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ORIGINAL PAPER

Wild yellow dung fly females may not select sperm based on dung pat microclimate but could nevertheless benefit from polyandry

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Abstract Molecular techniques have substantially improved our knowledge of post-copulatory sexual selection. Nevertheless, studies examining sperm utilization in natural populations of nonsocial insects are rare, support for sperm selection (biased use of stored sperm, e.g. to match offspring genotypes to prevailing environmental conditions) is elusive, and its relevance within natural populations unknown. We performed an oviposition site choice experiment in the field where female yellow dung flies *Scathophaga stercoraria* could deposit eggs into three different microenvironments on a dung pat (the east–west ridge, north- or south-exposed side), and genotyped the offspring and sperm remaining in storage after oviposition. Females exhibited plasticity in the number of eggs deposited according to pat age. Additionally, temperature strongly influenced egg placement: the warmer the temperature, the higher the proportion of eggs laid into the north-exposed side of dung. The number of ejaculates in storage differed amongst spermathecae, and females stored sperm from more males than fathered their offspring (2.11 sires vs. 2.84 males within sperm stores). Mean last male paternity was 83.4%, roughly matching previous laboratory estimates. Importantly, we found no evidence that females selectively lay eggs of different genotypes, by biasing paternity towards certain males, depending on offspring's microclimate. Thus, while we show female choice over number of eggs and where these are deposited, there was no evidence for sperm selection. We further revealed

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positive effects of multiple mating on total number of offspring and proportion of offspring emerging from the dung. We argue that the integration of field studies and laboratory experiments is essential to promote our understanding of polyandry and cryptic female choice.

Keywords Postcopulatory sexual selection · Cryptic female choice · Sperm competition · *Scathophaga* · *Scatophaga*

Introduction

The fitness benefits of multiple mating are much more obvious for males than for females: mate number usually covaries strongly with reproductive success in males, but not necessarily in females (Bateman 1948). In addition, multiple mating can be quite costly for females (Arnqvist and Rowe 2005). Nevertheless, females of many organisms are polyandrous (i.e. mate with more than one male). Direct and indirect (genetic) fitness benefits are commonly invoked as explanatory factors in this context, but despite much theoretical and empirical work, our understanding of the causes and consequences of polyandry is still fragmentary (Tregenza and Wedell 2000; Simmons 2005). The fact that research on polyandry is primarily focused on laboratory experiments, even though we often do not know how well these reflect natural conditions, impedes progress in this field of research. More studies of polyandry in wild populations are clearly needed (Bretman and Tregenza 2005; Rodriguez-Muñoz et al. 2010).

Measuring the degree of polyandry by tracking females and directly observing matings can be difficult for insects, but sperm storage in female insects is almost ubiquitous, and genotyping sperm stores can provide useful information on female mating frequency in natural populations (Demont et al. 2011). Several studies have investigated how sperm storage patterns translate into paternity and the number of fathers contributing to a clutch (Demont 2010; Rodriguez-Muñoz et al. 2010; Simmons and Beveridge 2010). Such studies are particularly important for evaluating if sperm utilization patterns demonstrated using double matings in the laboratory are sustained following multiple matings in the wild. The existing data are equivocal on this subject: some studies show the same last-male paternity for double and multiple matings (Cobbs 1977; Simmons 2001), while in other cases sperm are used differently after multiple matings than after only two (LaMunyon 1994; Zeh and Zeh 1994; Simmons et al. 2007). Most of the previous work in this area has been conducted on social insects (Simmons 2001, references therein), but a few recent studies have broken new ground in extending this work to other taxa (Bretman and Tregenza 2005; Simmons et al. 2007; Simmons and Beveridge 2010; Rodriguez-Muñoz et al. 2010). More work integrating these studies or extending them to new systems is clearly needed (Simmons 2001).

Polyandry can give rise to postcopulatory sexual selection, and numerous mechanisms of sperm competition and cryptic female choice have been described (Eberhard 1996; Simmons 2001; Birkhead and Pizzari 2002; Snook 2005; Birkhead et al. 2009). Sperm competition is generally seen as a strong selective agent (Parker 1970a), but the prevalence and importance of some cryptic choice mechanisms are subject of considerable debate. The most controversial of these is sperm selection, the selective use of certain sperm by females at the time of fertilization, when they have a mixture of sperm from different males in their sperm store(s) (Simmons and Siva-Jothy 1998; Simmons 2001). Conclusive evidence for sperm selection is extremely scarce. One barrier is doubtless the need for a precise

understanding of all episodes during which cryptic choice could act: sperm reception, transport within the female reproductive tract, storage and utilization. Additionally, a convincing demonstration of adaptive sperm selection would require demonstrating indirect benefits (e.g. good genes or compatible genes), which is difficult in its own right.

One of the most compelling systems for which there is some evidence of sperm selection is the yellow dung fly, *Scathophaga stercoraria* (Ward 2000). The yellow dung fly is a naturally polyandrous species. Experiments with singly versus doubly mated yellow dung fly females have revealed no simple benefits or costs of multiple mating (Tregenza et al. 2003), but a study in which females mated once or three times revealed a longevity cost to females that copulated with more males (Hosken et al. 2002). There is also some evidence that indirect benefits could offset this cost of mating: males that were more successful in sperm competition also had offspring that developed faster (Hosken et al. 2003). *Scathophaga stercoraria* has further been the subject of experimental evolution in this context, with experimentally enforced polyandry and monogamy rapidly leading to strong evolutionary responses (Hosken 2001; Hosken and Ward 2001; Hosken et al. 2001; Martin et al. 2004). Specifically, flies from polyandrous lines invest more in reproductive tissue such as testes and female reproductive accessory glands (Hosken and Ward 2001; Hosken et al. 2001), yet have decreased immune function (Hosken 2001). Furthermore, a study of the fitness consequences for females evolving under contrasting monogamy versus polyandry regimes suggests that sexual conflict rather than good genes may predominantly drive evolution under polyandry in this system (Martin et al. 2004).

