Natural Variation and Genetic Determinants of *Caenorhabditis elegans* Sperm Size

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ABSTRACT The diversity in sperm shape and size represents a powerful paradigm to understand how selection drives the evolutionary diversification of cell morphology. Experimental work on the sperm biology of the male-hermaphrodite nematode *Caenorhabditis elegans* has elucidated diverse factors important for sperm fertilization success, including the competitive superiority of larger sperm. Yet despite extensive research, the molecular mechanisms regulating *C. elegans* sperm size and the genetic basis underlying natural variation in sperm size remain unknown. To address these questions, we quantified male sperm size variation of a worldwide panel of 97 genetically distinct *C. elegans* strains, allowing us to uncover significant genetic variation in male sperm size. Aiming to characterize the molecular genetic basis of *C. elegans* male sperm size variation using a genome-wide association study, we did not detect any significant quantitative trait loci. We therefore focused on the genetic analysis of pronounced sperm size differences observed between recently diverged laboratory strains (N2 *vs.* LSJ1/2). Using mutants and quantitative complementation tests, we demonstrate that variation in the gene *nurf-1* underlies the evolution of small sperm in the LSJ lineage. Given the previous discovery that this same *nurf-1* variation was central for hermaphrodite laboratory adaptation, the evolution of reduced male sperm size in LSJ strains likely reflects a pleiotropic consequence. Together, our results provide a comprehensive quantification of natural variation in *C. elegans* sperm size and first insights into the genetic determinants of *Caenorhabditis* sperm size, pointing at an involvement of the NURF chromatin remodeling complex.

KEYWORDS Androdioecy; *Caenorhabditis*; male function; *nurf-1*; NURF chromatin remodeling complex; sperm competition; sperm dimorphism

SPERM morphology can show extreme variation and is often associated with variation in competitive ability and thus male reproductive success (Smith 1984; Birkhead and Moller 1998; Snook 2005; Birkhead *et al.* 2009; Pitnick *et al.* 2009; Ramm *et al.* 2014) . Furthermore, disparities of specific sperm traits, such as cell size or flagellum length, are not only common among species, but also within species (Pitnick *et al.* 2009). Numerous studies focusing on intraspecific variation, through comparison of sperm traits across populations or by using artificial selection on sperm traits, have uncovered extensive levels of heritable variation in diverse sperm characteristics (Ward 1998; Morrow and Gage 2001a; Joly *et al.* 2004; Pitnick *et al.* 2009; Simmons and Moore 2009). Despite such studies on diverse invertebrate and vertebrate taxa, the quantitative and molecular genetic architecture of sperm traits associated with competitive ability remain largely undescribed. Therefore, although the developmental genetics of spermatogenesis has been elucidated in great detail from model organisms, such as the fly *Drosophila melanogaster* (Demarco *et al.* 2014) or the nematode *Caenorhabditis elegans* (Ellis and Stanfield 2014), it is largely unknown whether uncovered genes are also a substrate for evolution to affect intraspecific variation in sperm characteristics relevant for competitive ability.

Here we aimed to quantify and characterize intraspecific genetic variation of a well-defined sperm trait, cell size, known

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to modulate sperm competitive ability in *C. elegans*. In this androdioecious (male-hermaphrodite) species, both males and hermaphrodites produce sperm, so that hermaphrodites can either self-fertilize or outcross with males. *C. elegans* shows a pronounced sperm size dimorphism: male sperm are larger and consistently outcompete smaller hermaphrodite sperm when both types of sperm are present in the hermaphrodite reproductive tract (Ward and Carrel 1979; LaMunyon and Ward 1995). Male sperm size is also critical when multiple males compete for fertilization, with larger sperm often outcompeting smaller sperm (LaMunyon and Ward 1998; Murray *et al.* 2011). In addition, increased levels of male-male competition in experimental contexts can lead to the evolution of larger male sperm size (LaMunyon and Ward 2002; Palopoli *et al.* 2015), consistent with the relevance of sperm size for male competitive ability. Although self-fertilization is the predominant mode of *C. elegans* reproduction, with rare occurrence of males and outcrossing events in natural populations (Jovelin *et al.* 2003; Barrière and Félix 2005; Sivasundar and Hey 2005), ample natural variation in diverse male traits exists (Hodgkin and Doniach 1997; Teotónio *et al.* 2006; Palopoli *et al.* 2008, 2015; Morran *et al.* 2009; Anderson *et al.* 2010; Noble *et al.* 2015; Alcorn *et al.* 2016), including male sperm size (Ward and Carrel 1979; LaMunyon and Ward 1995, 1998, 1999, 2002; Murray *et al.* 2011; Palopoli *et al.* 2015). Moreover, gonochoristic (male-female) *Caenorhabditis* species exhibit, on average, much larger male sperm than the three androdioecious species, *C. briggsae*, *C. elegans*, and *C. tropicalis*, in which male-male competition is much weaker (LaMunyon and Ward 1999; Vielle *et al.* 2016).

