The genetic basis and fitness consequences of sperm midpiece size in deer mice

Citation
Fisher, Heidi S., Emily Jacobs-Palmer, Jean-Marc Lassance, and Hopi E. Hoekstra. 2016. “The genetic basis and fitness consequences of sperm midpiece size in deer mice.” Nature Communications 7 (1): 13652. doi:10.1038/ncomms13652. http://dx.doi.org/10.1038/ncomms13652.

Published Version
doi:10.1038/ncomms13652

Permanent link
http://nrs.harvard.edu/urn-3:HUL.InstRepos:29739160

Terms of Use
This article was downloaded from Harvard University’s DASH repository, and is made available under the terms and conditions applicable to Other Posted Material, as set forth at http://nrs.harvard.edu/urn-3:HUL.InstRepos:dash.current.terms-of-use#LAA

Share Your Story
The Harvard community has made this article openly available. Please share how this access benefits you. Submit a story.

Accessibility
The genetic basis and fitness consequences of sperm midpiece size in deer mice

Heidi S. Fisher¹, Emily Jacobs-Palmer¹, Jean-Marc Lassance¹ & Hopi E. Hoekstra¹

An extensive array of reproductive traits varies among species, yet the genetic mechanisms that enable divergence, often over short evolutionary timescales, remain elusive. Here we examine two sister-species of Peromyscus mice with divergent mating systems. We find that the promiscuous species produces sperm with longer midpiece than the monogamous species, and midpiece size correlates positively with competitive ability and swimming performance. Using forward genetics, we identify a gene associated with midpiece length: Prkar1a, which encodes the R1α regulatory subunit of PKA. R1α localizes to midpiece in Peromyscus and is differentially expressed in mature sperm of the two species yet is similarly abundant in the testis. We also show that genetic variation at this locus accurately predicts male reproductive success. Our findings suggest that rapid evolution of reproductive traits can occur through cell type-specific changes to ubiquitously expressed genes and have an important effect on fitness.

¹ Department of Organismic & Evolutionary Biology, Department of Molecular & Cellular Biology, Museum of Comparative Zoology, Howard Hughes Medical Institute, Harvard University, 16 Divinity Ave, Cambridge, MA 02138, USA. † Present address: Department of Biology, University of Maryland, College Park, MD 20742, USA. Correspondence and requests for materials should be addressed to H.E.H. (email: hoekstra@oeb.harvard.edu).
The remarkable diversity of male reproductive traits observed in nature is often attributed to the evolutionary forces of sexual conflict, sperm competition and sperm precedence. However, the genetic mechanisms that enable reproductive traits to respond to changes in selective regime are often unknown. Moreover, because most genes expressed in reproductive organs (for example, testis) are also expressed elsewhere in the body, genetic changes that result in reproductive trait modification can potentially lead to negative pleiotropic consequences in either the opposite sex or in other tissues. Despite these constraints, reproductive phenotypes show striking and often rapid divergence, and can promote speciation.

Two closely related Peromyscus rodents with highly divergent mating systems show marked variation in male reproductive traits. Within the genus, the deer mouse, *P. maniculatus*, is considered one of the most promiscuous species: both sexes mate with multiple partners, often in overlapping series just minutes apart, and females frequently carry multiple-paternity litters in the wild. By contrast, its sister species, the old-field mouse, *P. polionotus*, is strictly monogamous as established from both behavioural and genetic data. Moreover, relative testis size is roughly three times larger in *P. maniculatus* than in *P. polionotus*, consistent with the well-documented relationship between relative testis size and level of sperm competition in rodents. Therefore the competitive environments experienced by sperm of *P. maniculatus* and *P. polionotus* males represent divergent selective regimes.

The factors that regulate mammalian reproductive success are numerous and complex, yet when sperm from multiple males compete for a limited number of ova, the quality of each male's sperm can influence who will sire offspring. Under intense competition, sperm motility can be a critical determinant of success. Previous studies have shown that *P. maniculatus* sperm swim with greater velocity than *P. polionotus*. A primary energy source for motility is acquired by oxidative phosphorylation in the mitochondria, which are located within the sperm midpiece. The size of the midpiece is thus predicted to positively influence flagellar thrust and sperm velocity and, indeed, evidence across multiple taxa supports the relationship between relative testis size and level of sperm competition in rodents. The size of the midpiece is thus predicted to positively influence flagellar thrust and sperm velocity and, indeed, evidence across multiple taxa supports the relationship between relative testis size and level of sperm competition in rodents. The size of the midpiece is thus predicted to positively influence flagellar thrust and sperm velocity and, indeed, evidence across multiple taxa supports the relationship between relative testis size and level of sperm competition in rodents.

In this study, we examine the relationship between sperm midpiece length, swimming performance and reproductive success in *P. maniculatus*, *P. polionotus*, and a hybrid population. We then identify a single gene of large effect that regulates the phenotypic difference in sperm midpiece length between the two focal species, and show how allelic variation at this locus influences sperm swimming velocity and ultimately, male fertility.

