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Competition drives cooperation among closely-related sperm of deer mice

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Abstract

Among the extraordinary adaptations driven by sperm competition is the cooperative behaviour of spermatozoa1. By forming cooperative groups, sperm can increase their swimming velocity and thereby gain an advantage in intermale sperm competition1,2. Accordingly, selection should favour cooperation of the most closely related sperm to maximize fitness3. Here we show that sperm of deer mice (genus *Peromyscus*) form motile aggregations, then we use this system test predictions of sperm cooperation. We first show that sperm aggregate more often with conspecific than heterospecific sperm, suggesting that individual sperm can discriminate based on genetic relatedness. Next, we provide evidence that the cooperative behaviour of closely-related sperm is driven by sperm competition. In a monogamous species lacking sperm competition, *P. polionotus*, sperm indiscriminately group with unrelated conspecific sperm. In contrast, in the highly promiscuous deer mouse, *P. maniculatus*, sperm are significantly more likely to aggregate with those obtained from the same male than sperm from an unrelated conspecific donor. Even when we test sperm from sibling males, we continue to see preferential aggregations of related sperm in *P. maniculatus*. These results suggest that sperm from promiscuous deer mice discriminate among relatives and thereby cooperate with the most closely-related sperm, an adaptation likely driven by sperm competition.

In species where females mate promiscuously, sperm competition, in which ejaculates of multiple males compete for fertilization within the female reproductive tract4,5, can drive the evolution of physiological, morphological and behavioural adaptations5. While fertilization success is largely determined by the relative number of spermatozoa inseminated by competing males, additional sperm traits can also improve fertilization ability6. Sperms wimming velocity, for example, is positively correlated with fertilization success in a number of vertebrate species7–13. Morphological adaptations can contribute to improved speed14, or more rarely, individual sperm form cooperative aggregates as they move through the female tract3. Spermatozoa of muroid rodents seem uniquely suited for

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this task; most possess a falciform head with an apical hook15 that is thought to facilitate the formation1 and/or stabilization16 of sperm aggregations. In the wood mouse (*Apodemus sylvaticus*)1 and Norway rat (*Rattus norvegicus*)16 sperm form groups or 'trains' of up to hundreds of cells that exhibit increased swimming velocity *in vitro*. Here we report cooperation in the sperm of *Peromyscus* mice and describe a unique adaptive behaviour: the ability to recognise sperm based on genetic relatedness and preferentially cooperate with the most closely-related sperm.

Upon initial release from the cauda epididymis, spermatozoa of deer mice, *Peromyscus maniculatus*, are highlymotile (≥90%progressively motile) single cells, yet within one minute the cells begin forming motile aggregations of 2–40 cells(Supplemental Movie 1), and continue forming groups for approximately one hour *in vitro*. Aggregates begin to disperse after approximately 40 minutes, and by 3 hours dispersal is complete. Sperm cells form groups by attaching to one another at the sperm head (Fig. 1a)or head hook to midpiece (Fig. 1b). Aggregates display significantly greater swimming velocity (127.4 μm s^{-1} ± 3.8 s.e.m., $n_{individuals}$ = 10, $n_{total\ aggregates}$ = 50) than single cells (109.8 μm s^{-1} ± 3.7 s.e.m., $n_{individuals}$ = 10, $n_{total\ cells}$ = 50; t = 3.028, P = 0.0039).Thus, in this species characterized by a highly promiscuous mating system17,18 and multiple-paternity litters19, sperm groups may gain a fertilization advantage in competitive environments as they are able to migrate through the female reproductive tract at a greater speed. Cooperation, however, may also be a risky strategy for sperm since a portion of cells in a motile aggregation may undergo a premature acrosome reaction rendering them unable to fertilize the oocytes1. While a sperm achieves the greatest fitness advantage with a successful fertilization (direct fitness), it can still improve the probability of transmitting its genes by aiding related sperm (indirect fitness)3. The benefit of aggregating should therefore depend on the genetic relatedness of the cells involved; sperm that are able to recognise relatives, and preferentially associate with them, should gain a selective advantage in a competitive environment.

If sperm are able to identify and group with related cells, this should be most pronounced in interspecific pairings, thus we first investigated the ability of sperm to discriminate between conspecific and heterospecific sperm. In an *in vitro* assay we mixed live sperm obtained from a *P. maniculatus* male and a male from its sister species, the oldfield mouse (*P. polionotus*; Fig 1c), each uniquely labelled with a fluorescent probe. Approximately 83% of aggregates included both *P. maniculatus* and *P. polionotus* sperm (e.g., Fig 1d), however we found that overall groups were composed of significantly more conspecific sperm than expected at random (t_{14} = 8.68, P < 0.0001, n = 15;Fig. 2a). Spermatozoa of the two species are morphologically similar, yet not identical20, and both are capable of cross-fertilization and hybridization21. Although these species no longer naturally co-occur, for sympatric species the ability to identify and cooperate with related sperm may provide a mechanism for conspecific sperm precedence whereby conspecific sperm, presumably adapted to the female reproductive tract, cooperate and outcompete heterospecifics22.

