

(same buffer containing 4.5 mg protein ml⁻¹ (10¹⁰–10¹¹ cells ml⁻¹), 5 mM ammonium and nitrite, 100 µM hydrazine) and consumption of ammonium and nitrite and production of nitrate and incorporation of ¹⁴C-CO₂ was measured as described previously^{16,22}. Purity was assessed by microscopic counting, electron microscopy and FISH.

Electron microscopy. Pelleted cell flocs were processed by a cryosubstitution protocol before embedding, sectioning and section staining, all using methods described in ref. 7. Cells were negatively stained using 1% uranyl acetate + 0.4% sucrose, after dispersal of flocs.

Fluorescent microscopy. FISH and DAPI staining were done as described¹⁰. Formamide concentration versus specificity of all probes was determined with the appropriate reference organism for each probe and with the biofilm enrichment culture and several undefined, heterogeneous environmental samples. All probes were at least 18 nucleotides long.

Phylogeny. Treeing and phylogenetic analysis was done using the ARB software package¹⁸. The sequence was obtained after DNA isolation, PCR amplification (primers 27 or 519 forward and 1,390 reverse; annealing at 46 °C, melting at 94 °C, 30 cycles), ligation of the product in vector pGEMT (Promega) and transformation into competent DH5α *E. coli* cells. The clone library was screened using restriction digestion, and representative clones of each restriction pattern were sequenced. The anammox sequence (GenBank accession number AJ131819) always grouped with the other planctomycete sequences, independent of treeing algorithm (neighbour joining, distance matrix or parsimony), inclusion of other bacterial phyla in the tree or the choice of the outgroup (*Thermotoga*, *Aquifex* or the Archaea).