While the size of amoeboid *Caenorhabditis* sperm can be a critical factor for sperm competitive ability, recent work has uncovered crucial roles of genetic factors in *Caenorhabditis* sperm competition that act independently of sperm size (Thomas *et al.* 2012; Ting *et al.* 2014, 2018; Fierst *et al.* 2015; Hansen *et al.* 2015; Yin *et al.* 2018; Yin and Haag 2019). Most prominently, genome shrinkage observed in the three androdioecious *Caenorhabditis* species involves a strong bias in the loss of male-expressed genes, including the parallel loss of the male secreted short (*mss*) gene family, which is critical for sperm competitive ability in gonochoristic species (Thomas *et al.* 2012; Fierst *et al.* 2015; Yin *et al.* 2018). Hence, not only sperm size, but also diverse cellular and genetic components jointly determine sperm competitive ability in *C. elegans* and other *Caenorhabditis* species. One question emerging from this updated view on *C. elegans* sperm competition is thus whether natural variation in *C. elegans* sperm size is indeed closely linked to variation in male fertilization success and male-male competitive ability.

Another large gap in our understanding of *C. elegans* sperm competition is the absence of information on the molecular genetic determinants of sperm size and its natural variation. Although the genetic regulation of spermatogenesis has been elucidated in great detail (L'Hernault 2006; Geldziler *et al.* 2011; Chu and Shakes 2013; Ellis and Stanfield 2014), no specific genes regulating *C. elegans* sperm size have so far been identified. Spermatogenesis of *C. elegans* hermaphrodites and males seem essentially identical: meiosis is initiated by the formation of primary spermatocytes, followed by two rapid, mostly symmetrical divisions resulting in four haploid spermatids and an anucleate residual body (Ward *et al.* 1981; Shakes *et al.* 2009; Vielle *et al.* 2016). The cell size of the primary spermatocyte is a key determinant of final spermatid size (Vielle *et al.* 2016). Therefore, sperm size seems to be mostly determined prior to or at the time of primary spermatocyte formation. However, it remains unknown how genetic factors contribute to this process. In addition, it is unclear to what extent the differential presence or activity of such potential genes explain reported differences in sperm size across sexes, genotypes, or species.

In this study, we therefore focused on the characterization of natural variation and its genetic basis in *C. elegans* male sperm size, using a worldwide collection of nearly 100 wild isolates (Andersen *et al.* 2012; Cook *et al.* 2017). First, we tested how well observed natural variation in male sperm size correlates with variation in male reproductive performance, as well as with morphological traits of both males and hermaphrodites, such as body size and hermaphrodite sperm size. Second, we aimed to characterize the molecular genetic basis of intraspecific variation in *C. elegans* male sperm, by performing a genome-wide association study and an in-depth genetic analysis of recently diverged laboratory strains that display strong sperm size differences.

Materials and Methods

Strains and culture conditions

All strains were maintained at 20° on 2.5% agar Nematode Growth Medium (NGM) plates seeded with the *Escherichia coli* strain OP50 (Stiernagle 2006). The following strains/genotypes were used in this study: 95 *C. elegans* wild isolates (Supplemental Material, Table S1) (Andersen *et al.* 2012; Cook *et al.* 2017) and laboratory strains N2, LSJ1, LSJ2, CX12311(*kyIR1* V, CB4856 > N2; *qqIR1* X, CB4856 > N2), CX13248 (*kyIR84* II, LSJ2 > N2), *nurf-1(n4295)* (MT13649), *isw-1(n3294)* (MT17795), *isw-1(n3297)* (MT16012), *pyp-1(n4599)* IV/*nT1 [qIs51]* (MT14910), PD4790, and *C. plicata* (SB355). Males homozygous for *pyp-1* die during larval stages and were thus scored as heterozygotes. Hermaphrodites of the mutant *fog-2(q71)* (strain CB4108) do not produce any self-sperm, *i.e.*, they are effectively females (Schedl and Kimble 1988), which were used for certain mating assays. The strain PD4790 contains an integrated transgene [*mls12* (*myo-2*::GFP, *pes-10*::GFP, *F22B7.9*::GFP)] in the N2 reference genetic background, expressing green fluorescent protein (GFP) in the pharynx. Additional information on wild isolates is available from *C. elegans* Natural Diversity Resource (CeNDR; http://elegansvariation.org) (Cook *et al.* 2017)