Next we examined intraspecific sperm recognition and took advantage of variation in *Peromyscus* mating systems to test the prediction that sperm competition drives preferential cooperation among closely-related sperm. In *P. maniculatus*, males often copulate with a female in overlapping series, in semi-natural enclosures copulations with multiple males can

occur less than 1 min apart18, providing an opportunity for sperm of different males to interact. When we mixed sperm from two unrelated conspecific *P. maniculatus* males, each labelled with a unique fluorescent probe, we found that sperm group significantly more often with sperm of the same male than expected at random ($t_7 = 11.963$, $P < 0.0001$, $n = 8$; Fig. 2b). In contrast to the promiscuous deer mouse, its monogamous sister-species, *P. polionotus*, experiences little if any sperm competition23. In a study of 220 wild-caught *P. polionotus* females, none showed genetic evidence of multiple paternity24. Moreover, relative testes size is three times smaller in *P. polionotus* than in *P. maniculatus*, consistent with the well-established relationship between relative testis size and sperm competition25. In contrast to the behaviour of *P. maniculatus* sperm, we found that aggregations form indiscriminately in assays involving sperm of two unrelated conspecific *P. polionotus* males($t_7 = 0.627$, $P = 0.547$, $n = 8$; Fig. 2c). Our data, therefore, support the prediction that sperm competition, and thus mating system, drives the evolution of preferential cooperation among related sperm cells.

Why then do sperm of *P. polionotus* aggregate at all if the species is strictly monogamous and lacks sperm competition? Sperm cooperation may benefit monogamous males if the increased swimming velocity of aggregated sperm allows them to migrate faster through a potentially hostile female tract3 or manoeuvre around obstacles while travelling to the fertilization site26. Consistent with these theories, in the wood mouse, *Apodemus sylvaticus*, >95% of sperm found in the uterine lumen following natural matings were aggregates, not single cells, in over half of the females tested1. Alternatively, it is possible that promiscuity is the ancestral reproductive strategy in *Peromyscus* and sperm aggregation arose before the divergence of *P. maniculatus* and *P. polionotus*, yet that the discriminating ability arose after the divergence.

Due to limited dispersal and typically high population densities of *P. maniculatus* in nature27, a female often may mate with males that are closely-related to one another. To examine the extent of discriminatory ability of *P. maniculatus* sperm, we tested the interaction of sperm from full-sibling littermates. Again we found a greater proportion of sperm from the same male grouped together than was expected at random ($t_7 = 3.782$, $P = 0.007$, $n = 8$; Fig. 2d). Moreover, we found that the average proportion of aggregated cells from the same male does not differ significantly when we mixed sperm of two siblings versus two unrelated conspecifics ($t_7 = 1.447$, $P = 0.191$; Fig. 2, horizontal line) or two heterospecifics ($t_7 = 0.412$, $P = 0.693$; Fig. 2, horizontal line), suggesting that *P. maniculatus* sperm discriminate equally against sperm of a brother and a heterospecific. Such highly selective aggregations are similar to cooperative phenotypes seen in social amoebas (*Dictyostelium discoideum*)28 and budding yeast (*Saccharomyces cerevisiae*)29. In these microbes, a single gene encodes for a homophilic adhesion protein28,29 suggesting that sperm aggregation may also operate under a simple genetic mechanism.

In competitive environments, the male (diploid genome) benefits if any one of his sperm fertilizes the egg; thus selection should favour adaptations that help his sperm reach the egg, such as sperm aggregations. The addition of any motile sperm, related or not, to an aggregate should increase the speed at which his sperm reach the egg; however, since all but one sperm fail to fertilise each oocyte, the chance that his sperm will fertilise the egg

decreases as number of unrelated cells join the group. From the sperm's perspective (the haploid genome), there is also a benefit to joining a group of related or unrelated sperm to improve its swimming speed. However if that sperm is unable to fertilize the egg, it can still increase the probability of transmitting its genes by aiding related sperm (inclusive fitness). Thus selection on both the diploid and haploid genomes should favour recognition and cooperation among related cells if fitness benefits (e.g., direct and indirect fitness) outweigh costs (e.g., sperm incapacitation due to a premature acrosome reaction)2,3. Although it is unclear whether the genotype of the diploid male or haploid sperm30 determines the observed aggregation phenotype, our results suggest that relatedness matters for cooperative behaviour in *P. maniculatus* sperm. In this system, sperm discriminate against those from a sibling where the probability of sharing a gene is 25%, and preferentially aggregate with sperm from the same male where the probability is 50%. By contrast, in the monogamous *P. polionotus*, sperm group indiscriminately with unrelated conspecifics. Our results, therefore, support the long-standing prediction that sperm competition drives the evolution of sperm cooperation, and most importantly, cooperation among closely-related cells. Here we show that the temporary alliances among sperm are not passively formed, rather they represent a complex discriminatory behaviour driven by sexual selection.