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1. Jetten, M. S. M., Horn, S. J. & van Loosdrecht, M. C. M. Towards a more sustainable wastewater treatment system. *Wat. Sci. Tech.* **35**, 171–180 (1997).
2. Strous, M., Van Gerven, E., Ping, Z., Kuenen, J. G. & Jetten, M. S. M. Ammonium removal from concentrated waste streams with the anaerobic ammonium oxidation process. *Water Res.* **31**, 1955–1962 (1997).
3. Van de Graaf, A. A., de Bruijn, P., Robertson, L. A., Jetten, M. S. M. & Kuenen, J. G. Metabolic pathway of anaerobic ammonium oxidation on basis of ¹⁵N-studies in a fluidized bed reactor. *Microbiology* **143**, 2415–2421 (1997).
4. Broda, E. Two kinds of lithotrophs missing in nature. *Z. Allgem. Mikrobiol.* **17**, 491–493 (1977).
5. Schlesner, H. & Stackebrandt, E. Assignment of the genera *Planctomyces* and *Pirella* to a new family *Planctomycetaceae* fam. nov. and description of the order *Planctomycetales* ord. nov. *Syst. Appl. Microbiol.* **8**, 174–176 (1986).
6. Fuerst, J. A. & Webb, R. I. Membrane-bound nucleoid in the eubacterium *Gemmata obscuriglobus*. *Proc. Natl Acad. Sci. USA* **88**, 8184–8188 (1991).
7. Lindsay, M. R., Webb, R. I. & Fuerst, J. A. Pirellulosomes: a new type of membrane-bounded cell compartment in planctomycete bacteria of the genus *Pirellula*. *Microbiology* **143**, 739–748 (1997).
8. Fuerst, J. A. The planctomycetes: emerging models for microbial ecology, evolution and cell biology. *Microbiology* **141**, 1493–1506 (1995).
9. Vergin, K. L. *et al.* Screening of a fosmid library of marine environmental genomic DNA fragments reveals four clones related to members of the order *Planctomycetales*. *Appl. Environ. Microbiol.* **64**, 3075–3078 (1998).
10. Neef, A., Amann, R. L., Schlesner, H. & Schleifer, K. H. Monitoring a widespread bacterial group: in situ detection of planctomycetes with 16S rRNA-targeted probes. *Microbiology* **144**, 3257–3266 (1998).
11. Hugenholtz, P., Goebel, B. M. & Pace, N. R. Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. *J. Bacteriol.* **180**, 4765–4774 (1998).
12. Byers, H. K., Stackebrandt, E., Hayward, C. & Blackall, L. L. Molecular investigation of a microbial mat associated with the Great Artesian Basin. *FEMS Microbiol. Ecol.* **25**, 391–403 (1998).
13. Zarda, B. *et al.* Analysis of bacterial community structure in bulk soil by in situ hybridization. *Arch. Microbiol.* **168**, 185–192 (1997).
14. Liesack, W., König, H., Schlesner, H. & Hirsch, P. Chemical composition of the peptidoglycan-free cell envelopes of budding bacteria of the *Pirella/Planctomyces* group. *Arch. Microbiol.* **145**, 361–366 (1986).
15. König, E., Schlesner, H. & Hirsch, P. Cell wall studies on budding bacteria of the *Planctomyces/Pasteuria* group and on a *Prosthecomicrobium* sp. *Arch. Microbiol.* **138**, 200–205 (1984).
16. Strous, M., Heijnen, J. J., Kuenen, J. G. & Jetten, M. S. M. The sequencing batch reactor as a powerful tool for the study of slowly growing anaerobic ammonium oxidizing microorganisms. *Appl. Microbiol. Biotechnol.* **50**, 589–596 (1998).
17. Fuqua, C. & Greenberg, E. Self perception in bacteria: quorum sensing with acylated homoserine lactones. *Curr. Opin. Microbiol.* **1**, 183–189 (1998).
18. Ludwig, W. *et al.* Bacterial phylogeny based on comparative sequence analysis. *Electrophoresis* **19**, 554–568 (1998).
19. Delong, E. F., Franks, D. G. & Alldredge, A. L. Phylogenetic diversity of aggregate-attached versus free-living marine bacterial assemblages. *Limnol. Oceanogr.* **38**, 924–934 (1993).
20. Fuerst, J. A. *et al.* Isolation and molecular identification of planctomycete bacteria from postlarvae of the giant tiger prawn *Penaeus monodon*. *Appl. Environ. Microbiol.* **63**, 254–262 (1997).
21. Woese, C. R. Bacterial evolution. *Microbiol. Rev.* **51**, 221–271 (1987).
22. Van de Graaf, A. A., Debruijn, P., Robertson, L. A., Jetten, M. S. M. & Kuenen, J. G. Autotrophic growth of anaerobic ammonium oxidizing microorganisms in a fluidized bed reactor. *Microbiology* **142**, 2187–2196 (1996).

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Sperm competition between *Drosophila* males involves both displacement and incapacitation

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Females in almost all animal groups copulate with multiple males^{1,2}. This behaviour allows different males to compete for fertilization³ and gives females the opportunity to mediate this competition⁴. In many animals and most insects, the second male to copulate with a female typically sires most of her offspring^{1,5,6}. In *Drosophila melanogaster*, this second-male sperm precedence has long been studied^{7–15} but, as in most species, its mechanism has remained unknown. Here we show, using labelled sperm in doubly mated females, that males can both physically displace and incapacitate stored sperm from earlier-mating males. Displacement occurs only if the second male transfers sperm to the female, and in only one of her three sperm-storage organs. Incapacitation can be caused by either fertile or spermless second males, but requires extended intervals between matings. Sperm from different males are not ‘stratified’ in the storage organs but mix freely. Many animal species may have multiple mechanisms of sperm competition like those observed here, and revealing these mechanisms is necessary to understand the genetic and evolutionary basis of second-male sperm precedence in animals.

The consequences of multiple mating are well known in *D. melanogaster*. Females store sperm in a long tubular seminal receptacle and two mushroom-shaped spermathecae¹⁰. The proportion of offspring produced after the second mating that are sired by the second male, P_2 , is typically above 0.8 in laboratory experiments¹⁶. Most wild-caught females carry sperm from multiple males¹⁷, and females in nature may copulate when they still carry a sizeable sperm load from previous matings¹⁸. If a sperm-carrying female remates, she produces fewer offspring sired by the first male than she would have if she had not remated¹⁶. At least some of this reduction in first-male reproductive success occurs if the second male transfers only seminal fluid and no sperm^{12,14}. Seminal fluid therefore plays some role in second-male sperm precedence¹⁵, but the extent of this role is unclear because some studies^{10,19} have not revealed a ‘seminal-fluid effect’. To investigate this discrepancy, we looked for a seminal-fluid effect in experiments carried out at two different time intervals.

