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Impacts of starvation on male reproductive success in *Tribolium castaneum*

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ABSTRACT

Background: Starvation is known to decrease male reproductive success in *Tribolium castaneum*. Starved males transfer less sperm, but we do not know whether reduced reproductive success is caused by lower oviposition rates of females or more frequent deposition of unfertilized eggs.

Study organism: The red flour beetle *Tribolium castaneum* (Coleoptera: Tenebrionidae).

Questions: Does the nutritional state of a male influence female oviposition rate and/or fertilization capability? Does sperm use by females at fertilization reflect reduced numbers of sperm transferred by starved males?

Hypothesis: If the number of sperm released by females reflects the number of sperm stored, we would expect the number of sperm per egg to be inferior if mated with starved males.

Methods: We assessed female oviposition rate and fertilization capability in females mated with fed versus starved males. We also quantified sperm use via microscopical counts of DAPI-stained sperm heads on freshly deposited eggs.

Results: Females mated with starved males were less likely to deposit eggs and deposited fewer eggs. Furthermore, the eggs of females mated with starved males had fewer sperm, and those females laid a significantly higher proportion of unfertilized eggs. Based on our counts of sperm per egg, we estimate that for starved males, about a third less sperm come close to the site of fertilization.

Keywords: Coleoptera, condition dependence, fertility, sexual selection, sperm competition.

INTRODUCTION

Reproduction can be very costly for females (see, for example, Chapman *et al.*, 1995; Blanckenhorn *et al.*, 2002), and it is also becoming increasingly clear that reproduction is linked with considerable intrinsic costs for males (see Cordts and Partridge, 1996; Martin and Hosken, 2004). Spermatogenesis and maintenance of gonadal tissue is an energetically demanding process: Kenagy and Trombulak (1986) estimate that in small mammal males, costs can amount to 10% of the basal metabolic rate. High costs of ejaculate production are further evidenced by the fact that males allocate ejaculate strategically in numerous species (Gage, 1991; Simmons *et al.*, 1993;

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Martin and Hosken, 2002; reviewed in Wedell *et al.*, 2002). Indeed, ejaculate traits are known to be sensitive to male condition, further underlining the fact that producing ejaculates is definitely not a cheap activity (e.g. Dewsbury, 1982; Simmons and Kotiaho, 2002).

More generally, it has been shown that condition (Schulte-Hostedde and Montgomerie, 2006; Perry and Rowe, 2010) and nutritional status (Blay and Yuval, 1997; Janicke *et al.*, 2011), as well as environmental factors such as temperature (Blanckenhorn and Hellriegel, 2002) affect a range of reproductive traits. Furthermore, condition cannot simply be viewed as an entirely separate issue from environmental effects, as environmental stressors will impact strongly on individual condition (see discussion in Bussière *et al.*, 2008). For instance, environmental fluctuations can directly affect availability of food or water and potentially lead to starvation or desiccation (Hoffmann and Parsons, 1991; Himuro and Fujisaki, 2010). Beyond considerable direct effects, environmental changes can hence clearly also cause heterogeneity in resources available to individuals. Food stress has been shown to affect various facets of reproduction, such as mating behaviour (e.g. Hingle *et al.*, 2001). Overall, environmental variables and related impacts on individual condition may thus have major impacts on reproduction (see Fricke *et al.*, 2009), so precise knowledge of how such factors could affect populations is valuable.

The red flour beetle *Tribolium castaneum* is a widespread stored product pest with a global distribution. Due to *T. castaneum* being a pest and the temporally and spatially highly heterogeneous environment this involves, variations in food abundance and quality are likely to have played a major role in its evolution. Its importance as a pest and the desire to counter economic impacts has led to intense research. Many facets of the biology of *Tribolium* beetles are now known, including developmental biology, ecology, and genetics (e.g. fully sequenced and annotated genome). *Tribolium castaneum* is also a major model for the study of pre- and post-copulatory sexual selection (reviewed in Fedina and Lewis, 2008; Pai and Bernasconi, 2008) and sexual conflict (Michalczyk *et al.*, 2011). Previous studies on sperm competition in *T. castaneum* indicate that there is strong last male sperm precedence (Arnaud *et al.*, 2001), as is generally commonly found in insects (Simmons, 2001). In particular, two studies focusing on the impact of male nutritional status have further shown that starved males (1) are inferior to fed males in sperm offence ability (Fedina and Lewis, 2006), and (2) transfer fewer sperm per spermatophore (Fedina, 2007).

Here we investigate reproductive success of *T. castaneum* males subjected to starvation, focusing in particular on the fate of sperm after copulation. Specifically, we allow females to mate with males subjected to two nutritional treatments (i.e. fed vs. starved males), and assess how male starvation affects oviposition rates and sperm use by females at fertilization. We assume that sperm numbers released by females reflect the number of sperm stored (see Sbilordo *et al.*, 2009 and references therein). Therefore, we expect that females receiving less sperm from starved mates also use less sperm per egg. In parallel, we measure effects of male starvation on sperm offence (P2) and male mating behaviour, to allow comparisons to be made with previous work (Fedina and Lewis, 2006; Fedina, 2007), and further assess defence (P1) for the first time.