Male yellow dung flies aggregate on and around dung pats where copulations take place. There is intense male–male competition, and several studies have found strong mating advantages for large males (e.g. Jann et al. 2000, references therein). During subsequent oviposition on cow pats, the males guard their mates. Females prefer to lay their eggs on small hills on the dung surface and avoid depressions and sharply elevated points where eggs may suffer a higher risk of drowning or desiccation, respectively, and such female choice of suitable oviposition sites increases female reproductive success (Ward et al. 1999). Although Ward et al. (1999) found that oviposition was not influenced by the presence of other eggs, a recent study reported that females do respond to egg density by decreasing clutch size on crowded pats (Buser et al. unpublished). Intriguingly, females seem not only to choose where to lay their eggs, and how many eggs to lay, but also the parentage of those eggs. In a series of studies, Ward and coworkers suggested that females are able to match the phosphoglucomutase (PGM) genotypes of their offspring to the prevailing environmental conditions (Ward 1998, 2000; Ward et al. 2002). Females collected in the field and allowed to oviposit in the laboratory produced offspring of different PGM genotypes depending on environmental conditions: one PGM allele was relatively more common if eggs had been laid in simulated sunshine (light bulb), and another PGM allele was relatively more common if the eggs had been laid in simulated shade (no light bulb) (Ward 1998). In the same study, Ward (1998) showed that heterozygotes at the PGM locus grew better (i.e. attained a higher pupal weight) in a variable temperature treatment, while homozygotes grew better at constant temperature. These data on larval performance suggest that females could potentially increase the fitness of their offspring by biasing paternity towards males with certain PGM genotypes, depending on the environment in which females lays their eggs. This prediction was supported by a study in which females homozygous for the most common PGM allele were mated with two homozygous males of the same or different genotype as the female (i.e. one of each genotype per female). Males with the same genotype indeed gained greater paternity with females that were exposed to constant temperature. However, homozygous males with a

different genotype (that would consequently produce heterozygous offspring) did not achieve greater paternity than males with the same genotype (producing homozygous offspring) in the variable environment (Ward 2000). Nevertheless, these experiments suggested that sperm selection might occur in yellow dung flies, although to date it appears that the phenomenon is restricted to a subset of females and environmental circumstances (Ward 1998, 2000). Comparing these laboratory findings with a natural population, Ward et al. (2002) found that PGM alleles from egg samples were non-randomly distributed between north and south slopes and between shaded and sunny parts of artificial cow pats in the field. However, Ward et al. (2002) could not determine whether the same females laid eggs of different genotypes in different places by selectively choosing their paternity, or whether females of different genotypes laid their eggs in different places.

Studies on sperm competition and sperm utilization in the laboratory often preclude us from extrapolating results to nature, because of the methodologies applied in most experiments: (1) Mates are randomly assigned, eliminating precopulatory sexual selection; (2) outcomes of copulation and fertilization are observed in isolation (e.g. no other animals present); (3) mating pairs are disturbed during copulation or oviposition (e.g. transferring mating pairs during copulation to oviposition substrate). Here we aimed to minimize these potential influences on the outcome of postcopulatory sexual selection in order to study sperm storage and paternity patterns in as natural a situation as possible. In particular, we addressed the following questions: (i) Do females alter oviposition behaviour depending on temperature or pat age? (ii) Do females select sperm based on dung pat microclimate? (iii) Do females benefit from polyandrous behaviour (i.e. number of ejaculates detected within their sperm stores) in terms of reproductive success? (iv) Do patterns of sperm storage and paternity in the field resemble laboratory estimates?

Materials and methods

Field work

A total of 22 dung fly females were sampled on 4 days in May 2006 on a pasture in Fehraltorf, near Zurich, Switzerland (8.55°E, 47.37°N). We collected fresh cow dung on the pasture, homogenised dung from several cow pats, formed small artificial dung pats on Petri dishes (diameter: 9 cm), and distributed these dishes throughout the pasture. Because the aim was to vary the microclimate of these artificial pats to mimic the natural situation, all dung pats had a "roof shape". We oriented the raised ridge in the middle along the east–west axis to maximize the difference between southern and northern aspects in temperature. Consequently, females could lay their eggs into three distinct areas (micro-environments) on the pat: the ridge, the south exposed surface, or the north exposed surface. The Petri dishes were carefully covered with a cage (dimensions: 29 × 29 × 29 cm) as soon as a female started oviposition on the artificial dung pat. Note that gravid females fly to dung pats in order to mate and lay their eggs, so no procedure was necessary to induce oviposition. Thus males and females were not assigned to each other, nor were copulations disturbed, and other males were present during copulation and oviposition. The use of a cage prevented further males from arriving on the pat and decreased the probability of take-overs (Parker 1970b). However, note that this does not impact on the question investigated in this study, as this remains the same with or without take-overs: whether non-random sperm utilization based on dung pat microclimate occurs. Dung pat age was defined as the time between distributing the pats on the pasture and the time when

oviposition started. Temperature in the sun (not shade) was measured close to the dung pat during oviposition. After oviposition, a collecting vial was passed through a sleeve in the cage to collect the focal female(s) (in two cases, two females were present on the pat at the same time) and one or more associated males. We recorded the dung pat from which flies were captured, and flies and dung pats were subsequently brought to the laboratory.