Rematings to fertile second males produced the usual result of second-male sperm precedence and resulted in significant reduction of first-male reproductive success when females remated after either two or seven days (Fig. 1a). When females were remated in exactly the same manner to infertile XO males transferring only seminal fluid (see Methods), there was a significant reduction in first-male offspring when females were remated after seven days, but only a slight and non-significant reduction when they were remated after two days (Fig. 1a). Similar results were found when this experiment was repeated with Ives males instead of *bw^D* males (carrying the brown-dominant mutation) as first mates (data not shown). These results confirm that at least some of the second male’s precedence can be ascribed to his seminal fluid, and, because this effect was not seen until seven days after remating, indicate that first-male sperm

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must remain in storage for a few days before they become susceptible to the harmful effects of second-male seminal fluid. Second-male precedence at shorter remating intervals apparently requires the transfer of sperm. This indicates at least two mechanisms of sperm precedence in *D. melanogaster*, only one of which requires sperm in the ejaculate.

The loss of first-male productivity caused by second-male seminal fluid was not accompanied by any loss of first-male sperm from storage (Fig. 1b). Although females that copulate with XO males seven days after their first mating produce about one-third as many offspring as they would have if they had not remated (Fig. 1a), XO-remated females have no fewer sperm in storage than do singly mated females (Fig. 1b). This indicates that the seminal fluid of a second male inhibits the use of stored sperm without removing them. Moreover, we found no evidence that the paucity of first-male progeny can be attributed to developmental problems in eggs fertilized by first-male sperm. Mean (\pm s.e.) egg hatchability for the first 48 h after mating was 0.85 (\pm 0.05; $n = 14$) for females singly mated to an Ives male; 0.84 (\pm 0.03; $n = 28$) for females singly mated to a *bw^D* male; and 0.87 (\pm 0.03; $n = 18$) for females mated first to a *bw^D* and then to an Ives male. Thus, interference with the effectiveness of first-male sperm occurs before and not after fertilization. We refer to this interference as 'sperm incapacitation'⁶.

Each of the experiments reported above was also performed in *Drosophila simulans*, a sister species of *D. melanogaster*, with similar results (data not shown). This indicates that incapacitation of stored sperm after extended remating intervals may be widespread in *Drosophila*.

To study the mechanics of sperm precedence when second males are fully fertile, we mated females to males that express green fluorescent protein (GFP) on their sperm tails²⁰ and then to males that lack GFP expression but carry a dominant eye-colour mutation, brown-dominant (*bw^D*). In this way, each offspring produced and each sperm stored by such a female could be assigned to one of her two mates (Fig. 2). Preliminary experiments confirmed that GFP males transfer only labelled sperm to females. In six females singly mated to GFP males, we found 516 GFP-labelled sperm and no unlabelled sperm. When GFP males copulate first, sperm precedence is high and within the normal range for *D. melanogaster* (Fig. 3a). Furthermore, GFP males suffer a net reproductive loss when females remate to *bw^D* males after either four or seven days (Fig. 3a). Most of the stored sperm in these females comes from the second male (Fig. 3b). Regardless of the interval between matings, doubly mated females have fewer first-male sperm in the seminal receptacle than do single-mated females (Fig. 3c). In contrast, first-

male sperm stored in the paired spermathecae show a much smaller and non-significant diminution after double matings (Fig. 3d).

It has been suggested that females lack the space to store two ejaculates without expelling first-male sperm¹⁰. There is, in fact, more than enough space for two ejaculates, and we observed loss of first-male sperm from the seminal receptacle despite this excess capacity. Females dissected up to 48 h after a single mating with a *bw^D* male had a mean of 256 (\pm 26.9; $n = 15$) and as many as 377 sperm in their seminal receptacles. On the fourth day after mating, those females had a mean of 63.4 (\pm 16.3; $n = 5$) sperm, and on the seventh day only 24.3 (\pm 8.2; $n = 5$) sperm in their seminal receptacles. If a female can store over 250 sperm, but carries an average of only 24 sperm at the time of remating, sperm displacement is not required to make space for the second ejaculate, nor even for second-male sperm to make up the majority in the seminal receptacle (in such a case with no displacement, P_2 would be $250/(24 + 250) = 0.91$).

