Haploid selection within a single ejaculate increases offspring fitness

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An inescapable consequence of sex in eukaryotes is the evolution of a biphasic life cycle with alternating diploid and haploid phases. The occurrence of selection during the haploid phase can have far-reaching consequences for fundamental evolutionary processes including the rate of adaptation, the extent of inbreeding depression, and the load of deleterious mutations, as well as for applied research into fertilization technology. Although haploid selection is well established in plants, current dogma assumes that in animals, intact fertile sperm within a single ejaculate are equivalent at siring viable offspring. Using the zebrafish *Danio rerio*, we show that selection on phenotypic variation among intact fertile sperm within an ejaculate affects offspring fitness. Longer-lived sperm sired embryos with increased survival and a reduced number of apoptotic cells, and adult male offspring exhibited higher fitness. The effect on embryo viability was carried over into the second generation without further selection and was equally strong in both sexes. Sperm pools selected by motile phenotypes differed genetically at numerous sites throughout the genome. Our findings clearly link within-ejaculate variation in sperm phenotype to offspring fitness and sperm genotype in a vertebrate and have major implications for adaptive evolution.

biphasic life cycle | sperm selection | sperm genotype | sexual reproduction | gametic selection

Sperm within an ejaculate exhibit remarkable phenotypic variation (1), but little is known about the causes and consequences of such variation and selection among sperm produced by one male [hereafter referred to as "sib sperm" (2, 3)]. The key reason for this lack of knowledge is the current assumption that performance of sperm produced by a male is under diploid control (4–6), a notion that is further supported by the apparent lack of association between the phenotypic variation among sib sperm and their genetic content (7, 8). Nevertheless, some empirical evidence shows that genes may be expressed at the haploid stages of spermatogenesis and that the transcripts of these genes are not always perfectly shared through cytoplasmic bridges among haploid spermatids (9, 10). Furthermore, the lack of perfect symmetry in sharing of transcripts among haploid cells suggests that phenotypic variation within an ejaculate may have a genetic or epigenetic basis and hence be under selection (11, 12).

Theory predicts that genetic/epigenetic variation among sib sperm may lead to competition between different sperm phenotypes for the fertilization of eggs and may translate into differential fitness effects in the offspring (3). In fact, two recent studies suggested a possible link between sperm phenotype and offspring phenotype: In a broadcast spawning ascidian, *Styela plicata*, longer-lived sperm sired offspring with higher early-life survival (13), and in the Atlantic salmon, *Salmo salar*, sperm with intermediate longevity sired faster-hatching offspring (14). However, no published study to date has separated sperm aging from the underlying genetic or epigenetic variation among sib sperm or has provided insights into the long-term fitness effects of variation in sperm phenotypes within a single ejaculate. Therefore our current understanding of the importance of selection at the gametic stage for Darwinian fitness continues to be incomplete.

Results and Discussion

Here we demonstrate that different cohorts of sperm phenotypes and genotypes, which exhibit varying levels of longevity and differentially affect offspring fitness, coexist within the ejaculate of a single male. We used the externally fertilizing zebrafish *Danio rerio* for a series of experiments using in vitro fertilizations (IVF) in which we selected on sperm phenotypes based on their longevity. Zebrafish gametes activate upon contact with water, and IVF allows precise control over the activation and fertilization of gametes as well as gamete numbers. Selection on sperm longevity was performed by experimentally manipulating the timing between sperm activation and fertilization. We divided the ejaculate of a male and the eggs of a female into two cohorts each and exposed each sperm cohort to one of two treatments. Sperm were activated with water; then, in the "short activation time" (SAT) treatment, one of the sperm cohorts was immediately added to one of the egg cohorts. In the "long activation

Significance

Diploid organisms produce haploid gametes for sexual reproduction, resulting in a biphasic life cycle. Although selection during the diploid phase is well understood, selection during the haploid gametic stage and its consequences are largely ignored despite its potential importance for fundamental evolutionary processes, including the rate of adaptation and inbreeding depression, as well as for applied research into fertilization technology. A current dogma assumes that in animals selection on the haploid gametic genotype is minimal. We examined the importance of haploid selection in the zebrafish and found strong fitness consequences of selection on sperm phenotype in the resulting offspring. Genomic data support the idea that these effects may well be the consequence of selection on the haploid sperm genotype.

