. v. veluchianus</i>			<i>P. v. minor</i>		
	n	mean	SE	n	mean	SE	n	mean	SE	n	mean	SE	n	mean	SE
Male body mass (g)	50	0.78	0.01	25	0.65	0.01	48	0.44	0.01	107	0.66	0.01	70	0.33	0.06
Female body mass (g)	50	0.85	0.02	10	0.83	-	50	0.56	0.01	206	0.74	0.01	50	0.43	0.01
Spermatophore mass (g)	9	0.04	0.01	11	0.05	0.00	20	0.12	0.01	10	0.16	0.01	19	0.06	0.00
Relative spermatophore mass (% MBM)	50	6.1	-	11	7.2	-	17	33.42	0.02	10	24.5	-	19	17.1	-

Population mating frequency

To determine female mating frequency at least 150 female adults (up to 300) of each species, (except for *P. thessalicus* 1997, where no sub-adults were found upon reaching the population), were taken from the field typically on the day or the day after the female population started undergoing their imago moult. Adult individuals were weighed and marked with individual numbers and reflective tape to facilitate night recapture (for details of the marking technique see Heller & Helversen 1990). The animals were then re-released into the original site.

The time that it took females to consume the spermatophore in the field was recorded, (except for *P. erimanthos* which had been measured previously in 1990), to determine the minimum time interval to use between field mating-frequency observations (*P. laevissimus*, 101min (n=12, SE 10.7); *P. erimanthos* (1990), 90min (n=39); *P. thessalicus* (1997), 930min (n=24, SE 48.8); *P. v. minor*, 399min (n= 6, SE. 74.8); *P. v. veluchianus*, 590min (n=24, SE 46.9)). Field populations were then checked periodically within the minimum spermatophore consumption interval throughout the entire mating season. All numbered females that were located in each observation were scored for the presence of a spermatophore, an ampulla only, or neither (for details see Heller & Helversen (1990)). These observations were made nightly for *P. thessalicus* (1997) and *P. laevissimus* and three-nightly for *P. v. veluchianus*, *P. v. minor* and *P. thessalicus* (1998). Observations on the diurnally active *P. erimanthos* were also made every three days.

Mating frequencies were calculated by taking the number of females observed each evening with a new spermatophore or ampulla and dividing by the total number

of females observed. The mean mating proportion was then taken for all observations to give the mean daily population mating frequency. Modifications were made to this basic plan depending on the species. For example, observations made early in the mating season, early at night or late in the morning, or during excessive wind or rain were excluded to ensure that the mating observations were made in peak season and in optimal conditions. Due to the risk of over- or under-estimating mating frequency in *P. laevissimus* (where the minimum checking time was close to or shorter than the time it took to check the population), only two full observation nights which clearly showed typical mating frequencies were used (for further details on mating frequency observations see Heller & Helversen (1990)). Number of female lifetime matings was estimated as the population mating frequency multiplied by the mature female life expectancy (see below).

Mature female longevity

The dates that bush-crickets from each population started their first imago moults were noted. The female lifespan was determined from the first 50 marked females used in the mating frequency observations. For all species, except *P. v. veluchianus*, longevity was recorded as the number of days from the beginning of the population imago moult to the day that each of these females was last recorded, adding half of the interval between the last observation when each female was seen alive and the next observation. Females do not become sexually receptive until several days after their final moult so the receptive period was estimated by recording the date that the majority of females started to mate. In all cases this date was clear because the mating frequency went from less than 10% in an observation period to a (near) maximum

mating frequency in the next. This was further substantiated with sperm counts from the spermatheca of five randomly chosen females from each population.

Large population movements after marking and subsequent lack of detailed data in 1998 for *P. v. veluchianus* precluded analysis on these data. Instead I used longevity data from Heller & Helversen (1990) that were collected from the same population and in the same manner as described above. Heller & Helversen (1990) estimate female longevity to be around 15 days and results from the present study indicate females take around four days to reach sexual maturity from the last moult, meaning that female reproductive life is estimated to be 11 days.

Daily egg batch laying frequency

Egg batch laying frequency was determined by collecting between five and 20 marked females from each species that had mated at least four times (except *P. thessalicus* which could only be mated two or three times). These were taken from the mating frequency field populations and placed individually in 400ml insect containers. About 1.5cm of laying substrate taken from the ground of the field population was placed at the bottom of each container. Each day, the containers were misted with fresh water and fresh leaves, and flowers from each species' preferred plants were added. All containers were checked daily for eggs by sifting the substrate. Egg batches were individually wrapped in tissue, labelled, and stored in cool, dark breathable containers. Egg batch laying frequency was taken as the number of batches for each female divided by the total number of days she was laying.

Egg mass and hatching success

Once shipped back to the laboratory, all egg batches were unwrapped and placed separately in petri-dishes containing sterile sand that covered the eggs. All dishes were placed in an artificial temperature controlled room on 12h dark/light cycle (for details see Achmann *et al.* (1992)). The dishes were checked daily for hatched nymphs and misted with fresh water. The number of eggs laid per mating was calculated as daily egg laying frequency divided by the population mating frequency of that species. To obtain egg mass, approximately twenty eggs were taken randomly from different batches from each species and weighed to the nearest 0.1 mg. Relative egg mass was calculated as the mean egg mass for each species divided by the mean female body mass.

When all hatching had finished the number of hatched nymphs and remaining unhatched eggs for each batch were summed to give the number of eggs produced per batch. The mean number of eggs that hatched in each batch was divided by the mean number of eggs per batch to give the proportion of eggs hatched.

Male per-mating reproductive fitness

To estimate male mating success, egg mass per mating (mean eggs laid per mating multiplied by the mean egg mass), batches laid per mating (daily batch laying frequency divided by the daily population mating frequency), and the number of eggs hatched per mating (proportion of eggs hatched multiplied by the number of eggs laid per mating), were calculated.

Female lifetime reproductive fitness and total lifetime spermatophore material received

Female reproductive output was estimated by calculating the lifetime number of eggs laid (daily egg laying frequency multiplied by mature female life expectancy), investment in egg mass (number of eggs multiplied by egg mass), and egg hatching success (number of eggs multiplied by the proportion of eggs hatched). In order to calculate the total investment in spermatophore production that males make over their lifespan, the number of female lifetime matings was multiplied by the relative spermatophore mass to give total relative lifetime spermatophore investment (Table 3), and can be understood as the equivalent volume of body mass that males of each species invest in spermatophore production.

Analysis

As with the calculations of male per-mating investment, means were used for these calculations and intraspecific variances could not be calculated. Regression analyses across all species between relative spermatophore size and components of female fitness were performed in order to understand the relationship between male and female reproductive investments.

To account for body mass, the term ‘relative’ was used throughout analyses to define that the stated factor was divided by the body mass of the sex from which that factor was derived. For example, relative spermatophore mass was determined by dividing spermatophore size by the male body mass, and egg data were divided by female body mass. Dependent variables were either \log_{10} or square-root of the arcsine-transformed to improve normality where appropriate and residual plots checked for

normality. Unless otherwise stated, all analyses were general linear regression analyses. All statistical analyses were performed using Minitab 15.

Phylogenetic independent comparisons

In conjunction with the regression analyses on relative spermatophore mass against male per-mating fitness (egg mass per mating, egg batches laid per mating and number of eggs hatched per mating), and female lifetime fitness (lifetime number of eggs laid, lifetime investment in egg mass and lifetime egg hatching success), corresponding regression analyses were also performed with phylogenetic independent contrasts (Felsenstein 1985) as dependent variables in order to control for relatedness. 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.

A cladogram of the species used in this study was constructed using the literature on the phylogeny for these *Poecilimon* taxa (see Fig. 1 for references) and the computer package PDAP (Maddison & Maddison, 2006) (Fig. 1). The mating and egg 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 male per mating investment response variable and the female lifetime reproductive success response variable. All inferential regressions involving phylogenetically-independent contrasts were forced through the origin (Garland *et al.* 1992).

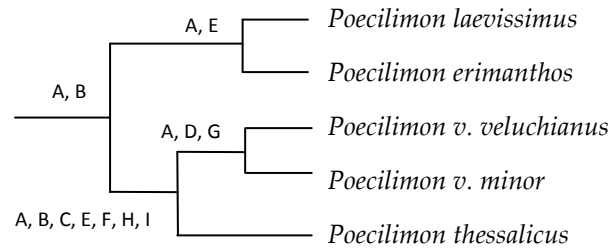


Figure 1. Cladogram representing the phylogenetic relationships between the five *Poecilimon* taxa used in this study. Letters at nodes indicate that subsequent daughter branches are based on information derived from the literature. References cited: A. Ulrich *et al.* in press, B. Heller (1984), C. 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).

Results

Body mass and spermatophore mass

Male body mass ranged between 330 mg for the smallest species, *P. v. minor* (Table 1), and 781 mg for the largest species, *P. laevissimus*. Similarly, the female body mass for *P. v. minor* was also the lowest at 425 mg and *P. laevissimus* was the largest at nearly twice the size, at 848 mg (Table 1). Despite the largest and smallest taxa having the largest and smallest respective spermatophores, there was no overall significant relationship between male body mass and spermatophore mass ($F_{1,4} = 0.00$, $p = 0.949$, $r^2 = 0.20$).

Population mating frequency

The remating frequency of females closely ties to the size of the nuptial gift that is consumed in *Poecilimon*. Population mating frequency was almost five-fold faster in the smaller spermatophore-producing *P. laevissimus* than it was in the larger spermatophore-producing *P. thessalicus* (Table 2). Small to medium spermatophore-producing species (*P. laevissimus*, *P. erimanthos* and *P. v. minor*) mated at least every day

or two, while species that produced relatively large spermatophores (*P. v. veluchianus* and *P. thessalicus*) mated approximately every four and eight days respectively. While there was a strong negative relationship between relative spermatophore size and population mating frequency (Fig. 2), it was just outside statistical significance ($F_{1,4} = 6.01$, $p = 0.092$, $r^2 = 0.67$). Taking into account female longevity, a stronger negative relationship between relative spermatophore mass and lifetime number of matings was observed, although this, too, was just outside statistical significance ($F_{1,4} = 8.23$, $p = 0.064$, $r^2 = 0.73$), (controlling relatedness; $F_{1,3} = 2.08$, $p = 0.286$). There is little power in a five species comparison, so it is not surprising that the results are not statistically significant, but the results are consistent with the hypothesis that female re-mating is influenced by male spermatophore investment. *P. erimanthos* is the main outlier in this data set and without it the relationship is significant (not controlling for relatedness; $F_{1,3} = 47.09$, $p = 0.021$, $r^2 = 0.96$).