Each of the experiments reported above was repeated with the mating order of GFP and *bw^D* males reversed. The GFP males are not as good at sperm offence as the *bw^D* males, and little sperm precedence was found with four days between matings ($P_2 = 0.54 \pm 0.10$; $n = 12$). However, normal sperm precedence was found with seven days between matings ($P_2 = 0.77 \pm 0.04$; $n = 15$). Comparing the number of first-male sperm stored in females remated after seven days with the number stored in singly mated females at the same time, we find that GFP second males caused physical loss of *bw^D* sperm from the seminal receptacle (one-tailed Mann-Whitney *U*, $P = 0.002$) but not from the spermathecae (one-tailed Mann-Whitney *U*, $P = 0.378$). The magnitude of the loss of sperm from the seminal receptacle was compared to the loss from the spermathecae using a two-tailed *t*-test²¹ for each of the four double matings ($P = 0.050$ for GFP then *bw^D* 4-day interval; $P = 0.264$ for GFP then *bw^D* 7-day interval; $P = 0.059$ for *bw^D* then GFP 4-day interval; $P = 0.036$ for *bw^D* then GFP 7-day interval). Combining the probabilities from all four independent comparisons²², the loss of sperm from the seminal receptacle was significantly greater than the loss of sperm from the spermathecae ($P = 0.0072$).

None of the dissections of doubly mated females supports the hypothesis²³ that sperm are layered in storage, so that second-male sperm are closer to the site of fertilization. Sperm masses were removed intact from the storage organs, and GFP and non-GFP sperm were always found closely intertwined.

Most proposed mechanisms of second-male sperm precedence in *D. melanogaster* have sought to explain how second-male sperm

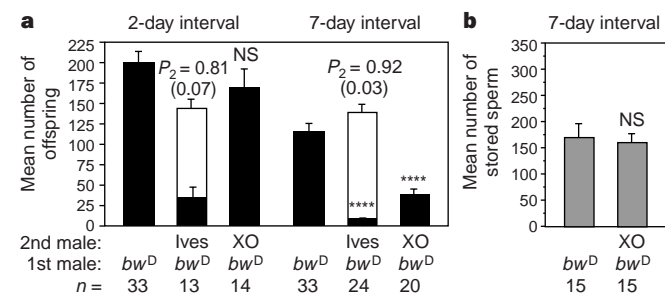


Figure 1 The mechanism of sperm competition depends on mating interval. **a**, Mean (\pm s.e.) number of offspring per female from first (solid bars) and second (open bars) matings. Offspring from single matings are those produced after the doubly mated females remated. P_2 (s.e.) is the proportion of second-male offspring produced after remating. **** $P < 0.0001$; NS: $P = 0.113$ (one-tailed Mann-Whitney *U* test, compared to the singly mated control). **b**, Mean (\pm s.e.) number of sperm stored per female in all organs. Data are pooled from females dissected 7, 8 and 9 days after the first mating. $P = 0.459$ (one-tailed Mann-Whitney *U*-test).

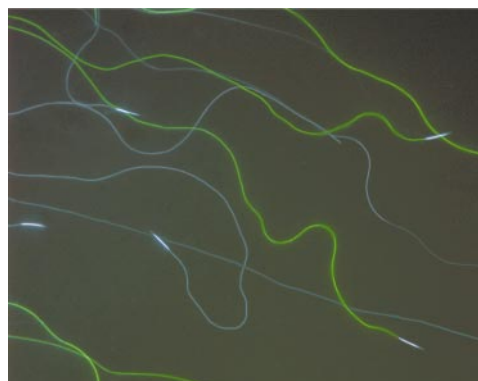


Figure 2 Differentially labelled sperm. DAPI-stained sperm from a GFP male (green tails) and a *bw^D* male (blue tails), dissected from the seminal receptacle of a single doubly inseminated female.