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Data deposition: The sequencing data from sperm samples and finclip reported in this paper have been deposited in the European Nucleotide Archive (accession no. PRJEB21611). All data on offspring fitness have been deposited on Dryad (doi:10.5061/dryad.7248c).

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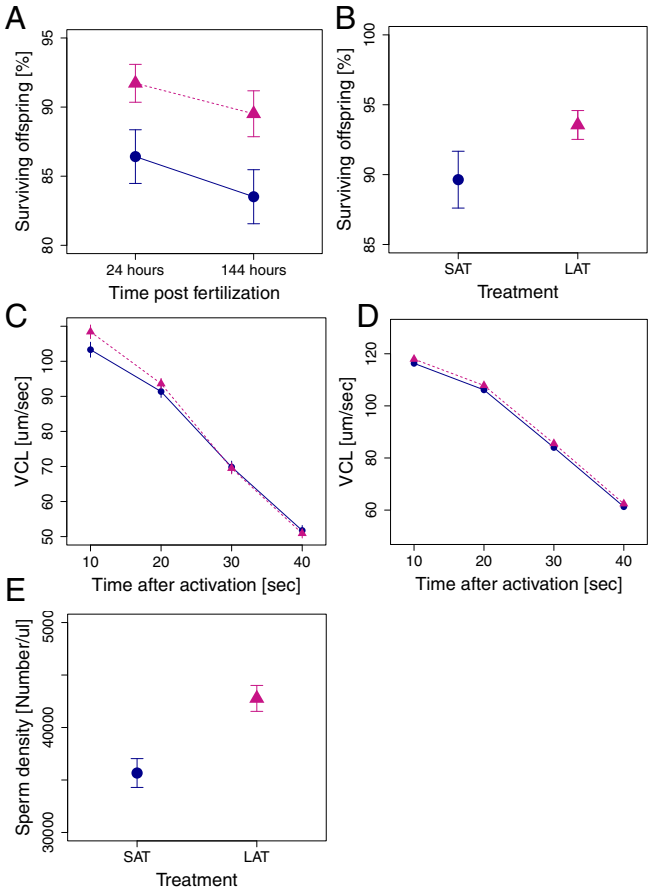

Fig. 1. Effect of sperm selection on offspring survival and sperm traits. Selection on sperm longevity results in an increase in offspring survival (*A* and *B*) and in increased sperm swimming VCL (*C* and *D*) and sperm density (*E*) in the resulting male offspring. Dark-blue circles and solid lines represent SAT; violet-red triangles and dotted lines represent LAT. Mean values ± SEs are shown.

time" (LAT) treatment, the sperm in the other cohort were held until about 50% were no longer motile, and then the sperm were added to the second egg cohort. Thus, the LAT treatment directly selected against short-lived sperm. So that the fertilization opportunity was equal in the two treatments, we doubled the amount of sperm present in the LAT treatment to compensate for the nonmotile sperm (see *SI Materials and Methods* for more details). To avoid any effect of egg aging, eggs were used within 1 min after collection, and previously activated sperm from both treatments were added to each of the two egg clutches at the same moment.

Our first aim was to describe any association between variation in sperm longevity and offspring fitness and to estimate its importance (experiment 1 A and B). Using the split design described above, we performed IVF and measured fitness traits of the resulting offspring from early development to adulthood. In experiment 1A, we measured sperm longevity for every male and calculated the time until 50% of sperm were no longer motile. We evaluated the effect of sperm selection on early offspring survival in 57 families and found that offspring sired by LAT sperm exhibited a 7% increase in survival compared with offspring sired by SAT sperm (treatment: $\chi^2_1 = 15.93$; $P < 0.0001$, time: $\chi^2_1 = 6.24$, $P = 0.012$) (Fig. 1*A*). Moreover, when measuring sperm swimming velocity in one to three sons from each of 35 families ($n = 108$ sons), we observed that sons sired by LAT sperm produced significantly faster-swimming sperm than their brothers sired by SAT sperm (about 5 μm/s faster in LAT males at 10 s postactivation) [curvilinear velocity (VCL): treatment: $\chi^2_1 = 14.55$, $P = 0.00013$; time: $\chi^2_1 = 189.71$, $P < 0.0001$; time^2: $\chi^2_1 = 145.89$, $P < 0.0001$; treatment × time: $\chi^2_1 = 29.66$, $P < 0.0001$; treatment × time^2: $\chi^2_1 = 11.50$, $P = 0.0007$] (Fig. 1*C*).