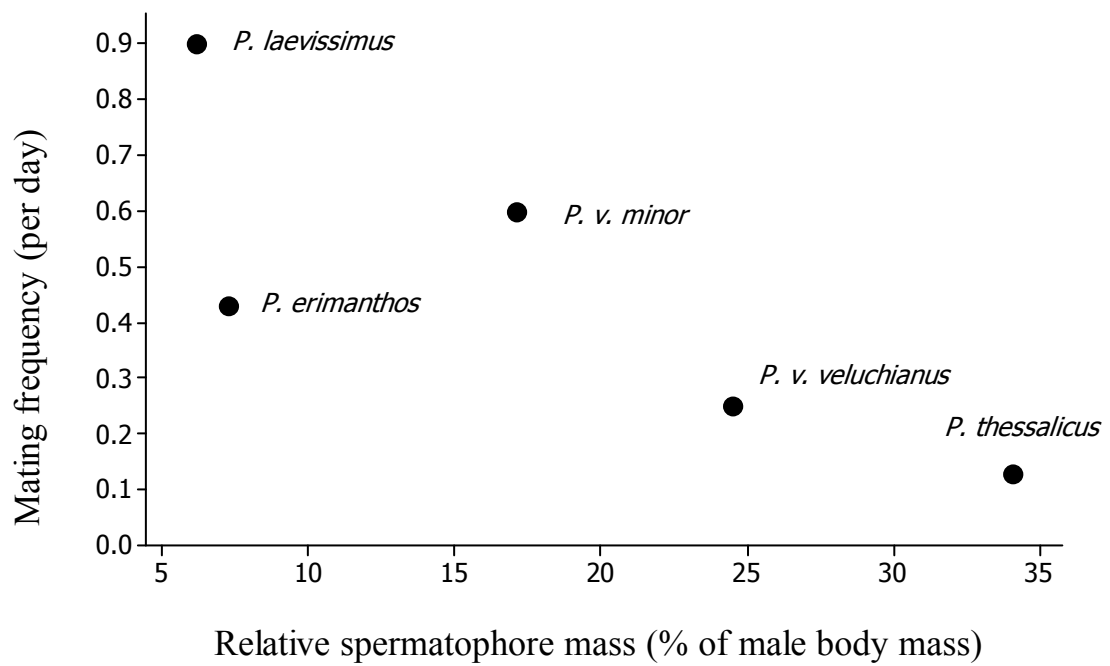


Figure 2. The relationship between spermatophore size and mating frequency among five field-observed *Poecilimon* species.

Table 2. Adult female life expectancy, mating frequency and egg production of five different *Poeciliimon* species.

	<i>P. laevisissimus</i>			<i>P. erimanthos</i>			<i>P. thessalicus</i>			<i>P. v. veluchianus</i>			<i>P. v. minor</i>		
	n	mean	SE	n	mean	SE	n	mean	SE	n	mean	SE	n	mean	SE
Proportion mated (n = nights of observation)	2	0.90	0.03	4	0.43	0.09	14	0.13	0.01	6	0.25	0.02	6	0.60	0.04
Mature female longevity (days) (n=number of females)	50	10.46	0.72	50	11.64	0.67	50	11.14	0.71	393	11	-	50	10.47	0.8
Number of female lifetime matings	-	9.41	-	-	5.06	-	-	1.45	-	-	2.75	-	-	6.28	-
Daily batch laying frequency (n=days)	5	0.63	0.04	19	0.46	0.02	13	0.62	0.07	16	0.67	0.05	20	0.66	0.04
Egg mass (mg wet weight)	20	0.99	0.027	20	1.03	0.025	20	0.59	0.022	20	0.53	0.011	11	0.45	0.021
Relative egg mass (% female body mass) (n=eggs)	20	1.16	-	20	1.23	-	20	1.05	-	20	0.72	-	20	1.06	-
Mean number of eggs per batch (n=batches)	36	3.67	0.48	87	3.70	0.28	68	3.01	0.25	104	2.42	0.16	156	1.87	0.12
Daily egg laying frequency (n=batches)	36	2.31	-	87	1.70	-	68	1.87	-	106	1.61	-	156	1.23	-
Mean number of eggs hatch per batch (n=batches)	36	1.22	0.28	87	1.92	0.18	68	1.72	0.24	106	2.21	0.14	155	1.53	0.11
Proportion of eggs hatched (n=batches)	36	0.32	0.06	80	0.52	0.04	65	0.50	0.05	104	0.82	0.03	139	0.76	0.03

Mature female longevity

Despite large variations in body size between species a remarkable consistency in the number of days that females of different species are available for mating was found (mature female longevity $F_{1,4} = 0.73$, $p = 0.534$), (Fig. 3). Females of all species, including *P. v. veluchianus* (which could not be included in the analysis, see Methods section), had a mating lifespan of around 11 days (Table 2). No relationship between relative spermatophore size and female longevity was observed when controlling for relatedness ($F_{1,3} = 0.25$, $p = 0.649$), or not controlling for relatedness ($F_{1,3} = 0.02$, $p = 0.887$, $r^2 = 0.008$).

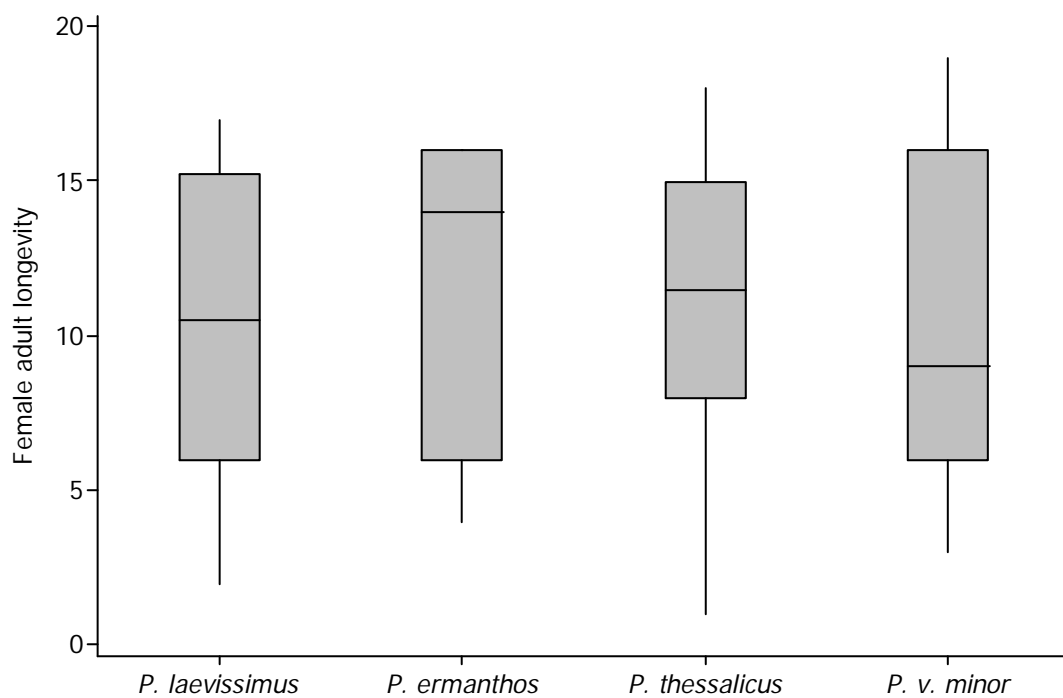


Figure 3. Little variation is found between the number of days that females from four *Poecilimon* taxa are available to mate (mature female longevity).

Daily egg batch laying frequency

A significant difference in the average daily egg-batch laying frequency between species was observed (ANOVA; $F_{1,4} = 4.83$, $p = 0.002$), however most species laid at similar rates (around 0.65 batches per day; Table 2), with the diurnal mating *P. erimanthos* (which laid at around 0.45 batches per day) the exception (Fig. 4). With *P. erimanthos* removed, there was no significant difference in daily egg batch laying frequency (ANOVA; $F_{1,3} = 0.23$, $p = 0.872$). However, *Poecilimon* lay eggs at night, so it is likely that all species generally lay on alternate nights.

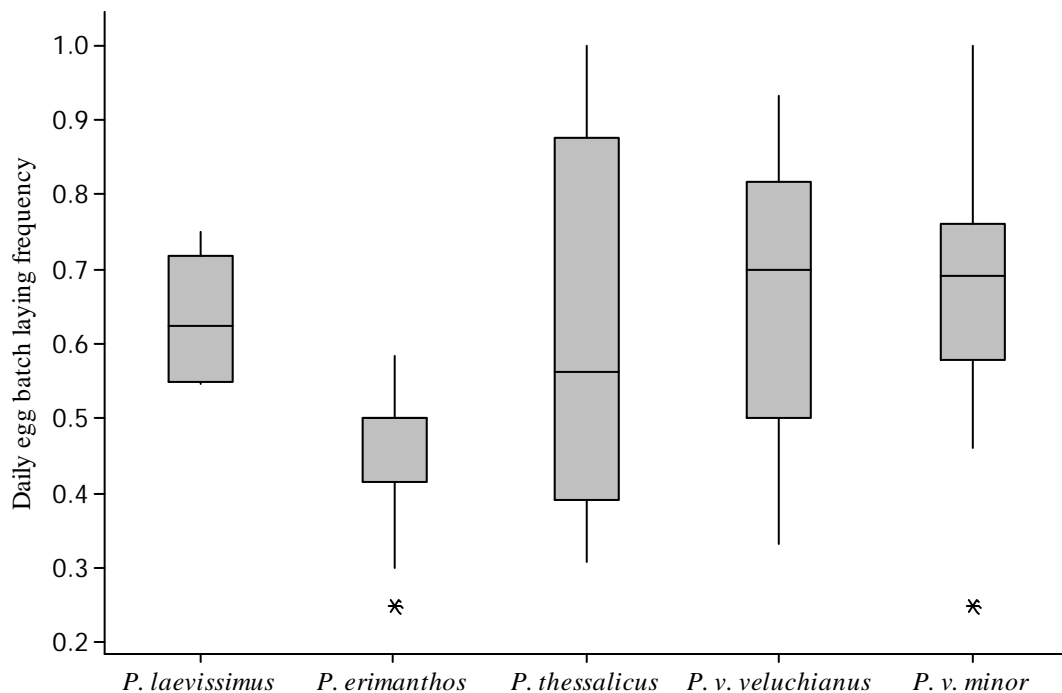


Figure 4. A similar daily egg batch laying frequency is found across the majority of tested *Poecilimon* species.