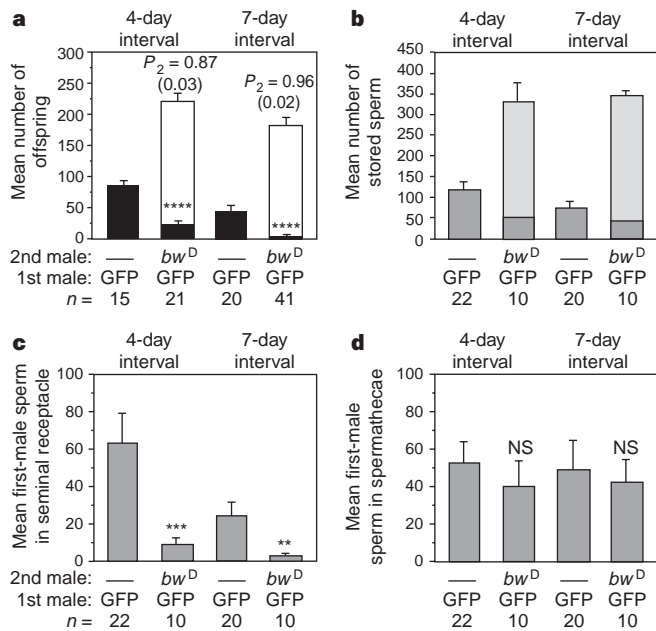


Figure 3 Physical displacement of first-male sperm. **a**, Mean (+ s.e.) number of offspring per female from first (solid bars) and second (open bars) matings. $****P < 0.0001$. **b**, Mean (+ s.e.) number of sperm stored per female in all organs from first (dark bars) and second (light bars) matings. **c**, Mean (+ s.e.) number of sperm stored per female in the seminal receptacle. $***P = 0.001$; $**P = 0.006$. **d**, Mean (+ s.e.) number of sperm stored per female in the spermathecae. $P = 0.412$ for the 4-day interval; $P = 0.387$ for the 7-day interval (one-tailed Mann-Whitney U -tests).

achieve numerical superiority in storage^{9–11,14}. It is often supposed that patterns of paternity directly reflect such a numerical advantage, implying that, once sperm storage is complete, females use the available sperm at random to fertilize eggs. We confirm here that the majority of stored sperm after double matings are second-male sperm (Fig. 3b), and attribute this in part to physical displacement of first-male sperm (Fig. 3c). These observations do not, however, rule out the possibility that females preferentially use first- or second-male stored sperm to fertilize their eggs.

Table 1 compares the number of sperm a female stores from a given male with the number of offspring ultimately produced by that male, and estimates the proportion of each type of stored sperm that is actually used to fertilize eggs. Seven days after a single mating to a bw^D male, females use about 68% of their remaining stored sperm to produce offspring (Table 1). If that female is remated to an XO male seven days after mating to the bw^D male, she then uses only about 24% of her stored sperm to produce offspring. This reflects

the sperm incapacitation described above. Females who copulate first with a GFP male and then after four days with a bw^D male subsequently use about 48% of their first-male sperm to produce offspring (Table 1). However, with seven days between matings, females use only about 7% of the stored first-male sperm. While the number of first-male sperm stored by females remated after seven days does not differ significantly from the number stored by females remated after four days, the former produce significantly fewer offspring from the first male (Table 1). Sperm precedence is thus very high after the seven-day remating ($P_2 = 0.96 \pm 0.02$; $n = 41$), almost certainly because many first-male sperm are displaced from the seminal receptacle (Fig. 3c), and because most of the sperm that survive displacement are incapacitated. Again, the incapacitation effect is seen only with the longer interval between matings.

