Received Date : 15-Dec-2015

Initial Acceptance Date: 11-Feb-2016

Final Acceptance Date:16-Mar-2016

Editor:T. Tregenza

Article type : Research Paper

Male mating success and the effect of mating history on ejaculate traits in a facultatively parthenogenic insect (*Extatosoma tiaratum*)

Yasaman Alavi[1] (corresponding author), Mark Adrian Elgar[1] (m.elgar@unimelb.edu.au), Therésa Melanie Jones[1] (theresa@unimelb.edu.au)

School of BioSciences, University of Melbourne, Victoria 3010, Australia[1]

Total number of words = 4694

Author for Correspondence
Yasaman Alavi
School of BioSciences, University of Melbourne,
Victoria 3010, Australia
+61 (3) 83 44 79 86
Email: yasaman.s.alavi@gmail.com

Abstract

Males can typically increase their lifetime reproductive success by mating with multiple females. However, recent studies across a broad range of species have demonstrated physiological constraints on male multiple mating. In this study, we investigate male mating capacity in *Extatosoma tiaratum*, a facultative parthenogenetic phasmatid. Sperm limitation is thought to be one factor favouring the evolution and maintenance of parthenogenetic reproduction, but studies on male mating ability in facultative parthenogenetic species are extremely rare. To explore whether male mating success varies with mating history, we provided males with weekly mating opportunities with different females throughout their lives. We then observed mating success, and the variation in ejaculate size and quality within each mating. We showed that most, but not all, males can mate multiply, however the amount of ejaculate produced is variable and depends upon male body mass and mating history.

Key words. Ejaculate size, Sperm viability, Spermatophore mass, mating interval facultative parthenogenesis, Extatosoma tiaratum

Introduction

Traditionally, mating was assumed to be inexpensive for males, due to the relative small size of sperm compared with the larger eggs produced by females (Trivers 1972). Accordingly, males were assumed to have an unlimited supply of sperm allowing them to mate with all available fertile females (Bateman 1948). Moreover, studies documenting male multiple mating and even extreme male mating frequencies for a range of taxa were relatively common (Andersson 1994). However, mounting evidence demonstrates that both sperm production and copulation are energetically costly, and thus male reproductive potential may be physiologically constrained (Dewsbury 1982; Wedell *et al.*, 2002; Scharf *et al.*, 2013). Thus, while in theory selection should favor high male mating frequency, the reality is that this may not always be physiologically possible, even in the absence of female discrimination (Dewsbury 1982).

Empirical evidence for physiological constraints on male mating, in terms of declines in ejaculate size and quality or reduced male lifespan has been documented for both vertebrates and invertebrates (vertebrates e.g. Huber *et al*., 1980; Nakatsuru & Kramer 1982; Warner *et al*., 1995; Preston *et al*., 2001; invertebrates e.g. Christenson 1989; Van Voorhies 1992; Rigaud & Moreau 2004). In the insects, sperm depletion following multiple mating is taxonomically widespread and both the quantity of sperm (Watanabe *et al*., 1998; Torres-Vila & Jennions 2005; Marcotte *et al*., 2005; Wedell 2010; Elzinga *et al*., 2011; Paukku & Kotiaho 2005; Rönn *et al*., 2008; Damiens *et al*., 2002; Damiens & Boivin 2005; Partridge & Farquhar 1981; Pitnick 1993, Jones 2001; Oliver & Cordero 2009) and quality of sperm (Dowling & Simmons 2012) may decline across successive matings. In cases with moderate or no decrease in ejaculate production, male mating history may affect other aspects of male reproductive success, such as longevity or mating frequency (e.g. Oliver & Cordero 2009, Lewis *et al*., 2011; Salehialavi *et al*., 2011).

Facultative parthenogenesis, in which females have the ability to reproduce both sexually and, if they remain unmated, parthenogenetically, is taxonomically widespread (Kramer & Templeton 2001; Matsuura *et al*., 2004; Booth *et al*., 2012), and unusually common among some insect orders, such as the Phasmatodea (Maynard Smith 1978). The adaptive significance of facultative parthenogenesis is poorly understood, but it has been proposed as a potential adaptation to male- or sperm-limitation (Matsuura & Nishida 2010; Schneider & Elgar 2010; Schwander *et al*., 2010; Elzinga *et al*., 2011). However, few, if any, studies of facultatively parthenogenetic species have investigated either the degree of male multiple mating or the capacity of males to produce sperm at each mating attempt, yet such data are pivotal for understanding the evolution and maintenance of parthenogenetic reproduction.

