Sperm size and number variation in the red flour beetle

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Disruptive selection between large, nutritive gametes and numerous, competing gametes may have driven the evolution and maintenance of anisogamy. Sperm competition can explain why there are so many tiny sperm because numerical competition between rival gametes drives males to maximize sperm number and this may be achieved by minimizing sperm size. Since males operate within a finite reproductive budget and ejaculate production is limited, we might predict that, when variation in sperm size exists, males must trade increases in sperm size against a decrease in sperm number. We use *Tribolium castaneum* as our model to investigate the existence of a sperm size–number trade-off. We sampled 14 different populations that have been isolated for different periods (up to 39 years) and find across this sample of 70 males that there is significant variation in both sperm length and ejaculate sperm number between males. Despite this significant variance, we find no evidence for any relationship between sperm size and number across males. There is some evidence for a trade-off when we analyse across 14 population means, but this relationship is not robust and disappears when a single outlier is omitted. We conclude that sperm size and ejaculate sperm number vary independently, but that differential allocation to gonadal tissue and/or ejaculation frequency would permit this independent variation.   © 2001 The Linnean Society of London

ADDITIONAL KEY WORDS: *Tribolium castaneum* – sperm competition – gamete – ejaculate – anisogamy.

INTRODUCTION

The evolution of anisogamy may have arisen via disruptive selection for individuals that produced either nutritive or competitive gametes (Parker, Baker & Smith, 1972; Parker, 1982). Theoretical modelling illustrates that numerical competition between smaller gametes for sexual fusion with the nutritive gametes generates a bimodal evolutionarily stable strategy where the competitive gametes are selected to be numerous while the nutritive gametes are selected to be large to maximize zygote survival (Parker *et al.*, 1972). Accordingly, this disruptive selection for the two gamete strategies gives rise to anisogamy and the male–female phenomenon. Because, fundamentally, competition between rival male gametes will operate numerically, selection acts on males to maximize sperm number and, within a fixed resource budget, one way this can be

achieved is through minimizing sperm size (Parker, 1982). Sperm competition may therefore explain why there are so many tiny sperm, and intergametic competition may have generated the initial evolution, and subsequent maintenance, of anisogamy (Parker *et al.*, 1972; Parker, 1982).

Sperm competition is now recognized as a widespread phenomenon across the animal kingdom, responsible for the evolution of a diversity of behavioural, anatomical and physiological reproductive strategies (Parker, 1970; Smith, 1984; Birkhead & Møller, 1998). The focus of selection from sperm competition is at the gamete level, and there is abundant comparative and experimental evidence that sperm competition selects on males to partition increased investment towards spermatogenesis and to produce greater numbers of sperm (reviewed in Birkhead & Møller, 1998). There is good experimental and comparative evidence that sperm number is an important determinant of sperm competition success (e.g. Simmons, 1987, and reviewed in Birkhead & Møller, 1998); however, less is known of

the importance of individual sperm size, despite wide variation in this trait across taxa (e.g. Gage, 1998). One prediction, therefore, of the role of sperm competition in the evolution of numerous tiny sperm is that, within a fixed male reproductive budget, there may be a trade-off between investment in sperm size and number (Parker *et al.*, 1972; Parker, 1982). For female gametes, optimal gamete size depends on a balance between the size and number of offspring where gamete size is up-shifted to maximize offspring survival (Smith & Fret-well, 1974). For males, this balance is polarized by sperm competition so that males are selected to max-imize gamete number for competition, by minimizing the size of each gamete unit.