There was a significant difference in the mean number of eggs produced per batch among the five *Poecilimon* taxa (ANOVA; $F_{1,4} = 14.81$, $p < 0.001$), and positive but non-significant relationships between female body mass and the mean number of eggs produced per batch ($F_{1,4} = 5.66$, $p = 0.098$, $r^2 = 0.65$) which did not improve with the exclusion of *P. erimanthos* ($F_{1,3} = 2.54$, $p = 0.252$, $r^2 = 0.56$) and daily egg laying frequency ($F_{1,4} = 2.30$, $p = 0.227$, $r^2 = 0.43$).

Egg mass and hatching success

A significant difference in egg mass was observed between species (ANOVA; $F_{1,4} = 137.04$, $p < 0.001$), (Fig. 5), however, despite a strong relationship with female body mass, it was not significant ($F_{1,4} = 7.00$, $p = 0.077$, $r^2 = 0.70$). After controlling for female body mass, a large variation in egg mass remained with an almost two-fold difference between species (Table 2).

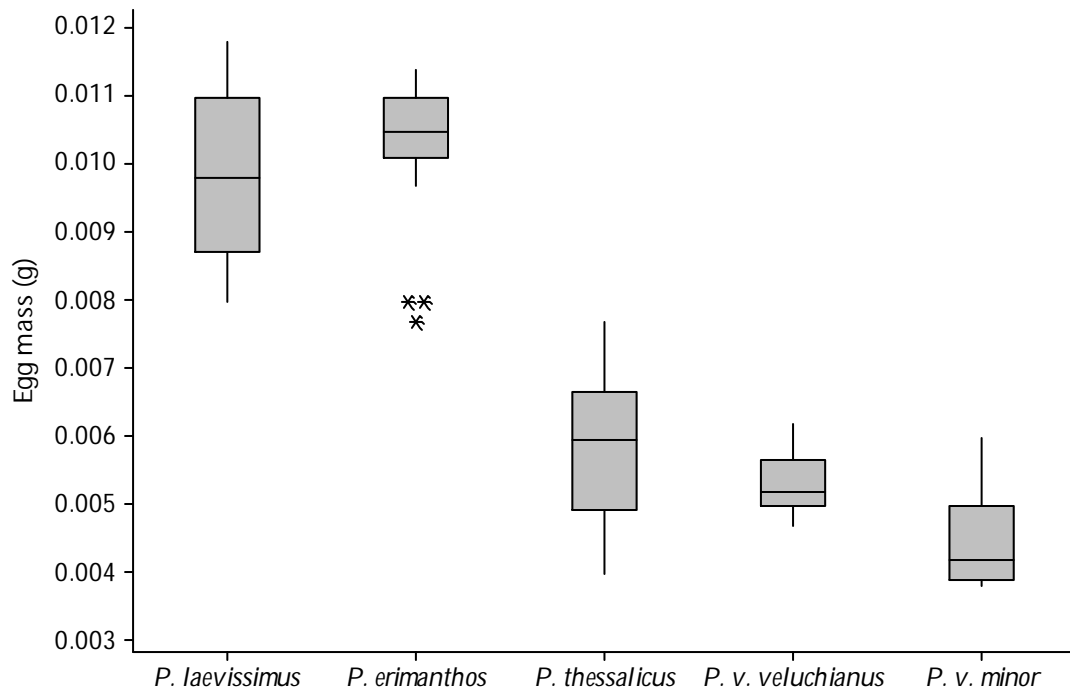


Figure 5. A significant difference in egg mass is found across five *Poecilimon* species.

While a significant difference was found in the number of eggs that hatched among species (ANOVA; $F_{1,4} = 4.18$, $p = 0.002$) (Fig. 6), hatchling number was not correlated with female body mass ($F_{1,4} = 0.02$, $p = 0.887$, $r^2 = 0.08$). The hatching success between species only ranged between just over one and two eggs per batch for the two largest species *P. laevissimus* and *P. v. veluchianus*. However, two of the largest number of eggs hatching per batch was observed in the two largest relative spermatophore-producing taxa, *P. thessalicus* and *P. v. veluchianus*.

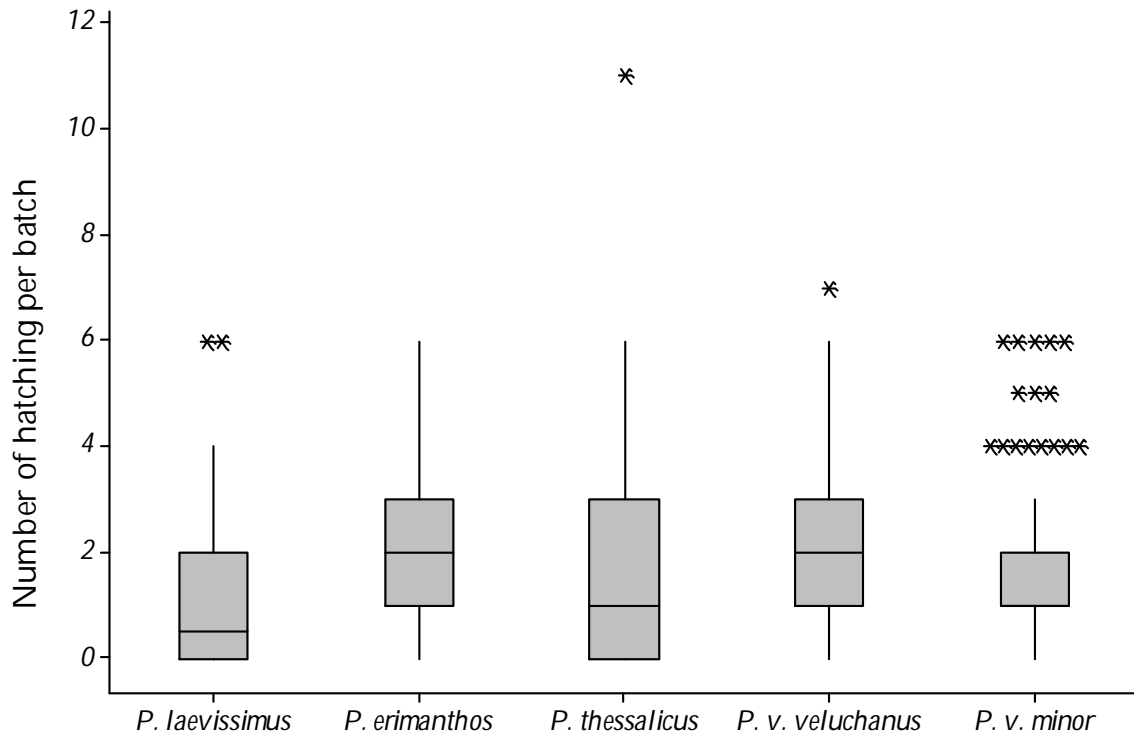


Figure 6. A significant difference in hatching success is found across five *Poecilimon* species.

There was also a weak but significant difference in the proportion of eggs that hatched (ANOVA; $F_{1,4} = 24.45$, $p = 0.000$, $r^2 = 0.18.9$), although this relationship also does not appear to be related to body size ($F_{1,4} = 1.01$, $p = 0.389$, $r^2 = 0.25$).

Male per-mating reproductive fitness (eggs laid, egg mass and number of eggs hatched per mating)

Without post-hoc data manipulation, analyses could not be performed on the differences in the number of eggs laid per mating (or differences in egg mass and number of eggs hatched per mating). However, a large variation existed between taxa in the estimated averages, with an almost five-fold difference between the largest and

smallest relative spermatophore-producing taxa (Table 3). *Poecilimon laevisissimus* and *P. erimanthos*, species producing the smallest relative spermatophore along with *P. v. minor* which produces a medium-large relative spermatophore, laid around two eggs per mating. This translates to around two eggs per day for *P. laevisissimus* and *P. erimanthos* and around one egg per day for *P. v. minor*. The relatively low number of eggs laid by *P. v. minor* is due to the low per-mating output, not the inter-mating refractory periods which appear to be in proportion with the relative size of the spermatophores. The relatively large spermatophore-producing taxa, *P. thessalicus* and *P. v. veluchianus*, laid over six and 14 eggs per mating; translating also to around two eggs per day, but in these taxa there is a lengthened inter-mating period in females.

Table 3. Male per-mating and female lifetime mating reproductive fitness of five *Poecilimon* taxa.

	<i>P. laevisissim us</i>	<i>P. erimanth os</i>	<i>P. thessalic us</i>	<i>P. v. veluchianus</i>	<i>P. v. minor</i>
	Mean	mean	mean	mean	mean
Eggs laid per mating	2.57	3.91	14.36	6.46	2.06
Egg mass per mating (mg)	2.54	4.01	8.43	3.45	0.93
Number of eggs hatched per mating	0.82	2.03	7.24	5.29	1.56
Lifetime number of eggs laid	24.18	19.81	20.79	17.76	12.92
Lifetime investment in egg mass (mg)	23.87	20.31	12.21	9.49	5.84
Lifetime egg hatching success	7.74	10.30	10.49	14.56	9.82
Total relative lifetime spermatophore receipt (% of male body mass)	57.43	36.45	48.4	67.38	107.04

Per-mating egg mass also varied to a large extent between species (Table 3); *P. thessalicus*, one of the smallest species with the largest relative spermatophore size and a long mating interval of around eight days, produced the largest mass of eggs per mating (8.4 mg), yet *P. v. minor*, the smallest species with a medium-large spermatophore relative to male body mass and a mating interval of approximately two days, produced less than 1 mg of eggs per mating. On the other hand, *P. laevissimus* and *P. erimanthos*, the two largest species, with small relative spermatophores and remating intervals of one and two days respectively, produced only 2.5 mg and 4 mg of eggs respectively for each mating. The relatively large spermatophore of the smaller species and the relatively small spermatophores of the large species appear to have a pronounced influence over remating intervals and the resulting respective egg mass produced.