**Results**

**Sperm morphology and performance.** We first measured four sperm traits of mice taken from our laboratory colonies of the two focal species, *P. maniculatus* and *P. polionotus* (Fig. 1a). We found that sperm head size does not differ between these species (Fig. 1bc), but *P. maniculatus* sperm have longer flagella than *P. polionotus* (Fig. 1d; t-test: *P* = 8 × 10⁻¹⁰, df = 9, *n* = 10 sperm/male). More specifically, the midpiece region of the flagellum is significantly longer in *P. maniculatus* sperm than in *P. polionotus* (Fig. 1e; t-test: *P* = 3 × 10⁻⁷, df = 9, *n* = 10 sperm/male). These data are consistent with morphological differences in sperm from wild-caught *P. maniculatus* and *P. polionotus*.

Indeed, sperm from the promiscuous *P. maniculatus* males swim with greater velocity (straight-line velocity [VSL]) than sperm of the monogamous *P. polionotus* (t-test: *P* = 0.0017, df = 8, *n* = 76–549 sperm/male), consistent with our previous results. Two other means of measuring sperm swimming performance, curvilinear velocity (VCL; t-test: *P* = 0.0024, df = 8, *n* = 76–549 sperm/male) and average path velocity (AVP; t-test: *P* = 0.0039, df = 8, *n* = 76–549 sperm/male), showed a similar difference as in the VSL results. Since all three velocity measures were consistent and are non-independent measurements, we focused on the most conservative estimate, VSL, in subsequent analyses (Supplementary Fig. 1).

To assay the relationship between *Peromyscus* midpiece length and swimming performance in a competitive context, we next conducted a series of swim-up assays, a clinical technique used to screen for highly motile spermatozoa that are most likely to achieve fertilization. We tested sperm with variable midpiece lengths by centrifuging cells and collecting sperm best able to swim towards the surface through a viscous media. We first competed sperm from two heterospecific males (*P. maniculatus* versus *P. polionotus*), which as a single mixed sample offers the greatest range of sperm morphology, and found that the most motile sperm had a significantly longer midpiece (Fig. 2; t-test: *P* = 3.28 × 10⁻⁴, df = 11; *n* = 20 sperm/trial). We found a strikingly similar result when the competitions involved sperm...
from two unrelated conspecific males (P. maniculatus versus P. maniculatus), representing a more biologically relevant competition (Fig. 2; t-test: $P = 0.001$, $df = 14$; $n = 20$ sperm/trial), and even within-male competitions (P. maniculatus) in which all sperm were harvested from a single male for each trial but still showed variation in midpiece length (Fig. 2; t-test: $P = 0.014$, $df = 18$; $n = 20$ sperm/trial). In total, these results suggest that sperm with larger midpiece regions are more motile, and thus more likely to achieve fertilization, whether they are competing against heterospecific, conspecific or even of other sperm produced by the same male.

**Genetic mapping of sperm midpiece length.** Next, to dissect the genetic basis of adaptive differences in sperm morphology, we performed a genetic intercross between P. maniculatus and P. polionotus to produce 300 second-generation hybrid (F2) male offspring. We then genotyped each F2 male at 504 anonymous loci throughout the genome. We identified a single chromosomal region significantly associated with midpiece length variation on linkage group 4 (LG4; Fig. 3; on the basis of logarithm of odds [LOD], significance determined by a genome-wide permutation test with $\alpha = 0.01$). This single region of the genome explains 33% of sperm midpiece length variation in the F2 hybrids, and largely...
recapitulates differences in midpiece length observed between the pure species (Fig. 4). Furthermore, we found that F2 males carrying at least one P. maniculatus allele at this locus have a significantly longer midpiece than those with none (Fig. 4; t-test: $P = 4.44 \times 10^{-15}$, df = 49), suggesting the P. maniculatus allele acts in a dominant fashion. Thus, a single large-effect locus explains much of the difference in sperm midpiece length between these two species.

While we found a single significant peak associated with sperm midpiece length on linkage group 4 (Fig. 3), we found no significant quantitative trait loci (QTL) for sperm total flagellum length (Fig. 3) or any measure of sperm velocity (VSL, VCL, VAP). The lack of significant QTL for total flagellum length or velocity in this cross does not suggest that variation in these traits lacks a genetic basis, rather the result may be due to measurement error or an inability to detect genes of small phenotypic effect; due to the complex nature of these traits, it is likely that they are controlled by multiple genes. Moreover, velocity is a composite trait likely influenced by sperm morphology as well as other factors. We performed post-hoc scans for each of these phenotypic traits (midpiece, total flagellum, VSL, VCL and VAP with 1,000 permutations, $\alpha = 0.05$) with each other trait, and with the genetic marker of highest linkage, as covariates; we found no additional significant QTL.

To further refine the single QTL for sperm midpiece length and identify a causal gene, we enriched marker coverage in the 20 cM region surrounding the marker of highest association by genotyping each F2 male for eight additional single nucleotide polymorphisms (SNPs; Supplementary Table 1). The increased marker density improved our QTL signal and reduced the 1.5-LOD support interval to 3.3 cM and the 99% Bayes credible interval to a single locus containing the Prkar1a gene (Fig. 3). We then confirmed this association with a genetic breakpoint analysis that included two additional SNPs flanking the marker of highest association, thereby narrowing the 3.3 cM interval to 0.8 cM around Prkar1a (Fig. 5). The Prkar1a gene encodes the R1 regulatory subunit of the Protein Kinase A (PKA) holoenzyme and is the only gene within the broader 3.3 cM confidence interval previously implicated in male fertility, sperm morphology or sperm motility24,25 (Supplementary Table 2). Therefore, Prkar1a represents a strong candidate for further functional analyses.