Methods Summary

We obtained *Peromyscus maniculatus bairdii* and *P. polionotus subgriseus* from the Peromyscus Genetic Stock Center (University of South Carolina). Laboratory-reared males were weaned at 25 days postpartum, housed individually, then paired with a sexually-mature virgin female at 60 days postpartum for 15 days. We harvested cauda epididymal sperm by making a single cut at the edge of the vas deferens and incubating epididymides in 2ml of Biggers-Whitten-Whittingham (BWW) medium31 for 10 min at 37°C to release sperm. We observed cells using phase-contrast microscopy(Axio Scope.A1, Carl Zeiss Incorporated) and assessed straight-line velocity with Axio Vision tracking software (Carl Zeiss Incorporated). Opportunistic observations of ejaculated sperm (*P. maniculatus*, $n = 3$; *P. polionotus*, $n = 1$) collected at time of sacrifice showed identical aggregation behaviour as those collected from cauda epididymides.

For each assay, we labelled two 1mL aliquots of live sperm with a unique fluorescent probe (25nM MitoTracker Green FM and 25nM MitoTracker Red 580; Invitrogen Corporation). We incubated aliquots for 10 min, centrifuged at 500g for 5 min, resuspended in 2ml BWW, centrifuged and resuspended again, all at 37°C. We combined equal amounts of live sperm from one male (labelled green) and a second male (labelled red) and incubated for 30 min at 37°C. To control for aggregates formed during the labelling process, we also made a mixture containing sperm from each male labelled with both red and green probes. We fixed sperm in 4% formalin and systematically scored 25 aggregates (mean size = 12.76 cells ± 1.52 s.e.m,) (Fig. 1d). To measure the relative amount of aggregation between sperm of different males, we calculated the proportions of red and green sperm in each group and then compared the higher of these two values (Fig 2; black bars) to the expected proportion as seen in the control assays (Fig 2; white bars). Thus for each test male, we compared how his sperm grouped when mixed with unrelated cells (from a heterospecific or conspecific male)and closely-related cells(other sperm from the same male).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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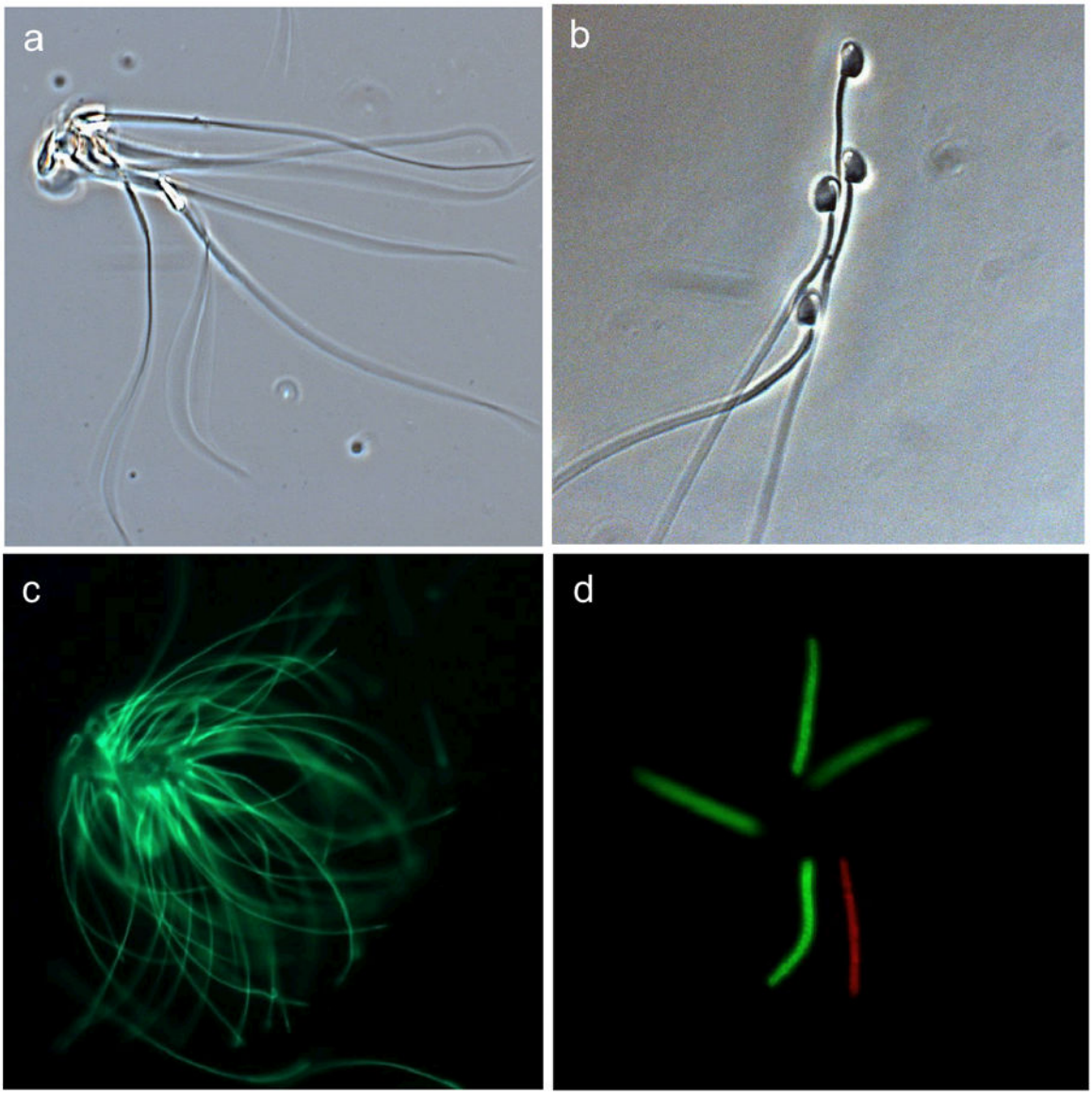