Upon arrival in the laboratory, adult flies were immediately frozen at −80°C, and the number of eggs laid in the north (N), south (S) and ridge (R) areas was counted. Eggs were then transferred according to their microclimate origin into 200 ml plastic rearing containers (one container per clutch and origin). All transferred eggs were raised in climate chambers at constant 20°C, 60% relative humidity, and 13 h light: 11 h dark regime. We decided to apply this experimental procedure to ensure standardized rearing conditions, but acknowledge that our method does not control for the possibility that applied conditions were unsuitable for flies originating from a certain microclimate (see also "Discussion"). The containers were checked for emerged adults every day until no individuals emerged for 3 weeks. We checked containers for such a long time to be absolutely sure that we did not miss any emerging flies. All emerged flies were immediately frozen at −80°C and subsequently genotyped.

Dissections

Sperm were extracted from the spermathecae using a method described by Tripet et al. (2001) and applied in yellow dung flies before (Bussière et al. 2010; Demont et al. 2011). We separated the abdomens of the dung fly females from the rest of the body and stored them for 48 h in 70% ethanol. Under a stereo microscope (Leica MZ-12, Leica Microsystems GmbH, Wetzlar, Germany), we carefully removed the posterior part of the female reproductive tract (including the common oviduct, spermathecae, spermathecal ducts, accessory glands, and the bursa copulatrix) by grasping the genital valves with forceps and tearing them from the abdomen (Bussière et al. 2010; Demont et al. 2011). Next, the three spermathecae were separated from the rest of the reproductive system and individually transferred to a drop of distilled water. For every female, we could easily distinguish the singlet spermatheca from the middle and outer doublet spermathecae (regardless of the side of the body on which it is found, Hosken et al. 1999). We removed all tissue surrounding each spermatheca and then applied soft pressure to the spermathecal capsule to carefully break it open. As storage in 70% ethanol causes the ejaculate in the spermatheca to coagulate, we were able to remove the sperm pellet from every single spermatheca (Tripet et al. 2001). The three sperm pellets from each female, each originating from a different spermatheca, were transferred to 180 µl of buffer solution (ATL buffer from the QIAamp® DNA Micro Kit, Qiagen; see below) and immediately stored at −80°C for subsequent DNA extraction. We photographed and measured the hind tibiae of all flies under a stereo microscope with the software ImageJ 1.37v (Wayne Rasband, National Institutes of Health, USA, http://rsb.info.nih.gov/ij/).

Extraction, amplification and analysis of DNA

DNA extraction was performed from sperm pellets according to Bussière et al. (2010): we used a kit designed for small amounts of DNA (QIAamp® DNA Micro Kit, Qiagen AG, Switzerland) to extract the potentially very low number of DNA copies from sperm pellets, and added carrier RNA to buffer AL (1 µl dissolved carrier RNA in 200 µl buffer AL). Note that carrier RNA does not dissolve in buffer AL; it must first be dissolved in buffer

AE and then added to buffer AL. We used the minimum recommended amount of elution buffer AE (20 µl) to retain the highest possible concentration of sperm DNA. The QIAGEN® Multiplex PCR Kit (Qiagen AG, Switzerland) was used to simultaneously amplify seven microsatellite loci: SsCa1, SsCa3, SsCa16, SsCa21, SsCa24, SsCa26, and SsCa30 (Garner et al. 2000; Demont et al. 2008). Total PCR reaction volume for the sperm was 30 µl (cf. Bussière et al. 2010 used only 24 µl): 5 µl DNA template, 15 µl QIAGEN Multiplex PCR Master Mix, 7 µl distilled water and 3 µl microsatellite primer mix (100 µM). Cycling conditions for the sperm were as follows: 95°C for 15 min, then 30 cycles of 94°C for 30 s, 60°C for 3 min and 72°C for 45 s, and finally 60°C for 30 min. These cycling conditions did not produce large stutter peaks for six of the applied markers. Locus SsCa21 was the exception, consistently showing stutter. This was not a problem for paternity analyses since we could match offspring genotypes to parental genotypes. In contrast, stutter peaks were able to potentially cause problems for quantifying sperm storage patterns (i.e. number of males detected within spermathecae). We therefore excluded SsCa21 from sperm storage analyses.

We used a Chelex extraction method to extract DNA from the heads of all flies (parents, offspring, and other flies that were collected from the artificial cow pat). Heads were transferred into 96-well PCR plates and kept on ice. We then pipetted 100 µl of 6% Chelex suspension (Chelex 100®, Na^+-form, particle size 50–100 mesh, Fluka) into each well using wide-ended tips. Subsequently we covered the plate with a plastic mat, carefully shook it, and spun down the heads to ensure samples were submerged in Chelex suspension. We used a thermocycler to incubate plates for 60 min at 55°C, boil for 9 min at 100°C, and cool down to 20°C. After taking samples out of the thermocycler we again shook them carefully and spun them down, stored the plate at 4°C for 10–20 h and froze it at −20°C for at least 24 h before DNA extractions were used for subsequent processing. DNA template amount (1 µl), total PCR reaction volume (6 µl), and cycling parameters (number of cycles: 27) for the heads were the same as in Bussière et al. (2010).