METHODS

Experimental animals

In the experiments described below, we used the standard *T. castaneum* wild-type strain Ga1 (Georgia 1, collected in 1980 and kept in culture by Richard Beeman, USDA, Manhattan,

KS). Stocks and experimental animals were maintained continuously at 30°C. Experimental animals were collected as pupae to ensure virginity. Pupae were separated by sex and kept individually in the compartments of square plastic boxes (25 compartments per box, area of 1.8 cm^2; Sterilin, UK). All females used in the experiment were standard fed Ga1 females kept on organic white flour supplemented with 10% brewer's yeast. Male condition was varied via two contrasting food treatments. Collected male pupae were divided equally into two groups:

- *Starved* males were kept without food for 12 days before the experiments (so factoring in mean duration of pupal stage, adult males experienced ~8 days of starvation).
- *Fed* males were supplied with standard food (see above).

These treatments were chosen to mirror previous work on male starvation in this species (Fedina and Lewis, 2006; Fedina, 2007). These authors starved males for a comparable period of 7 days. However, we used the wild-type laboratory stock Ga1 (competed vs. Reindeer, see below), whereas Fedina and Lewis (2006) used Berkeley (competed vs. Chicago black). Reproductive traits, such as remating behaviour, are known to differ markedly between *T. castaneum* strains (see, for example, Pai *et al.*, 2007). Thus, assessing repeatability of male starvation results across strains – using a different pair of focal and tester strains – is generally worthwhile (see, for example, Pai *et al.*, 2007). This is especially true because responses to stresses, such as susceptibility to starvation, are also known to differ markedly across populations within species [e.g. in *Drosophila* (Sisodia and Singh, 2010)].

Oviposition rate and sperm use

Experimental animals and assessment of oviposition rates

Twelve days after collection, pupae virgin females were placed individually in 5 cm diameter Petri dishes with filter paper circles on the bottom. To form pairs, either a *fed* or a *starved* male was added to each of the females. The males were marked with 'Queen marker' pens (Bienen-Meier, Künten, Switzerland) on the thorax 5 days before starting the experiment to allow separation from the females. A total of 259 beetle pairs were set up to assess female oviposition during the first 24 h after removing males. Altogether, 188 pairs consisted of females with starved males and the remaining 71 consisted of fed males and females. More pairs involving starved males were started to counter potentially greater mortality and/or oviposition failure rates. Pairs had the opportunity to mate for approximately 24 h. Males were then removed and females were provided with 1.25 mL standard flour-yeast mix. During the 24 h that pairs were together, one or both partners died in 33 pairs. These pairs were excluded from the analysis, resulting in a final sample size of 157 pairs with starved males and 69 pairs with fed males.

To collect eggs, the flour was sieved (mesh aperture 280 μm; Retsch GmbH, Haan, Germany) and all eggs deposited within the first 24 h after removal of the male were counted. The filter paper circles were inspected under a dissection microscope to detect potentially adhering eggs. In addition, we selected a subsample of females for comparing egg-laying rates over time in more detail. As a selection criterion, only females were used, which had deposited at least one egg during these 24 h. This more detailed study of egg-laying patterns over time was carried out for 14 days after removing the male and

involved counting all eggs deposited during this time. Specifically, the assay consisted of 19 females mated with starved males and 23 females caged with fed males. Eggs of these females were prepared for sperm counts to assess the presence of sperm and numbers of sperm used by females at fertilization (for details, see below).

Egg preparation and sperm count

On days 1, 2, 3, 6, and 14 after removing the male, the numbers of sperm adhering to the eggs were counted under the microscope. On the days before these sperm counts, the females were supplied with a portion of flour to ensure eggs for microscopy were fresh. For the preparation of the microscope slides we followed the protocol in Sbilordo *et al.* (2009) developed for the eggs of the dung fly *Scathophaga stercoraria* with some modifications to adjust the protocol for *Tribolium*. Eggs were removed from the sieve with the help of a fine paintbrush and placed on a glass coverslip (18×18 mm or 21×26 mm) into a drop of 80% ethanol. To clean the eggs of flour, they were carefully swirled in the ethanol with the brush. The ethanol was then allowed to evaporate and a drop of mounting medium containing DAPI (4,6-diamidino-2-phenylindole; medium: VECTASHIELD VC-H-1500, Vector, Burlingame, CA) was added to the preparation. Subsequently, the preparations were covered with a second coverslip held in place with a drop of nail polish. The preparations were examined under a fluorescence microscope (Nikon Eclipse 600) from both sides. Although the washing step usually did not remove any of the sperm sticking to the egg surface, the whole preparation was checked for free sperm. In the rare instances where individual sperm had been washed off onto the slide, these were included in the counts. Sperm heads were counted at a magnification of 600× . Some eggs contained either too many sperm or sperm were too densely clumped to distinguish single sperm. In these cases, we stopped counting at 300 sperm (= maximum). For sperm counts, 84 of 418 eggs burst during handling before the microscope slides could be produced. This reduced the sample size to 334 eggs, 237 of them derived from females maintained with a fed male ($n = 23$) and 97 from females with a starved male ($n = 17$). Digital photographs of eggs and sperm for illustration were taken with a ProgRes C5 camera.

Male reproductive success: sperm defence and offence abilities

A classic way of studying the outcomes of sperm competition is to mate females with two males sequentially. Once females have produced offspring, the paternity shares of the two potential sires can be determined via male phenotype or genotype. With this kind of experimental set-up, paternity of the first male is termed P1 or sperm defence, and the second male's share is P2 or sperm offence (Boorman and Parker, 1976). Several lines of evidence suggest that it is critically important to assess both sperm defence and offence abilities (discussed in Fricke *et al.*, 2010). Hence we chose to assess both facets of sperm competitive ability.