In this study, we investigated male multiple mating in Macleay's Spectre, *Extatosoma tiaratum*, a facultatively parthenogenetic Australian insect (Phasmatodea). *E. tiaratum* occurs in the rainforests of tropical and subtropical Queensland and northern New

South Wales (Gurney 1947). Females oviposit continuously throughout their adult lifespan, and un-fertilized eggs develop into female offspring (Carlberg 1983). Parthenogenetic reproduction appears to be a strategic response to the absence of males, since females delay egg laying when reared with males at the juvenile stages (Schneider & Elgar 2010). Copulations typically last up to 19 hours, during which time a male attaches a spermatophore to the female's terminal genital segment (Carlberg 1983). To explore male multiple mating, we provided males with a mating opportunity each week, and asked whether the variation in ejaculate size and quality depended upon male mating history. We expected males to mate with multiple females, as male biased sex ratios, a condition required for the evolution of monogyny (male monogamy), is not expected in facultative parthenogenetic populations (Fromhage *et al.*, 2005). However, in line with previous data on other invertebrates, including insects, we also expected that males would be limited in the amount of sperm they could transfer and thus we predicted declines in ejaculate characteristics over successive matings.

Methods

Experimental animals

A stock population was established from individuals obtained from various insect breeders across the Melbourne region (Victoria, Australia). Males and females were maintained in mesh cages (46 × 46 × 91 cm^3) in one of two climate-controlled laboratories under identical conditions (24-26° C; 50% humidity; 12:12hr light:dark cycle). Males and females were reared in different laboratories to ensure there were no pheromone-derived influences on reproductive behavior that could affect the current mating environment (see Schneider & Elgar 2010). All individuals were provided with the leaves of various species of *Eucalyptus*, *ad libitum*, which were lightly sprayed with water daily and replaced regularly as required. Twenty final instar experimental male offspring were selected from the stock population. Experimental males were maintained until approximately four weeks after moulting to adulthood. Following their final moult, adult males were uniquely marked with a drop of non-toxic acrylic paint on their left tarsus and kept in a single mesh cage.

Experimental design

Two weeks after reaching adulthood, each of the 20 adult males was allocated a weekly mating opportunity (every 5-7 days) for the duration of his life. At each mating opportunity, a male was transferred to a mating cage containing at least ten sexually mature females (virgin females were added to mating cages weekly to maximize female receptivity). This experimental protocol ensured that males had the opportunity to find preferred mates and intra-sexual competition between males did not interfere with the opportunity to mate. The cages were monitored five hours after dark and males were removed if they had not mounted any females. Copulating pairs were checked every 30 minutes for a successful mating (defined as spermatophore transfer), and the externally transferred spermatophore was removed from the female's genitalia using fine forceps once it was fully produced but prior to any sperm transfer. Each spermatophore was weighed (to the nearest 0.0001g) and digital images of each spermatophore were obtained from three different perspectives. The diameter was measured using ImageJ (1.46r) software, and the volume was estimated by assuming a spherical shape. Each copulating pair was kept in a separate container until the male detached. Males were weighed before and after mating, and tarsus length was also measured as an estimate of body size.

Preparation of the sperm solution

The sperm solution was prepared by first cutting the neck of the spermatophore using micro-scissors. The spermatophore was transferred into a 1.5 mL microcentrifuge tube containing 80 μl of 0.04 Beadle saline (128.3 mM NaCl, 4.7 mM KCl, and 23 mM $CaCl_2$) and squeezed gently before being left for one hour to ensure complete transfer of sperm into Beadle solution. The solution was then gently mixed and sperm density and viability was measured via two separate methods.

Sperm density assay

A total of 1 μl of the sperm solution was pipetted into a 200 μl microcentrifuge tube and diluted 1:100 in distilled water. 10 μl of the diluted sperm solution was pipetted on the well of a haemocytometer. Sperm were visualized using light microscopy ® Leica DM 2500 (Leica Microsystems GmbH, Wetzlar, Germany), at 200× magnification, with all sperm within five predetermined grid squares counted. Sperm density was calculated by multiplying mean haemocytometer count by its dilution factor to calculate sperm density. Total sperm count was calculated as the product of spermatophore volume and sperm density.

