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Evolutionary insight from a humble fly: sperm competition and the yellow dungfly

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Studies of the yellow dungfly in the 1960's provided one of the first quantitative demonstrations of the costs and benefits associated with male and female reproductive behaviour. These studies advanced appreciation of sexual selection as a significant evolutionary mechanism, and contributed to the 1970's paradigm shift toward individual selectionist thinking. Three behaviours in particular led to the realization that sexual selection can continue during and after mating: (i) female receptivity to remating, (ii) sperm displacement, and (iii) post-copulatory mate guarding. These behaviours either generate, or are adaptations to sperm competition, cryptic female choice and sexual conflict. Here we review this body of work, and its contribution to the development of post-copulatory sexual selection theory.

1. INTRODUCTION

The yellow dungfly, *Scathophaga* (= *Scatophaga*) *stercoraria* L. holds a special place in the history of sperm competition research. Yellow dungflies can be found associated with cattle throughout the cooler temperate regions of Europe, Asia and North America. The flies arrive en masse, within minutes of the dung being deposited [1, 2]. Males arrive more quickly than females, and in significantly greater numbers. Females arrive from downwind, flying over the dropping and alighting in the grass beyond, and it is here that males concentrate their search for females and seize them immediately upon detection [3, 4]. Pairs copulate, usually in the grass, after which they walk or fly to dung-pats where females oviposit [5]. The male remains mounted on the female during egg laying [6]. Once eggs are laid females fly away while males remain on the dropping to search for additional mating opportunities.

The breeding behaviour of yellow dungflies provided an early quantitative test of sexual selection theory. Sexual selection should favour males that search in areas where fitness gains are greatest. During the first twenty minutes after deposition, the arrival rate of females is greatest on and 20-30 cm upwind of a dropping. The distribution of searching males on and around the dung matches the female arrival rate so that the male fitness gain at various locations is equal, following an ideal free distribution [3]. As a dropping ages, new female arrivals decrease and mated pairs begin to accumulate on the dung for oviposition. Accordingly, males switch from searching in the grass to searching on the dung, via aggressive take-over attempts of ovipositing females from their attendant males [7]. If successful in a struggle over a female, the winning male will copulate with her before she continues oviposition. Copulation takes less time, and thus the rate of fitness return is greater, on the dung surface, so pairs will remain on the dung following a take-over. However, as the density of searching males on the dung increases, so does the risk of further take-overs. As

predicted, the probability of emigrating to the surrounding grass to copulate increases with the density of searching males [8].

Males vary in their ability to compete for females. Large males have a competitive advantage over small males in aggressive interactions [9], resulting in a non-random distribution of male sizes around the dropping; in high density populations, large males dominate at sites of maximum female arrival with small males displaced to less profitable search locations [10, 11]. Indeed, the smallest males may adopt alternative mate searching tactics, searching for and copulating with females at foraging sites far from droppings [12, 13]. Since females invariably mate on arrival at the dung, this alternative tactic yields lower reproductive returns than searching for and mating with females at the site of oviposition, so that small males make the best-of-a-bad-job in avoiding direct competition with their larger conspecifics, fertilizing the small proportion of eggs that are not fertilized by the last male to mate. Because large males monopolize the most profitable mate locating sites and are more successful in achieving and resisting take-overs, there is significant pre-mating sexual selection acting on male size, both within and among populations [14, 15]. Variation in the strength of pre-mating sexual selection among genetically isolated fly populations is reflected in the evolutionary divergence in male size among populations [16]. Early work on the dungfly mating system thus provided quantitative support for the role of sexual selection at a time when this aspect of Darwinian evolution was largely dismissed as unimportant.