Sperm competition offers a unique opportunity to study adaptations shaped by the interacting forces of natural, sexual and sexually antagonistic selection²⁴. Future work in these species must explain, both mechanistically and evolutionarily, why first-male sperm become more susceptible to incapacitation the longer they remain in storage. This phenomenon may explain how males avoid incapacitating the sperm in their own ejaculate, despite their ability to displace and/or incapacitate their own sperm once it has been stored in a female¹⁵. Females may play a role in sperm incapacitation if they gradually alter the physiology of the sperm they store. Genetic differences among females in the ability to alter sperm in this way might explain why female genotypes differ widely in the degree of last-male precedence²⁵. Moreover, if females evolve differences in the way they alter stored sperm and males evolve ways to protect their sperm from alteration by particular females, this might explain the observation that a first male's success depends on both his genotype and that of his mate²⁶. Sperm competition in animals other than *Drosophila* may involve mechanisms similar to those described here, and any general explanation of the ubiquity of second-male sperm precedence among animals must consider that multiple mechanisms may have evolved within a single species. □

Methods

Matings. Stock maintenance, mating observations and the rearing of offspring were performed as described²⁷. All females came from the Ives laboratory population²⁸ and males were taken as noted from the Ives, brown-dominant (bw^D) or *djGFP II/CyO* (GFP) stock²⁰. XO males were produced by crossing virgin Ives females to males from an attached-X, attached-XY stock. All male offspring from this cross lack a Y chromosome, and produce normal seminal fluids but no sperm^{29,30}. Females were transferred to fresh food vials every three days until they either stopped laying fertile eggs or were chosen at random for dissection. All offspring were reared to adulthood and scored for paternity, determined by the presence of brown or wild-type eyes.

Dissections. For each mating type, 5–10 females were dissected per day at timed intervals after the end of their last copulation. Females were etherized and their reproductive tracts removed in a drop of phosphate buffered saline (PBS). The spermathecae, seminal receptacle and uterus were each transferred

Table 1 Proportion of sperm used to produce offspring

1st male	2nd male	Days	First male			Second male		
			Offspring produced	Sperm stored	Sperm used (%)	Offspring produced	Sperm stored	Sperm used (%)
bw^D	—	—	114.1 (12.1)	168.8 (26.9)	68%	—	—	—
bw^D	XO	7	37.9 (7.3)	157.8 (22.3)	24%	—	—	—
GFP	bw^D	4	23.7 (4.2)	49.8 (35.0)	48%	197.5 (13.6)	300.2 (87.6)	66%
GFP	bw^D	7	3.0 (0.7)	43.2 (14.3)	7%	183.2 (13.1)	338.4 (19.5)	54%

Days are the number of days between the first and second mating. Per cent sperm used is calculated by dividing the mean number of offspring produced by the mean number of sperm stored. Mean (s.e.) of offspring and sperm are from data shown in Figs 1 and 3. Data for the single mating are from beyond the first seven days after mating. One-tailed Mann-Whitney U -tests: $****P < 0.0001$; NS (non-significant) $P > 0.4$.

to a separate drop of PBS to prevent cross-contamination of sperm from different organs. The sperm from each organ were then removed with insect pins and minimal manipulation. Slides to be scored for GFP vs non-GFP sperm were dissected in 0.5 $\mu\text{g ml}^{-1}$ DAPI (4,6-diamidino-2-phenylindole) in PBS, covered immediately with a coverslip and scored with an epifluorescent microscope within 2 h of dissection. Slides that required only simple sperm counts were dried after dissection at 60 °C for 5–10 min, fixed in 3:1 methanol and glacial acetic acid for 5 min, rinsed three times in PBS and labelled with DAPI in glycerol (0.5 $\mu\text{g ml}^{-1}$). All sperm in each of the three storage organs of every female were counted. To ensure precision, 10% of the slides were counted at least twice by different people.

Egg hatchability. Females were allowed to lay eggs on small plastic spoons filled with grape-juice-tinted medium for 24 h, and then either transferred to a fresh spoon or discarded. Spoons were stored at 24 °C for 28 h before hatched and unhatched eggs were counted. Brown eggs, which may indicate zygotes that died early in development, were observed only rarely.

