Competition drives cooperation among closely related sperm of deer mice

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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 to test predictions of sperm cooperation. We find that sperm aggregate more often with conspecific than heterospecific sperm, suggesting that individual sperm can discriminate on the basis of 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, Peromyscus polionotus, sperm indiscriminately group with unrelated conspecific sperm. In contrast, in the highly promiscuous deer mouse, Peromyscus maniculatus, sperm are significantly more likely to aggregate with those obtained from the same male than with 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 to have been 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 adaptations6. Although fertilization success is largely determined by the relative number of spermatozoa inseminated by competing males, additional sperm traits can also improve fertilization ability6. Sperm swimming 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 tract1. Spermatozoa of muroid rodents seem uniquely suited for this task; most possess a falciform head with an apical hook15 that is thought to facilitate the formation7 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 recognize 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, P. maniculatus, are highly motile (≥90% progressively motile) single cells, yet within one minute the cells begin forming motile aggregations of 2–40 cells (Supplementary Movie 1), and continue forming groups for approximately one hour in vitro. Aggregates begin to disperse after approximately 40 min, and by 3 h 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 ± 3.8 μm s⁻¹ (± s.e.m.), nindividuals = 10, ntotal aggregates = 50) than single cells (109.8 ± 3.7 μm s⁻¹ (± s.e.m.), nindividuals = 10, ntotal 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, as a portion of cells in a motile aggregation may undergo a premature acrosome reaction, rendering them unable to fertilize the oocytes1. Although 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.

Figure 1 | Images of Peromyscus sperm in BWW medium. a, b, Phase contrast image (1,000×) of P. maniculatus sperm aggregates attached at sperm heads (a) and head hook to midpiece (b). c, Image (400×) of motile P. polionotus sperm aggregate stained with 400 nM Tubulin Tracker. d, Image (1,000×) 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).
sperm (indirect fitness). The benefit of aggregating should therefore depend on the genetic relatedness of the cells involved; sperm that are able to recognize 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 (for example, Fig. 1d), however we found that overall groupings were composed of significantly more conspecific sperm than expected at random (t_{4,6} = 8.68, P < 0.0001, n = 15; Fig. 2a). Spermatozoa of the two species are morphologically similar, yet not identical, and both are capable of cross-fertilization and hybridization. These species no longer naturally co-occur, yet 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 heterospecifics.

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; and in semi-natural enclosures, copulations with multiple males can occur less than 1 min apart, 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 competition.

In a study of 220 wild-caught P. polionotus females, none showed genetic evidence of multiple paternity. Moreover, relative testis size is three times smaller in P. polionotus than in P. maniculatus, consistent with the well-established relationship between relative testis size and sperm competition. 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_{5} = 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 tract or manoeuvre around obstacles while travelling to the fertilization site. Consistent with these theories, in the wood mouse, A. sylvaticus, >95% of sperm found in the uterine lumen following natural matings were aggregates, not single cells, in over half of the females tested. 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 the discriminating ability arose after the divergence.

Owing to limited dispersal and typically high population densities of P. maniculatus in nature, a female may often mate with multiple 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 litters. Again we found a greater proportion of sperm from the same male grouped together than was expected at random (t_{8} = 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_{8} = 1.447, P = 0.191; Fig. 2, horizontal line) or two heterospecifics (t_{8} = 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) and budding yeast (Saccharomyces cerevisiae). In these microbes, a single gene encodes for a homophilic adhesion protein, 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, as all but one sperm fail to fertilize each oocyte, the chance that his sperm will fertilize the egg decreases as the 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 (direct and indirect fitness) outweigh costs (for example, sperm incapacitation due to a premature acrosome reaction). Although it is unclear whether the genotype of the diploid male or haploid spem 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 have shown 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 *P. maniculatus bairdii* and *P. polionotus subgriseus* from the Peromyscus Genetic Stock Center (Univ. 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 2 ml of Biggers-Whitten-Whittingham (BWW) medium for 10 min at 37 °C to release sperm. We observed cells using phase-contrast microscopy (Axio Scope.A1, Carl Zeiss) and assessed straight-line velocity with AxioVision tracking software (Carl Zeiss). 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 1-ml aliquots of live sperm with a unique fluorescent probe (25 nM MitoTracker Green FM and 25 nM MitoTracker Red 580; Invitrogen). We incubated aliquots for 10 min, centrifuged at 500 g for 5 min, resuspended in 2 ml 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 control assay (Fig. 2; white bars). Thus for each test male, we compared how his proportion of red and green sperm in each group and then compared the higher 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 value with the expected proportion as seen in the control assay (Fig. 2; black bars). Thus for each test male, we compared how his sperm grouped when mixed with unrelated cells (from a heterospecific or conspecific male) and with closely related cells (other sperm from the same male).

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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