Sperm viability assay

We used the ® LIVE/DEAD assay (Molecular Probes, Sigma, Australia, L-7011) to estimate sperm viability (see Damiens *et al.*, 2002). We pipetted 5 μl of the diluted sperm onto a glass slide and added 10 μl of 1:50 diluted 1mM SYBR14. The slide was incubated at room temperature in the dark for ten minutes before adding 2 μl of 2.4 mM Propidium Iodide followed by an additional 10 minute incubation. Samples were observed under fluorescent microscopy 30 minutes after staining (blue excitation filter at λ= 490 nm; ×20 magnification). Ten images were taken from different field views (200× magnification) on each slide and the proportion of live to dead sperm was quantified. At least 500 live spermatozoa were counted per sample.

Statistical analyses

We used general linear mixed models (GLMM including male ID as a random effect) in JMP version 12 to examine the effect of mating number on ejaculate size (spermatophore mass and sperm density). To investigate the effect of mating number on sperm viability, we used the non-parametric Wilcoxon test weighted by the total number of sperm counted per sample. To remove the potential problem of the first mating interval being recorded as zero, and thus biasing models where we specifically needed to include the first interval, we added seven days (the minimum number of days we permitted a male to rest between mating opportunities) to the time until successful production of a spermatophore following the first mating opportunity. Thus, if a male mated and transferred a spermatophore on his first mating opportunity, his first mating interval would be recorded as 7; however if he failed to transfer a spermatophore on this attempt and mated on his subsequent attempt (approximately seven days later) his first mating interval would be recorded as 7+7 = 14 days. For all subsequent mating intervals, the actual number of days between the current and the previous spermatophore produced was taken as the mating interval. Spermatophores that were not removed immediately after production were excluded from analyses of sperm quantity and quality (11 spermatophores from the first mating and one from the third mating). As few males mated more than five times, spermatophores from the fifth and any subsequently matings were pooled for the spermatophore mass and sperm density analyses. For sperm viability analysis we excluded 6+ spermatophores, as the models with and without these spermatophores were similar and we were unable to include male ID as a random effect in the nonparametric model. Where possible, we included mating interval (the total number of days between two consecutive matings), male body mass before each mating, male age and tarsus length as covariates in all models; terms were dropped where $P > 0.10$. Data were transformed where necessary to improve normality. Unless otherwise stated, all presented averages are means ± standard errors.

Results

Male mating history and survival

During their adult lifespan of 16.6 ± 3.7 weeks, males mated on average 4 ± 0.4 times (minimum = 1 mating, maximum = 8 matings, median = 4 matings, interquartile range = 3.2 matings). Nine of twenty virgin males mated at their first mating opportunity and the average age at first mating was 23.9 ± 1.8 days post final moult (minimum = 14 days, maximum = 48 days, median = 22 days, interquartile range = 8 days). On average, males lost 6.4 ± 0.7 % of their body mass during copulation and transferred a spermatophore that was roughly 1.4 ± 0.04 % of their body mass. Male body size (tarsus length) did not influence either the total number of spermatophores produced (GLM with Poisson error distribution and log-link: $F_{1, 17} = 0.20$, $P = 0.66$), or the age at first mating ($F_{1, 17} = 0.95$, $P = 0.34$). Average male mating interval was 16.2 ± 1.2 days (minimum = 7 days, maximum = 48 days, median = 13 days, interquartile range = 14 days). Proportional hazards survival analysis revealed that male survival was comparable for all males regardless of the number of spermatophores produced ($\chi^2_{1, 16} = 0.44$, $P = 0.50$), their average body mass ($\chi^2_{1, 16} = 0.16$, $P = 0.69$), and the average mass of spermatophores produced ($\chi^2_{1, 16} = 0.6$, $P = 0.44$). Male identity (random effect) did not account for more than 14% of the explained variation in any of our models.

Effects of multiple mating on ejaculate size and quality

Spermatophore mass –Spermatophore mass varied significantly with spermatophore number (Table 1a). Post-hoc Tukey's tests revealed that, on average, the mass of spermatophores 1-3 were comparable but that spermatophore mass declined between the third and subsequent spermatophores (Fig. 1a). Spermatophore mass was positively related to male body mass (Fig 2a), male mating interval (Fig 2b) and male age (Table 1a).

Sperm density – Sperm density varied significantly with spermatophore number and was positively correlated with mating interval (Table 1b, Fig 2c). Post hoc Tukey's tests revealed sperm density was highest in the first and second spermatophores produced and lowest in the fifth or more spermatophores (Fig. 1b). We repeated the above analysis using total sperm count (sperm density multiplied by spermatophore volume) and achieved qualitatively similar results (results not presented).