The role of Prkar1a in Peromyscus reproduction. We found that R1\(\alpha\) is abundant and localized in the sperm midpiece in Peromyscus (Fig. 6a, Supplementary Fig. 2; and in contrast to another potential causal gene within the 0.8 cM QTL peak, the predicted gene LOC102911133, which we found localizes to the...
sperm head (Supplementary Note 1; Supplementary Fig. 3), consistent with studies in humans26 and rats27. To confirm the influence of Prkar1a on midpiece length, we examined Mus musculus C57BL/6 animals with only a single functional copy of the gene (homozygous knockouts are inviable)28 and found that the midpiece of Prkar1a+/− males is significantly shorter than wild type brothers (Fig. 6b; t-test: \( P = 4.54 \times 10^{-8} \), \( df = 7 \), \( n = 20 \) sperm/male). These findings therefore strongly implicate the Prkar1a gene as a major determinant of sperm midpiece length differences observed in Peromyscus.

We next investigated how the PKA R1α subunit differed between our two focal species. First, we found no non-synonymous differences between P. maniculatus and P. polionotus in the coding exons of Prkar1α mRNA (1,146 bp), suggesting that both species produce similar R1α proteins. This protein is expressed throughout male germ cell development29. In whole testis samples, we found no significant differential expression of Prkar1α mRNA and R1α protein levels (mRNA: Supplementary Fig. 4, Bayes factor = 0.071, Posterior Probability = 0.008, \( n = 8 \) males; protein: Fig. 7; t-test: \( P = 0.664 \), \( n = 5 \) males), possibly because testis samples contain cells of multiple developmental stages. However, in mature, fully developed sperm released from the caudal epididymis, we found P. polionotus expresses significantly more R1α protein than P. maniculatus (Fig. 7; t-test: \( P = 0.028 \), \( n = 6 \) males). These data, in combination with the knockout phenotype in Mus, suggest that changes in the expression of R1α can lead to changes in sperm midpiece length.

**Linking genotype, phenotype and fitness in a hybrid population.** In addition to genetic mapping, our genetically heterogeneous F2 hybrid population also enables us to test for statistical associations among traits. First, to test the prediction that sperm midpiece length influences swimming performance, we compared the mean straightline velocity (VSL) to both mean midpiece length and flagellum lengths of sperm from F2 males using a linear regression, with each trait as the independent variable. While we found no significant association with flagellum length \( [R^2 = 0.003, P = 0.29, df = 232] \), midpiece length and VSL show a significant positive correlation \( [Fig. 8a; R^2 = 0.028, P = 0.0057, df = 232] \), which remains significant after considering flagellum length as a covariate \( [R^2 = 0.028, P = 0.012, df = 232] \), midpiece was the only coefficient with \( P < 0.05 \). Moreover, we found no significant association between midpiece length and total flagellum length in F2 males \( (R^2 = 0.004, P = 0.14, df = 232) \), which suggests that these two traits are genetically independent. These results show that VSL is correlated with midpiece length in Peromyscus, either because increase in midpiece length leads to

---

**Figure 6 | PKA R1α localization and effect on midpiece length.** (a) PKA R1α localization in mature P. maniculatus sperm cell (×400); immunofluorescence of anti-PKA R1α (red) and DAPI (blue). (b) Mean ± s.e. midpiece length of wild-type Prkar1α+/+ (red) and heterozygous Prkar1α+/− (pink) Mus musculus C57BL/6 sperm (t-test: \( n = 8 \) males, \( n = 20 \) sperm/male). Note truncated y axis.

**Figure 7 | PKA R1α.** Mean ± s.e. PKA R1α protein expression as measured by percentage of relative abundance (%RA) in testis (\( n = 5 \)) and epididymal sperm (\( n = 6 \)) samples from P. polionotus (white bars) and P. maniculatus (black bars) males. \( NS = P > 0.05, t\)-test.

**Figure 8 | Sperm performance and male fertility in F2 hybrids.** (a) Association between midpiece length and straight-line velocity of F2 hybrid males (linear regression: \( n = 233 \) males, \( n = 10 \) sperm/male). For reference, midpiece length of each parental species is plotted as a dashed line. (b) Mean ± s.e. sperm midpiece length of F2 males that did (\( n = 85 \)) and did not (\( n = 173 \)) sire offspring (t-test). Note truncated y axis.
increased speed (that is, variation in these two traits share a pleiotropic genetic basis), or less likely, these two traits are influenced independently by tightly linked genes.