Three aspects of the yellow dungfly's mating behaviour led to the initial insight into post-copulatory sexual selection [17]. First, females are highly polyandrous, retaining their sexual receptivity after mating. When arriving at fresh dung females almost always carry sperm in their sperm stores [5, 18, 19] and can fertilize at least four successive egg-clutches without remating [20]. Nevertheless females mate each time they visit a dropping to oviposit, and when subject to take-over(s) will mate twice (or more) on a single visit [5]. Second,

males are eager to copulate and do not discriminate between newly arriving and recently mated females [5] or between virgin and mated females [19]. Lengthy copulations are costly for males, reducing the time available to mate with other females [20]. Finally, males remain with females after copulation and during oviposition (originally termed the “passive phase”), a significant time cost that could also be spent searching for additional females. These male time investment strategies make adaptive sense in the light of post-copulatory sexual selection via sperm competition.

2. The adaptive significance of female receptivity

Females arriving at a dropping are ready to oviposit and can do so within 16 mins [5]. Copulation takes an average of 36 minutes, and as females have already mated before arriving, they suffer a copulation time cost that could otherwise be spent foraging for the resources to manufacture a subsequent clutch of eggs. Selection should favour unreceptivity to mating unless the advantages of receptivity outweigh the costs of mating. Females can and do reject copulations: copulations with non-gravid females away from dung [13, 21] and with females immediately after they have completed oviposition at the dropping [5], are both shortened by the violent side-to-side shaking movements of unreceptive females. However, unreceptivity would subject an ovipositing female to repeated harassment by searching males if not guarded by the last male to mate. The time costs associated with full receptivity at the dropping were estimated as the time spent copulating with a single male plus the average time spent copulating due to take-overs, being some fraction less than a single copulation that is dependent on the probability of take-over [5]. The time cost of rejecting a male was estimated from the reduction in copula duration, some 15 minutes, experienced by males mating with post-oviposition females resisting copulation. The time cost difference between full receptivity and rejection depends strongly on the density of males on the dropping, and

therefore the number of male mating attempts an ovipositing female can expect to receive [5]. At the average density of searching males, full receptivity females were calculated to gain some 50 mins for each oviposition cycle relative to females resisting copulation, explaining why females retain receptivity when visiting droppings. We now also know that females can gain significant genetic advantages for their offspring by mating with multiple males [22, 23] so that retaining sexual receptivity is an adaptive strategy adopted by females.

3. The adaptive significance of copula duration: sperm allocation and displacement

Sperm allocation in the yellow dung fly is possibly the only case where *quantitative* predictions of sperm allocation have been examined against observed allocations. These calculations maximize a male's fertilization gain per minute of reproductive activity, and are possible because: (i) sperm transfer to the female is directly proportional to copula duration, (ii) paternity gain shows diminishing returns with time spent copulating, and (iii) average time spent searching for and guarding a female can be estimated from field data [1]. Thus the marginal value of continuing copulation with the present female can be balanced against the value of searching for an alternative female. This trade-off between post-copulatory gains from a present mating and gains from expected future matings set the scene for the development of sperm allocation models (Parker this issue).

(a) Initial approaches

Early experiments using interrupted matings showed that a male's fertilization gains when mating with a previously-mated female follow diminishing returns with copula duration [20]. Since the winning sperm allocation strategy was expected to depend on the strategies of other males in the population, the first model took an early evolutionarily stable strategy (ESS [24]) approach. It calculated expected egg gains to mutant individuals deviating in strategy in

populations fixed at a given copula duration, and demonstrated that the observed duration lay within the predicted range [20], offering a plausible explanation for the observed sperm allocation pattern.

A later, simplified model assumed that since payoffs are only weakly frequency dependent, the optimal copula duration can be approximated by Marginal Value Theorem [25, 26]. It predicted a 42 min copula duration compared with the observed duration of around 36 min. A simple ESS modification yielded a similar result [19], and Marginal Value Theorem has been used in most subsequent models.