Sperm viability – Overall, the proportion of viable sperm was relatively high (Median= 0.96, interquartile range = 0.21, N = 20 males and 4 ± 4.4 spermatophores produced per male). The proportion of viable sperm was not correlated with spermatophore number (Fig 1c), male body mass, or mating interval (Table 1c). However, the proportion of viable sperm increased with male age.

Discussion

Our results showed that while males of *E. tiaratum* are capable of multiple mating, they did not mate with every opportunity: males typically mated in less than 30% of the weekly mating trials in which they were provided with access to females. The data also suggest that males are limited in the amount of ejaculate they are able to invest over successive matings: both spermatophore mass and sperm density decreased with increasing number of matings.

Our results add to the growing body of evidence for male physiological constraints on ejaculate production. Firstly, there was variation in the amount of ejaculate transferred across matings. Spermatophore size was the largest in the second mating and on average declined 23.22 ± 3% in the final mating. Sperm density was the highest at the first and second mating and again significantly declined in later matings. Secondly, the positive relationship between both spermatophore mass and sperm density, with mating interval (Fig 1b and c) suggests that males need time to replenish their sperm reserves following mating. Contrary to the results for ejaculate quantity, we found no evidence for reduction in ejaculate quality: sperm viability was comparable across all spermatophores produced. The latter result is perhaps unsurprising as selection on sperm viability is often strong in polyandrous insects (Hunter & Birkhead 2002; García-González & Simmons 2005). This lack of a difference may have arisen due to small sample sizes, but it is also possible that other components of ejaculate quality such as sperm size and velocity or any tradeoffs between sperm traits (not measured here), were affected by male mating history.

Our data do not fully support the traditional view of male reproductive success (*sensu* Bateman 1948): *E. tiaratum* males were limited in both the number of matings achieved and the amount of ejaculate provided to each mate. The former result is particularly interesting given that males were provided with ten females simultaneously. We suggest it is unlikely that a lack of available females could explain the observed low mating frequency. We instead suggest two mutually non-exclusive alternatives: either, all ten females found the male unattractive at a given mating opportunity and rejected his attempt, or males were physiologically constrained and thus unable to produce an ejaculate and/or copulate successfully. While we are unable to discount either mechanism entirely, the second explanation seems the more parsimonious, given the positive correlations between mating interval with spermatophore mass and sperm density, and the fact that more than half of the virgin males did not mate when first presented with a mating opportunity. Although we note that the females may also have discriminated against sperm-depleted males and thus avoided them. While *E. tiaratum* females cease sexual signaling (releasing a sex pheromone) once they commence egg laying (Schenider & Elgar 2010), female receptivity does not appear to be directly correlated with sexual signaling because ovipositing females will mate if they encounter a male. Accordingly, we do not expect ovipositing to influence male mating frequency.

Lifetime male mating success was not related to male body size, suggesting that females do not discriminate between males according to their size, a common sexually selected trait (Jennions *et al.*, 2001). However, males appear to be sexually immature following their final moult to adulthood. Few males mated in their first attempt, and the average age at first mating was 23.9 ± 1.8 days post final moult. Whether this latency period prior to the first mating is a product of the need to acquire somatic resources, or to ensure sperm maturation following sexual maturity at the adult stage of the lifecycle is unknown. However, males typically lost 6.4% of their body mass following each mating, highlighting that mating is physically costly for males both directly in terms of investment in the mating act and spermatophores themselves, and indirectly through lost foraging opportunities (males do not feed during copulation, which lasts up to 19 hours). This may have significant consequences for *E. tiaratum* males in particular, as their natural diet predominantly comprises *Eucalyptus* leaves with low nutritional value (Moore *et al*., 2004).

Sperm limitation may be a more significant factor favouring the maintenance of parthenogenesis than is generally appreciated, since mating with sperm depleted males can influence female reproductive success (Wedell & Ritchie 2004; Jones *et al*., 2006; Lauwers & Van Dyck 2006; Elzinga *et al*., 2011). Males of *E. tiaratum* are capable of multiple mating, consistent with the likely female-biased populations in facultative parthenogenetic species. However, male physiological constraints may affect female fertilization success either because males are unwilling to mate, or they transfer insufficient sperm. Such constraints are likely more important in populations with low mate encounter rates (due to stochastic changes in population densities or environmental factors, Gascoigne *et al.*, 2009). Theoretically, parthenogenetically produced offspring may have reduced fitness compared with sexually produced offspring (Maynard Smith 1986, Kondrashov 1988), however parthenogenesis may rescue maternal fitness if the alternative is mating failure. By producing female offspring through parthenogenesis, females will increase their fitness, especially if some of their daughters can find a mate and reproduce sexually. Future research might be profitably aimed at investigating potential links between sperm limitation and parthenogenetic reproduction by studying egg fertilization patterns and mate encounter rates in natural, ecologically varied, populations.