Similarly, the low variation in hatching success among *Poecilimon* taxa, combined with the effect that spermatophore size had on the egg mass and number of eggs laid per mating, meant that taxa producing the relatively large spermatophores, *P. thessalicus* and *P. v. veluchianus*, produced over eight times more hatched eggs than the smallest spermatophore-producing species *P. laevissimus* (Table 3).

Differences between species in female lifetime reproductive fitness

An almost two-fold difference in variation exists between *Poecilimon* taxa in the lifetime number of eggs laid (Table 3). The largest species, *P. laevissimus* produces the greatest number of eggs throughout their adult life, around 24, and the smallest taxon, *P. v. minor*, produces the least at around 13. However, *P. v. veluchianus*, the second largest

species produces the second fewest eggs behind *P. v. minor*, despite the females receiving one of the largest relative spermatophores of the taxa presented here.

Female lifetime investment in egg mass also varied considerably between species (Table 3) with the two *P. veluchianus* taxa producing the two lowest egg masses (*P. v. veluchianus*, 9.49 mg; *P. v. minor*, 5.84 mg). The larger *P. laevissimus* and *P. erimanthos* produced the largest egg mass at around 28 mg and 24 mg respectively, around four-fold more than *P. v. minor*. *Poecilimon thessalicus* produced a little less than the larger species at an egg mass of 22 mg.

Even though the lifetime investment in egg number and egg mass was not directly predictable from spermatophore size, taxa with smaller spermatophores did tend to have fewer hatching eggs over the females' lifetime. *P. laevissimus* for example, with the smallest relative spermatophores, produced the greatest lifetime number and biomass of eggs, but produced the fewest live offspring. On the other hand, *P. v. veluchianus* produced the second least lifetime egg number and mass yet had the highest number of hatched eggs, twice that of *P. laevissimus*.

Male per-mating reproductive fitness

A positive correlation was found between relative spermatophore size and relative number of eggs laid per mating ($F_{1,4} = 9.35$, $p = 0.055$, $r^2 = 0.76$). This relationship was strengthened after controlling for relatedness ($F_{1,3} = 94.01$, $p = 0.002$), (Fig. 7). A significant relationship is observed between relative spermatophore mass and relative egg mass per mating, when controlling for relatedness ($F_{1,3} = 18.64$, $p = 0.023$), although it is not significant when relatedness is not controlled ($F_{1,4} = 4.46$, $p = 0.125$, $r^2 = 0.60$), (Fig. 7). While the relative number of eggs that hatch per mating is strongly associated

with spermatophore size ($F_{1,4} = 44.64$, $p = 0.007$, $r^2 = 0.94$; controlling for relatedness $F_{1,3} = 24.36$, $p = 0.016$), (Fig. 7), relative spermatophore size does not appear to influence the proportion of eggs that hatched ($F_{1,4} = 0.12$, $p = 0.748$, $r^2 = 0.04$).

Taken together, these results indicate that males of taxa with larger spermatophore investments potentially have higher per mating success than the males of taxa that produce smaller spermatophores. Assuming a high last male sperm precedence pattern, males of taxa that produce relatively large spermatophores sire a larger number of offspring per mating than those taxa producing relatively small spermatophores.

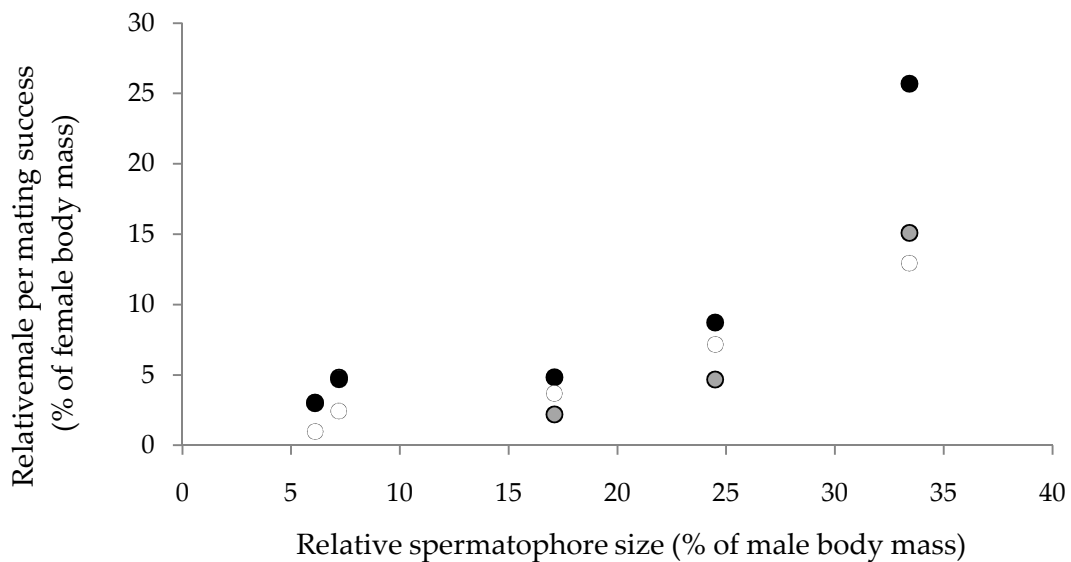


Figure 7. A positive relationship exists between relative spermatophore mass and three measures of per-mating male fitness across five *Poeciliimon* taxa (black circles = relative egg number laid per mating; grey circles = relative egg mass per mating; open circles = relative number of eggs hatched per mating).

***Female lifetime reproductive fitness, spermatophore size
and total lifetime spermatophore material received***

The total relative spermatophore volume that females receive varied almost three-fold between larger *P. erimanthos*, receiving only 37% of the male's body mass over their entire life, and the smallest species, *P. v. veluchianus*, which received the equivalent of 107% of male body mass in spermatophore material (Table 3). However, the remaining three species received between 50 and 70% of relative male body mass in spermatophore material. Controlling for relatedness, no overall relationship between relative spermatophore size and total relative lifetime spermatophore receipt was observed ($F_{1,3} = 0.46$, $p = 0.545$), which was confirmed with a low, non-significant relationship when relatedness was not controlled ($F_{1,4} = 0.03$, $p = 0.881$, $r^2 = 0.09$).

No significant relationships were observed between relative spermatophore mass received and relative lifetime number of eggs produced ($F_{1,4} = 0.17$, $p = 0.718$, $r^2 = 0.08$; controlling for relatedness $F_{1,3} = 1.02$, $P = 0.388$), relative lifetime egg mass ($F_{1,4} = 0.94$, $p = 0.403$, $r^2 = 0.23$; controlling for relatedness; $F_{1,3} = 0.06$, $p = 0.816$), or relative lifetime egg hatching rates ($F_{1,4} = 2.58$, $p = 0.206$, $r^2 = 0.46$; controlling for relatedness, $F_{1,3} = 0.56$, $p = 0.508$).

Discussion

Males of large spermatophore-producing *Poecilimon* obtain large per-mating fitness benefits, particularly hatching success, from producing larger spermatophores, while at the same time appearing to increase female mating interval. As a result, females of taxa receiving larger gifts do not gain any net benefits that may be associated with larger

nuptial gifts (greater material and genetic benefits reviewed in Gwynne 2008; Vahed 2007a). This is the first comparative test to show that the males of larger spermatophore-producing taxa apparently manipulate females while increasing their own fitness.

Given that larger relative spermatophore-producing species, such as *P. thessalicus* and *P. veluchianus*, often show indications of paternal investment (reviewed in McCartney *et al.* 2008), it may seem unusual that larger spermatophore-receiving *Poecilimon* taxa do not show any signs of increased fitness over relatively smaller spermatophore-producing species. This may be partly explained by the fact that females of four out of the five *Poecilimon* taxa tested here, including the two largest and two smallest relative spermatophore-producing species, receive somewhat similar amounts of total spermatophore volume across their lifespan (between 37% and 67% of male body weight). At least two factors affect this finding. Spermatophore size corresponds to male mating rates. While larger relative spermatophore-producing *Poecilimon* males generate larger spermatophores per mating, female inter-mating refractory period is lengthened proportionally so males incur the subsequent effects of a reduced mating frequency (Heller & Helversen 1991; Vahed 2007b). The second factor is that of female longevity (Parker & Simmons 1989). In other cricket species, spermatophore consumption may positively influence female fitness by increasing female longevity (reviewed in Vahed 1998; Arnqvist & Nilsson 2000), and thus allowing more time to produce offspring. Female longevity showed little variation between *Poecilimon* taxa, and the variation that was found was not explained by differences in spermatophore mass. Thus, overall similarities in female longevity and

receipt of spermatophore volume over the lifespan are likely to determine similar outcomes in offspring fitness and fecundity obtained by females.

The negative correlation between spermatophore size and female mating frequency observed across *Poecilimon* taxa support the findings of other comparative studies across bush-cricket species (Wedell 1993a; Vahed 2006). Recent studies interpret this relationship to mean mating frequency is negatively impacted by spermatophore size (reviewed in Arnqvist & Nilsson 2000; Gwynne 2008; Vahed 2007a). However, this may not demonstrate that males either ‘manipulate’ females, nor that spermatophore size imposes sub-optimal re-mating latencies in females, given that a negative impact of spermatophore size on female fitness was not observed. While there is increasing evidence that conflict occurs between the sexes over mating rate (reviewed in Arnqvist & Nilsson 2000; Parker 2006; Gwynne 2008; Vahed 2006, 2007a,b), recent literature has detailed the difficulty in determining female reproductive re-mating optima (reviewed in Gwynne 2008; see also Vahed 2006, who highlights the difficulty in determining cause and effect between spermatophore components and mating frequency). Sub-optimality, as Gwynne (2008) points out, is not measured by mating latency or polyandry, rather by decreased fitness. It may be that female refractoriness is male-influenced or even male-imposed, but unless females suffer a fitness cost, sub-optimality is not concomitant. Respective species’ spermatophore size and remating latency may alternatively represent differences in mating strategies where, *ceteris parabus*, the females of each species obtain sufficient genetic and material benefits required for optimal reproductive fitness (reviewed in Hosken & Stockley 2003; Zeh & Zeh 2003; Simmons 2005; Gwynne 2008; Vahed 2007a).