To assess sperm competition, we used the phenotypic marker strain Reindeer (Rd, supplied by the Beeman lab). Rd beetles have a dominant mutation causing antennae with a markedly different shape so that offspring can be scored easily by eye. Crucially, Rd is sufficiently viable when mating with Ga1 to be useful in sperm competition assays, and has also been applied successfully in other studies (Michalczyk *et al.*, 2010, 2011). Five days before the sperm competition experiments, hatched Ga1 males were again marked to facilitate separation from the females. Since Rd males look clearly distinct, marking was not required.

The experiments incorporated four combinations, two to investigate sperm defence (P1) and two for the assessment of sperm offence (P2). To assess P1, females were mated with either a starved ($n = 20$) or a fed ($n = 33$) male and then a Rd male (paternity share of focal male = P1, sperm defence). To assess P2, females were mated with a Rd male first and then with either a starved ($n = 21$) or a fed ($n = 39$) male (paternity share of focal male = P2, sperm offence). For all sperm competition trials, the first males were placed with individual tester females for 24 h and then replaced with the allocated second males (mirroring the protocol in Michalczyk *et al.*, 2010). After a further 24 h, second males were removed and females transferred to Petri dishes containing flour-yeast mix topped with rolled oats. Offspring were sieved after 35 days (i.e. when adult offspring had emerged) and counted, with P1 and P2 scored as relative numbers of Ga1 versus Rd offspring.

As a complement to the sperm competition success experiment outlined above, we investigated effects of male condition on mating behaviour. Fed males might acquire a greater share of paternity simply because they copulate more frequently with females. We exposed females (fed Ga1 beetles) simultaneously to a fed and a starved male in mating chambers (both males marked). In total, 68 such mating trials were performed, each lasting for 10 min. During this time we observed mating interactions and scored latency to mount and the number of mountings.

Statistical analyses

Statistical analyses were performed with R v.2.11.1 (R Developmental Core Team, 2010). Female oviposition behaviour (i.e. whether females oviposited on the first day) was analysed using a generalized linear model (GLM) with a binary response, quasi-binomial errors and logit link function, and male type (fed or starved) as the explanatory variable. The total number of eggs deposited by females (during the 14 days of the experiment) was analysed with a GLM with quasi-Poisson errors, log link function, and male type as the explanatory variable. The number of sperm on single eggs was analysed with a linear mixed model (LMM) with male type (fed or starved), day, and their interaction as the explanatory variables. Before analysis, the number of sperm per egg + 1 was log-transformed (as in Sbilordo *et al.*, 2009). Single eggs were nested within day within female to control for pseudo-replication. We further applied generalized linear mixed models (GLMMs) with Poisson and binomial errors to determine whether sperm were detected on an egg or not and the number of sperm per egg. We analysed these models with the glmmPQL function from the MASS package (Venables and Ripley, 2002). The explanatory variables in both models were male type (fed or starved), day, and the interaction between male type and day. In addition, we analysed the number of sperm on single eggs using only data for the first day (i.e. where we had access to the largest number of females) with a linear mixed model (LMM) with male type (fed or starved) as the explanatory variable. Again eggs were nested within female and the same transformation was performed on the response variable (log of sperm number + 1).

Analyses designed to contrast the sperm competitive abilities (P1 and P2) of fed versus starved experimental males were implemented as generalized linear models (GLMs). Specifically, we analysed P1 and P2 with GLMs with quasi-binomial errors and a logit link function. The explanatory variable was the focal male type (fed/starved). Generalized linear models were fitted with the glm function from the stats package (R Development Core Team, 2010).

Mating behaviour (latency to mount and frequency of mountings) of fed versus starved males was analysed using paired *t*-tests. In addition, we assessed the influence of male type on the binary response variable mating 'yes' (i.e. if at least one copulation took place) or 'no' with a generalized linear mixed model (GLMM) in R (R Development Core Team, 2010). The GLMM was fitted with binomial errors and a logit link function by applying the glmmPQL function from the MASS package (Venables and Ripley, 2002).

RESULTS

Influence of male nutritional status (fed vs. starved) on female oviposition rates

Male starvation had a significant effect on the oviposition behaviour and oviposition rates of their mates. First, a GLM with quasi-binomial errors (dispersion parameter: 1.009) and logit link revealed that females maintained with starved males were less likely to oviposit eggs during the first 24 h after removing the male than females mated with fed males ($F_{1,224} = 33.639$, $P < 0.0001$). Specifically, 88 of 157 females caged with starved males refused to lay eggs the first day after removal of males. In contrast, only 11 of 69 females caged with fed males did not oviposit during the first 24 h. Second, females not only differed in their propensity to oviposit, but also in the number of eggs deposited on the first day if they oviposited: females with starved males ($n = 69$) deposited 2.1 (S.D. = 1.19) eggs versus 3.76 (S.D. = 1.97) eggs for females caged with fed males ($n = 58$). This difference persisted in the subsample of females caged with males of the two treatments (fed = 23 vs. starved = 19) where we assessed egg counts over 14 days. A GLM with quasi-Poisson errors (dispersion parameter: 7.381) and log link shows that females housed with starved males deposited significantly fewer eggs than females housed with fed males ($F_{1,40} = 23.802$, $P < 0.0001$; Fig. 1).