We next examined how variation at the Prkar1a locus predicts sperm morphology and performance in the F2 hybrid population. We found that F2 males carrying at least one P. maniculatus Prkar1a allele (AA or Aa, as defined by the Prkar1a SNP) produce sperm with significantly longer midpiece, as mentioned earlier (Fig. 4), but their sperm also swim with greater velocity (VSL) than sperm from males homozygous for P. polionotus Prkar1a allele (aa; mean VSL ± s.e.: AA = 78.7 ± 1.9 µm/s, Aa = 77.5 ± 1.1 µm/s, aa = 73.1 ± 1.8 µm/s, t-test: $P_{AA-aa} = 0.040$, $P_{Aa-aa} = 0.044$, $df = 49$). Therefore, Prkar1a genotype is associated with both sperm morphology and swimming performance.

Finally, to understand how allelic and phenotypic variation influences male reproductive success, we scored which F2 males sired offspring in natural matings. We found that F2 hybrid males carrying at least one dominant P. maniculatus Prkar1a allele were more likely to sire offspring when paired with a female for 2 weeks than those homozygous for the Prkar1a (X$^2$:AA-Aa-aa = 6.35, $P = 0.042$, $df = 2$). This is a conservative estimate of male fertility because many false negatives were likely included due to pairs that failed to mate, infanticide, female infertility and other unrelated physiological or behavioural conditions of the animals, which make an effect more difficult to detect. Moreover, this result is consistent with lower reproductive success in pure P. polionotus matings compared to P. maniculatus under similar conditions ($z = 1.37$, $P = 0.0071$, $df = 19$). Furthermore, F2 males that sired pups had sperm with significantly longer midpiece regions than those that did not reproduce (Fig. 8b; $t$-test: $P = 0.041$, $df = 84$). Together, these analyses show that males carrying at least one copy of the P. maniculatus Prkar1a allele produce significantly faster sperm with longer midpieces, and also benefit from greater reproductive success, suggesting a direct link between fitness, phenotype and genetic variation at the Prkar1a locus.

Discussion

When females mate with multiple partners within a reproductive cycle, males can continue to compete for reproductive success long after mating has occurred as their sperm compete for the fertilization of a limited number of ova. The strength of post-copulatory sexual selection is therefore largely determined by female mating strategy. In this study, we examined two species of Peromyscus mice with divergent mating systems. Female P. maniculatus mate with multiple males in a reproductive cycle, allowing for sperm of different males to compete within the female reproductive tract for fertilization success; in contrast, P. polionotus females mate monogamously and thus sperm competition is limited. Theory predicts that these differing competitive regimes may favour the evolution of trait divergence. Our results suggest that the difference observed in sperm midpiece length between P. maniculatus and P. polionotus confers an important reproductive advantage that improves sperm swimming performance. We first found that sperm with longer midpiece are more motile in a competitive swim-up assay. Second, within our hybrid population, we observed a positive relationship among sperm midpiece length, swimming velocity and male reproductive success. The targets of post-copulatory sexual selection can vary tremendously across taxa, but in this system, our results are consistent with the hypothesis that selection favours sperm with longer midpiece regions, consistent with findings in other species$^{20–22}$.

If a simple relationship between midpiece length and fitness exists, it is puzzling why a monogamous species would not share the same sperm morphology as its promiscuous sister-species. The functional relevance of the midpiece, in both evolutionary and human fertility studies, is controversial$^{20,21}$, and many closely related species vary extensively in this trait$^{22–34}$. Nonetheless, while drift and/or selection acting on pleiotropic traits could lead to shorter midpiece, sperm cells with more or larger mitochondria afforded by the larger midpiece also may experience greater oxidative stress, which is known to increase mutagenesis in germ cells$^{25}$. Therefore, when sperm competition is absent, such as in P. polionotus, the benefits conferred by producing faster sperm may not outweigh the associated costs. However, in the highly competitive environment that P. maniculatus sperm experience, even small increases in sperm performance could differentiate those that reproduce and those that do not. Thus, the balance between negative and positive effects of cellular respiration in sperm may determine the relative roles of natural and sexual selection as drivers of interspecific midpiece variation seen in Peromyscus, and across animals more generally.

Using a forward-genetics approach, we identified a single gene of large effect on midpiece size, Prkar1a, which encodes for the R1z regulatory subunit of the PKA. We then corroborated the role of this gene by demonstrating that lab mice with a dominant negative mutation in Prkar1a have shorter midpiece than their wild-type brothers. Successful fertilization requires precise temporal and spatial regulation of PKA activity, the main downstream effector of cellular cyclic AMP concentrations, and is in part, regulated by R1z$^{29}$. The R1z subunit is known to influence gross sperm morphology, motility and fertility in humans presenting Carney Complex (a disease associated with mutations in Prkar1a) and in Mus musculus Prkar1a mutants$^{24}$. While most reports on Prkar1a implicate negative consequences for male fertility and are associated with large-scale changes in R1z expression$^{24,29,36–38}$, our results suggest that the subtle tuning of the expression of this regulatory subunit may also confer beneficial effects. In Peromyscus, we found that R1z is similarly abundant in the testis of the two focal species, yet is differentially expressed in mature sperm of P. maniculatus and P. polionotus. Considering that the heterogeneous nature of testis tissue contains germ cells at various stages of their differentiation process and Sertoli cells, it is likely that subtle difference in Prkar1a mRNA abundance or R1z protein may not be detectable if expressed in a limited number of cell types. Our analysis of mature spermatozoa, however, suggests that cell type-specific R1z expression differences present during later stages of Peromyscus spermatogenesis are likely to regulate midpiece length.