(b) Male size

Studies from the 1990s onwards examined optimal copula duration in terms of phenotypic variation. Although on average the percentage of eggs fertilized by the last male to mate is in the region of 80%, regardless of the number of previous matings by the female, the variation about the average varies considerably, depending largely on copula duration but also on male size [27-31]. Smaller males copulate for longer, which appears to be adaptive, resulting from two different effects: (i) a male's sperm input rate to the female increases with his size, and (ii) the time taken for a male to find and guard a female decreases with his size, due to the size advantage in gaining take-overs in struggles for females [28]. As a result, the expected total sperm allocation (and hence paternity gains) should remain roughly constant with male size [32], which matches observations [28]. Because small males have lower input rates, to achieve equal total sperm input they copulate for longer than large males.

Last male precedence in dungflies was first thought to result from sperm input into the female's spermathecae directly displacing an equivalent output. An exponentially diminishing returns model of direct displacement with rapid mixing of sperm in the spermathecae gave a good fit with the observed relationship between paternity gains and

copula duration. Competitive PCR techniques have also confirmed that average last male paternity matches the mean proportion of his sperm in the spermathecae [33]. When used to predict the relationship between optimal copula duration and male size, concurrence with the data was good except for very small males, where predicted durations were considerably longer than observed [28]. However, independent histological [34] and isotope-labeling investigations [29] indicated that sperm displacement is indirect. Sperm are input at a constant rate to the female's bursa, and are transferred to her spermathecae via movements of the spermathecal ducts. Sperm displacement therefore results from an interaction between male and female activities. A simulation of indirect transfer using observed parameters generated a predicted relation between male size and copula duration close to that observed across all natural male sizes [35]. Male size-dependent sperm displacement can thus be explained quantitatively as adaptive phenotype plasticity in response to fertilization rate maximization.

(c) Female quality

Female quality varies in two ways that affect sperm allocation: for gravid females arriving to oviposit, egg content increases linearly with female body size [20], and for females taken over during oviposition, egg content depends on eggs remaining to be laid. Copula duration increases both with gravid female size, and eggs remaining to be laid after take-over [36]. However, to model optimal sperm allocation, two complications were considered. First, spermathecal volumes (but not spermathecal duct diameters) increase with female size, suggesting decreasing paternity with increasing female size at a given copula duration (due to decreased displacement rate as a proportion of spermathecal sperm). This is matched by data [36]. Second, while a male's expected gains in future clutches exert only a small effect on optimal copula duration with gravid females, it can affect matings after take-overs, depending

on numbers of unlaidd eggs and on female survival between clutches. Accounting for these factors, the indirect displacement model predicts a positive relationship that matches (both slope and intercept) the observed increase in copula duration with gravid female size [36]. The data suggest, therefore, that males assess female size, rather than egg content directly. For matings after take-over, the model prediction also fits the observed increase in copula duration and eggs remaining, but with slightly steeper slope than that observed. Males successful at take-over may assess egg content by how much the female's abdomen is distended, which decreases markedly during oviposition.

(d) Matings away from droppings

All the above investigations relate to matings around droppings, where large males dominate the dung surface, with smaller and intermediate-sized males more often in the surrounding grass [11]. However, matings also occur away from dung (extra-dung matings [8]); experimental copulations with dung absent are 10% longer than those with dung present [27]. The optimal copula duration for extra-dung matings depends on whether they form an alternative mating tactic, and whether virgin females occur equally in the two localities [37]. If each male encounters virgins equally frequently in both localities, extra-dung matings are predicted to be shorter than dung matings because of the reduced paternity gains when the female remates before ovipositing. But if dung and extra-dung matings are alternative tactics [12, 13], then it seems likely that extra-dung matings should be longer (as found experimentally [27]), because the search time to find a female may be longer.

4. The adaptive significance of post-copulatory mate guarding

Searching for ovipositing females on the dropping makes adaptive sense, given that females retain receptivity to mating while they have eggs to lay, and although highly variable, on

average the last male to copulate can fertilize the majority of the eggs laid. Sperm competition was therefore proposed to have favoured the evolution of post-copulatory mate guarding to prevent sperm displacement by rivals [6].