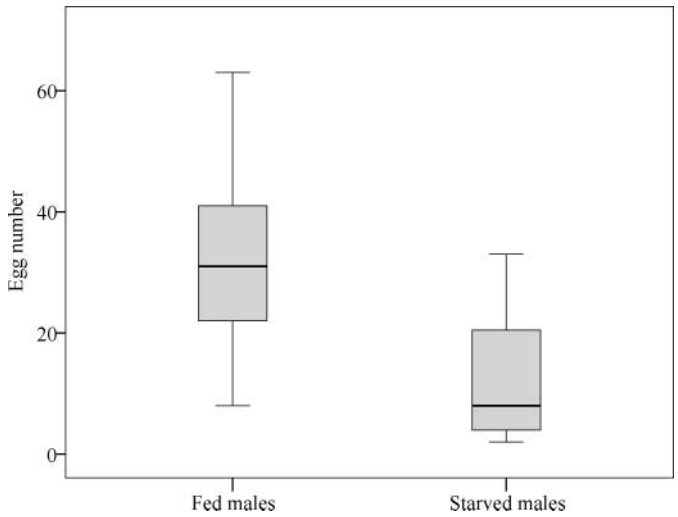

Fig. 1. Number of eggs deposited by females mated with fed versus starved males. Females mated with starved males deposited significantly fewer eggs over a period of 14 days. See main text for statistics.

Influence of male nutritional status (fed vs. starved) on sperm numbers used by females to fertilize individual eggs

Figure 2 shows a fluorescence microscope image of a freshly deposited egg (a: overview), with parts (c) and (d) illustrating how DAPI-stained sperm heads appear on preparations used to count sperm. The general external morphology of *T. castaneum* eggs has been described (e.g. Le Cato and Flaherty, 1974). However, further details such as the location of sperm entrance sites or whether a micropyle facilitates sperm entrance are, to our knowledge, still lacking.

Females caged with starved males more often deposited eggs with no (visible) sperm on their surface than females with fed males (GLMM: $t_{38} = -2.906$, $P = 0.006$). Neither day nor the interaction between male type and day influenced whether sperm was found on eggs or not (both $P > 0.10$). In the full dataset, 2 of 23 females caged with a fed male versus 7 of 17 females confined with a starved male only ever produced eggs without visible sperm. We cannot completely rule out the possibility that these females did not mate, or that females ejected the ejaculate (see Lewis and Jutkiewicz, 1998) of starved males more frequently. So, in the interest of being conservative, only females that produced at least one egg with visible sperm were used in the final analyses of the number of sperm used (see below).

Females mated with a starved male used significantly fewer sperm to fertilize eggs than females mated to a fed male (LMM: $F_{1,29} = 11.084$, $P = 0.002$; Fig. 3). In addition, sperm counts per egg decreased significantly with day ($F_{1,78} = 12.733$, $P < 0.001$; Fig. 3), while the interaction between male type and day was not significant ($P = 0.99$). GLMM analysis with sperm per egg as the response variable delivered the same qualitative results, with both male type and day being highly significant (results not shown). Similarly, LMM analysis based on data from the first day only highlighted significant differences due to male type. Residuals for both the linear models (LMMs) applied were normally distributed (both Shapiro-Wilk tests, $P > 0.29$).

Male reproductive success: sperm defence and offence abilities

We analysed sperm defence (P1) and offence (P2) using generalized linear models with quasi-binomial errors (dispersion parameter: P1 as response, 38.58; P2 as response, 22.10) and logit link. Starvation clearly decreased male sperm competitive abilities, as starved males had both lower sperm defence (P1: $F_{1,51} = 9.155$, $P = 0.004$; starved 38.23% (S.D. = 45.05) vs. fed 72.32% (S.D. = 37.81)) and lower sperm offence (P2: $F_{1,58} = 88.298$, $P < 0.0001$; starved 21.56% (S.D. = 34.85) vs. fed 90.16% (S.D. = 20.97)) than fed males.

Mating behaviour

Results of the paired *t*-tests comparing latency to mount ($t_{27} = -0.5232$, $P = 0.61$) and frequency of mountings ($t_{67} = -0.4367$, $P = 0.66$) between fed and starved males revealed no significant differences. Note that a linear mixed model with latency to mount as the response, and a generalized linear mixed model with frequency of mountings as the response provided qualitatively the same non-significant results. In addition, a generalized linear mixed model with mating 'yes' or 'no' as the binary response indicated that male type did not influence whether a copulation took place or not ($t_{67} = 0.379$, $P = 0.71$).