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1. Smith, R. L. (ed.) *Sperm Competition and the Evolution of Animal Mating Systems* (Academic, London, 1984).
2. Birkhead, T. R. & Møller, A. P. (eds.) *Sperm Competition and Sexual Selection* (Academic, London, 1998).
3. Parker, G. A. Sperm competition and its evolutionary consequences in the insects. *Biol. Rev.* **45**, 525–567 (1970).
4. Eberhard, W. G. *Female Control: Sexual Selection by Cryptic Female Choice* (Princeton Univ. Press, Princeton, 1996).
5. Birkhead, T. R. & Møller, A. P. *Sperm Competition in Birds: Evolutionary Causes and Consequences* (Academic, London, 1992).
6. Simmons, L. W. & Siva-Jothy, M. T. in *Sperm Competition and Sexual Selection* (eds Birkhead, T. R. & Møller, A. P.) 341–434 (Academic, London, 1998).
7. Nonidez, J. F. The internal phenomena of reproduction in *Drosophila*. *Biol. Bull.* **39**, 207–230 (1920).
8. Lobashov, M. E. Mixture of sperm in case of polyandry in *Drosophila melanogaster*. C. R. (*Doklady Acad. Sci. de l'URSS*) **23**, 827–830 (1939).
9. Kaufmann, B. P. & Demerec, M. Utilization of sperm by the female *Drosophila melanogaster*. *Am. Nat.* **76**, 445–469 (1942).
10. Lefevre, G. & Jonsson, U. B. Sperm transfer, storage, displacement, and utilization in *Drosophila melanogaster*. *Genetics* **47**, 1719–1736 (1962).
11. Newport, M. E. A. & Gromko, M. H. The effect of experimental design on female receptivity to remating and its impact on reproductive success in *Drosophila melanogaster*. *Evolution* **38**, 1261–1272 (1984).
12. Scott, D. & Richmond, R. C. Sperm loss by remating *Drosophila melanogaster* females. *J. Insect Physiol.* **36**, 451–456 (1990).
13. Scott, D. & Williams, E. Sperm displacement after remating in *Drosophila melanogaster*. *J. Insect Physiol.* **39**, 201–206 (1993).
14. Harshman, L. G. & Prout, T. Sperm displacement without sperm transfer in *Drosophila melanogaster*. *Evolution* **48**, 758–766 (1994).
15. Gilchrist, A. S. & Partridge, L. Male identity and sperm displacement in *Drosophila melanogaster*. *J. Insect Physiol.* **41**, 1087–1092 (1995).
16. Gromko, M. H., Gilbert, D. G. & Richmond, R. C. in *Sperm Competition and the Evolution of Animal Mating Systems* (ed. Smith, R. L.) 371–426 (Academic, London, 1984).
17. Imhof, M., Harr, B., Brem, G. & Schlötterer, C. Multiple mating in wild *Drosophila melanogaster* revisited by microsatellite analysis. *Mol. Ecol.* **7**, 915–917 (1998).
18. Gromko, M. H. & Markow, T. A. Courtship and remating in field populations of *Drosophila*. *Anim. Behav.* **45**, 253–262 (1993).
19. Gromko, M. H., Newport, M. E. A. & Kortier, M. G. Sperm dependence of female receptivity to remating in *Drosophila melanogaster*. *Evolution* **38**, 1273–1282 (1984).
20. Santel, A., Winhauer, T., Blümer, N. & Renkawitz-Pohl, R. The *Drosophila* *don juan* (*dj*) gene encodes a novel sperm specific protein component characterized by an unusual domain of a repetitive amino acid motif. *Mech. Dev.* **64**, 19–30 (1997).
21. Snedecor, G. W. & Cochran, W. G. *Statistical Methods* (Iowa State Univ. Press, Ames, 1967).
22. Sokal, R. R. & Rohlf, F. J. *Biometry* (Freeman, New York, 1995).
23. Nachtsheim, H. Eine Method zur Prüfung der Lebendauer genotypisch verschiedener Spermien bei *Drosophila*. Verhandl. V. Intern. Kongr. Verebungsw., 1143–1147. *Z. Ind. Abst. Vereb. (Suppl. II)* (1927).
24. Rice, W. R. Sexually antagonistic male adaptation triggered by experimental arrest of female evolution. *Nature* **381**, 232–234 (1996).
25. Clark, A. G. & Begun, D. J. Female genotypes affect sperm displacement in *Drosophila*. *Genetics* **149**, 1487–1493 (1998).
26. Clark, A. G., Begun, D. J. & Prout, T. Female \times male interactions in *Drosophila* sperm competition. *Science* **283**, 217–220 (1999).
27. Price, C. S. C. Conspecific sperm precedence in *Drosophila*. *Nature* **388**, 663–666 (1997).
28. Coyne, J. A., Aulard, S. & Berry, A. Lack of underdominance in a naturally occurring pericentric inversion in *Drosophila melanogaster* and its implications for chromosome evolution. *Genetics* **129**, 791–802 (1991).
29. Ingman-Baker, J. & Candido, E. P. Proteins of the *Drosophila melanogaster* male reproductive system: two-dimensional gel patterns of proteins synthesized in the XO, XY, and XYY testis and paragonial gland and evidence that the Y chromosome does not code for structural sperm proteins. *Biochem. Genet.* **18**, 809–828 (1980).
30. Kiefer, B. I. Ultrastructural abnormalities in developing sperm of X/O *Drosophila melanogaster*. *Genetics* **54**, 14441–1452 (1966).