As noted above, the rate at which searching males encounter ovipositing pairs rises rapidly with increasing male density, with an encounter rate around one per minute at the average density [7]. Encounters typically involve a searching male pouncing towards the pair, upon which the guarding male will raise one or both middle legs to fend off the attacker [7]. If the attacker persists, the guarding male adopts a “standing” posture whereby he straightens his front legs, pushing the attacker away and the female closer to the dung surface, while raising his middle legs to further fend off the attacker. The standing posture has the effect of doubling the distance between attacker and female and preventing him from detecting her presence. When the guarding male is large relative to the attacker, such responses are sufficient to ward off attackers in seconds. However, should the attacker detect the female, a struggle will ensue during which he attempts to leverage himself between the guarding male and female so as to engage her in genital contact. Struggles can last up to a minute or more, depending on the outcome.

Dungfly struggles conform to assessment strategies in which successful take-overs depend on asymmetries between guarding and attacking males [9]. Thus, guarding males have an advantage in struggles, with only 20-25% resulting in a successful take-over, consistent with contest asymmetries in favour of the owner. First, when the guarding male wins, the struggle duration decreases with the guarding male’s resource holding power (body size relative to attacker), consistent with contest theory predictions that opponents assess their ability to take-over and persist for longer when this is high. Second, struggles involving a take-over decrease in duration as the female oviposits, suggesting that the guarding male has information that her resource value has declined, and so invests less in resisting take-over [9].

When a take-over occurs, the successful male copulates with the female, displacing rival sperm before allowing the female to continue oviposition.

For males, guarding represents time lost that could be spent searching for and copulating with additional females. Nevertheless, provided that the average rate of egg gain for a male investing in guarding exceeds that expected from searching for additional mates, selection should favour the guarding strategy. The average eggs fertilized per minute of reproductive activity for each strategy (guarding or non-guarding) can be calculated [6]. For a guarding male, this decreases with increasing density of searching males, because of the increasing encounter rate with searching males and subsequent egg loss due to take-overs. However, for a mutant non-guarding male who saves 16 mins of guarding time, the rate of egg gain falls far more rapidly because a female unguarded after copula is likely to be quickly captured and remated by a searching male [6].

Quantification of the time costs and benefits of mate guarding, and how they translate into fertilization rates, led to the conclusion that post-copulatory sexual selection would favour the evolution of mate guarding whenever: (i) mating immediately precedes fertilization, (ii) pre-copulatory sexual competition for females at the location of mating is high, (iii) following female remating the last male to mate can gain a reasonable proportion of fertilizations, and (iv) males have a reasonable probability of retaining females during take-over attempts. These conditions for the evolution of post-copulatory mate guarding hold true in several theoretical treatments [38-40].

5. Cryptic female choice and sexual conflict

Work on yellow dungflies in the 1990's contributed to the growing debate over the evolutionary potential of cryptic female choice [41]. This occurs when, after the initiation of copulation, females manipulate their mates' siring success based on male phenotype and/or

genotype [42]; essentially the continuation of mate-choice after copulation commences. It was suggested that standard experimental protocols used to investigate dungfly sperm competition, which employed short delays between matings by competing males, and between mating and egg laying, afforded females with limited opportunity to influence paternity [41]. Female dung flies were proposed to exercise cryptic female choice following a series of experiments that purportedly controlled for male effects and that allowed females to lay multiple clutches of eggs without remating between them [41]. This was because larger, more “preferred” males, tended to have greater siring success as the second of two mates (P_2), and that the P_2 of smaller males increased over 3 clutches of eggs. Unfortunately, to “control” for male effects, the experimental protocol had artificially restricted the copula duration of all competing males to 20 minutes – roughly half the normal copula duration [41].