Acknowledgments

We thank Ellie Michaelides for her help in developing sperm staining methods and Dr. Gareth Hopkins and Luke Barrett for their comments on the manuscript. YA was funded by the Holsworth Wildlife Research Endowment 2013-2014.

References

Andersson, M.B. 1994: Sexual selection. Princeton Univ. Press. Princeton, NJ.

Bateman, A.J. 1948: Intra-sexual selection in Drosophila. Heredity, 2(3), 349–368.

Booth, W., Smith, C. F., Eskridge, P. H., Hoss, S. K., Mendelson, J. R. & Schuett, G.W. 2012: Facultative parthenogenesis discovered in wild vertebrates. Biol. Lett. 8(6), 983–985.

Carlberg, U. 1983: Copulation in Extatosoma tiaratum (MacLeay)(Insecta: Phasmida). Zool. Anz. 5(6), 340-356

Christenson, T., 1989: Sperm depletion in the orb-weaving spider Nephila clavipes (Araneae, Araneidae). J. Arachnol. 115–118.

Damiens, D., Bressac, C., Brillard, J. P. & Chevrier, C. 2002: Qualitative aspects of sperm stock in males and females from Eupelmus orientalis and Dinarmus basalis (Hymenoptera : Chalcidoidea) as revealed by dual fluorescence. Physiol. Entomol. 27(2), 97–102.

Damiens, D. & Boivin, G., 2005: Male reproductive strategy in Trichogramma evanescens: sperm production and allocation to females. Physiol. Entomol. 30(3), 241–247.

Dewsbury, D.A., 1982: Ejaculate cost and male choice. Amer. Nat. 119(5), 601–610.

Dowling, D.K. & Simmons, L.W., 2012: Ejaculate economics: testing the effects of male sexual history on the trade-off between sperm and immune function in Australian crickets. PLoS One. 7: e30172.

Elzinga, J.A. Chevasco, V., Grapputo, A. & Mappes, J. 2011: Influence of male mating history on female reproductive success among monandrous Naryciinae (Lepidoptera: Psychidae). Ecol. Entomol. 36(2), 170–180.

Fromhage, L., Elgar, M.A. & Schneider, J.M., 2005: Faitful without care: the evolution of monogamy. Evolution. 59(7), 1400–1405.

García-González, F. & Simmons, L.W., 2005: Sperm viability matters in insect sperm competition. Curr. Biol. 15(3), 271–275.

Gascoigne, J., Berec, L., Gregory, S. & Courchamp, F. 2009: Dangerously few liaisons: a review of mate-finding Allee effects. Popular Ecol. 51, 355–372.

Gurney, A.B. 1947: Notes on some remarkable Australasian walkingsticks, including a synopsis of the genus Extatosoma (Orthoptera: Phasmatidae). Ann. Entomol. Soc. Am. 40(3), 373–396.

Gwynne, D.T. 2008: Sexual conflict over nuptial gifts in insects. Annu. Rev. Entomol. 53, 83–101.

Huber, M. H. R., Bronson, F. H. & Desjardins, C. 1980: Sexual activity of aged male mice: correlation with level of arousal, physical endurance, pathological status, and ejaculatory capacity. Biol. Rep. 23(2), 305–316.

Hunter, F. M. & Birkhead, T. R. 2002: Sperm viability and sperm competition in insects. Curr. Biol. 12(2), 121–123.

Jennions, M. D., Moller, A. P. & Marion, P. 2001: Sexually Selected Traits and Adult Survival: A Meta-Analysis. Q. Rev. Biol. 76(1), 3–36.

Jones, T.M. 2001: A potential cost of monandry in the lekking sandfly Lutzomyia longipalpis. J. Insect. Behav. 14(3), 385–399.

Jones, T. M., McNamara, K. B., Colvin, P. G. R, Featherson, R. & Elgar, M. A. 2006: Mating frequency, fecundity and fertilization success in the hide beetle, Dermestes maculatus. J. Insect. Behav. 19(3), 357–371.