Few studies have explored the relationships between sperm size and number. A major problem arises in that ejaculate sperm number is an inherently variable trait. Pitnick (1996) in a carefully controlled study across 11 species of *Drosophila* Meigen showed that there was a trade-off between sperm length and ejaculate sperm number. Within species, Gage, Stockley & Parker (1998) found no evidence for a relationship between stripped ejaculate sperm number and sperm size in Atlantic sal-mon. Radwan (1996) found no correlation between sperm size and number in the bulb mite *Rhizoglyphus robini* Claparède, although the relative size of the amoeboid sperm that each male produced dictated his success in sperm competition. A similar large-sperm ad-vantage was discovered in the nematode *Caeno-rhabditis elegans* (Maupas) (LaMunyon & Ward, 1998). Although numerical competition theoretically drives males to maximize sperm number by, perhaps, min-imizing sperm size, there may be mechanisms of com-petition in which individual sperm size is selected to increase. If sperm competition follows the scenario of a race and/or sperm must compete for access to storage or fertilization sites, competition may select for sper-matozoa which can achieve greater velocities or may generate increased flagellar forces. There is some evid-ence that increased sperm length may result in longer flagella which can propel sperm faster and/or generate increased flagellar forces (Katz & Drobnis, 1990; Go-mendio & Roldan, 1991). These factors may explain com-parative evidence that sperm competition relates to increasing sperm length across mammals (Gomendio & Roldan, 1991, but see Harcourt, 1991, and Dixson, 1993), birds (Briskie & Montgomerie, 1992; Johnson & Briskie, 1999), butterflies (Gage, 1994) and moths (Mor-row & Gage, 2000). The positive relationships are coun-ter to predictions of a trade-off between sperm size and number. In support of a trade-off, Stockley *et al.* (1997) found that increasing sperm competition was associated with decreasing sperm length and increasing sperm numbers across fishes.

In this paper, we examine relationships between sperm size and sperm number across males from dif-ferent populations within a single species. A within-species study alleviates many of the confounding problems of phylogenetic association and uncontrolled factors that are inherent in cross-species investigations (Felsenstein, 1985; Harvey & Pagel, 1991). *Tribolium castaneum* (Herbst) (Coleoptera, Tenebrionidae) is a widely distributed beetle that is a major pest of stored products (Sokoloff, 1974). The global distribution and the fact that different *T. castaneum* populations have gone through a wide range of population bottlenecks, diverse selection regimes and a variety of levels of isol-ation (Sokoloff, 1974) mean that this beetle is an ideal candidate for exploring divergence and variation in im-portant reproductive traits such as sperm size and num-ber. *T. castaneum* has a polygamous mating pattern and generates high levels of sperm competition that can be dependent on the local population structure (Haubruge *et al.*, 1999) which itself varies widely between different populations (Sokoloff, 1974). Furthermore, the patterns and mechanisms of sperm precedence have received concerted investigation to make *T. castaneum* a model species for investigating sperm competition (Schlager, 1960; Wool & Bergerson, 1979; Lewis & Austad, 1990, 1994; Bloch Qazi, Herbeck & Lewis, 1996; Bloch Qazi, Aprille & Lewis, 1998; Lewis & Jutiewicz, 1998; Hau-bruge *et al.*, 1999; Edvardsson & Arnqvist, 2000; Ar-naud, Gage & Haubruge, 2001). Therefore with this established information, and the variance in the degree of isolation and structure of different populations, we are able to explore how sperm size has diverged in re-lation to ejaculate sperm number in *T. castaneum*. Ejaculate size is recognized as a relatively plastic unit that can be proximately influenced by a number of fac-tors (e.g. mating history (Bloch Qazi *et al.*, 1996), male–male competition (Gage, 1991), female fecundity (Gage, 1995)). Importantly, therefore, we raise all males under standard conditions and maintain and mate all males with random females under identical protocols to min-imize uncontrolled variance in this trait.

We make no assumptions about differences in the mating pattern between different populations but have sampled a range of strains that have been in isolation for between 8 and 39 years. These levels of isolation present an excellent opportunity for differences in cul-ture management and maintenance to generate variant selection regimes that could direct variance in sperm size and number. We therefore measure the degree of this variation across males and then determine whether a relationship exists between sperm size and number.

MATERIAL AND METHODS

THE INSECTS

Fourteen strains of the red flour beetle originating from different geographic areas and with a range of

Table 1. Populations of the red flour beetle, *Tribolium castaneum*, and their origin

Population	Origin	Year of collection
Argyle	Manitoba, Canada	1992
Asm	Abidjan, Ivory Coast	1989
Ex-Maff	Maff, UK	1991
Ga-1	Georgia, USA	1980
Japan	Japan	unknown
Kano	Nigeria	1961
Lab-S	Kansas, USA	1960
Landmark	Manitoba, Canada	1991
Mozambique	Mozambique	1976
Pakistan	Karachi, Pakistan	1989
Paulo d'Amico	British Columbia, Canada	1976
PRm	Naphin, Philippines	1976
Rio Desago	New Brunswick, Canada	1976
Waseco	Minnesota, USA	1982

culture histories were selected for investigation (Table 1). Beetles were cultured in a dark incubator at $30 \pm 3°C$ and $65 \pm 5\%$ relative humidity with wheat flour and brewer's yeast (10:1 by weight) as rearing medium. Beetles were sexed as pupae and maintained individually (to ensure their virginity) in small vials ($5.5 \, cm^3$) with $0.5 \, g$ of the medium. Adults were 1 month old when used in the experiments.