Figure 1.

Images of *Peromyscus* sperm in BWW medium. (a) 1000X phase contrast image of *P. maniculatus* sperm aggregate attached at sperm heads and (b)head hook to midpiece. (c) 400X image of motile *P. polionotus* sperm aggregate stained with 400nM Tubulin Tracker (Invitrogen Corp.). (d) 1000X image of aggregated sperm observed in a mixture containing sperm from one *P. maniculatus* male and one *P. polionotus* male (midpiece of *P. maniculatus* sperm is stained with MitoTracker Red 580 and midpiece of *P. polionotus* sperm with MitoTracker Green FM).

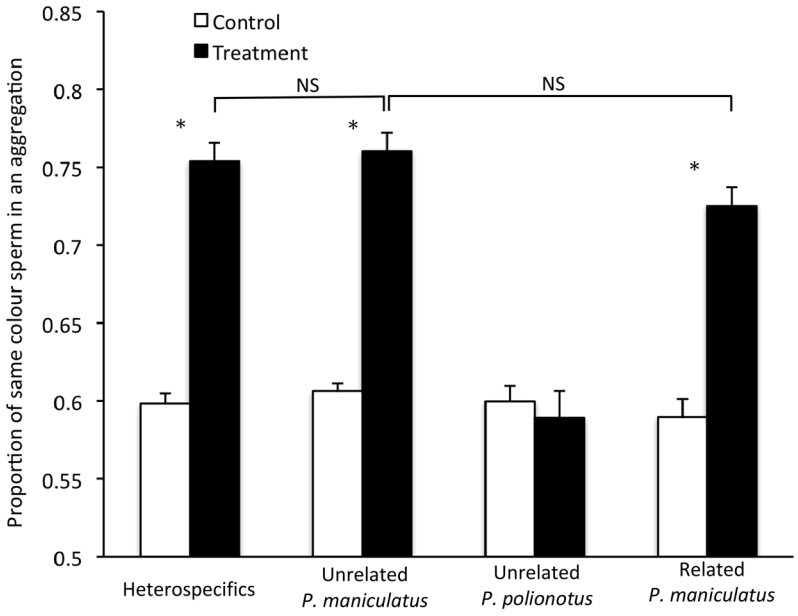

Figure 2.

Preferential sperm aggregations. Mean ± s.e.m. proportion of cells in a sperm aggregate labelled with a single probe. Black bars indicate treatments in which sperm of one male is labelled green and sperm of another male is labelled red; white bars indicate controls in which sperm from a single male is labelled with both red and green probes. Pairwise comparisons between treatment and control groups are by paired two-tailed t-test with Bonferroni correction, asterisks indicate $P < 0.01$. Treatments include: (a) heterospecific mixtures containing live sperm from one *P. maniculatus* male and one *P. polionotus* male ($n = 15$), (b) conspecific mixtures of sperm from two unrelated males of the promiscuous *P. maniculatus* species ($n = 8$), (c) conspecific mixture of sperm from two unrelated males from the monogamous *P. polionotus* ($n = 8$), and (d) conspecific mixture of sperm from two full-sibling *P. maniculatus* males ($n = 8$). Horizontal lines above bars indicate comparisons between aggregations of sperm by unpaired two-tailed t-tests with Bonferroni correction (NS = non-significant).