All PCR products from sperm and heads were separated on a capillary sequencer (Applied Biosystems 3730 DNA Analyzer), and the output analysed using Applied Biosystems GeneMapper® software. Genotypes from heads were simple to score. Sperm samples were more challenging because of the number of alleles present. To avoid artificial inflation of our estimate of the number of alleles and males present in the sperm stores, we did not consider small peaks on either side of a large peak. As the only exception we counted small peaks (alleles) that were also found in the offspring. We obtained the *number of alleles* present in every spermatheca by counting the alleles after discarding all alleles that could potentially come from the female (in case of incomplete removal of female tissue during dissection). We obtained the *number of males* present in every spermatheca as follows: In cases where maternal alleles were present in the array of alleles, these alleles were discounted. We then identified the alleles from the last male in the array and subtracted them. Afterwards we divided the remaining alleles by two (rounding up) because every male could potentially be heterozygous. This resulting number plus 1 (i.e. the last male) represents our estimate of the minimum number of males (cf. Demont et al. 2011). We therefore obtained separate estimates for the minimum number of males present in a spermatheca from the six microsatellite loci amplified (i.e. locus SsCa21 excluded). The largest of these numbers was our estimate of the minimum number of males present in any given spermatheca.

P_{last} (the proportion of paternity assigned to the last male mated to a female) was determined by subtracting which alleles were passed on by the male. If offspring had the same genotype as the mother, then the exact paternal contribution for that locus is unclear

(e.g. one or the other allele could be contributed by the male), so we denoted both alleles as possibly coming from the father. We assigned offspring to the last male if all paternal alleles (one or two per locus) at all seven loci were found in the multilocus genotype of the last male. Note that matching offspring multilocus genotypes to last male multilocus genotype was absolutely certain in all cases, even when more than one male was present on cow pats. We estimated the minimum number of males contributing to a clutch of a female with the software GERUD 1 (Jones 2001).

Statistical analyses

Statistical modelling was performed as recommended by Crawley (2007): we started with a maximal model that included all factors, covariates, interactions, and quadratic terms that could be of interest (i.e. that we measured and that are likely to be biologically relevant), and simplified it in a stepwise manner on the basis of deletion tests (e.g. F tests or chi-squared tests) to the minimal adequate model. Hence, we only included an explanatory variable in a model if it significantly improved the fit of the model (Crawley 2007). All analyses were performed with R 2.6.2 (R Development Core Team 2008). Linear models were fitted with the *lm* function from the *stats* package, generalized linear models with the *glm* function from the *stats* package, and linear mixed-effects models with the *lmer* function from the *lme4* package (Bates and Maechler 2008).

We analysed clutch size (i.e. total number of eggs laid) and total number of emerged flies with linear models and square-root transformed response. The maximal model included female size, size of the last male, number of yellow dung fly males on cow pat (besides the copulating pair), pat age, temperature, and number of alleles or males detected within sperm storage organs as explanatory variables. The proportion of eggs deposited in the north exposed side of the cow pat and the total proportion of emerged flies were analysed using generalized linear models with quasibinomial errors and logit link. We used quasibinomial error structures because models were overdispersed. Explanatory variables were chosen as in the linear models described above. We investigated if proportion of emerged flies (untransformed and arcsin square-root transformed) differed between eggs originating from different microenvironments by applying paired *t*-tests in a pairwise fashion (N vs. S, N vs. R, and S vs. R). We analysed the minimum number of fathers of a clutch (obtained from the software GERUD 1) using generalized linear models with quasipoisson errors and log link. We used a dispersion parameter since the model was underdispersed. The maximal model included female size, size of the last male, number of alleles or males detected within sperm storage organs, and two-way interactions as explanatory variables. We analysed sperm storage patterns with linear mixed models and log10 transformed number of males detected within each spermatheca as the response. We initially included spermathecal identity, female size, size of the last male, and all two-way interactions as fixed explanatory variables, and female identity as a random effect. We additionally compared the number of different alleles and males (i.e. sires) detected in the offspring to the number of alleles and males detected within the spermathecae with paired *t*-tests. We also used paired *t*-tests to compare the number of alleles and males present across the different spermathecae. Residuals in all linear models were normally distributed (all Kolmogorov–Smirnov tests: $P > 0.10$). We investigated if females could bias paternity to match the genotypes of their offspring to different environments by comparing last male paternity across the three different environments (N, S, and R). This was done by applying binomial proportions tests *prop.test* from the *stats* package in R. We compared last male paternity in a pairwise fashion (N vs. S, N vs. R, and S vs. R) for every female.

Results

Sample sizes

Analyses of clutch size, the proportion of eggs laid in the northern exposed side of the cow pat, and sperm storage patterns are based on a sample size of 22 females. In three clutches no flies emerged, presumably because of very wet dung resulting from rainfall that started during oviposition. Therefore, emergence, paternity and our comparison of sperm storage and paternity were analysed with a sample size of 19 females. Statistically significant terms and their p-values indicated below are in each case for the minimal adequate model.

Oviposition

Mean clutch size (±SE) was 33.27 ± 2.29 eggs for all 22 females collected in the field, and 34.21 ± 2.59 eggs for the 19 females for which we had paternity data. We analysed clutch size (i.e. total number of eggs laid) using linear models and a square-root transformed response. Clutch size significantly increased with female size ($F_{1,18} = 14.633$, $P = 0.001$; Fig. 1a). The significant quadratic term for pat age ($F_{1,18} = 8.353$, $P = 0.009$; Fig. 1b) indicated that clutch sizes were largest in the middle of the range of time that dung was offered for oviposition. The linear term for pat age in the minimal adequate model was not significant ($F_{1,18} = 0.239$, $P = 0.63$). Model simplification revealed that the size of the last male, the number of other yellow dung fly males on the cow pat, temperature, and all the interactions included should not be retained in the model as explanatory terms (all $P > 0.10$).