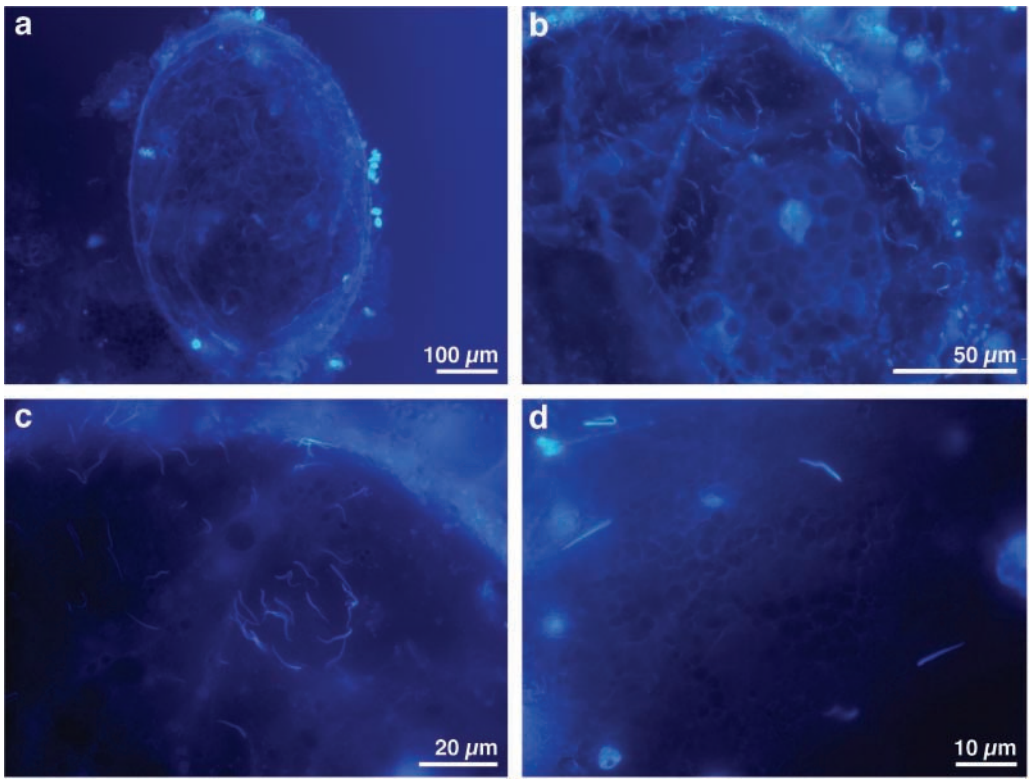

Fig. 2. Fluorescence microscope images of a freshly deposited *Tribolium castaneum* egg. To enable sperm counts for female sperm use at fertilization, sperm heads were stained with DAPI (4,6-diamidino-2-phenylindole). (a) Overview of a whole egg; (b) sperm attached to the surface of an egg; (c, d) close-up views of the attached sperm heads.

DISCUSSION

Food availability (access to normal food vs. starvation) and hence male condition clearly had strong effects on male reproductive success. Females mated with starved males were far less likely to lay eggs. Of these females, those that did deposit eggs oviposited many fewer eggs. From the microscopical analysis of sperm numbers in the eggs, it was also apparent that the eggs of mates of starved males contained fewer sperm per egg. In addition, eggs of these females were more likely not to contain any sperm. Sperm counts per egg decreased significantly over time, as observed in other species (Birkhead and Fletcher, 1994; Birkhead *et al.*, 1994). However, this decline appears independent of male treatment. Considering the inferior numbers of sperm found, it is clear that there must be differences in either sperm quantity or quality of starved versus fed males. The former appears more likely, as previous work found no difference in the viability of sperm in the seminal vesicles of starved and fed males (Fedina and Lewis, 2006). Furthermore, the number of sperm released by females may generally reflect the number of sperm in storage (see Sbilordo *et al.*, 2009). Overall, it is probable that decreased sperm numbers used by females reflects decreased numbers transferred by males initially.

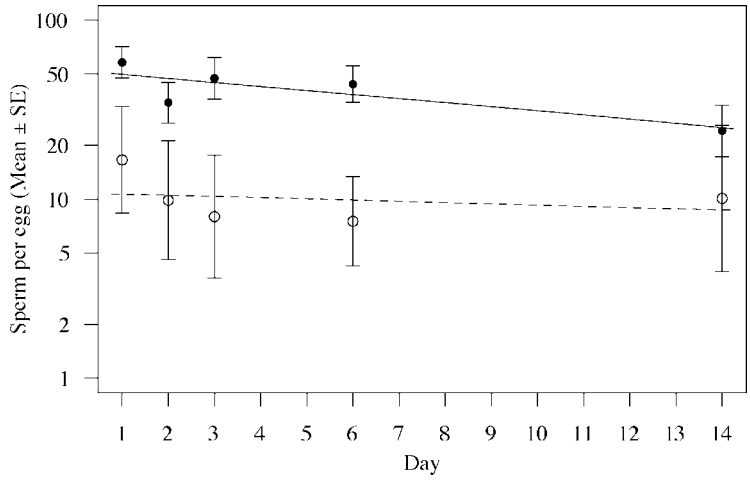

Fig. 3. Mean number of sperm per egg deposited by females mated with fed versus starved males over time. The mean number of spermatozoa (y-axis log scale) found on eggs was assessed on five separate occasions (once on each of the three days immediately following mating to assess short-term sperm use by females, plus about a week and two weeks after mating for longer term use). Sperm numbers were significantly lower when females had mated with starved (open symbols) compared with fed (solid symbols) males. This effect was found both overall and also when only data from day 1 were analysed separately. See main text for statistics.