We repeated this experiment with a different selection protocol to verify the robustness of our results and to perform further fitness assays on offspring. Because the variation in sperm longevity among males observed in experiment 1A was relatively small, we simplified our protocol to standardize the time postactivation in the LAT treatment to 25 s; this change resulted in a reduction of motile sperm to ∼50% in the LAT treatment (experiment 1B). Using a total of 39 families, we found that offspring from the LAT treatment exhibited a 5% increase in viability at 24 h postfertilization (hpf) (treatment: $\chi^2_1 = 30.86$, $P < 0.0001$) (Fig. 1*B*), supporting our original findings. We measured sperm from three to five sons from each of 34 families in the two treatments ($n = 264$ sons). Sons resulting from the LAT treatment produced ejaculates that again exhibited

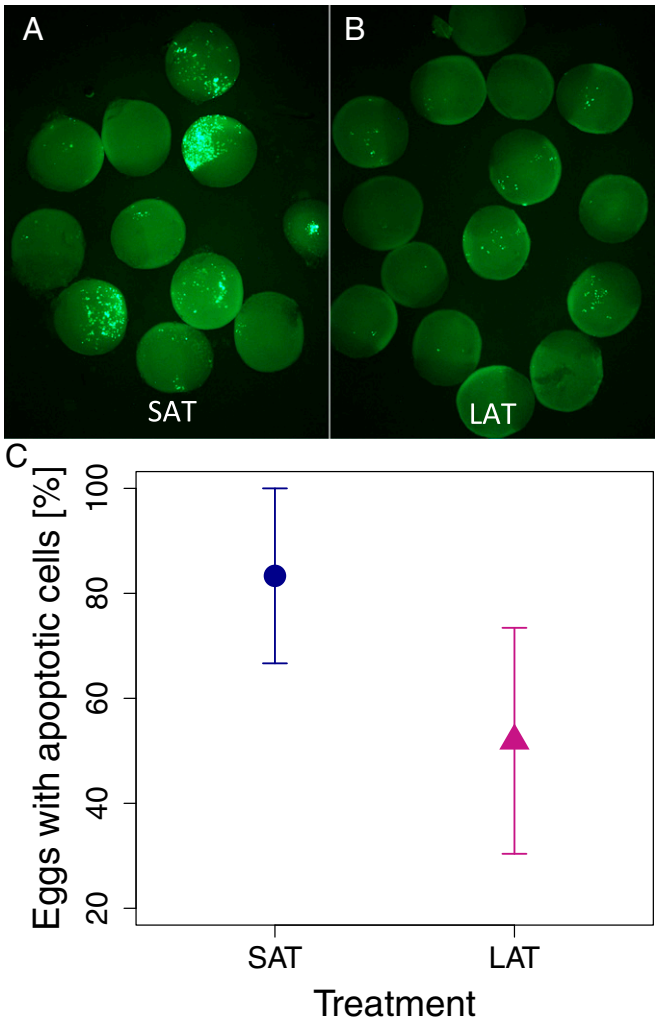

Fig. 2. Differential cell apoptosis at 8 hpf in response to sperm selection. (*A* and *B*) Examples of different levels of apoptosis in eggs fertilized by SAT sperm (*A*) and by LAT sperm (*B*) resulting from differential numbers of apoptotic cells, which are marked with green fluorescent dye. (*C*) Significantly more eggs exhibited signs of cell apoptosis when fertilized by SAT sperm (dark-blue circle) than when fertilized by LAT sperm (violet-red triangle). Mean values ± SEs are shown.

faster-swimming sperm (on average about 3 μm faster than in males resulting from the SAT treatment) (treatment: χ^2_1 = 3.93, P = 0.047; time: χ^2_1 = 370.79, P < 0.0001; time^2: χ^2_1 = 678.18, P < 0.0001; time^3: χ^2_1 = 456.38, P < 0.0001) (Fig. 1*D*) and a 20% higher sperm density (χ^2_1 = 728.8, P < 0.0001) (Fig. 1*E*) than their SAT brothers.