In fact, despite large variations in mating frequency, the fact that the female *Poecilimon* tested here receive similar total quantities of spermatophore material over their lifespan, suggests that overall spermatophore material attained by females is similarly optimal in all species (see also Heller & Helversen 1991). Indeed, given that females are almost invariably the choosier sex, spermatophore size and corresponding mating frequency variations between species are more likely to reflect a combination of environmental variations in seasonal food supply and population densities (competition), and may ultimately be selected by females. Female *Poecilimon* receiving large spermatophores, for example, benefit by a reduction in the costs of polyandry associated with mate searching (Heller 1992). Females of *Poecilimon* taxa that receive larger gifts typically respond to male calls and perform risky phonotaxis. Females of taxa receiving smaller spermatophores respond acoustically and wait for the approaching male, and are exposed to lower levels of predation (Heller 1992; McCartney *et al.* submitted manusc.). In a meta-analysis, Arnqvist & Nilsson (2000) found that egg and offspring production, and female longevity, were all positively correlated with polyandry. While this was not tested directly here, remating latency is highly correlated to spermatophore size and showed no effects on offspring fitness. Arnqvist & Nilsson (2000) suggested that their results indicate nuptial gifts may in fact have evolved as a result of “sexual conflict, manipulation and extortion” as they appear to “represent manipulative and sinister superstimuli”; this remains to be seen across *Poecilimon*. Indeed, until the costs of larger spermatophores over smaller spermatophores can be observed across *Poecilimon* taxa the assertion that larger gifts manipulate females in a suboptimal manner remains unsubstantiated.

The increased male fitness I found among taxa that produce larger spermatophores supports the view that females are not manipulated by larger spermatophores. Male fitness is predicted to increase when males mate multiply, whereas female fitness is typically maximised with few matings and males are not guaranteed the same genetic investment in their offspring (Bateman 1948; Trivers 1972; Alexander & Borgia 1979; Wickler 1985). Lower levels of sexual conflict are expected for males that invest paternally (Gwynne 2008; Vahed 1998). While males of *Poecilimon* taxa that invest more in larger spermatophores are therefore predicted to either sire fitter or a greater number of offspring or have an increased parental certainty, none of these outcomes need to occur at a cost to the female. The large spermatophores produced by *P. v. veluchianus* males increase offspring fitness (Reinhold & Heller 1993; Reinhold 1999), and the large spermatophores of male *P. thessalicus* are likely to have a paternal investment function because they transfer a spermatophylax gift that is four times larger than necessary to obtain total sperm transfer (McCartney *et al.* submitted manuscript). While indirect genetic influences remain to be tested across *Poecilimon*, male *Poecilimon* studied here sire a greater number of fitter offspring per mating. Furthermore, the two species with larger spermatophores show indications of paternal investment, so it is likely that sexual conflict is not a major determining factor in spermatophore size variation across *Poecilimon*.

An ejaculate protection function for the nuptial gift is well documented for bush-crickets (reviewed in Vahed 1998; Gwynne 2001) including in *Poecilimon* (reviewed in McCartney *et al.* 2008). However, in order to determine fitness increases in males (as well as paternal investment) as a direct result of the donating male's

spermatophore, it is necessary to show how sperm is utilised by the female and that the offspring males sire receive nutrients from the donating male's spermatophore. While this is clear for *P. v. veluchianus* (Reinhold & Heller 1993; Reinhold 1999), this remains to be investigated in the other *Poecilimon* taxa studied here. The phylogenetic proximity of taxa studied here to those *Poecilimon* species showing pronounced last-male precedence patterns suggests that all species presented here are likely to show a high last male precedence pattern (see Fig. 1 and references cited therein). However, spermatophore component size is evolutionary labile in *Poecilimon* (McCartney *et al.* 2008) and this may also be the case for their sperm precedence pattern. The only bush-cricket species that appears to have a first male pattern (i.e., all offspring are sired by the first male to mate), that is not a result of active sperm removal (e.g., Helversen & Helversen 1991), appears to be *Requena verticalis* (Gwynne & Snedden 1995). However, *R. verticalis* typically produce offspring batches sired by three fathers (Simmons *et al.* 2007). First male patterns are reduced from 100 to 81% if females oviposit between the first two matings (Gwynne & Snedden 1995). Furthermore, *R. verticalis* males prefer younger females that have a higher probability of being virgins (Simmons *et al.* 1994) and decrease spermatophylax gift size when mating with older females. In contrast to the reduced gift size of *R. verticalis* when mating with an older female, *P. thessalicus* has a spermatophylax gift size that increases toward the end of the season to almost twice the size it was at the beginning (McCartney *et al.* 2010). Furthermore, *P. thessalicus* produces a spermatophylax-gift that is almost four times larger than necessary to protect the transfer of sperm (McCartney *et al.* submitted manuscript). This suggests a

significant waste in expensive spermatophylax gift unless utilised paternally for offspring fitness or securing a last-male sperm advantage.

Given that the mating frequency and egg laying frequency of each species is well understood, knowledge of the sperm precedence pattern may not be so important. *P. thessalicus* and *P. v. veluchianus* females appear to mate on average approximately twice in their lifetime, the operational sex ratio of *P. veluchianus* (and another *Poecilimon* species *P. affinis*; Heller & Helversen (1991)) is typically male-biased so fewer matings on average per male can be expected. A similar outcome might also be predicted if total sperm mixing occurs, such as that observed in *Decticus verrucivorus* (Wedell 1991). Irrespective of the sperm precedence pattern however, it is likely that the donating males of these species sire the majority of offspring and, given the lengthened delay before female re-mating, are likely to have nutrients from their own spermatophylax-gift incorporated into the eggs they sire (Reinhold 1999).

Poecilimon laevisissimus and *P. erimanthos* are unlikely to show a first-male precedence pattern. It appears that female *P. laevisissimus*, for example, remate constantly throughout the season to replenish sperm reserves that stay at a constant level (McCartney unpub. data), and prefer virgin males (McCartney & Heller 2008) which transfer a higher number of sperm. Male *P. laevisissimus* transfer around 700,000 sperm per mating (McCartney *et al.* 2008), assuming a similarly small number of sperm are required to fertilise each egg as that observed for *P. v. veluchianus* (around 75,000 or fewer: CI 95% 0-250,000; K. Reinhold pers. comm.), then *P. laevisissimus* only supply enough sperm to fertilise around one egg batch per mating. This effectively nullifies any potential effects of sperm precedence; males only deposit enough sperm to

inseminate the eggs laid by the female until she remates. Despite the fact that spermatophore nutrients may be an important source of nutrition for females (Bowen *et al.* 1984; Wedell 1993b; Voigt *et al.* 2006) and may be assimilated within hours of consumption (Voigt *et al.* 2008), and increases in egg mass in bush-crickets have been noted within 24 hours of spermatophore consumption (Simmons 1990), it remains to be seen however, whether the nutrients of the smaller spermatophore-producing species have enough time to transfer across to their own eggs.

Males of relatively large spermatophore-producing *Poecilimon* taxa sire a greater number of hatched offspring per mating than *Poecilimon* species that produce relatively smaller spermatophores. While relative spermatophore size corresponds to female polyandry, relative spermatophore volume received through the lifespan of females was similar across most species. This was mainly due to the similar adult female mating lifespan across *Poecilimon* species, and indicates that increased polyandry does not affect females' longevity or offspring fitness. No differences were found between the total egg number, total egg mass and hatching success across female *Poecilimon* despite differences in relative spermatophore volume received between taxa. It therefore remains to be seen whether female *Poecilimon* are manipulated beyond their mating optima or whether sexual confluence and female selection determine spermatophore size. While indirect genetic benefits and the effects of polyandry on sex-specific fitness are not tested directly here, sexual conflict appears, at this stage, to have little influence over spermatophore size variation across *Poecilimon* bush-crickets.

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Chapter 9

Discussion and conclusions

Jay McCartney



A female *Poecilimon laevissimus*.

The behavioural factors that influence nuptial gift size variation across bush-crickets illustrate the complex relationship between sex-specific investments in reproduction (reviewed in Gwynne 2001; Vahed 1998a; McCartney et al. 2008 [Ch 2]). Rooted in environmental and physiological variation, these behavioural influences dictate reproductive fitness, facilitate speciation and drive evolution. They are the core of this thesis.

The selective pressures that form the basis of my thesis: operational sex ratios, reproductive effort, mating effort, parental investment, ejaculate protection, sperm competition, mate choice, sexual conflict and reproductive fitness, are discussed here in relation to the specific results of each chapter and with relation to how they influence spermatophore size variation across *Poecilimon*.

Poecilimon as a model taxon

There is ongoing debate over the function of nuptial feeding in insects (McCartney et al. 2008 [Ch 2]). Nuptial gifts may function via sexual selection for mating effort to maximize ejaculate transfer, or via natural selection for paternal investment where nutrients from the nuptial gift can be transferred into the donating male's offspring. While these hypotheses are not mutually exclusive, there is overwhelming support showing nuptial gifts are more likely to function to protect the ejaculate (reviewed in Vahed 1998; Gwynne 2001; McCartney et al. 2008 [Ch 2]). The larger gifts of three species, however, have been observed to additionally function as paternal investment (*Requena verticalis*, Gwynne 1984, 1988; *Kawanaphilla nartee*, Simmons 1990; Simmons

and Bailey 1990; *Poecilimon veluchianus*, Reinhold and Heller 1993; Reinhold 1999; reviewed in Vahed 1998; Gwynne 2001; McCartney et al. 2008 [Ch 2]). While these studies have their respective strengths – for example, when combined they show that the ejaculate protection function is likely to account for the evolution and maintenance of the nuptial gift – comparative support comes from quite disparate bush-cricket species that vary greatly in relatedness. The variety of species used means that any differences between taxa (such as diet, body size, spermatophore component size, population density, sperm precedence pattern, etc) along with the variation in relatedness, cloud the interpretation of gift size variation. Comparative studies across a wide range of species have helped increase our understanding of, for example, gift function (Wedell 1993a, 1994a,b; Vahed and Gilbert 1996; Del Castillo and Gwynne 2007; Vahed 2006, 2007a), however all studies neglected in some way to control confounding factors such as diet (Wedell 1993a, 1994b; Vahed and Gilbert 1996; Vahed 2006, 2007a; Del Castillo and Gwynne 2007) or relatedness (Wedell 1993a, 1994a,b), and all have used at least some laboratory-reared species (Wedell 1993a, 1994a,b; Vahed and Gilbert 1996; Del Castillo and Gwynne 2007; Vahed 2006, 2007a).