Regardless of whether quantity or quality contributes more to decreased male fitness, here starvation clearly impacted strongly on the outcome of sperm competition. When competing with normally fed counterparts, starved males were inferior in both sperm defence (P1) and offence ability (P2). The reduction in P2 observed is in line with the findings of Fedina and Lewis (2006). However, these authors did not assess sperm defence, so our results further show that starvation has a similarly drastic impact on P1. This is important, as sperm defence and offence are not at all trivially related – both are associated with measures of lifetime reproductive success, although not necessarily in exactly the same manner (see Fricke *et al.*, 2010). The effect of male condition on sperm competitive ability is strong, and affects P2 more strongly than P1. This pattern is similar to the negative effect of inbreeding already described in this species (Michalczyk *et al.*, 2010), as here again P2 was more clearly reduced than P1. Michalczyk *et al.* (2010) used the same protocol and laboratory strain, and found that outbred males outperformed inbred males in both sperm defence (18.3% vs. 11.9%) and offence (95.2% vs. 73.3%). Together, these results suggest that P2 could be more sensitive to male quality, perhaps requires more energetic investment, or is more strongly dependent on accessory gland proteins (Acps). Indeed, extensive work in *Drosophila melanogaster* has shown that particular Acps are associated to differing degrees with P1, P2 or both (e.g. Clark *et al.*, 1995; Fiumera *et al.*, 2005). It has also been reported that male nutritional status can play a role in shaping female responses to Acps (Fricke *et al.*, 2008). In addition, due to the prevailing general pattern of the last sperm precedence pattern in *T. castaneum* (Arnaud *et al.*, 2001), the range of values for P1 is possibly more constrained, automatically providing less scope for variation. Together, these studies (Fedina and Lewis, 2006; Michalczyk *et al.*, 2010; present study) serve to underline the importance of male quality (whether

determined genetically or environmentally) in shaping male reproductive success in *Tribolium*.

Based on our assessment of male mating behaviour, the demonstrated inferior sperm competition (both P1 and P2) of starved males does not seem to be simply due to decreased mating effort. There were no significant differences in any of the aspects of mating behaviour assessed. This is in agreement with previous work by Fedina and Lewis (2006), although these authors did find an indication that copulation duration was shorter for starved males. This aspect was not assessed here but considering that effects on P2 were similar in both strains used [Georgia 1 in the present study vs. Berkeley in Fedina and Lewis (2006) and Fedina (2007)], it is plausible that copulation duration might also differ. In the study of Michalczyk *et al.* (2010), decreased sperm competitive ability of inbred males was also not associated with differences in mating effort or viability of their offspring. These findings mirror our results and again point to an effect of male quality on either sperm quality or more likely quantity.

Indeed, previous research on *Tribolium* indicates that starved males do transfer less sperm per spermatophore, strengthening suggestions that starvation affects sperm quantity received by females. Fedina (2007) found that normally fed *T. castaneum* males transfer ~300,000 sperm, whereas starved males transfer ~160,000 sperm. Thus, starvation leads to a reduction of ~47% in the quantity of sperm transferred per spermatophore, the initial maximal set entering the female (Fedina, 2007). Females may be actively involved both in reducing the number of sperm transferred by starved males (Fedina 2007), and in controlling sperm quantities entering storage (Bloch-Qazi *et al.*, 1998). Based on our data on sperm numbers found on eggs, we show that for starved males approximately a third less sperm come close to the site of fertilization. These estimations are in close agreement, and together show compellingly that the effects of starvation are drastic. Despite the large numbers of gametes involved, combined with the fact that only ~4% of individual ejaculates ever make it into storage in the spermatheca (Bloch-Qazi *et al.*, 1996), there is no compensation. Sperm numbers initially transferred by starved males, even if in excess of what females store or use, have knock-on consequences for numbers used by females even in the absence of competition. Sperm use hence seems to be a fair reflection of available sperm, or suggestive of female involvement in biasing against sperm from starved males [i.e. via cryptic female choice (see Fedina, 2007)]. It is therefore not at all surprising that the decreased numbers of sperm entering competition and the higher incidence of eggs with no sperm play a major role in post-copulatory sexual selection.

Finally, focusing on the role played by non-sperm components of the ejaculate, previous work showed that starved males also had smaller accessory glands (Fedina and Lewis, 2006). This suggests that starved males might also transfer less accessory gland products. Little is known of the action of these substances in *Tribolium* to date. However, work on other species (reviewed in Gillott, 2003), including in-depth study of *Drosophila* (e.g. Chen, 1996; Chapman, 2001; Wolfner, 2002; Wigby and Chapman, 2005), has established that such products have strong effects. In particular, they can serve to increase male chances in sperm competition or reproductive success (e.g. via decreasing female remating rates or increasing egg-laying rates). If similar mechanisms were present in *T. castaneum*, an inferior ability to transfer accessory gland products could contribute to decreasing success in sperm competition. According to this rationale, decreased sperm competitive ability would be a product of inferior sperm numbers compounded by smaller quantities of accessory gland products. Future work should address the role played by accessory gland products in *Tribolium*.

CONCLUSIONS

In conclusion, we confirm that male starvation – and hence male condition – drastically affects reproductive success. Females mated with starved males were less likely to lay eggs and also deposited far fewer eggs. In addition, microscopical analysis of sperm on deposited eggs demonstrates impacts on sperm use by females: eggs of mates of starved males contained fewer sperm per egg, and also frequently contained no sperm at all. We confirm that food availability strongly impacts on male reproductive success, with starved males inferior in assays of both sperm defence and sperm offence. Based on our assessment of sperm use by females at fertilization, there appear to be very clear differences in either sperm quantity or quality, with the former being far more likely. It would be interesting to assess whether the observed consequences of short-term starvation for male fitness can be reversed [i.e. via subsequent feeding (see, for example, Parthasarathy and Palli (2011) for females)]. In addition, future research should focus on how male condition interacts with other important determinants of male competitive ability (e.g. inbreeding, environment).