To understand further how embryo development may be affected by sperm selection during the first 24 hpf, we examined the occurrence of apoptotic cells (15), which are a potential indicator of embryonic fitness (16, 17), in embryos at the age of 8 hpf (experiment 4) (Fig. 2 *A* and *B*). We used a total of six pairs to perform split IVFs as described for experiment 1. More eggs exhibited apoptotic cells when fertilized by SAT sperm than when fertilized by LAT sperm (treatment: χ^2_1 = 6.56, P = 0.010) (Fig. 2*C*). This difference may reflect either a general increase in apoptosis in SAT embryos or a shift in the timing of apoptosis events as part of normal development.

We then measured the reproductive success of adult male and female offspring resulting from experiment 1B by assessing number and quality of offspring resulting from natural matings with nonexperimental fish by setting up pairs comprising one experimental fish and one nonexperimental fish of the opposite sex. Here we used a total of 26 families and two to four offspring of either sex in each family in both treatments (n = 202 offspring). We found no difference between LAT and SAT females in fertilization success (χ^2_1 = 1.77, P = 0.18) (Fig. 3*A*) or in the total number of eggs produced (χ^2_1 = 2.40, P = 0.12) (Fig. 3*B*). However, fertilization success was higher in LAT males by about 4% (χ^2_1 = 31.87, P < 0.0001) (Fig. 3*A*), and females mated to LAT males produced about 20% more eggs (fertilized and unfertilized) than females mated to SAT males (χ^2_1 = 17.19, P < 0.0001) (Fig. 3*B*). The difference in fertility in females mated to experimental males is likely induced by behavioral patterns based on sperm numbers available. In zebrafish, pairs spawn in bouts initiated by the male; females release several (5–20) eggs during each bout until the female has run out of eggs or the male stops courting (18). Furthermore, we found a higher survival rate in offspring of LAT females (treatment: χ^2_1 = 4.43, P = 0.035) (Fig. 3*C*) but not males (treatment: χ^2_1 = 0.28, P = 0.60) and a higher percentage of normal embryos among the offspring from matings between LAT offspring of both sexes and nonexperimental fish than among matings between SAT offspring of both sexes and nonexperimental fish (treatment: χ^2_1 = 129.82, P < 0.0001; sex: χ^2_1 = 20.00, P < 0.0001) (Fig. 3*D*). Our results show that fitness traits were strongly affected by sperm selection not only in the immediate offspring but also in the F2 generation when crossed with nonexperimental fish. The finding of sex differences in fitness effects—that LAT males have a clear fitness advantage, producing more offspring than SAT males, but LAT females do not have this advantage over SAT females—is intriguing and may provide a possible explanation for the maintenance of variation in sperm phenotypes.

Our LAT treatment resulted in a decrease in fertilization success by about 5% despite doubling the amount of sperm in experiments 1 A and B (Fig. S1; see *SI Materials and Methods* for details), but this difference in fertilization success is unlikely to cause the fitness differences observed between the two treatments for two reasons. First, decreasing fertilization success and resulting potential selection among eggs for sperm did not have any fitness effects on offspring in a similar setup (14). Second, in our outcrosses between experimental fish and wild-type fish, the fertilization success was about 4% higher in the LAT treatment than in the SAT treatment in males (Fig. 3*A*), i.e., opposite the pattern found in experiment 1 A and B. However, the survival rate was again higher in the offspring from LAT fish than from SAT fish (Fig. 3*C*). We therefore conclude that fertilization success has no impact on our results.