On average, females laid most of their eggs in the northern exposed side of a cow pat. Mean (±SE) proportions off eggs laid into N, S, and R were: 0.65 ± 0.07, 0.09 ± 0.03, and 0.26 ± 0.06 for all 22 females and 0.65 ± 0.08, 0.10 ± 0.04, and 0.25 ± 0.06 for the 19 females with emerging offspring, respectively. The proportion of eggs laid in the northern side of the cow pat was analysed using generalized linear models with quasibinomial errors and logit link. The minimal adequate model contained just two parameters: the intercept and temperature. The proportion of eggs laid in the northern exposed side of the cow pat significantly increased with temperature ($F_{1,20} = 12.797$, $P = 0.002$; Fig. 2). Model simplification provided no justification for retaining female size, size of the last male, number of other dung fly males on the cow pat, pat age, or any interaction in the model (all $P > 0.10$).

Adult emergence

Mean (±SE) number of emerged flies per clutch was 23.95 ± 2.47 (n = 19 females). We analysed the total number of emerged flies using linear models and square-root transformed response. Total number of emerging flies increased significantly with female size ($F_{1,16} = 12.220$, $P = 0.003$) and the total number of alleles present in the spermathecae ($F_{1,16} = 5.666$, $P = 0.03$; Fig. 3a). The size of the last male and all interactions were not significant and omitted during the process of model simplification. Mean (±SE) proportion of emerged flies was 0.69 ± 0.04 (n = 19 females), and did not differ between flies originating from the three microenvironments (proportion emerged flies: all $P > 0.35$; proportion emerged flies arcsin square-root transformed: all $P > 0.25$). The proportion of emerged flies (analysed using generalized linear models with quasibinomial errors and

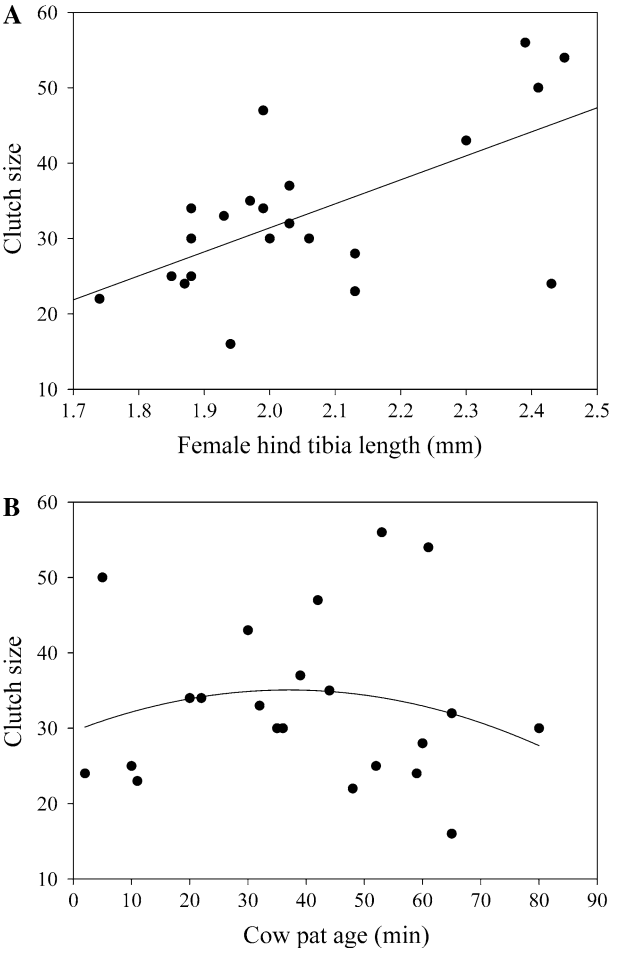

Fig. 1 Clutch size as a function of **a** female size (hind tibia length) and **b** dung pat age

logit link) significantly increased with increasing number of alleles detected in the spermathecae ($F_{1,15} = 5.143$, $P = 0.039$; Fig. 3b). Additionally, the proportion of emerged flies increased with female size only when the female mated last with a large male (significant female size by last male size interaction: $F_{1,14} = 5.514$, $P = 0.034$). The main effects for female size ($F_{1,17} = 0.101$, $P = 0.76$) and the size of the last male ($F_{1,16} = 0.018$, $P = 0.89$) did not significantly influence the proportion of emerging flies. The total number of emerged flies and proportion of emerged flies also had a tendency to increase with increasing number of males (instead of alleles) detected within spermathecae, but not significantly ($P = 0.06$ and $P = 0.12$, respectively).