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REFERENCES

Arnaud, L., Haubruge, E. and Gage, M.J.G. 2001. The dynamics of second- and third-male fertilization precedence in *Tribolium castaneum. Entomol. Exp. Appl.*, **99**: 55–64.

Birkhead, T.R. and Fletcher, F. 1994. Sperm storage and release of sperm from the sperm storage tubules in Japanese quail *Coturnix japonica. Ibis*, **136**: 101–105.

Birkhead, T.R. Sheldon, B.C. and Fletcher, F. 1994. A comparative study of sperm–egg interactions in birds. *J. Reprod. Fertil.*, **101**: 353–361.

Blanckenhorn, W.U. and Hellriegel, B. 2002. Against Bergmann's rule: fly sperm size increases with temperature. *Ecol. Lett.*, **5**: 7–10.

Blanckenhorn, W.U., Hosken, D.J., Martin, O.Y., Reim, C., Teuschl, Y. and Ward, P.I. 2002. The costs of mating in the dung fly *Sepsis cynipsea*: I. Costs of copulation. *Behav. Ecol.*, **13**: 353–358.

Blay, S. and Yuval, B. 1997. Nutritional correlates of reproductive success of male Mediterranean fruit flies (Diptera: Tephritidae). *Anim. Behav.*, **54**: 59–66.

Bloch-Qazi, M.C., Herbeck, J.T. and Lewis, S.M. 1996. Mechanisms of sperm transfer and storage in the red flour beetle (Coleoptera: Tenebrionidae). *Ann. Entomol. Soc. Am.*, **89**: 892–897.

Bloch Qazi, M.C., Aprille, J.R. and Lewis, S.M. 1998. Female role in sperm storage in the red flour beetle, *Tribolium castaneum. Comp. Biochem. Physiol. A*, **120**: 641–647.

Boorman, E. and Parker, G.A. 1976. Sperm (ejaculate) competition in *Drosophila melanogaster*, and reproductive value of females to males in relation to female age and mating status. *Ecol. Entomol.*, **1**: 145–155.

Bussière, L.F., Hunt, J., Stölting, K.N., Jennions, M.D. and Brooks, R. 2008. Mate choice for genetic quality when environments vary: suggestions for empirical progress. *Genetica*, **134**: 69–78.

Chapman, T. 2001. Seminal fluid-mediated fitness traits in *Drosophila. Heredity*, **87**: 511–521.

Chapman, T., Liddle, L.F., Kalb, J.M., Wolfner, M.F. and Partridge, L. 1995. Costs of mating in *Drosophila melanogaster* females is mediated by male accessory gland products. *Nature*, **373**: 241–244.

Chen, P.S. 1996. The accessory gland proteins in male *Drosophila*: structural, reproductive, and evolutionary aspects. *Experientia*, **52**: 503–510.

Clark, A.G., Aguade, M., Prout, T., Harshman, L.G. and Langley, C.H. 1995. Variation in sperm displacement and its association with accessory-gland protein loci in *Drosophila melanogaster*. *Genetics*, **139**: 189–201.

Cordts, R. and Partridge, L. 1996. Courtship reduces longevity of male *Drosophila melanogaster*. *Anim. Behav.*, **52**: 269–278.

Dewsbury, D.A. 1982. Ejaculate cost and male choice. *Am. Nat.*, **119**: 601–610.

Fedina, T. 2007. Cryptic female choice during spermatophore transfer in *Tribolium castaneum* (Coleoptera: Tenebrionidae). *J. Insect Physiol.*, **53**: 93–98.

Fedina, T. and Lewis, S.M. 2006. Proximal traits and mechanisms for biasing paternity in the red flour beetle *Tribolium castaneum*. *Behav. Ecol. Sociobiol.*, **60**: 844–853.

Fedina, T. and Lewis, S.M. 2008. An integrative view of sexual selection in *Tribolium* flour beetles. *Biol. Rev. Camb. Phil. Soc.*, **83**: 151–171.

Fiumera, A.C., Dumont, B.L. and Clark, A.G. 2005. Sperm competitive ability in *Drosophila melanogaster* associated with variation in male reproductive proteins. *Genetics*, **169**: 243–257.

Fricke, C., Bretman, A. and Chapman, T. 2008. Adult male nutrition and reproductive success in *Drosophila melanogaster*. *Evolution*, **62**: 3170–3177.

Fricke, C., Perry, J., Chapman, T. and Rowe, L. 2009. The conditional economics of sexual conflict. *Biol. Lett.*, **5**: 671–674.

Fricke, C., Martin, O.Y., Bretman, A., Bussière, L.F. and Chapman, T. 2010. Sperm competitive ability and indices of lifetime reproductive success. *Evolution*, **64**: 2746–2757.

Gage, M.J.G. 1991. Risk of sperm competition directly affects ejaculate size in the Mediterranean fruit fly. *Anim. Behav.*, **42**: 1036–1037.