Kondrashov, A. S. 1988: Deleterious mutations and the evolution of sexual reproduction. Nature. 336, 435-440.

Kramer, M. G. & Templeton, A. R. 2001: Life history changes that accompany the transition from sexual to parthenogenetic reproduction in *Drosophila Mercatorum*. Evolution. 55(4), 748–761.

Lauwers, K. & Van Dyck, H. 2006: The cost of mating with a non-virgin male in a monandrous butterfly: experimental evidence from the speckled wood, *Pararge aegeria*. Behav. Ecol. Sociobiol. 60(1), 69–76.

Lewis, Z., Sasaki, H. & Miyatake, T. 2011: Sex starved: do resource-limited males ensure fertilization success at the expense of precopulatory mating success? Anim. Behav. 81(3), 579–583.

Marcotte, M., Delisle, J. & McNeil, J.N. 2005: Impact of male mating history on the temporal sperm dynamics of *Choristoneura rosaceana* and *C. fumiferana* females. J. Insect. Physiol. 51(5), 537–544.

Matsuura, K., Fujimoto, M. & Goka, K. 2004: Sexual and asexual colony foundation and the mechanism of facultative parthenogenesis in the termite *Reticulitermes speratus* (Isoptera, Rhinotermitidae). Insectes Sociaux. 51(4), 325–332.

Matsuura, K. & Nishida, T. 2001: Comparison of colony foundation success between sexual pairs and female asexual units in the termite Reticulitermes speratus (Isoptera: Rhinotermitidae). Pop. Ecol. 43(2), 119–124.

Maynard Smith, J. 1986: Contemplating life without sex. Nature. 324, 300-301.

Maynard Smith, J. 1978: The evolution of sex, New York: Cambridge Univ. Press.

Moore, B. D., Wallis, I. R., Wood, J. T. & Foley, W. J. 2004: Foliar nutrition, site quality, and temperature influence foliar chemistry of tallowwood (*Eucalyptus microcorys*). Ecol. Monographs. 74(4), 553–568.

Nakatsuru, K. & Kramer, D. L. 1982: Is sperm cheap? Limited male fertility and female choice in the lemon tetra (Pisces, Characidae). Science. 216(4547), 753–755.

Oliver, C. & Cordero, C. 2009: Multiple mating reduces male survivorship but not ejaculate size in the polygamous insect *Stenomacra marginella* (Heteroptera: Largidae). Evol. Ecol. 23(3), 417–424.

Partridge, L. & Farquhar, M. 1981: Sexual activity reduces lifespan of male fruitflies. Nature. 294, 580-582.

Paukku, S. & Kotiaho, J. S. 2005: Cost of reproduction in Callosobruchus maculatus: effects of mating on male longevity and the effect of male mating status on female longevity. J. Insect. Physiol. 51(11), 1220–1226.

Pitnick, S. 1993: Operational sex ratios and sperm limitation in populations of *Drosophila pachea*. Behav. Ecol. Sociobiol. 33(6), 383–391.

Preston, B. T, Stevenson, I. R., Pemberton, J. M. & Wilson, K. 2001: Dominant rams lose out by sperm depletion. Nature. 409, 681–682.

Rigaud, T. & Moreau, J. 2004: A cost of Wolbachia–induced sex reversal and female–biased sex ratios: decrease in female fertility after sperm depletion in a terrestrial isopod. Proc. R. Soc. Lond. Ser. B. Biol. Sci. 271, 1941–1946.

Rönn, J. L., Katvala, M. & Arnqvist, G. 2008: Interspecific variation in ejaculate allocation and associated effects on female fitness in seed beetles. J. Evol. Biol. 21(2), 461–470.

Salehialavi, Y., Fritzsche, K. & Arnqvist, G. 2011: The cost of mating and mutual mate choice in 2 role–reversed honey locust beetles. Behav. Ecol. 22(5), 1104–1113.

Scharf, I., Peter, F. & Martin, O.Y. 2013: Reproductive trade-offs and direct costs for males in arthropods. Evol. Biol. 40(2), 169–184.

Schneider, A. & Elgar, M.A. 2010: Facultative sex and reproductive strategies in response to male availability in the spiny stick insect, *Extatosoma tiaratum*. Aust. J. Zool. 58(4), 228–233.