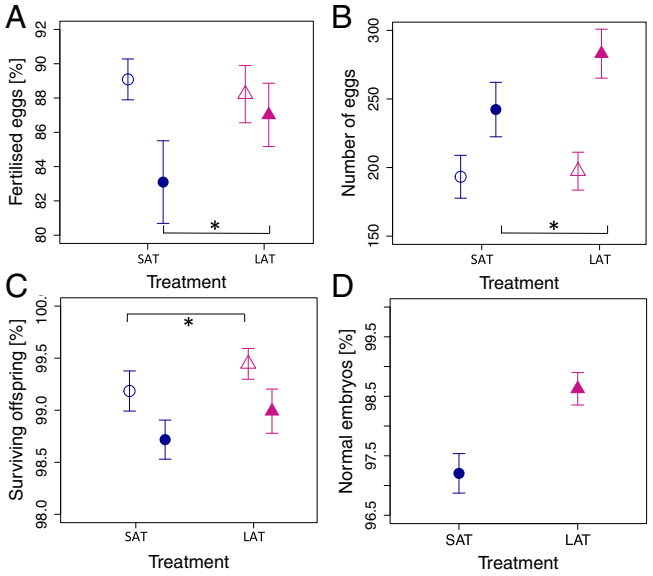

Fig. 3. The effect of sperm selection on reproductive success in male and female offspring. Although there was no difference in fertilization success or in the number of eggs in female offspring (*A* and *B*), males from LAT treatments fertilized more eggs (*A*), and females produced more eggs in matings with such males (*B*). (*C* and *D*) The resulting offspring were more viable in LAT females than in SAT females, whereas the difference between LAT and SAT males was not significant (*C*), and offspring of both sexes exhibited a higher percentage of normal embryos (*D*). Mean values ± SEs are shown. In *A*–*C* results are shown for males and females separately; empty symbols indicate females, filled symbols indicate males, dark-blue circles indicate SAT progeny, and violet-red triangles indicate LAT progeny; significant differences between treatments are indicated by a connecting bar; *P < 0.0001 (see Tables S1–S3 for statistics with both sexes included). In *D*, results for sexes are combined.

An immediate question arising from these results is whether the effects observed in experiment 1 A and B are the result of the selection of sperm cohorts differing in phenotype or are the result of sperm aging. Sperm aging, before or after ejaculation, may affect the epigenetic composition of the sperm (19) and/or the quality of sperm DNA by inducing deleterious mutations (20, 21) that may, in turn, affect the development of the resulting zygote. For experiment 2, we investigated the possibility of preejaculation sperm aging by collecting consecutive sperm subsamples, each containing 0.8 μL of ejaculate, until the male had no more sperm; thus the first subsample contained the oldest sperm, and the last sample contained the youngest (see *SI Materials and Methods* for details). This procedure resulted in a maximum of three subsamples per male from 11 different males. We found no evidence that the subsample identity and hence preejaculation sperm age had any differential effect on offspring viability (subset effect: χ^2_1 = 0.063, P = 0.97). In many animals, males continuously release unused sperm, apparently to avoid preejaculation sperm aging (22–24), and this continuous release may explain the lack of an effect.

To test for a possible postejaculation sperm-aging effect on offspring performance, we reduced the osmotic stress on sperm by increasing the ratio of Hank's balanced salt solution (HBSS) to water during sperm activation before fertilization to extend sperm lifespan (experiment 3). We divided the ejaculate of a male and the clutch of eggs of a female (n = 23 pairs) and performed IVF as described above but activated the sperm 25 s or 50 s before fertilization. Offspring viability during the first 24 hpf did not differ between the treatments (25 s: dead = 6.6% ± 0.5 SD; 50 s: dead = 6.3% ± 0.6 SD; treatment: χ^2_1 = 1.36, P = 0.24). Thus,

postejaculation sperm aging has no impact on offspring performance in this system, and the increase in the viability of offspring sired by LAT sperm is not a result of sperm aging. We conclude that our experimental protocol allows selection on sperm cohorts within an ejaculate that differ in fertilization success and longevity. This conclusion is further supported by the observation that selection for long-lived sperm resulted in increased offspring fitness in every trait that we examined, the opposite of what would be predicted if degradation arising from sperm aging had occurred.

A possible mechanism underlying the observed differences between LAT and SAT treatments is a trade-off between sperm swimming speed and sperm longevity (25). Swimming speed is assumed to play a major role in fertilization success in external fertilizers (26), and if such a trade-off occurred in our system the fast, short-lived sperm could fertilize eggs in our SAT treatment, whereas the slow, longer-lived sperm could fertilize eggs in our LAT treatment. This assumption also would imply that slower, longer-lived sperm sire offspring with increased fitness. However, when looking for such a trade-off within the ejaculates of six males, we found no evidence for any significant association between these two traits when tracking individual sperm over time (Fig. S2; see *SI Materials and Methods* for details). Therefore an alternative and more likely scenario, independent of swimming speed, is that SAT offspring may be sired by both short-lived and long-lived sperm, whereas LAT offspring are sired only by long-lived sperm. Of course, the possibility that other traits may determine variation in fertilization success among sib sperm needs to be explored carefully.