Sperm storage and number of mates

In total we genotyped sperm from 66 spermathecae (22 females × 3 spermathecae). One outer doublet spermatheca provided an unreadable array of alleles and was excluded from analyses. The last male to mate with the female was always found in all three

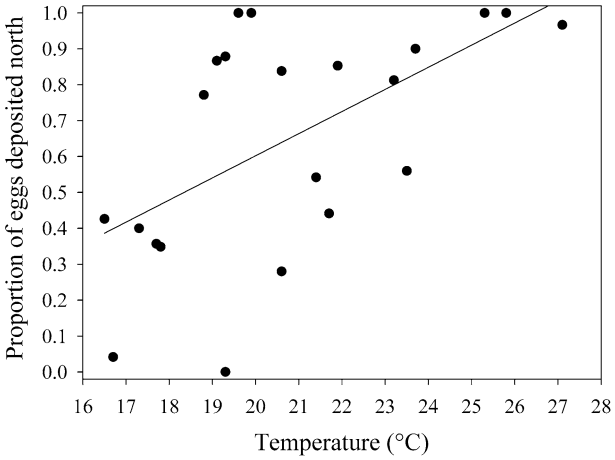

Fig. 2 Proportion of eggs deposited on the north-facing side of a dung pat as a function of temperature

spermathecae. Twenty-one out of 22 females stored sperm from two or more males. On average, females stored sperm from 2.82 ± 0.20 males (n = 22 females; the average was 2.84 ± 0.23 for the 19 females from whose clutches offspring emerged). We found a significant effect of spermathecal identity on the number of males represented in the sperm store (Markov Chain Monte Carlo $P = 0.002$, n simulations = 10,000). This indicated a consistently lower number of ejaculates present in the singlet spermatheca compared to the middle and outer doublet spermatheca (Fig. 4). Paired *t*-tests supported this and showed that there was no significant difference in the number of ejaculates between the middle and outer doublet spermathecae: singlet spermatheca vs. middle doublet spermatheca: $t = -3.250$, $df = 21$, $P = 0.004$; singlet vs. outer doublet: $t = -2.905$, $df = 20$, $P = 0.009$; middle doublet vs. outer doublet: $t = 0$, $df = 20$, $P = 1$. Linear mixed models revealed no significant influence of female size, last male size or any interaction on sperm storage patterns. Mixed model analyses using number of alleles (instead of number of males) as the response variable and paired *t*-tests based on alleles provided qualitatively the same results as analyses with number of males. Sperm storage patterns based on alleles are shown alongside the pattern for males in Fig. 4.

Paternity

Last male paternity and the minimum number of sires for all analysed clutches are given in Table 1. Of the 19 analysed clutches, four clutches featured only eggs laid in the northern exposed side of the cow pat (i.e. no eggs in the S and R portions of the artificial dung pat). This restricted analyses of differences in last male paternity across N, S, and R to only 15 females. Binomial proportions tests revealed no females that showed differences in last male paternity across N, S, and R (one $P = 0.08$, all other $P > 0.16$). Note that the 15 clutches also include four clutches with complete last male sperm precedence (i.e. all offspring were from the last male). The minimum number of fathers contributing to a clutch was estimated with the software GERUD and is given in Table 1. We analysed the minimum number of fathers with generalized linear models with quasipoisson errors and log link. The minimum number of fathers estimated for a specific clutch significantly increased with increasing female size ($F_{1,17} = 6.186$, $P = 0.025$), increasing last male size

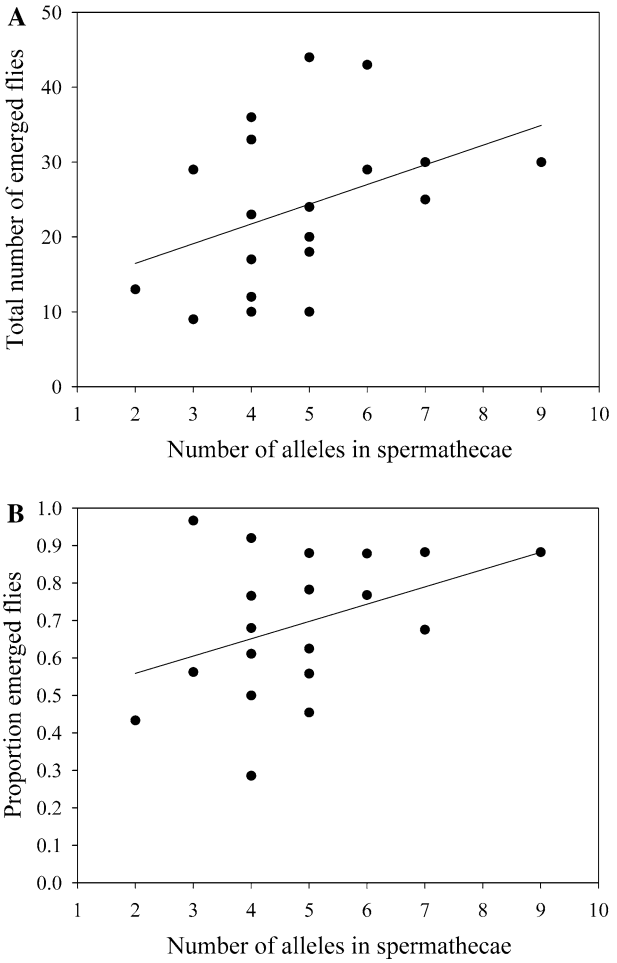

Fig. 3 **a** Total number of emerging flies and **b** proportion of emerging flies as a function of the total number of alleles detected within the sperm storage organs (spermathecae) of wild females

($F_{1,16} = 7.641$, $P = 0.014$), and the number of males detected within the spermathecae ($F_{1,15} = 19.419$, $P < 0.001$). Generalized linear model analyses with number of alleles detected within spermathecae (instead of number of males) as the explanatory variable provided the same results. Female size, last male size and number of alleles within spermathecae all had significant positive effects on the number of fathers that contribute to a clutch.