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Vanilloid receptors on sensory nerves mediate the vasodilator action of anandamide

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The endogenous cannabinoid receptor agonist anandamide¹ is a powerful vasodilator of isolated vascular preparations^{2–4}, but its mechanism of action is unclear. Here we show that the vasodilator response to anandamide in isolated arteries is capsaicin-sensitive and accompanied by release of calcitonin-gene-related peptide (CGRP). The selective CGRP-receptor antagonist 8-37 CGRP (ref. 5), but not the cannabinoid CB1 receptor blocker SR141716A (ref. 7), inhibited the vasodilator effect of anandamide. Other endogenous (2-arachidonylglycerol, palmitylethanolamide) and synthetic (HU 210, WIN 55,212-2, CP 55,940) CB1 and CB2 receptor agonists¹ could not mimic the action of anandamide. The selective ‘vanilloid receptor’ antagonist capsazepine^{6,7} inhibited anandamide-induced vasodilation and release of CGRP. In patch-clamp experiments on cells expressing the cloned vanilloid receptor (VR1)⁸, anandamide induced a capsazepine-sensitive current in whole cells and isolated membrane patches. Our results indicate that anandamide induces vasodilation by activating vanilloid receptors on perivascular sensory nerves and causing release of CGRP. The vanilloid receptor may thus be another molecular target for endogenous anandamide, besides cannabinoid receptors, in the nervous and cardiovascular systems.

Anandamide (arachidonylethanolamide) was originally isolated from brain as an endogenous cannabinoid receptor ligand⁹. Bio-synthetic pathways for anandamide are also present outside the central nervous system, for example, in vascular endothelium and macrophages^{10,11}. It has been suggested that anandamide induces hypotension in anaesthetized rats by inhibiting peripheral sympathetic neurotransmission¹². Macrophage-derived anandamide is implicated in haemorrhagic shock¹³ and endotoxin-induced hypotension¹⁴. Although CB1 receptor messenger RNA has been detected in sympathetic nerves, vascular endothelium and smooth muscle^{10,15,16}, the involvement of cannabinoid receptors in the vasodilator effects of anandamide in isolated vascular preparations has been questioned^{2,4,17}. Anandamide is structurally related to capsaicin and olvanil (*N*-vanillyloleamide), compounds that all have an amide bond and an aliphatic side chain. Capsaicin and olvanil activate a subpopulation of primary sensory neurons, which can then become refractory to subsequent stimuli (desensitization)⁷. As such nerves mediate vasodilation¹⁸, we hypothesized that the vascular effects of anandamide and capsaicin have a common mechanism, involving excitation of primary sensory nerves in the vessel wall and consequent release of vasodilator neuropeptides such as CGRP.

To test this proposal, we examined the effects of capsaicin and the selective CGRP-receptor antagonist 8-37 CGRP (ref. 5) on anandamide-induced relaxation in rat hepatic and small mesenteric arteries and guinea-pig basilar artery. Pretreatment with capsaicin to cause desensitization and/or neurotransmitter depletion in perivascular sensory nerves abolished anandamide-induced relaxation in all