SPERM SIZE AND EJACULATE SPERM NUMBERS

When successful sperm transfer was recorded, the entire female reproductive tract was isolated by severing its associations with the median oviduct and hindgut and placed on a cavity slide with $100 \, \mu l$ of modified Barth saline (Gage & Cook, 1994). The reproductive tract was then ruptured in several parts and teased apart with fine entomological needles and this allowed the ejaculate sperm mass to disperse. Even dispersal was encouraged by gentle mixing in the slide's cavity. The whole procedure was observed under $40 \times$ magnification to ensure full dispersal and that there were no spermatozoal agglutinations. The sperm–buffer solution was then washed off the slide using $c.\ 5 \, ml$ Barth saline into a $50 \, ml$ Falcon tube and then diluted up to $15 \, ml$ with deionized water. Rotation of this solution ensured even dispersal of the sperm in the diluent. Four $20 \, \mu l$ subsamples were retrieved using a micropipette from each $15 \, ml$ sperm solution and each $20 \, \mu l$ sample was placed as a smear on a flat slide and allowed to air dry under a dust cover. Ejaculates from five males were screened from every population, giving a total of 70 males.

Sperm counts

Every sperm in each of the $20 \, \mu l$ dried smears was counted under $200 \times$ magnification using dark-field phase contrast microscopy (Olympus BX-50). The total numbers of sperm per ejaculate were calculated by multiplying the mean sperm count from each male's four smears by the ejaculate's dilution factor.

Sperm length

Sperm on the dried smears lie in a flat two-dimensional plane on the slide. Dark-field phase contrast images of intact sperm were relayed at $400 \times$ magnification via a JVC video camera to a flat-screen monitor. The entire length of each intact sperm was traced onto an acetate film fixed to the monitor screen. These images were then measured using a digital map-measurer and lengths converted to micrometres. Fifteen sperm per male were measured. This technique of direct measurement is accurate and repeatable (Morrow & Gage, 2000).

RESULTS

EJACULATE SIZE

Ejaculate sperm number summary data for each population are presented in Table 2. In total, the mean ejaculate size for *T. castaneum* was 123 000 (± 3000 SE, $N = 70$) sperm. The coefficient of variation (CV) of sperm number was 45.6%. Our findings are consistent with Bloch Qazi *et al.* (1996) who found that approximately 133 000 ($\pm 19\,200$ SE, $N = 10$) sperm are transferred to the female and that the CV of sperm number was 46.0% (recalculated from data in Bloch Qazi *et al.*, 1996).

Repeated-measures ANOVA showed that ejaculate sperm number varied significantly between the 14 populations ($F_{13,56} = 2.40$, $P = 0.012$), and was also variant between individual males within populations ($F_{56,210} = 32.93$, $P < 0.0001$). There was no significant

Table 2. Mean ejaculate size in 14 populations of the red flour beetle, *Tribolium castaneum*

Population	Mean ejaculate size ($\pm$SE)
Argyle	129 525 ($\pm$14 584)
Asm	128 550 ($\pm$12 159)
Ex-Maff	211 950 ($\pm$12 638)
Ga-1	112 725 ($\pm$9187)
Japan	82 162 ($\pm$8 224)
Kano	114 975 ($\pm$12 547)
Lab-S	118 463 ($\pm$11 779)
Landmark	97 650 ($\pm$6032)
Mozambique	129 300 ($\pm$9585)
Pakistan	104 700 ($\pm$12 307)
Paulo D'Amico	108 637 ($\pm$10 018)
PRm	169 388 ($\pm$7305)
Rio Desago	86 475 ($\pm$8235)
Waseco	123 412 ($\pm$7952)

difference in the standard deviation of ejaculate size between populations (Levene's test, $F_{13,69}=0.48$, $P=0.926$). These results therefore provide opportunities for exploring how ejaculate size variation relates to variance in sperm size between populations and individual males.