I have gathered previously unpublished data and extracted data from the literature to make comparative analyses across 37 *Poecilimon* taxa. In addition, I intensively studied five taxa in the field for specific focal comparisons. In doing so, my first aim was to compile this information so that the genus *Poecilimon* may be viewed as an ideal model system for understanding gift-giving mating systems (reviewed in McCartney et al. 2008 [Ch 2]; but see also Lehmann and Lehmann 2007, 2008a,b, 2009; McCartney and Heller 2008 [Ch 3]; Voigt et al. 2008; McCartney et al. 2010 [Ch 5]). To

this point, there appears to be no other field-observed data-sets on gift-giving species that rival the *Poecilimon* model system.

Controlling for natural variations in a closely related taxon

Spermatophore size variation is driven by environmental, phylogenetic and behavioural (e.g., diet) factors. Diet and relatedness, for example, are elemental in shaping the selective forces dictating behavioural influences on spermatophore size variation and function. While diet and relatedness in bush-crickets have received some attention (e.g., Gwynne 1985; Boggs 1990; Simmons and Bailey 1990; Wedell 1994a), the control of natural variations among taxa by observing closely related species within their natural environments has been largely ignored (Maklakov et al. 2005; Simmons et al. 2007; McCartney et al. 2008 [Ch 2]). Utilising field observations on closely related monophyletic *Poecilimon* taxa is central to the methodology of this thesis and has a major influence on the degree to which this work is a novel contribution to the study of mating systems, and thus deserves specific mention here.

Comparisons among closely related species offer insights into the evolution of labile traits such as gift size, ampulla volume and sperm number. The genus *Poecilimon* represents a closely related, monophyletic group and while the group contains around 140 species (Eades and Otte 2008), the physiology of *Poecilimon* remains similar among them. Comparisons among closely related species from different environments provide us with an approximation of the extent to which a character has been influenced by relatedness (Harvey and Pagel 1991). While comparative analyses using

phylogenetic data are often used to understand evolutionary changes across distantly related groups, closely related species should be treated as independent if differences reflect either the adaptive outcome of stabilizing selection, or adaptive responses to similar environments. Failure to recognise similarities between relatives as ecological will unnecessarily increase the likelihood of diagnosing a positive genetic relationship when none exists (Westoby et al. 1995; Carvalho et al. 2006). Indeed, the validity of phylogenetic tests on some comparative data has been questioned (e.g., Weathers and Siegel 1995; Ricklefs and Starck 1996; Björklund 1997; Mazer 1998; Abouheif 1999; Gillooly and Ophir 2010), because phylogenetic correction procedures can inappropriately mask interspecific differences related to stabilization and adaptive selection as well as phylogenetic niche conservatism (Gittleman and Luh 1994; Westoby et al. 1995; Gittleman et al. 1996).

My research has relied heavily on the close phylogenetic relationship found across *Poecilimon* to control for external factors such as diet and physiology. Largely due to the availability of a comprehensive phylogeny for *Poecilimon* (Ulrich et al. in press), a phylogenetic hypothesis for this taxon could later be constructed. Related individuals are more likely to have traits that are similar (Gwynne 1990a, 1995b; Heller and Reinhold 1994; McCartney et al. 2008 [Ch 2]) and consequently, comparative data on spermatophore component size variation between species should not be treated independently without accounting for this relatedness (Ridley 1983, 1989; Harvey and Pagel 1991; Gwynne 1995a).

For these reasons a comparison between the traits under study was first analysed without controlling for relatedness, to set a baseline understanding of

Poecilimon spermatophore size variation (e.g., McCartney et al. 2008 [Ch 2]). These relationships were then tested again taking into account *Poecilimon* phylogeny (e.g., McCartney and Heller submitted manusc. [Ch 7]; see also McCartney submitted manusc. [Ch 8]). Ultimately, I was able to support previous findings from McCartney et al. (2008 [Ch 2]), and show that evolutionary history explained little of the variation in spermatophore size within the genus (McCartney and Heller submitted manusc. a [Ch 7]).

Laboratory studies are typically preferred over field studies when observing insect mating systems because of the greater environmental control they offer. However, laboratory results are potentially unreliable because they may not represent the natural variation observed within wild populations. In fact, the majority of research appears to be based on, or incorporate, data from laboratory-reared species (see, for example, Gwynne 1986a, 1990; Wedell and Arak 1989; Gwynne and Simmons 1990; Heller and Helversen 1991; Simmons and Gwynne 1991; Wedell 1993a,b, 1994a,b, 2008; Reinhold 1994; Simmons 1995a; Vahed and Gilbert 1996; Vahed 1997, 1998b; Lehmann and Lehmann 2000a, 2007; Bateman 2001; McCartney et al. 2008 [Ch 2]). Recent reviews (Maklakov et al. 2005; Simmons et al. 2007; McCartney et al. 2008 [Ch 2]), have pointed out the shortcomings of such research, especially given the variable nature of insect mating behaviour and labiality in which spermatophore component variation, such as sperm number (Reinhold 1994; McCartney et al. 2008 [Ch 2]), and spermatophylax size (Heller and Helversen 1991), respond to environmental pressures. Laboratory research typically lacks ecological validity. To the strength of this thesis, I conducted all behavioural research in natural populations.

Combined, the use of natural population field studies and the collection of data on a model taxon with a known phylogenetic history are central tenets running throughout my thesis.

*Mating effort, paternal investment, ejaculate protection
and sperm competition*

To understand the spermatophore size variation I controlled for relatedness and diet across 33 field-observed *Poeciliimon* species (McCartney et al. 2008 [Ch 2]). Collecting 62 independent observations, published data was combined with novel and unpublished data so that family-wide variations in spermatophore size could be understood with respect to mating effort and paternal investment (McCartney et al. 2008 [Ch 2]). In support of previous comparative research on ejaculate protection (Wedell 1993a; Vahed and Gilbert 1996), controlling for male body mass, I found positive relationships between spermatophylax-gift mass and ampulla mass, as well as between ampulla mass and sperm number (McCartney et al. 2008 [Ch 2]). Both relationships were further supported using a phylogenetic comparative analysis (McCartney and Heller submitted manusc. [Ch 7]), indicating that the nuptial gift functions to protect the sperm and ejaculate as it transfers into the female. However, regression analysis between spermatophore components generally revealed that there was a lot of variation between components that could not be explained by the model. Relationships between spermatophylax mass and two spermatophore components, sperm number and ampulla mass, for example, explained less than 50% of the variation. Further, I showed (McCartney et al. 2008 [Ch 2]) that there is no relationship

between relative spermatophore component size and body mass, indicating that spermatophore component size is evolutionarily labile and likely reflects differences between species in mating effort and paternal investment.

To understand in detail the relative impacts of mating effort and paternal investment on spermatophore size variation I conducted two further studies. First, I explored these two factors across the lifespan of two *Poecilimon* species that differ in spermatophore size (McCartney et al. 2010 [Ch 5]. *Poecilimon thessalicus* has a spermatophore that generally ranges between 26-33% of male body mass, although it can occasionally reach a size of 40% of male body mass (McCartney submitted manusc. [Ch 8]), while *Poecilimon veluchianus minor* has a modest spermatophore of between 17-20% of male body mass (McCartney et al. 2008 [Ch 2]). *Poecilimon thessalicus* shows a large variation in body mass and gift size over the season, however, they consistently produce similar-sized ampullae and transfer constant numbers of sperm. In contrast, *P. v. minor* produces ampullae that vary in size, yet the body, spermatophylax mass and sperm number remain constant over the season (McCartney et al. 2010 [Ch 5]). The fact that spermatophylax-gift mass and sperm number are constant over the mating season in *P. v. minor* is expected under the ejaculate protection hypothesis; these components are closely coupled in this species (Reinhold and Heller 1993; Heller and Reinhold 1994), and across *Poecilimon* species when phylogeny is controlled (McCartney and Heller submitted manusc. [Ch 7]; c.f. McCartney et al. 2008 [Ch 2]). Surprisingly, and in contrast, only spermatophylax mass and ampulla mass were size-coupled in *P. thessalicus* across the season. The spermatophylax-gift is much larger than necessary for full sperm transfer in *P.*

thessalicus (McCartney et al. submitted manusc. a [Ch 4]) which is likely explained by paternal investment (see below). However, the association between spermatophylax mass and ampulla mass, combined with the lack of association between spermatophylax and sperm number in *P. thessalicus*, may also be explained by protection of the ejaculate, but not protection of the sperm *per se* because the ejaculate, may manipulate the oviposition rate and mating frequency of females (reviewed in Vahed 2007a). The large increase in ampulla mass toward the end of the season in *P. v. minor* may also be explained by ejaculate protection. While I conclude (McCartney et al. 2010 [Ch 5]) that differences between the two species reflect within-species adjustments in order to maximize reproductive output, it became clear that in order to understand the relationship between spermatophore size and the relative influences on fitness, it was important to directly test the difference in timing between optimum sperm transfer and nuptial gift consumption time across *Poecilimon*.