Discussion

This study provides rare and useful information on sperm storage, paternity, and post-copulatory sexual selection in a natural population of yellow dung flies. Our experiment allowed females to exhibit plasticity in several different aspects of their oviposition

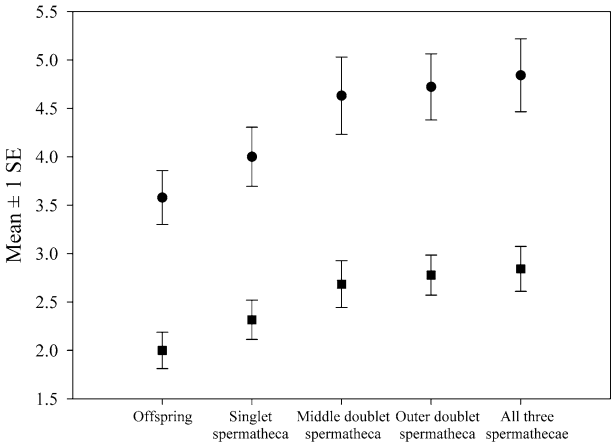

Fig. 4 Mean number of males (*squares*) and alleles (*circles*) detected within the offspring, each of the individual spermathecae, and all three spermathecae combined

Table 1 Last male paternity and minimum number of sires for 19 wild-caught female yellow dung flies *Scathophaga stercoraria*

Female	Last male paternity	Minimum number of sires
a	0.881	3
b	0.700	3
c	0.900	3
d	0.682	2
e	0.444	3
f	0.862	2
g	0.886	3
h	0.917	2
I	0.909	2
j	0.800	2
k	0.700	2
l	0.500	2
m	1.000	1
n	0.889	2
o	1.000	1
p	0.917	2
q	1.000	1
r	1.000	1
s	0.850	3
Mean	0.834	2.11

behaviour. We report strong evidence that females react to temperature and pat age in adjusting the number of eggs and their location. In contrast, we found no evidence for sperm selection: females did not appear to selectively lay eggs of different genotypes by biasing paternity towards certain males according to dung pat microclimate (cf. Ward 1998, 2000). There were nevertheless two findings consistent with the potential for females

to bias paternity even if it was not realized: the number of stored ejaculates differed between the singlet spermatheca and the doublet spermathecae, and females stored sperm from more males than sired their offspring. On average, females stored sperm from 2.84 males within their sperm stores, indicating high levels of sperm competition prevailing in the field. In summary, although wild yellow dung fly females do not appear to select sperm adaptively in the context provided in our experiment, they can benefit from polyandry via an increased fraction of emerging offspring.

No evidence of sperm selection based on dung pat microclimate

In the present field study, females could lay their eggs into three distinct areas (environments) on cow pats: the south exposed surface, the north exposed surface, or the ridge in between. Microclimate variation in these cow droppings seems to be substantial (Landin 1967; Ward et al. 2002). Nevertheless, we find no evidence that females match offspring genotypes to prevalent environmental conditions by biasing paternity toward certain fathers from which they have sperm in storage. Binomial proportions tests revealed that no single female showed differences in last male paternity across the three experimental cow pat environments. We concede that our sample size was modest. On the other hand, the strength of our test of sperm selection based on microclimate was that it did not make any assumptions about specific traits a female might prefer (e.g. a certain allozyme, other physiological trait, body size, morphology, etc.). Obtaining detailed knowledge of the exact male traits females exert preferences for is often difficult. Nevertheless, if females are capable of matching offspring genotypes to environmental conditions, they must do so by selecting sperm from certain males at fertilization. The binomial proportions tests we used above explicitly tested for this and found no indication of females using sperm differentially in the different environments available on a single pat. The last male that had mated with a specific female was always statistically equally successful, irrespective of the pat aspect on which females oviposited. However, one important methodological issue requires attention. Since paternity determination from freshly hatched larvae is difficult in yellow dung flies, and can result in DNA profiles of low quality that are difficult to interpret (personal experience), we determined paternity success at emergence. Importantly, the period between oviposition and emergence might be critical if there is genotype-to-environment matching. Because all offspring were reared under the same environmental conditions, we cannot rule out the possibility that conditions were unsuitable for certain flies (i.e. originating from a certain microenvironment) and that this potentially affects our paternity estimates. This problem is also not entirely erased by the fact that flies originating from the three microenvironments emerged equally well in the present study. Future studies in this vein could raise flies originating from different microenvironments at different temperatures to detect how this affects the interpretations of the present study.

Despite the enormous interest in postcopulatory sexual selection, convincing evidence for sperm selection remains elusive. Previous studies in yellow dung flies provided evidence of sperm selection based on phosphoglucomutase (PGM) alleles (Ward 1998, 2000). However, in these experiments, results were restricted to a small fraction of flies with certain PGM genotypes. The frequency of the most common allele is >85% in the field (Ward et al. 2002), thus strongly constraining the scope for choice. In addition, not all predictions concerning cryptic female choice could be confirmed (Ward 1998, 2000). Our present study did not support cryptic female choice concerning the paternity of eggs laid in particular environmental conditions, but this clearly is still consistent with the ability of females to make subtle decisions regarding the placement of eggs or number of eggs laid.

Previous work demonstrated that females prefer to lay eggs on small hills on the dung surface and avoid depressions and sharply elevated points where larvae suffer increased risks of drowning or drying out (Ward et al. 1999). Our findings additionally reveal that temperature and pat age strongly influence oviposition. Females laid more eggs at intermediate times after a cow pat had been deposited on a pasture, indicated by a significant quadratic effect of pat age on clutch size. The adaptive significance of this behaviour remains to be established. Furthermore, the proportion of eggs deposited into the northern portion of a cow pat strongly increased with increasing environmental temperature. The protection of eggs against the negative effects of elevated temperatures and/or desiccation seems the most likely explanation for this behaviour (Ward and Simmons 1990). Thus the present study strengthens the notion that modulation of the number of eggs deposited and the choice of suitable oviposition sites are much more pronounced than specific choices regarding paternity of the eggs laid (Buser et al. unpublished).