The rapid evolution of reproductive protein coding regions is a well-known response to post-copulatory sexual selection$^{39}$; we demonstrate here that expression changes in gametes of a broadly expressed gene can also be a target of selection. Over 50% of mammalian genes are expressed in the testis and most of these genes are also expressed in other tissues$^{30,31}$; therefore, cell type-specific changes in protein expression in reproductive tissues are likely to be a common mechanism by which selection in males can operate with swiftness and without deleterious effects in females or other tissues.

Methods

Mice. Wild derived Peromyscus maniculatus bairdii and Peromyscus polionotus sublineatus were originally obtained from the Peromyscus Genetic Stock Center at the University of South Carolina and have been maintained at Harvard University in accordance with guidelines established by Harvard’s Institutional Animal Care and Use Committee. Adult sexually mature P. polionotus and P. maniculatus males were used to collect data for cross-species comparisons. In addition, we bred four mice, two P. polionotus males and two P. maniculatus females, to produce 40 first-generation (F1) hybrids, and then intercrossed siblings to generate second-generation (F2) hybrid progeny. For genetic mapping, we used 300 F2 hybrid males...
and obtained genotypic and phenotypic data as described below. All males were sexually mature and were paired with a female prior to harvesting sperm. *Mus musculus* females for Prkar1a locus on C57BL/6 background were mated to wild type C57BL/6 males to produce wild type offspring. These mice were a generous gift of Dr Stanley McKnight at the University of Washington.

**Sperm analysis.** After sacrifice via carbon dioxide overdose, we immediately removed the left caudal epididymis of each F2 male with a single cut at the intersection of the urodeus (the pre-spin sample) to the sperm that were able to swim to the midpiece-principal piece boundary (see Fig. 1a) using the curve-spline tool in the AxioVision Image Analysis Software (Zeiss). Intraclass correlation coefficients among sperm within F2 hybrid males were significant and high (midpiece length; Supplementary Table 1; Supplementary Note 1). This region of the genome surrounding the marker most strongly associated with sperm midpiece length was the most important marker for the association between phenotype and genotype.

**Fitness assays.** We tested for an effect of sperm midpiece length on competitive success by performing three types of sperm swim-up assays: heterospecific competitions including the sperm of a *P. polionotus* male, conspecific competitions between the sperm of two *P. maniculatus* males, and within-male competitions between the sperm of a *P. maniculatus* male, and *P. polionotus* males were measured. We sequenced the protein-coding region by extracting mRNA from testes of F1 males that showed a recombination event in the surrounding region, and all F2 males that showed a recombination event in the surrounding region. We then PCR-amplified the remaining fragments ligated to sequencing adapters containing a unique barcode and amplified the remaining fragments ligated to sequencing adapters containing a unique barcode. Finally, we amplified the remaining fragments using a Phusion High Fidelity PCR Kit (Thermo-Fisher) and sequenced the resulting libraries on a Genome Analyzer IIx or a HiSeq 2500 (Illumina). We then designed 1,753 informative SNP markers that consistently differed between the two parental species and identified markers that were expected as heterozygous in the first generation hybrids (F1). We then pruned our marker set to exclude any markers genotyped in fewer than 100 individuals or with genotype information identical to another marker.