In McCartney et al. (submitted manusc. a [Ch 4]), I compared the match/mismatch between spermatophore consumption time and the time it takes for the majority of sperm to transfer into the female, in five *Poecilimon* species that vary markedly in spermatophore size. The ejaculate protection hypothesis predicts a high correlation between spermatophylax size and ampulla size (volume) or sperm number (Wedell 1993a; Vahed and Gilbert 1996; McCartney et al. 2008 [Ch 2]). However, the paternal investment hypothesis predicts that spermatophylax size and sperm number or ampulla mass vary independently, and that the spermatophylax may be much larger than necessary for sperm transfer (Wedell 1993a, 1994a,b; Vahed and Gilbert 1996; McCartney et al. 2008 [Ch 2]). The species with the smallest nuptial gift (*P.*

laevissimus at around 6.1% of male body mass (McCartney et al. 2008 [Ch 2])), appears to be too small to allow a maximum number of sperm to transfer into the female; females remove and consume the whole spermatophore with only about 10-15% of the total sperm being transferred. In *P. erimanthos*, with a spermatophore mass of around 10% of male body mass (McCartney et al. 2008 [Ch 2]), and the two *Poecilimon veluchianus* sub-species, which produce medium and large spermatophores respectively (*P. v. minor* at around 19% and *P. v. veluchianus* around 26% of male body mass (McCartney et al. 2008 [Ch 2]; McCartney et al. submitted manuscript a [Ch 4])), the nuptial gift size roughly corresponds to the time it takes for the majority of sperm to transfer into the female. However, *P. thessalicus* has a nuptial gift that takes almost four times as long to consume as it does to transfer the maximum amount of sperm. In addition, controlling for body mass and relatedness, I also found a positive relationship between relative spermatophore mass and the proportion of sperm to transfer into the female by the spermatophore consumption time.

Given a productive environment (food and water availability), and a lack of environmental constraints (e.g., predators or parasites (see for example Heller 1992; Lehmann and Lehmann 2000a,b)), increases in spermatophore size are predicted as a function of sperm competition and ejaculate protection (McCartney et al. 2008 [Ch 2]; McCartney et al. 2010 [Ch 5]). *Poecilimon* spermatophore size appears to reach a threshold whereby a decrease in female mating frequency occurs (and decreased sperm competition), which results in more time for gift nutrients to be transferred into the donating male's offspring (McCartney et al. 2010 [Ch 5]; McCartney et al. submitted manuscript a [Ch 4]; McCartney submitted manuscript. [Ch 8]). Subsequently, these males

benefit further from greater paternal assurance (Wickler 1985; Gwynne 1986b; McCartney et al. submitted manusc. a [Ch 4]).

Mating effort and paternal investment appear to be selective forces enhancing the size of the spermatophore in *Poecilimon* (McCartney et al. 2008 [Ch 2]; McCartney et al. 2010 [Ch 5]; McCartney et al. submitted manusc. a [Ch 4]; McCartney submitted manusc. [Ch 8]), and bush-crickets in general (reviewed in Vahed 1998; Gwynne 2001; McCartney et al. 2008 [Ch 2]). Spermatophores in bush-crickets, however, are not always large (reviewed in McCartney et al. 2008 [Ch 2]) and certain factors may mediate the extent to which spermatophore size may increase.

Reproductive effort

Resources employed in reproduction are energetically demanding (e.g., Simmons et al. 1992; Simmons 1993) and often limited, so trade-offs between alternative reproductive efforts may be expected (Halliday 1987; Simmons et al. 1992; Lehmann and Lehmann 2008; McCartney et al. 2010). Spermatophores are costly to produce; time, energy and a trade-off in future reproductive potential influence the degree to which a male may invest in spermatophore production (Dewsbury 1982; Simmons 1990; Heller and Helversen 1991; Hayashi 1993; Reinhold and Helversen 1997; Lehmann and Lehmann 2000b; Vahed 2007a). As a consequence, size variation in *Poecilimon* spermatophores may not only reflect selection for large spermatophores through mating effort and paternal investment, but also species-specific trade-offs between alternative mating investments.

To understand this premise in detail, I directly compared the reproductive efforts channelled into mate attraction and those spent in spermatophore production across 37 *Poecilimon* taxa (McCartney and Heller submitted manuscript. [Ch 7]). I hypothesised that increases in energy toward mate attraction would lead to decreases in gift expenditure, ultimately leading to a reduced net gain in fitness through ejaculate protection and paternal investment (McCartney and Heller submitted manuscript. [Ch 7]). First, controlling for male body mass and phylogeny, I corroborated the ejaculate protection function for the nuptial gift in *Poecilimon* (see McCartney et al. 2008 [Ch. 2]). This may be compensated for in males by attracting more or better females through mate attraction. However, in contrast, I observed a significant positive relationship between spermatophylax-gift size and two forms of auditory mate attraction (syllable number produced and tooth impact number), albeit with a negative relationship between peak carrier frequency and nuptial gift size. Larger spermatophore-producing species apparently increase their efforts in mate attraction. While the energetic relationship that peak carrier frequency has with gift size is difficult to interpret, these results contradict the majority of studies stating gifts are expensive to produce (Wedell 1994a; Simmons 1988; Gwynne 1990; Simmons and Bailey 1990; Simmons et al. 1992; Heller and Reinhold 1994; Reinhold and Helversen 1997; Vahed 2007a), and peak carrier frequency should be traded-off with calling efforts (e.g., Simmons 1992). My results show that factors selecting for larger gifts are not necessarily traded for alternate reproductive efforts such as mate attraction, but may in fact work in concert, enhancing spermatophore size (McCartney and Heller submitted manuscript. [Ch 7]). Interestingly, this finding supports a long standing, but previously

untested, hypothesis that calling in bush-crickets evolved in order to advertise costly resources to females (Alexander and Borgia 1979; Alexander et al. 1997).

In order to further understand if competing efforts in mate attraction could explain further variations in nuptial gift size, I explored the evolutionary relationship between long-distance calling and spermatophore size. *Poecilimon* appear to exhibit one of two pair-formation protocols: in some taxa females search for a calling male, in others, males call but females respond acoustically and the male subsequently approaches the female (Heller 1990, 1992; Heller and Helversen 1991, 1993; Heller et al. 2006; McCartney et al. submitted manuscript [Ch 6]). Three hypotheses were proposed to test the relationship between mate-calling and spermatophore size variation across 32 *Poecilimon* species: the resource-advertising hypothesis predicts that calling evolved to advertise resources important to females (Alexander and Borgia 1979; Alexander et al., 1997). Alternatively, males that perform the more risky behaviour, i.e., calling (Thornhill 1979; Alexander et al. 1997), may be selected by females. Lastly, phonotaxis may be energetically expensive and stationary males may conserve energy as well have more time to eat and accumulate energetic reserves. These males may therefore have more available energy to produce larger spermatophores. In support of the resource-advertising hypothesis, spermatophore and spermatophore component sizes are larger in taxa where males are stationary and the females search. In contrast to the predictions of the risky-calling hypothesis, larger spermatophores are unlikely to be selected by females because calling does not appear to be more risky than searching (Heller 1992). While my results are consistent with the idea that males investing in larger spermatophores also invest more in mate attraction (McCartney and Heller

submitted manuscript. [Ch 7]), they are not inconsistent with the idea that males in taxa that are stationary conserve energy for spermatophore production. However, if males are able to re-direct search effort (previously used in phonotaxis) into spermatophore production, it would be expected that male calling evolved first; this does not appear to be the case in tettigoniids (Gwynne 1995; Desutter-Grandcolas 2003).

A greater effort in mate attraction therefore seems to be coupled with larger spermatophore size. Larger spermatophore-investing males that have attained ejaculate protection, increase parental assurance and can invest paternally into their own offspring, would therefore benefit from increasing their abilities to attract females in order to advertise their greater resources (Alexander and Borgia 1979; Alexander et al. 1997; McCartney et al. submitted manuscript. b [Ch 6]). Males of these taxa may resultantly become the limiting sex thereby becoming less ardent, choosier and sought by females (e.g., Gwynne 1985; Heller 1992). In principle, selection for stationary calling would allow males to attain greater food resources while decreasing predation risk, and, in turn, allow an even greater investment in spermatophore production (McCartney et al. submitted manuscript. b [Ch 6]).

Along with increased ejaculate protection, paternal assurance and investment, investing larger amounts of energy and/or resources in alternative reproductive efforts do not limit the large investments that males make in spermatophore size across *Poecilimon*. In contrast, they appear to enhance spermatophore size further. However, while competition between males and trade-offs within males may not limit selection for larger spermatophores through ejaculate protection and paternal investment,

females are likely to be highly selective in their choice of partners; for example, females preferring a high mating frequency may select males with smaller nuptial gifts.

Mate choice

The potential for mate choice to influence spermatophore size variation is most likely seen through comparisons between male and female choice for larger mates or virgin status (McCartney and Heller 2008 [Ch. 3]). Male and female preference for larger partners is expected because body size is related to fecundity in females (e.g., Gwynne 1981, 1984, 1985; Thornhill and Alcock 1983; Simmons and Bailey 1990; Honek 1993; Vincent and Lailvaux 2006), as well as several measures of direct and indirect fitness in males (e.g., disease and parasite load, male and sperm vigour, good genes; reviewed in Wedell and Ritchie 2004). On the other hand, males may be expected to seek virgins in order to avoid sperm competition (e.g., Simmons and Achmann 2000; Simmons 2001) or increase the chance of mating with a younger female that may produce eggs at a greater rate or of better quality than older females (e.g., Rutowski 1982). Virgin males are also typically younger and are less affected by age-related conditions such as parasite load and disease, which negatively influence spermatophore production (for reviews see, Lehmann and Lehmann 2000b; Zuk and Stoehr 2002). Younger males may also produce higher quality sperm (e.g., Reinhardt and Siva-Jothy 2005). Despite the predicted benefits of preferences for larger body size or virgin status, there appears to be little research showing a preference for either in bush-crickets. Furthermore, there appears to be no published research on female preferences for males.

Exposing *Poecilimon laevissimus* males and females to a mate-choice test between a smaller virgin and a larger non-virgin of the opposite sex, I found that smaller virgins are preferred in all cases (McCartney and Heller [Ch 3]. In the one case where the virgin female was larger than the non-virgin female, the virgin was also chosen by the male. In another bush-cricket, *Requena verticalis*, it is youth, *per se*, that is preferred over virginity by males (Lynam et al. 1992; Simmons et al. 1993, 1994). This is understandable in this species because it is typically the first male to mate that sires the majority of the offspring (Gwynne 1988; Gwynne and Snedden 1995; Simmons 1995b; Simmons and Achmann 2000). In terms of selection by males on increasing spermatophore size, however, *P. laevissimus* is unlikely to show a first male sperm precedence pattern (McCartney et al. submitted manuscript a [Ch 4]; McCartney submitted manuscript [Ch 8]), and selection for virgin mates with the spermatophore at its current size is not likely to secure the male more offspring. Furthermore, *P. laevissimus* females may mate twice per day (unpub. data), and males produce spermatophores that are likely too small to afford him either paternal investment (McCartney et al. 2008 [Ch 2]; McCartney et al. submitted manuscript a [Ch 4]) or paternal assurance (McCartney et al. submitted manuscript [Ch 4]; McCartney submitted manuscript [Ch 8]). Males are therefore likely to prefer smaller virgin females as there is no risk of sperm competition, and virgin females may be more fecund than non-virgin females.