Sperm storage, paternity, and the potential for cryptic female choice

Genotyping sperm stores to estimate female mating frequency in natural populations is more useful than genotyping offspring because postcopulatory sexual selection may bias paternity toward certain mates, resulting in an underestimate of existing levels of polyandry in the wild (Bretman and Tregenza 2005; Simmons et al. 2007; Demont et al. 2011). We revealed high levels of polyandry (i.e. high sperm competition intensity) in the field: 21 out of 22 females (95.5%) stored sperm from two or more males, and on average 2.84 ejaculates competed within the sperm storage organs. A related study detected pronounced temporal changes in sperm competition intensity in the same population (Demont et al. 2011). Both the proportion of multiply mated females and the absolute number of competing ejaculates were consistent with previous findings for the same time period (i.e. May) in that study (Demont et al. 2011).

Theoretical work and laboratory studies suggest that females could bias paternity toward certain males by differentially storing sperm from different males in each spermatheca and subsequently choosing sperm (or a sperm mix) from a particular spermatheca (Hellriegel and Ward 1998; Hellriegel and Bernasconi 2000; Bussière et al. 2010). The present study revealed that sperm mixtures differ in wild yellow dung flies, as we found a significantly lower number of ejaculates in the singlet spermatheca compared with the doublet spermathecae. This result was also in accordance with a separate recent study (Demont et al. 2011), where in contrast to the present work, copulations were interrupted. Bussière et al. (2010) demonstrated that following double matings, the highest proportion of sperm from the second male (S2) was found in the singlet spermatheca. This accords well with the current work, where as a result of stronger sperm displacement, the fewest number of ejaculates were found in the singlet spermatheca. It remains to be precisely established why the singlet typically features higher sperm displacement (i.e. S2 values) than either doublet spermatheca (Bussière et al. 2010). In particular, it is unclear whether this is a result of female influence on sperm storage, or second (or later) males consistently filling spermathecae in the same order, starting with the singlet.

We additionally showed that females stored sperm from more males than sired their offspring. The recently developed competitive PCR technique for assessing the proportions of sperm from competing males within females' sperm stores assumes that all genotypes of the males involved are known (Bussière et al. 2010; Hall et al. 2010). Applying this technique, it has been shown that the amount of stored sperm from each male correlates well with achieved paternity success following double matings (Demont 2010). In this

study we only counted the different ejaculates present across the spermathecae. Since the genotypes of all involved males (except one) were unknown, we could not quantify the amount of stored sperm for each specific male. Therefore, the present study cannot relate success or failure of a specific male in obtaining paternity to its amount of stored sperm. More advanced techniques that allow the quantification of the different proportions of stored sperm following multiple matings when the candidate male genotypes are unknown will be a fruitful avenue for future research.

Benefits of polyandry

Several laboratory studies have documented benefits of polyandry (Tregenza and Wedell 2002; Zeh and Zeh 2006; Price et al. 2008). In contrast, only a few studies have examined polyandry in natural populations and reported benefits (Madsen et al. 1992; Fisher et al. 2006). In yellow dung flies, laboratory studies have shown that multiple mating is associated with longevity costs (Hosken et al. 2002), but that females also benefit from polyandry: more successful males in sperm competition sire offspring that developed faster (Hosken et al. 2003). Here we document benefits of polyandry in a natural population: the proportion and the total number of emerged offspring increased significantly with the number of alleles (our proxy for the number of mating partners) detected within the sperm stores of females. Analyses with the number of males (instead of the number of alleles) as the explanatory variable provided the same patterns, but were marginally non-significant. We used a multiplex PCR reaction consisting of seven (after exclusion of SsCa21: six) highly polymorphic microsatellite loci to avoid situations where males in the spermathecae share all their alleles. As a consequence, males will always be dissimilar at certain loci. This suggests that the number of mates per se and not genetic dissimilarity among mating partners is responsible for increased fertility with increasing number of alleles. Furthermore, because of the microsatellites applied, males will be heterozygous at least at some loci. This implies that the observed pattern also does not arise because some females mate with homozygous males and some with heterozygous males. The precise genetic mechanism (e.g. good genes vs. compatible genes) underlying the documented increase in reproductive success of polyandrous females in the field is unclear, as is whether the magnitude of expected benefits to polyandry are likely to outweigh the potential costs of multiple mating. In yellow dung flies, mating at least once before each oviposition bout is practically inevitable because dung pats tend to be so densely occupied by males, and so there is no necessary expectation that polyandry must be explained as a female adaptation. Further, larger females, who are more fecund, might get inseminated by more males, and this could partially explain the relationship between number of alleles and emergence.

In summary, our study showed that female yellow dung flies make subtle decisions regarding the placement of eggs or the number of eggs laid. There was no evidence of selective use of sperm from particular mating partners according to dung pat microclimate. However, we did find that sperm mixtures differed amongst spermathecae, and that females stored sperm from more males than sired their offspring. The present study further supports previous findings of intense sperm competition levels in the field, and indicates that polyandry has a positive effect on the number of offspring emerging. Precise mechanisms underlying the positive effect of multiple mating remain to be established. In this context, a better integration of field studies and controlled laboratory experiments seems a particularly promising way to advance our understanding.

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