Gillott, C. 2003. Male accessory gland secretions: modulators of female reproductive physiology and behavior. *Annu. Rev. Entomol.*, **48**: 163–184.

Himuro, C. and Fujisaki, K. 2010. Mating experience weakens starvation tolerance in the seed bug *Togo hemipterus* (Heteroptera: Lygaeidae). *Physiol. Entomol.*, **35**: 128–133.

Hingle, A., Fowler, K. and Pomiankowski, A. 2001. The effect of transient food stress on female mate preference in the stalk-eyed fly *Cyrtodiopsis dalmanni*. *Proc. R. Soc. Lond. B*, **268**: 1239–1244.

Hoffmann, A.A. and Parsons, P.A. 1991. *Evolutionary Genetics and Environmental Stress*. New York: Oxford University Press.

Janicke, T., Sandner, P. and Schärer, L. 2011. Determinants of female fecundity in a simultaneous hermaphrodite: the role of polyandry and food availability. *Evol. Ecol.*, **25**: 203–218.

Kenagy, G.J. and Trombulak, S.C. 1986. Size and function of mammalian testes in relation to body size. *J. Mammal.*, **67**: 1–22.

Le Cato, G.L. and Flaherty, B.R. 1974. Description of eggs of selected species of stored-product insects (Coleoptera and Lepidoptera). *J. Kansas Entomol. Soc.*, **47**: 308–317.

Lewis, S.M. and Jutkiewicz, E. 1998. Sperm precedence and sperm storage in multiply mated red flour beetles. *Behav. Ecol. Sociobiol.*, **43**: 365–369.

Martin, O.Y. and Hosken, D.J. 2002. Strategic ejaculation in the common dung fly *Sepsis cynipsea*. *Anim. Behav.*, **63**: 541–546.

Martin, O.Y. and Hosken, D.J. 2004. Copulation reduces male but not female longevity in *Saltella sphondylli* (Diptera: Sepsidae). *J. Evol. Biol.*, **17**: 357–362.

Michalczyk, L., Martin, O.Y., Millard, A.L., Emerson, B.C. and Gage, M.J.G. 2010. Inbreeding depresses sperm competitiveness, but not fertilization or mating success in male *Tribolium castaneum*. *Proc. R. Soc. Lond. B*, **277**: 3483–3491.

Michalczyk, L., Millard, A.L., Martin, O.Y., Lumley, A.J., Emerson, B.C. and Gage, M.J.G. 2011. Experimental evolution exposes female and male responses to sexual selection and conflict in *Tribolium castaneum*. *Evolution*, **65**: 713–724.

Pai, A. and Bernasconi, G. 2008. Polyandry and female control: the red flour beetle *Tribolium castaneum* as a case study. *J. Exp. Zool. (Mol. Dev. Evol.)*, **310B**: 148–159.

Pai, A. Feil, S. and Yan, G. 2007. Variation in polyandry and its fitness consequences among populations of the red flour beetle, *Tribolium castaneum. Evol. Ecol.*, **21**: 687–702.

Parthasarathy, R. and Palli, S.R. 2011. Molecular analysis of nutritional and hormonal regulation of female reproduction in the red flour beetle, *Tribolium castaneum. Insect Biochem. Mol. Biol.*, **41**: 294–305.

Perry, J.C. and Rowe, L. 2010. Condition-dependent ejaculate size and composition in a ladybird beetle. *Proc. R. Soc. Lond. B*, **277**: 3639–3647.

R Development Core Team. 2010. *R: A Language and Environment for Statistical Computing*. Vienna, Austria: R Foundation for Statistical Computing (http://www.R-project.org).

Sbilordo, S.H., Schäfer, M. and Ward, P.I. 2009. Sperm release and use at fertilization by yellow dung fly females (*Scathophaga stercoraria*). *Biol. J. Linn. Soc.*, **98**: 511–518.

Schulte-Hostedde, A.I. and Montgomerie, R. 2006. Intraspecific variation in ejaculate traits of the northern watersnake (*Nerodia sipedon*). *J. Zool.*, **270**: 147–152.

Simmons, L.W. 2001. *Sperm Competition and its Evolutionary Consequences in the Insects*. Princeton, NJ: Princeton University Press.

Simmons, L.W. and Kotiaho, J.S. 2002. Evolution of ejaculates: patterns of phenotypic and genotypic variation and condition dependence in sperm competition traits. *Evolution*, **56**: 1622–1631.

Simmons, L.W., Craig, M., Llorens, T., Schinzig, M. and Hosken, D. 1993. Bushcricket spermatophores vary in accord with sperm competition and parental investment theory. *Proc. R. Soc. Lond. B*, **251**: 183–186.

Sisodia, S. and Singh, B.N. 2010. Resistance to environmental stress in *Drosophila ananassae*: latitudinal variation and adaptation among populations. *J. Evol. Biol.*, **23**: 1979–1988.

Venables, W.N. and Ripley, B.D. 2002. *Modern Applied Statistics with S*, 4th edn. New York: Springer.

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

Wigby, S. and Chapman, T. 2005. Sex peptide causes mating costs in female *Drosophila melanogaster. Curr. Biol.*, **15**: 316–321.

Wolfner, M.F. 2002. The gifts that keep on giving: physiological functions and evolutionary dynamics of male seminal proteins in *Drosophila. Heredity*, **88**: 85–93.