This restriction has important consequences because the rate of sperm transfer to a female increases with male size (Section 3(b)); smaller males must copulate longer to transfer the same number of sperm as larger males [27-29, 43]. Thus, artificially capping copulation duration at a point mid-way through normal completion of copula allows larger males to transfer relatively more sperm to females: the putative cryptic female choice for larger males was more simply explained by a male size-dependent numerical sperm advantage without the need to invoke any female control over sperm use [44]. Rather than controlling for male effects by restricting copula duration, the approach simply guaranteed a larger male advantage in sperm competition [44]. Previous work had also shown that sperm competition outcomes were not impacted by delays between matings – the siring success of the last male to mate was always high [20]. Although this may contrast with the finding of increased P_2 of small males across clutches [41], the lack of copulation between bouts of egg-laying almost never occurs in nature [5, 13, 19, 20] so it is difficult to understand what this element of experimental design actually revealed.

It was also suggested that because females have three spermathecae rather than one, they might have control over sperm use [41]. A model assuming differential sperm storage across two or more spermathecae and spermicide at the site of insemination showed that males could *in theory* exert cryptic choice of sires [45]. Sperm first enter the bursa rather than the spermathecae [29, 34] (Section 3(b)); the spermathecae then fill somewhat sequentially rather than concurrently, generating some separation of ejaculates from different males [29, 33]. Additionally, when the nervous system of females was inhibited, normal patterns of sperm storage were disrupted [46, 47]. Nevertheless, although there is clearly female involvement in sperm storage, evidence for cryptic choice was still lacking because differential female responses to male phenotypes had not been found.

The next tests focused on an enzyme important in metabolism, phosphoglucosmutase (*pgm*) [46, 48]. Work in other systems showed that enzymes like *pgm* can affect fitness components, including sexual fitness [49]. Building on previous investigations of allozyme variation, it was reported that dungflies laid eggs of specific *pgm* genotypes to match the environmental conditions mothers experienced during egg-laying [46]. This work was conducted under simulated sunny or shady conditions in the laboratory with field-captured females. Females were proposed to detect the *pgm* genotype of sperm they had previously stored and to differentially fertilize eggs with specific *pgm*-carrying gametes, depending on the laying conditions [46]. Subsequent field studies that involved manipulating dung pats and allowing free-living females to oviposit naturally provided further evidence that egg-laying was non-random with respect to *pgm*-genotype [50]. Some *pgm* allozymes were found more frequently on sunny south-facing dung pats and others were more frequent on north-facing or shaded pats. Further laboratory experiments suggested that females homozygous for the commonest *pgm* allele adjusted their offspring *pgm*-genotype to the environment they

experienced (constant vs. variable), with a relative increase in offspring heterozygosity when females were reared in variable temperatures [48].

However, there is now considerable evidence to suggest that *p_{gm}* is unlikely to be the focus of cryptic female choice. Importantly, across European populations of yellow dungflies, *p_{gm}* is not under selection [51]. Additionally, in double-mating experiments, average paternity share mirrors sperm representation in storage and sperm from every detected male was found in every spermatheca, albeit with variation in the proportional representation of different males across spermathecae [33]. These findings match work with isotope-labeled ejaculates where paternity gains also mirrored increases in amounts of sperm moving into sperm stores, and the relative contribution of second-male ejaculate in storage increased with copula duration [29]. No compelling evidence exists for intra-ejaculate sperm sorting [18], and finally, earlier findings of *p_{gm}*-biased egg-laying with respect to dung-microhabitat could not be replicated [22, 52]. These studies again included dung-pat manipulations in the field. For example, one study sculpted pats to form sunny and shady sides, with a ridge area between the two, following which females were allowed to lay naturally. All females (and any associated males) and eggs were collected, genotyped and paternity assigned. There was no evidence that paternity varied across the microhabitats [22]. As an aside, experimental evolution also indicated that post-copulatory sexual selection does not increase female fitness [53], and hence does not offer the benefits necessary to maintain cryptic female choice [48].