Female *Ephippiger ephippiger* (Tettigoniidae) mate with younger males; youth in this species indicates mating history and males with fewer matings produce larger, more nutritious spermatophores and more sperm (Wedell and Ritchie 2004). Similarly

the nuptial gift mass in *P. laevisissimus* is relatively small compared to the volume of sperm produced (McCartney et al. 2008 [Ch 2]); females consume the ampulla far quicker than it takes for the majority of sperm to transfer into the female (McCartney et al. submitted manusc. a [Ch 4]). Female *P. laevisissimus* may therefore select virgin males in order to receive greater sperm loads. Males that produce large spermatophores in the closely related, large spermatophore-producing *Poecilimon zimmeri*, are similarly preferred by females (Lehmann and Lehmann 2008). Apparently, spermatophore resources are important for large and small spermatophore-receiving female *Poecilimon*. While it is difficult to tease apart the effects of female preferences for male size, virginity or youth, female *P. laevisissimus* may actually select for larger sperm loads (and probably spermatophylax-gifts) that are likely coupled with virginity and youth, but not body size, *per se*, in this species.

In contrast to the idea that females may select males that produce smaller spermatophores, this appears to be the first evidence showing that females select directly for virgin males which produce relatively larger sperm volumes. While this research is a preliminary analysis, and further samples and species are required to answer the questions of mate choice definitively, it appears that males of *Poecilimon* taxa that transfer small spermatophores are under strong female selection to enhance gift size. However, in a similar fashion to female mate choice, sexual conflict may be a stronger female-imposed force mediating the production of larger spermatophores.

Operational sex ratio, sexual conflict and reproductive fitness

The operational sex ratio of a population forms the basis of mating systems; the ratio of sexually available males compared to the availability of sexually fertilisable females dictates the level of sexual selection (Trivers 1974; Emlen 1976; Emlen and Oring 1977). In contrast, reproductive fitness is the ultimate measure of sex-specific investments (Clutton-Brock 1998). I consider both of these underlying principles in McCartney (submitted manuscript [Ch 8]). Because the operational sex ratio of a population is only a measure of the population's mating frequency, actual population mating frequency is the best direct measure of the strength that sexual selection has on one sex over the other (see for example, Heller and Helversen 1991). Using sexual conflict as a framework, I compared spermatophore size variations across five field-observed *Poecilimon* species. Specifically, I compared variations in population mating frequencies to male and female reproductive fitness in order to determine the overall level of sexual conflict in *Poecilimon*.

Sexual conflict is expected to occur in bush-crickets because spermatophore size influences female mating frequency (Vahed 2006; reviewed in Vahed 2007b; Gwynne 2007). Males of taxa producing larger spermatophores are predicted to increase their own fitness at a cost to the female by reducing her lifetime polyandry (reviewed in Vahed 2007b; Gwynne 2007). An increase in male fitness due to larger investments in spermatophore production predicts a conflict of interest between the sexes; as a result, *Poecilimon* females are likely to influence spermatophore size variation by selecting smaller spermatophores.

This is the first comparative test of how spermatophore size variation affects male and female fitness in gift-giving species. Controlling for body size and relatedness, I observed that males of taxa that produce relatively large spermatophores were able to secure more eggs, of a greater overall mass, and obtain relatively more hatched offspring per-mating than taxa producing smaller gifts. However, relative spermatophore size did not correspond to female longevity, relative lifetime production of egg number or egg mass, and could not explain variations in hatching success across the females of *Poecilimon* taxa. I therefore concluded that while males of large spermatophore-bearing taxa receive greater fitness, conflicts of interest are unlikely because overall female fitness remains unaffected. Spermatophore size variation therefore does not appear to be influenced by sexual conflict over mating rates or spermatophore size across *Poecilimon*.

In a comparative study across 19 bush-crickets Wedell (1994a) showed that large spermatophores may function as paternal investment. While this has been confirmed in focal species studies (Gwynne 1984, 1988; Simmons 1990; Reinhold and Heller 1993; Reinhold 1999), this is the first comparative research showing that male per-mating fitness increases as a result of producing larger spermatophores. Furthermore, I found no evidence that females are negatively influenced by decreases in polyandry, thus raising doubt about whether male *Poecilimon* 'manipulate' females. Because males of taxa producing larger spermatophores are not selected by females to produce smaller 'non-manipulating' spermatophores, it is likely that females obtain greater net benefits from the larger donations. For example, females in taxa that receive larger spermatophores typically move toward the stationary calling male (Heller 1990,

1992, 2006; Heller and Helversen 1991, 1993; McCartney et al. submitted manusc. b [Ch 6]). Moving individuals are exposed to higher rates of predation (Heller 1992). Lower mating frequencies therefore benefit females by reducing the predation risk that they are exposed to.

Female selection on males to produce smaller spermatophores appears unlikely; even in the small spermatophore species, *P. laevissimus*, larger spermatophore-producing virgin males are selected by females (McCartney and Heller 2008 [Ch 3]). Furthermore, the large spermatophore-receiving females in *P. v. veluchianus* produce heavier offspring (Reinhold and Heller 1993), and produce fitter offspring under food restricted conditions compared with females receiving no spermatophores (Reinhold 1999). While the spermatophore volume received over the lifetime across *Poecilimon* taxa appears to be similar, females that receive all their spermatophore material in the first mating can assimilate this quickly (Voigt et al. 2008) and it may be used to increase egg production over the female's lifespan, in comparison to females that receive small volumes consistently through-out their life. In any case, *Poecilimon* spermatophores do not seem to be selected by females to be small under predictions of sexual conflict, and again it seems, given the advantages obtained through ejaculate protection, paternal investment, and mate choice, that males, given optimum environmental conditions, are selected to produce larger spermatophores.

Future research

Each behavioural influence on spermatophore size variation presented here has suggested areas that need further exploration. While many of these suggestions were subsequently addressed in later manuscripts, much of this work simply called for increases in sample size (e.g., McCartney and Heller 2008 [Ch 3]; McCartney et al. submitted manusc. a [Ch 4]; McCartney submitted manusc. [Ch 8]), and are covered appropriately within the respective chapters. However, at least two factors highlighted in this thesis remain to be fully understood.

The conclusions drawn in McCartney (submitted manusc. [Ch 8]) are partly based on the assumption that all *Poecilimon* species have similar post-mating sperm utilisation patterns (see particularly McCartney submitted manusc. [Ch 8]; but see also McCartney et al. 2008 [Ch 2]; McCartney et al. submitted manusc. a [Ch 4]). While this assumption has a solid foundation, the study would have benefitted from a full understanding of the sperm precedence pattern and the number of sperm used to fertilise each egg from all taxa presented; spermatophore size may vary according to the precedence pattern (Achmann 1996). Future work on *Poecilimon* nuptial gift size variation would benefit from a better understanding of sperm utilisation patterns.

While studying spermatophore component size variation between two *Poecilimon* groups that differ in their pair-formation protocol (McCartney et al. submitted manusc. b [Ch 6]), I was unable to control for relatedness because wing loss is not a monophyletic trait across *Poecilimon*, i.e., some species where males call and females respond phonotactically are secondarily derived in the *Poecilimon* clade that

typically shows male phonotaxis. These species also appear to have large spermatophores (instead of small spermatophores typical of their clade (McCartney et al. submitted manusc. b [Ch 6])), but there are either no spermatophore data for these species or their phylogenetic relationship within the *Poecilimon* group is not well understood. This research would have been improved with the addition of spermatophore data or phylogenetic placement of these additional species so relatedness could be controlled.

Conclusions

Poecilimon spermatophore size varies markedly between species and may be viewed as a model system for investigating spermatophore size variation across Orthoptera (McCartney et al. 2008 [Ch 2]). The ejaculate protection and paternal investment functions of the spermatophore select for larger gifts in *Poecilimon* (McCartney et al. 2008 [Ch 2]; McCartney et al. 2010 [Ch 5]; McCartney et al. submitted manusc. a [Ch 4]). Furthermore, increases in paternal assurance (McCartney submitted manusc. [Ch 8]) may also allow for an increase in ability to advertise expensive gifts (McCartney et al. submitted manusc. b [Ch 6]). While a reduction in spermatophore size may be expected through female selection, opportunities for females to increase sperm receipt in small spermatophore-producing species conversely select for larger gifts (McCartney and Heller 2008 [Ch 3]). Furthermore, the sperm utilisation patterns of females do not appear to reduce male investment in spermatophore production (McCartney et al. submitted manusc. a [Ch 4]; McCartney submitted manusc. [Ch 8]),

and the majority of species, independent of gift size, match sperm transfer with female gift consumption time (McCartney et al. submitted manuscript. a [Ch 4]).

Further restrictions to spermatophore production are predicted through trade-offs between alternative reproductive efforts. However, in contrast to a trade-off, male *Poecilimon* advertise large spermatophore resources to females (McCartney et al. submitted manuscript. b [Ch 6]; McCartney and Heller submitted manuscript. [Ch 7]). Sexual conflict may also mediate spermatophore size because spermatophores are predicted to manipulate female polyandry in a dose-dependent manner (McCartney submitted manuscript. [Ch 8]). However, while male per mating fitness increased with increasing spermatophore size, there is no apparent direct cost of spermatophore magnitude on female fitness as a result of decreased polyandry in *Poecilimon*.

Ultimately, few behaviour-related factors seem to play a significant role in selecting against large spermatophore size. The direct effects of environmental conditions on spermatophore size variation and fitness needs to be studied among species and within species among populations, to fully understand spermatophore size variation across *Poecilimon*.

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