SPERM LENGTH

Mean sperm lengths for the different *T. castaneum* populations are shown in Table 3. Mean sperm length averaged across all males and all populations was 866.38 µm ($\pm$1.24 SE, $N=70$). Sperm length showed a CV of 3.8% across all populations.

As for ejaculate size, there were significant differences between sperm lengths of the different populations (repeated-measures ANOVA: $F_{13,56}=2.43$, $P=$

0.011). There was also significant variation between individual males within populations ($F_{56,980}=4.92$, $P<0.0001$). The standard deviation of sperm length did not differ between males of different populations (Levene's test, $F_{69,1049}=0.987$, $P=0.509$) and was not correlated with mean sperm length ($R=-0.086$, $P=0.480$, $N=70$).

SPERM SIZE VS SPERM NUMBER

We found no evidence for a trade-off or trade-up between sperm size and number across individual males. Sperm length showed no relationship with ejaculate sperm number across individual males ($R=-0.163$, $P=0.157$, $N=70$, Fig. 1). There was evidence for a significant negative relationship between sperm length and number across the 14 different populations ($R=0.6$, $P=0.022$, $N=14$). However, this relationship is driven by a single outlier (the Ex-Maff population), removal of which generates a non-significant result ($R=0.28$, $P=0.350$, $N=13$). Furthermore, if we use non-parametric regression analysis there is no evidence for a negative relationship across all 14 populations ($R_{sp}=-0.29$, $P=0.31$, $N=14$).

DISCUSSION

Our results reveal that there is significant variation in sperm length and ejaculate sperm number between both populations and individual males across *T. castaneum*. This data range provides fertile ground for exploring the existence of trade-offs or trade-ups between the two fundamental components that constitute the male fertilization unit: sperm size and sperm number. However, our central finding across 70 individual males is that there is no evidence for any significant

Table 3. Sperm length in populations of the red flour beetle, *Tribolium castaneum*. Sperm lengths are means and absolute values are in µm ($\pm$SE)

| Population	Sperm length ($\pm$SE)	CV	Maximum	Minimum
Argyle	878.18 ($\pm$3.59)	3.45$\pm$0.29	939.39	797.98
Asm	883.23 ($\pm$5.30)	4.89$\pm$1.00	969.70	702.02
Ex-Maff	837.64 ($\pm$4.06)	3.98$\pm$0.47	904.04	722.22
Ga-1	863.77 ($\pm$4.24)	3.95$\pm$0.38	934.34	717.17
Japan	877.37 ($\pm$3.85)	3.00$\pm$0.20	939.39	792.93
Kano	867.88 ($\pm$5.04)	4.12$\pm$0.54	944.44	671.72
Lab-S	850.71 ($\pm$4.86)	4.18$\pm$0.44	929.29	732.32
Landmark	887.47 ($\pm$3.99)	3.68$\pm$0.47	949.49	762.63
Mozambique	859.39 ($\pm$4.17)	3.85$\pm$0.57	919.19	727.27
Pakistan	857.04 ($\pm$4.16)	3.30$\pm$0.27	934.34	762.63
Paulo D'Amico	856.36 ($\pm$5.25)	4.44$\pm$1.01	1121.21	757.58
PRm	861.15 ($\pm$4.73)	3.95$\pm$0.60	944.44	686.87
Rio Desago	876.57 ($\pm$4.46)	3.55$\pm$0.76	939.39	732.32
Waseco	872.59 ($\pm$3.22)	2.71$\pm$0.16	939.39	777.78
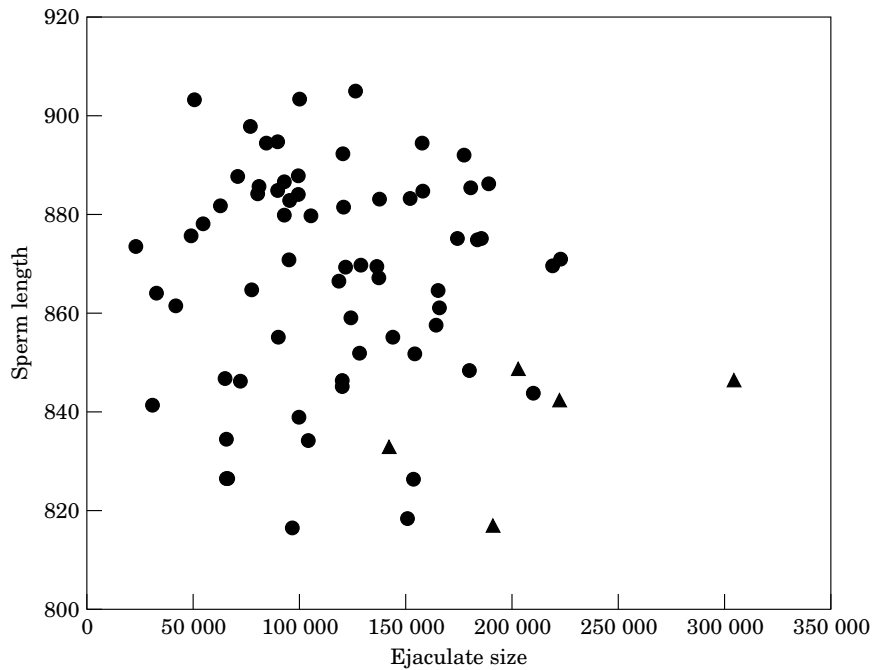
Figure 1. There is no significant relationship between ejaculate sperm number and sperm length (μm) across 70 male *Tribolium castaneum* ($R = -0.163$, $P = 0.157$). The outlying ex-MAFF males are labelled as triangles.