We then conducted all genetic mapping analyses using R/qtl software, an add-on package for R statistical software. To construct a genetic link map, we calculated linkage distances based on the fraction of recombination events and Logarithm of Odds (LOD) scores between SNP marker pairs. Next, we grouped markers by varying the recombination parameters until we recovered a map with 25 linkage groups containing at least five markers each (the karyotypes of both species are known [n = 24 chromosomes]; however, we are unable to recover the majority of the Y chromosome with the cross design employed in this study). Any markers not included in the 25 linkage groups were excluded. Finally, we refined this map by ordering the markers within each linkage group in overlapping windows of eight markers and determining the frequent flanking events between markers in each window. The resulting genetic link map contained 504 in 25 linkage groups. To identify QTL contributing to sperm morphology, we performed Haley-Knott regression and interval mapping analyses sequentially with 1,000 permutations and 25 linkage groups containing at least five markers each (the karyotypes of both species are known [n = 24 chromosomes]; however, we are unable to recover the majority of the Y chromosome with the cross design employed in this study). Any markers not included in the 25 linkage groups were excluded. Finally, we refined this map by ordering the markers within each linkage group in overlapping windows of eight markers and determining the frequent flanking events between markers in each window. The resulting genetic link map contained 504 in 25 linkage groups. To identify QTL contributing to sperm morphology, we performed Haley-Knott regression and interval mapping analyses sequentially with 1,000 permutations and 25 linkage groups containing at least five markers each (the karyotypes of both species are known [n = 24 chromosomes]; however, we are unable to recover the majority of the Y chromosome with the cross design employed in this study). Any markers not included in the 25 linkage groups were excluded. Finally, we refined this map by ordering the markers within each linkage group in overlapping windows of eight markers and determining the frequent flanking events between markers in each window. The resulting genetic link map contained 504 in 25 linkage groups. To identify QTL contributing to sperm morphology, we performed Haley-Knott regression and interval mapping analyses sequentially with 1,000 permutations and 25 linkage groups containing at least five markers each (the karyotypes of both species are known [n = 24 chromosomes]; however, we are unable to recover the majority of the Y chromosome with the cross design employed in this study). Any markers not included in the 25 linkage groups were excluded. Finally, we refined this map by ordering the markers within each linkage group in overlapping windows of eight markers and determining the frequent flanking events between markers in each window. The resulting genetic link map contained 504 in 25 linkage groups. To identify QTL contributing to sperm morphology, we performed Haley-Knott regression and interval mapping analyses sequentially with 1,000 permutations and 25 linkage groups containing at least five markers each (the karyotypes of both species are known [n = 24 chromosomes]; however, we are unable to recover the majority of the Y chromosome with the cross design employed in this study). Any markers not included in the 25 linkage groups were excluded. Finally, we refined this map by ordering the markers within each linkage group in overlapping windows of eight markers and determining the frequent flanking events between markers in each window. The resulting genetic link map contained 504 in 25 linkage groups. To identify QTL contributing to sperm morphology, we performed Haley-Knott regression and interval mapping analyses sequentially with 1,000 permutations and 25 linkage groups containing at least five markers each (the karyotypes of both species are known [n = 24 chromosomes]; however, we are unable to recover the majority of the Y chromosome with the cross design employed in this study). Any markers not included in the 25 linkage groups were excluded. Finally, we refined this map by ordering the markers within each linkage group in overlapping windows of eight markers and determining the frequent flanking events between markers in each window. The resulting genetic link map contained 504 in 25 linkage groups. To identify QTL contributing to sperm morphology, we performed Haley-Knott regression and interval mapping analyses sequentially with 1,000 permutations and 25 linkage groups containing at least five markers each (the karyotypes of both species are known [n = 24 chromosomes]; however, we are unable to recover the majority of the Y chromosome with the cross design employed in this study). Any markers not included in the 25 linkage groups were excluded. Finally, we refined this map by ordering the markers within each linkage group in overlapping windows of eight markers and determining the frequent flanking events between markers in each window. The resulting genetic link map contained 504 in 25 linkage groups.
Reverse Transcriptase (Thermo-Fisher) and a poly-T primer (T16). We amplified ARTICLE NATURE COMMUNICATIONS | DOI: 10.1038/ncomms13652 | www.nature.com/naturecommunications

We then washed the cells in phosphate-buffered saline with 0.1% Tween 20 (PBT) for

Sequence data from this study have been deposited in GenBank under the accession codes KF005595 and KF005596. RNAseq data have been deposited in the GenBank/EMBL/DBJ Sequence Read Archive under the accession code PRJNA343919.