Schwander, T. Vuilleumier, S., Dubman, J. & Crespi, B. J. 2010: Positive feedback in the transition from sexual reproduction to parthenogenesis. Proc. R. Soc. Lond. Ser. B. Biol. Sci. 277, 1435–1442.

Torres-Vila, L. M. & Jennions, M. D. 2005: Male mating history and female fecundity in the Lepidoptera: do male virgins make better partners? Behav. Ecol. Sociobiol. 57(4), 318–326.

Trivers, R. 1972: “Parental investment and sexual selection”. Sexual Selection & the Descent of Man, New York: Aldine de Gruyter.

Van Voorhies, W. A. 1992: Production of sperm reduces nematode lifespan. Nature 360, 456–458

Warner, R. R., Shapiro, D. Y., Marcanato, A. & Petersen, C. W. 1995: Sexual conflict: males with highest mating success convey the lowest fertilization benefits to females. Proc. R. Soc. Lond. Ser. B. Biol. Sci. 262, 135–139.

Watanabe, M., Wiklund, C. & Bon’no, M.1998: The effect of repeated matings on sperm numbers in successive ejaculates of the cabbage white butterfly *Pieris rapae* (Lepidoptera: Pieridae). J. Insect. Behav. 11(4), 559–570.

Wedell, N. 2010: Variation in male courtship costs in butterflies. Behav. Ecol. Sociobiol. 64(9), 1385–1391.

Wedell, N., Gage, M. J. G. & Parker, G. A. 2002: Sperm competition, male prudence and sperm-limited females. Trends. Ecol. Evol. 17(7), 313–320.

Wedell, N. & Ritchie, M. G. 2004: Male age, mating status and nuptial gift quality in a bushcricket. Anim. Behav. 67(6), 1059–1065

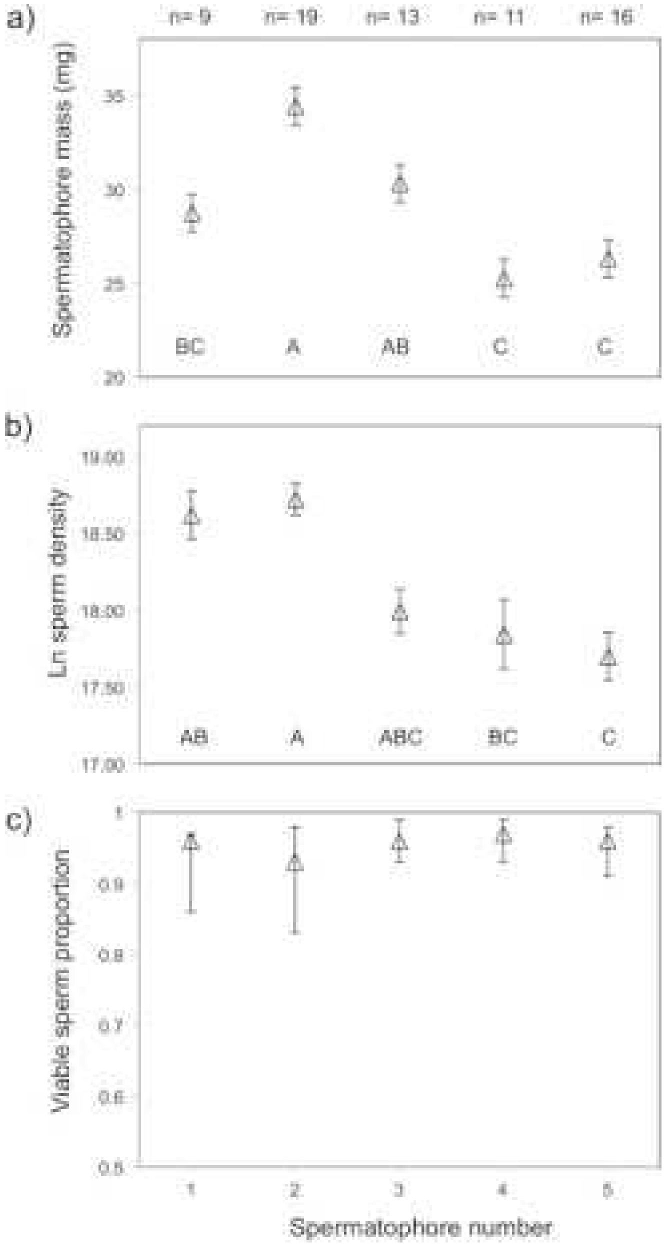
Table 1 The effect of male body mass, male age, mating number and mating interval on a) spermatophore mass, b) sperm density (Ln transformed) and c) viable sperm proportion across males.