An open question is whether within-ejaculate sperm variation is based on genetic mechanisms. To test for a genetic difference between haploid sperm phenotypes, we performed in vitro assays to separate sperm within an ejaculate according to their ability to survive and cover a certain distance throughout their motile phase. We then examined allele frequencies at heterozygous paternal sites throughout the genome, comparing the separated pools in three different males. We placed a sample of the ejaculate of one male in the center of a 280-μL water droplet harbored in a concave microscope slide. The droplet was framed with a concentrated glucose solution to provide a dilution gradient attracting sperm toward the edges of the droplet (27). Upon contact with water, sperm were activated and dispersed within the water droplet, and longer-lived sperm were expected to reach the outer edge of the water droplet more frequently than short-lived sperm. Although this selection regime is not identical to the selection regime in the experiments described above, we know that longer-lived sperm cover longer distances (see Fig. S3 and *SI Materials and Methods* for details), and hence sperm collected from the outer edges of the droplet will show phenotypic overlap with LAT sperm (Fig. S3). Sperm pools collected from the center will contain a mix of all sperm, including some nonmotile sperm, which would sire no offspring in our SAT treatment. The center and outer pools, each containing many thousands of sperm, as well as an untreated sperm pool and a finclip from each of the three males, were subjected to whole-genome sequencing to ~60× coverage after a PCR-free library preparation to reduce bias in allelic ratios (Table S4). We mapped reads to the *D. rerio* Zv9 reference assembly, determined heterozygous paternal sites using reads from finclips, and then conducted statistical tests of sperm pool allele frequencies at these paternal sites using 400-kbp half-overlapping windows throughout the genome of each male. We checked for two possible sources of allele frequency bias. First, we checked for allele transmission bias from male to sperm by comparing allele counts in finclip reads and untreated sperm pool reads using allele-frequency likelihood ratio tests (LRTs) (28). Second, we checked for handling bias possibly introduced by the in vitro gradient assay by comparing allele counts in untreated sperm pool reads and reads from the center-selected sperm pool using LRTs (28). We found no systematic evidence of transmission bias (Fig. S4) or handling bias (Fig. S5) for two males; the read pool for unselected sperm from a third male (male 32) had an undetermined technical error and could not be used.

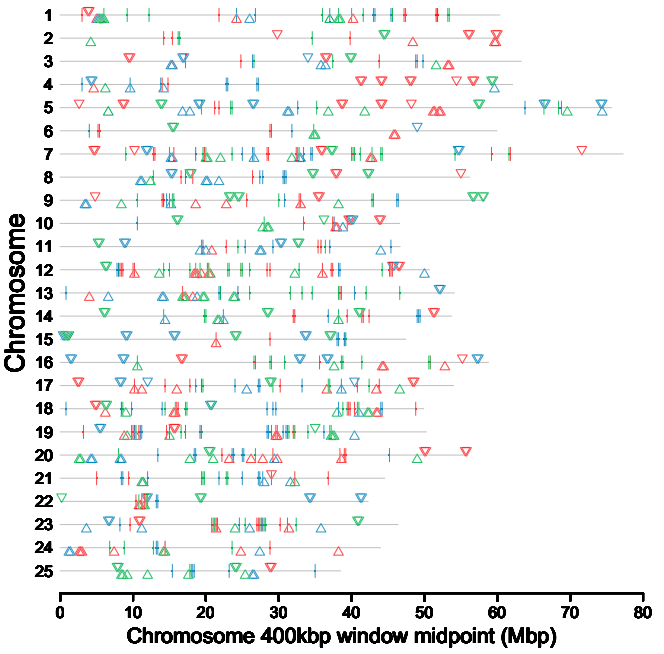

Fig. 4. Genetic differences between selected sperm pools from three males. Each symbol aggregates allele frequency comparisons at heterozygous sites within half-overlapping 400-kbp windows containing at least one site/10 kbp and shows windows in which the given test value is within the 99% quantile of its distribution for each male. Upward-pointing triangles indicate allele frequency assessed by LRT. Downward-pointing triangles indicate binomial tests showing skewed and opposed allele frequencies in selected sperm pools, via binomial LOD scores. Vertical lines indicate binomial tests showing skewed allele frequencies in either the central or outer selected sperm pools, via binomial LOD scores. Color indicates male identity: red, male 31; blue, male 32; green, male 34.