References

1. Chapman, T., Arroyost, G., Bangham, J. & Rowe, L. Sexual conflict. Trends Ecol. Evol. 18, 41–47 (2003).
2. Hosken, D. J. & Stockley, P. Sexual selection and genital evolution. Trends Ecol. Evol. 19, 87–93 (2004).
3. Snook, R. Sperm in competition: not playing by the numbers. Trends Ecol. Evol. 20, 46–53 (2005).
4. Howard, D. J. Conspecific sperm and pollen precedence and speciation. Annu. Rev. Ecol. Syst. 30, 109–132 (1999).
5. Chalmel, F. et al. The conserved transcriptome in human and rodent male gametogenesis. Proc. Natl Acad. Sci. USA 104, 8346–8351 (2007).
6. Shima, J. E., McLean, D. J., McCarry, J. R. & Griswold, M. D. The murine testicular transcriptome: characterizing gene expression in the tests during the progression of spermatogenesis. Biol. Reprod. 71, 319–330 (2004).
7. Birkhead, T. R., Hosken, D. J. & Pitnick, S. Sperm Biology: An Evolutionary Perspective (Academic Press, 2008).
8. Fisher, H. S. & Hoekstra, H. E. Competition drives cooperation among closely related sperm of deer mice. Nature 463, 801–803 (2010).
9. Fisher, H. S., Giomi, L., Hoekstra, H. E. & Mahadevan, L. The dynamics of sperm cooperation in a competitive environment. Proc. R. Soc. B: Biol. Sci. 281, 20140296 (2014).
10. Linzey, A. V. & Layne, J. C. Comparative morphology of spermatozoon of the rodent genus Peromyscus (Muridae). Am. Mus. Novit. 2531, 1–20 (1974).
11. Linzey, A. V. & Layne, J. C. Comparative morphology of the male reproductive tract in the rodent genus Peromyscus (Muridae). Am. Mus. Novit. 2355, 1–47 (1970).
12. Dewsbury, D. A. & Dewsbury, D. A. Interactions between males and their sperm during multi-male copulatory episodes of deer mice (Peromyscus maniculatus). Anim. Behav. 33, 1266–1274 (1985).
13. Birdsell, D. A. & Nash, D. Occurrence of successful multiple insemination of females in natural populations of deer mice (Peromyscus maniculatus). Evolution 27, 106–110 (1973).
14. Dewsbury, D. A. Exercise in the prediction of monogamy in the field from laboratory data on 42 species of muroid rodents. Biologist 63, 138–162 (1981).
15. Holt, D. W. Genetic evidence for long-term monogamy in a small rodent, Peromyscus polionotus. Am. Nat. 117, 665–675 (1981).
16. Ramn, S. A., Parker, G. A. & Stockley, P. Sperm competition and the evolution of male reproductive anatomy in rodents. Proc. R. Soc. B: Biol. Sci. 272, 949–955 (2005).
17. Simmons, L. W. & Fitzpatrick, J. L. Sperm wars and the evolution of male reproductive function. Proc. R. Soc. B: Biol. Sci. 265, 19–34 (2008).
18. Gage, M. J. G. Mammalian sperm morphology. Proc. R. Soc. B: Biol. Sci. 265, 103–108 (2003).
19. Cardullo, R. A. & Baltz, J. M. Metabolic regulation in mammalian sperm: mitochondrially determined sperm shape and flagellar beat frequency. Cell Motil. Cytoskeleton 19, 180–188 (1991).
20. Firman, R. C. & Simmons, L. W. Sperm midpiece length predicts sperm swimming velocity in house mice. Biol. Lett. 6, 513–516 (2010).
21. Vladić, T. V. Sperm quality as reflected through morphology in salmon alternative life histories. Biol. Reprod. 66, 98–105 (2002).
22. Lipold, S., Calhoun, S., Immler, S. & Birkhead, T. R. Sperm morphology and sperm velocity in passerine birds. Proc. R. Soc. B: Biol. Sci. 276, 1175–1181 (2009).
23. Holt, W. V., Hernandez, M., Warrell, L. & Satake, N. The long and the short of sperm selection in vitro and in vivo: swim-up techniques select for the longer and faster swimming mammalian sperm. J. Evol. Biol. 23, 598–608 (2010).
24. Burton, K. A. et al. Haplotypinsufficiency at the Protein Kinase A R12 gene locus leads to fertility defects in male mice and men. Mol. Endocrinol. 20, 2504–2513 (2006).
25. Matzuk, M. M. & Lamb, D. J. The biology of infertility: research advances and clinical challenges. Nat. Med. 14, 1197–1213 (2008).
26. Reiniton, N. et al. Localization of a novel human A-kinase-anchor protein, hAKAP220, during spermatogenesis. Dev. Biol. 225, 194–204 (2000).
27. Dahle, M. K., Reiniton, N., Orstavik, S., Taskén, K. & Taskén, K. Novel alternatively spliced mRNA (1c) of the Protein Kinase A R12; subunit is implicated in haploid germ cell specific expression. Mol. Reprod. Dev. 59, 11–16 (2001).
28. Willis, B. S., Niswender, C. M., Su, T., Amieux, P. S. & McKnight, G. S. Cell type specific expression of a dominant negative PKA mutation in mice. PLoS ONE 6, e18772 (2011).
29. Burton, K. A. & McKnight, G. S. PKA, germ cells, and fertility. Physiology 22, 40–46 (2007).
30. Mukai, C. & Okuno, M. Glycolysis plays a major role for adenosine triphosphate supplementation in mouse sperm flagellar movement. *Biol. Reprod.***71*, 540–547 (2004).

31. Piomboni, P., Focarelli, R., Stendardi, A., Ferramosca, A. & Zara, V. The role of mitochondria in energy production for human sperm motility. *Int. J. Androl.* **35**, 109–124 (2011).

32. Tourmente, M., Gomendio, M. & Roldan, E. R. Sperm competition and the evolution of sperm design in mammals. *BMC Evol. Biol.* **11**, 12 (2011).

33. Tourmente, M., Gomendio, M., Roldán, E. R. S., Giojalas, L. C. & Chiaraviglio, M. Sperm competition and reproductive mode influence sperm dimensions and structure among snakes. *Evolution***63*, 2513–2524 (2009).

34. Immler, S. & Birkhead, T. R. Sperm competition and sperm midpiece size: no consistent pattern in passerine birds. *Proc. R. Soc. B: Biol. Sci.***274*, 561–568 (2007).

35. Atifien, R. J. & Baker, M. A. Oxidative stress and male reproductive biology. *Reprod. Fertil. Dev.* **16**, 581 (2004).

36. Vijayaraghavan, S., Goueli, S. A., Davey, M. P. & Carr, D. W. Protein kinase A-anchoring inhibitor peptides arrest mammalian sperm motility. *J. Biol. Chem.* **272**, 4747–4752 (1997).

37. Witsenboer, P. et al. Male infertility as a component of Carney complex. *Andrologia***39*, 196–197 (2007).

38. Hwang, K. *et al.* Mendelian genetics of male infertility. *Ann. NY Acad. Sci.* **1214**, E1–E17 (2011).

39. Swanson, W. J. & Vacquier, V. D. The rapid evolution of reproductive proteins. *Nat. Rev. Genet.* **3**, 137–144 (2002).