Model Parameters	β ± SE	DF	Statistic	Probability
a) Spermatophore mass				
Male body mass	0.006 ± 0.002	1	F= 11.10	**0.002**
Male age	9.13 ± 4.12 × 10-5	1	F= 4.90	**0.03**
Spermatophore number		4	F= 10.40	**<0.0001**
Mating interval	0.0001± 4.43 × 10-5	1	F= 18.79	**<0.0001**
b) Sperm density				
Spermatophore number		4	F= 4.96	**0.002**
Mating interval	0.02 ± 0.007	1	F= 6.51	**0.01**
c) Sperm viability (individual non-parametric models)				
Male body mass		n = 59	$r_s = 0.09$	0.50
Male age		n = 59	$r_s = 0.27$	**0.04**
Spermatophore number		n = 59	$\chi_4^2 = 7.29$	0.12
Mating interval		n = 59	$r_s = -0.14$	0.28

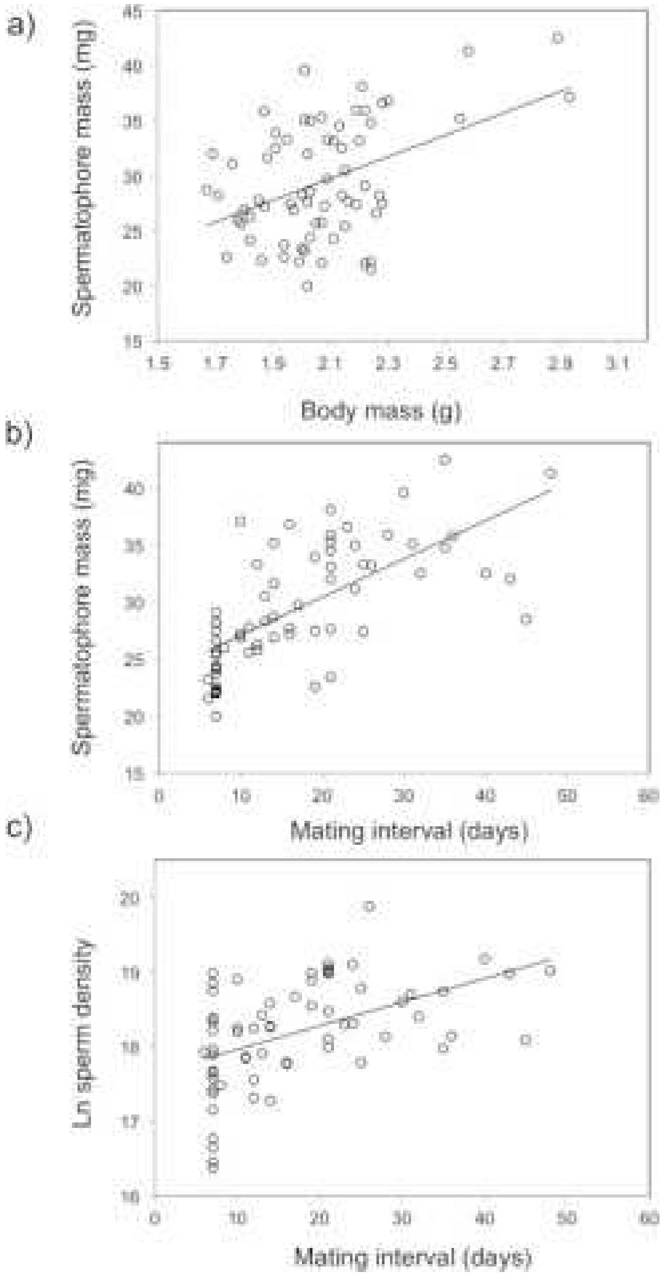
Figure legends

Fig 1. The relationship between spermatophore number and ejaculate traits; a) spermatophore mass, b) sperm density (Ln transformed), and c) viable sperm proportion; Levels not connected by same letters are significantly different based on post hoc Tukey's tests.

Fig 2. The relationship between a) spermatophore mass and body mass ($r^2 = 0.19$, P= 0.0002), b) spermatophore mass and mating interval ($r^2 = 0.45$, P <0.0001) c) sperm density (Ln transformed) and mating interval ($r^2 = 0.24$, P <0.0001)



eth_12497_f1.tif



eth_12497_f2.tif