Thus claims of cryptic female choice in dungflies remain unsubstantiated, despite compelling evidence of active female involvement in sperm storage [29, 46] and that sperm from different males can be stored disproportionately across spermathecae [33]. To unequivocally demonstrate cryptic female choice, female responses must vary with male phenotype and/or genotype [42]; detecting a female effect on sperm storage alone is not

sufficient. Nonetheless, there is definitely female-male coevolution for a range of reproductive characters across the Scathophagidae, including sperm stores and sperm [54] that require explanation. Prosaically, they may simply result from intra-specific intersexual genetic correlations for reproductive characters that represent ontogenetic constraints [55], though this seems unlikely.

Genetic correlations can also be indicative of underlying sexual conflict [55-57], a divergence in the evolutionary interests of the sexes [58]. Sexual conflict can result in evolutionary arms races over the outcomes of interactions (inter-locus sexual conflict) and evolutionary tugs-of-war over specific trait values (intra-locus sexual conflict) [56-59]. Furthermore, conflict should be greater in polyandrous than monogamous mating systems, because with polyandry, the fitness interests of the sexes are not completely coupled [60]. To demonstrate sexual conflict unequivocally, sexually antagonistic selection on some trait or interaction must be detected [56, 57].

Dungflies are extremely polyandrous [18, 20], generating opportunities for sexual conflict. Although mating may reduce the time costs for females that would be associated with unreceptivity [5], mating struggles between males for access to guarded females can also be costly for females; female wing-damage correlates positively with number of matings [18], and evidence exists that wing damage lowers fitness in the field [14]. Additionally, females that mate more can have reduced longevity [61]. When forced to evolve under monogamy or polyandry for 10 generations and then singly mated, females evolving under polyandry died faster and had lower fitness [53]. Ideally then, fewer matings might be beneficial for females even though they mate repeatedly to avoid harassment (Section 2). Thus it appears that there is sexual conflict over mating in dungflies and harm to females is likely to be a side-effect of competition among males.

Experimental evolution resulted in weaker immune system components [62] and larger testes [63] for males evolving under polyandry. Predictably, these males also enjoyed greater siring success as last males to mate (P_2) when in competition with males from populations evolving under enforced monogamy. P_2 also tended to be lower with females from monogamous populations [64], interpreted as polyandrous females being better able to circumvent the selfish interests of their second (of two) mates. However, it was not clear what fitness gain this generated for females within the experimental polyandrous treatment, except perhaps to decrease within-female variance in number of fathers, which has potential benefits under some circumstances [65]. It is also possible that the reduced P_2 was merely a consequence of the larger female accessory sex-glands evolved under polyandry [64]. These glands apparently produce secretion used during mating and sperm are found in them after copulation [66]. If larger glands resulted in more ejaculate “accidentally” entering the glands rather than the spermathecae, P_2 could be reduced under polyandrous evolution with no net advantage to females required. Finally, while the many intersexual genetic correlations detected in dungflies are potentially indicative of intra-locus sexual conflict [55], large body size, which is positively genetically correlated across the sexes [67], appears to be under positive selection in both males and females [14]. Thus despite pronounced sexual dimorphism, including size dimorphism, it is not yet clear what effect intersexual genetic correlations have on sex-specific evolution in this fly.

6. Concluding remarks

Darwin [68] discussed sexual selection up to ejaculation. However, the behavioural ecology of the yellow dung fly offered insight into the fact that females typically mate with multiple males and will generate post-copulatory sexual selection through sperm competition, cryptic female choice and sexual conflict. This work suggested that sperm competition should

generally favour the evolution of opposing adaptations in males that, on the one hand allow them to pre-empt the sperm stored by females from previous mates, while on the other protect a male's sperm from being pre-empted by rivals. The fly's copula duration and post-copulatory mate guarding can be explained quantitatively in the light of sperm competition. Work on the yellow dungfly provides a beautiful example of how empirical and theoretical approaches conducted in parallel can advance our understanding of behavioural evolution, the essence of the behavioural ecology approach. The behavioural ecology these flies prompted a literature survey that revealed abundant evidence for the occurrence of sperm competition in other insect species, and revealed many traits that could be explained by post-copulatory sexual selection [17]. And so the study of post-copulatory sexual selection was born.

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