relationship between sperm size and sperm number. Regression of the population mean sperm lengths against ejaculate sperm numbers provides some evidence for a trade-off between sperm size and number but, when a single outlying data point is removed, the relationship falls far from significance ($P = 0.350$). A robust test for the existence of a sperm size–number trade-off would be at the level of the individual male, particularly given that there is significant variance in both of these traits between males, and that a test across 70 individuals provides more power to be a representative analysis.

Ejaculate size can be a condition-dependent trait that may be affected by a number of factors (e.g. Gage, 1991, 1995; Gage & Cook, 1994; Birkhead *et al.*, 1995; Bloch Qazi *et al.*, 1996). However, there is no evidence that sperm length is a condition-dependent trait. For example, severe nutritional deprivation generated decreases in body and testis size and sperm numbers in the moth *Plodia interpunctella* but did not influence sperm length (Gage & Cook, 1994). Furthermore, while there is potential for ejaculate size to be variable, sperm length variance must be an inert trait in the short term because of the prolonged nature of spermiogenesis. Males should be under selection to produce an assemblage of sperm lengths that are optimized for the environment in which those sperm are to function. It is therefore counterintuitive that significant variation in sperm lengths exists between individual males and that each male produces a relatively narrow range

of sperm lengths (average CV = 3.79% compared with the variation in ejaculate size where CV = 45.6%). This significant variance in sperm size between males within populations has been documented in a range of other taxa, including humans (Ward, 1998; Morrow & Gage, 2001). The existence of this variance remains unexplained but its influence within sperm competition should be considered (Ward & Hauschteck-Jungen, 1993), particularly since much consistent, but unexplained, variance in sperm competition success exists between male *T. castaneum* (Lewis & Austad, 1990) and since sperm size can be an important determinant of sperm competition success in other taxa (Radwan, 1996; LaMunyon & Ward, 1998).

Spermatogenesis generates non-trivial costs and males operate within a limited energy budget for reproduction. Sperm is a limiting resource for *T. castaneum* males since consecutive matings result in declining ejaculate sperm numbers (Bloch Qazi *et al.*, 1996). How, therefore, do males partition finite resources between individual sperm size and sperm numbers? Sperm are produced in the testis and males may therefore vary investment to this organ so that investment in sperm size and numbers can be varied independently. Testis size does vary and is positively correlated with sperm competition risk, both across taxa (reviewed in Birkhead & Møller, 1998) and within species (e.g. Gage, 1995). Alternatively, males may vary the frequency of ejaculate production so that investment in sperm size and number can be in-

dependent. In this study, we found that a significant proportion of matings do not result in sperm transfer; males may go through apparently normal copula behaviour with up to five different females before transferring an ejaculate (unpubl. data). As observed by Lewis & Iannini (1995) and Arnaud & Haubruge (1999), male *T. castaneum* have evolved mechanisms to discriminate among potential mates, and such male mate choice may enable optimal allocation of limited ejaculates while allowing independent variation in the two components that constitute the ejaculate: sperm size and sperm number.

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