40. Biggers, J. D., Whitten, W. K. & Whittingham, D. G. in *Methods in Mammalian Embryology.* (ed. Daniel, J. C.) 86–116 (Freeman, 1971).

41. Wilson-Leedy, J. G. & Ingermann, R. L. Development of a novel CASA system based on open source software for characterization of zebrafish sperm motility parameters. *Theriogenology***67**, 661–672 (2007).

42. Calhim, S., Immler, S. & Birkhead, T. R. Postcopulatory sexual selection is associated with reduced variation in sperm morphology. *PLoS ONE***2*, e413 (2007).

43. Birkhead, T. R., Pellatt, E. J., Brekke, P., Yeates, R. & Castillo-Juarez, H. Genetic effects on sperm design in the zebra finch. *Nature***434*, 383–387 (2005).

44. Firman, R. E. C. & Simmons, L. W. Experimental evolution of sperm quality via postcopulatory sexual selection in house mice. *Evolution***64**, 1245–1256 (2009).

45. R Core Team. *R: A Language and Environment for Statistical Computing.* (Foundation for Statistical Computing, 2010).

46. World Health Organization. *WHO Laboratory Manual for the Examination and Processing of Human Semen* (2010).

47. Hayssen, V. D., Van Tienhoven, A. & Van Tienhoven, A. *Asdell’s Patterns of Mammalian Reproduction* (Cornell University Press, 1993).

48. Peterson, B. K., Weber, J. N., Kay, E. H., Fisher, H. S. & Hoekstra, H. E. Double digest RADSeq: an inexpensive method for *de novo* SNP discovery and genotyping in model and non-model species. *PLoS ONE***7*, e37135 (2012).

49. Broman, K. W., Wu, H., Sen, S. & Churchill, G. A. *R/qtl: QTL mapping in experimental crosses.* *Bioinformatics***19*, 889–890 (2003).

50. Ramsdell, C. M. *et al.* Comparative genome mapping of the deer mouse (*Peromyscus maniculatus*) reveals greater similarity to rat (*Rattus norvegicus*) than to the lab mouse (*Mus musculus*). *BMC Evol. Biol.* **8**, 65 (2008).

51. Karolchik, D. *et al.* The UCSC Genome Browser database: 2014 update. *Nucleic Acids Res.* **42**, D764–D770 (2014).

52. The UniProt Consortium. UniProt: a hub for protein information. *Nucleic Acids Res.* **43**, D204–D212 (2015).

53. Blake, J. A. *et al.* The Mouse Genome Database: integration of and access to knowledge about the laboratory mouse. *Nucleic Acids Res.* **42**, D810–D817 (2014).

54. Dobin, A. *et al.* STAR: ultrafast universal RNA-seq aligner. *Bioinformatics***29*, 15–21 (2013).

55. Turro, E., Goncalves, A., Coin, L. J. M., Richardson, S. & Lewin, A. Haplotype and isoform specific expression estimation using multi-mapping RNA-seq reads. *Genome Biol.* **12**, R13 (2011).

56. Turro, E., Astle, W. J. & Tavaré, S. Flexible analysis of RNA-seq data using mixed effects models. *Bioinformatics***30*, 180–188 (2014).

57. McAlister, G. C. *et al.* MultiNotch MS3 enables accurate, sensitive, and multiplexed detection of differential expression across cancer cell line proteomes. *Anal. Chem.* **86**, 7150–7158 (2014).

Acknowledgements

We thank S. McKnight for generously donating *Prkar1a*<sup>+/−</sup> mice; R. Mallarino, B. Peterson and J. Weber for experimental advice; A. Bree, E. Lievens, C. Scholes, J. Weaver and C. Xu, and for technical assistance; and S. McKnight, K. Peichel, M. Ryan and S. Suarez for comments on earlier versions of this manuscript. Research was funded by an NIH Ruth Kirschstein National Research Service Award (NIGMS084719) and an NIH Pathway to Independence Award to H.S.F. (NICHD071972); an NSF Graduate Research Fellowship and an NSF Doctoral Dissertation Improvement Grant to E.J.-P.; a European Molecular Biology Organization Postdoctoral Fellowship and a Human Frontier Science Program Long-term Fellowship to J.-M.L.; and an Arnold and Mabel Beckman Foundation Young Investigator Award to H.E.H. H.E.H. is an Investigator at the Howard Hughes Medical Institute.

Author contributions

H.S.F. and H.E.H. conceived of and designed the study; H.S.F. bred, processed and genotyped *Peromyscus*, performed *R/qtl* and fine-scale mapping, protein localization and quantification; E.J.-P. measured sperm morphology, bred and processed *Mus*; J.-M. L. designed and analysed the RNA-seq experiments; H.S.F. and H.E.H. interpreted the results and wrote the paper.

Additional information

Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

Competing financial interests: The authors declare no competing financial interests.

Reprints and permission information is available online at http://npg.nature.com/reprintsandpermissions/

How to cite this article: Fisher, H. S. *et al.* The genetic basis and fitness consequences of sperm midpiece size in deer mice. *Nat. Commun.* **7**, 13652 doi: 10.1038/ncomms13652 (2016).

Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.