We then tested for differences in allele frequency by comparing the allele counts in the reads from the two selected sperm pools (center and outer), using allele-frequency LRTs (28) with critical values set empirically as equal to or greater than the 99% quantile of the distribution of likelihood ratios for each male. We supplemented the LRTs with tests of significant allele-frequency skews using logarithm of the odds (LOD) scores calculated from binomial probabilities of allele counts in reads from each selected pool. Binomial tests could result in no allele-frequency skew in either pool, in a skew (binomial probability <0.01) in one pool only, or in opposed skews, i.e., alleles A and a being most frequent in different pools. As for LRTs, critical values for binomial tests were set empirically at equal to or greater than the 99% quantile of the LOD scores for each male (see *SI Materials and Methods* for further methodological details). In contrast to the bias checks, we found differences in allele frequency between selected sperm pools throughout the genome (Fig. 4 and Fig. S6), although there was considerable variation among males and between tests. One male (male 31) showed consistently elevated LRTs in the long arm of chromosome 4 (Fig. S6), which is unusually repeat-rich (29) and did not feature in either of its bias comparisons (Figs. S4 and S5). We will not speculate on the functional basis of these results with respect to specific genes or genomic regions at this point, because we need a stronger dataset with more males to support such

speculation. Nevertheless, there is clear evidence that genetic variation accompanies the phenotypic variation among selected sperm pools and that this variation is not the result of transmission or handling biases.

We provide clear evidence that variation in sperm produced by the same male in a single ejaculate has pronounced effects on several fitness-related traits throughout life and that this variation has a genetic basis. Selection on sperm within the ejaculate results in reduced occurrence of apoptotic cells during early development, more viable embryos, and more fit adult offspring. The sequenced sperm pools further suggest a link between sperm phenotype and sperm genotype. Such a link may have several nonmutually exclusive causes, and one possible explanation is variation in epistatic interactions and hence additive genetic effects of the different sperm haplotypes. This hypothesis provides a particularly plausible scenario for the variation in sites diverging among the sperm pools of the three males. Regardless of the exact genetic underpinning of our observations, our findings are likely to have major implications for key evolutionary processes including the rate of adaptation (30), the evolution of a sexually dimorphic recombination rate (31, 32), the load of deleterious mutations (33), and the extent of inbreeding depression (34). They also may account for hitherto unexplained patterns of non-Mendelian inheritance (35) and apparent discrepancies in observed mutation rates (36). In addition, our findings provide insights that are crucial for clinical and agricultural assisted-fertilization techniques such as IVF and intracellular sperm injection (ICSI). These techniques omit many if not all naturally occurring steps of within-ejaculate sperm selection, and the consequences of such omission need to be understood (1, 37). Future research therefore should focus on the consequences of gametic selection in a broad variety of taxa with both external and internal fertilization.

Materials and Methods

All experiments described here were performed in accordance with the guidelines and approved by the Swedish Board of Agriculture (Jordbruksverket approval number C341/11). For a detailed description of materials and methods, please see *SI Materials and Methods*.

In a first step we performed IVF experiments using the zebrafish *D. rerio* in which we split the male ejaculate and the female clutch of eggs into two halves. We exposed sperm to one of two treatments differing in the time from sperm activation to fertilization: SAT, 0 s; LAT, ~25 s. We repeated this experiment twice using slightly different selection criteria for SAT and LAT. We monitored offspring fitness by assessing differences in cell apoptosis during early developmental stages, embryo survival, sperm swimming velocity, sperm density, and reproductive success.

We tested for preejaculation sperm aging by collecting three successive sperm samples from each male with the first sample containing the oldest sperm and the third containing the youngest sperm and tested for post-ejaculation sperm aging by delaying the time between sperm activation and fertilization by 25 s or 50 s. Using IVF and a split-clutch design we tested for differences in embryo survival. Finally, we selected sperm for their swimming phenotype by placing them in a droplet surrounded by a glucose ring to let them swim toward the edges. We collected sperm from the center and the edges of the droplet and sequenced sperm collected from each site, sperm from an unselected droplet, and a finclip from each male.

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