Measurements of male and hermaphrodite sperm size

Males were collected from strain cultures at the L4 stage to be maintained on NGM plates containing only males to measure their spermatid size at stage L4 + 24 hr from synchronized and unmated males. Hermaphrodite spermatids were dissected from young virgin adults (at around mid-L4 + 24 hr), at which stage most individuals contained both spermatids and activated sperm (spermatozoa), the latter of which were excluded from analysis. To measure sperm size, male or hermaphrodite spermatids were released into sperm medium (50 mM HEPES, pH 7.8, 50 mM NaCl, 25 mM KCl, 5 mM $CaCl_2$, 1 mM $MgSO_4$, 1 mg/ml BSA) by needle dissection (Nelson and Ward 1980). Images of the spermatids were captured using Nomarski optics (×60 or ×63 objectives). ImageJ software (Rasband, 1997–2014) was used to calculate length and width of each spermatid to obtain measures of cross-sectional area assuming an ellipse shape: $\pi \times (\text{length}/2) \times (\text{width}/2)$ (Vielle *et al.* 2016).

Male mating ability

All mating assays (Figure 3 and Figure 4) were performed on mating plates (35 mm diameter NGM plates seeded with a spot of 20 μl OP50) using unmated males, *fog-2* females, or hermaphrodites that had been isolated at the L4 larval stage 24 hr (hermaphrodites and females) or 36 hr (males) prior to the assay. Following established protocols (Wegewitz *et al.* 2008; Murray *et al.* 2011), 10 virgin *fog-2* females were transferred onto a mating plate and allowed to roam for 30 min on the bacterial lawn. Next, a single male was added to the plate and allowed to mate for 8 hr, after which it was discarded. Females were left on the mating plate for an additional hour and were then picked as single animals onto fresh NGM plates. Females were scored as not fertilized if they did not contain any embryos in the uterus 24 hr later. Offspring production of fertilized females was followed over four consecutive days.

Male sperm number transferred during single insemination (ejaculate size)

We quantified the number of sperm transferred during single insemination events (Figure 2H and Figure S1, E–H). For each strain to be tested (N2, CB4856, LSJ1, JU561, CX11285, EG4946, JU393, and JU782), unmated *fog-2* females were individually mated with an excess of 20–30 males, aged 36 hr post-L4 larval stage, to increase the chance of mating. Mating was monitored every few minutes by observation through the stereoscope. When a male was engaged in mating, it was kept under constant surveillance for spicule insertion and visualization of sperm flow from the male vas deferens to the female uterus, until mating was completed. Immediately after the end of mating, the inseminated female was isolated and fixed in ice-cold methanol and the mated male was removed from the male pool. A new virgin female was then mated with the males. Next, fixed females were washed twice in M9 and mounted in DAPI-containing Vectashield (Vector Laboratories, Burlingame, CA). Sperm number was counted on images taken at ×40 magnification as Z-stacks covering the entire thickness of the gonad using an Olympus BX61 microscope with a CoolSnap HQ2 camera (Poullet *et al.* 2015, 2016).

Hermaphrodite-male sperm competition

We measured variation in competition between hermaphrodite and male sperm by measuring male fertilization success of the eight strains when mated to hermaphrodites of a tester strain (the wild isolate CB4856) (Figure 4A). L4 hermaphrodites of the CB4856 strain were isolated 24 hr prior to mating, and L4 males of the eight strains were isolated 36 hr prior to mating. On the next day, one single CB4856 adult hermaphrodite was mated with an excess of 20–30 males for each strain to be tested and kept under surveillance for single mating as described above. As soon as mating was completed, the male was discarded from the male pool and the hermaphrodite was isolated onto a fresh NGM plate. Offspring (and male) production was scored for 3 days after the mating assay (*i.e.*, until completion of the reproductive span).

Male-male sperm competition

We measured second-male sperm precedence of the eight *C. elegans* strains using a tester strain expressing GFP in the pharynx (PD4790), following previously used protocols (Murray *et al.* 2011) (Figure 2, E–G). *fog-2* females were first mated with PD4970 males, then with males of the eight strains. Males and *fog-2* females were isolated at the L4 stage and maintained in isolation for 36 hr prior to mating assays. Each mating plate ($N = 20$) was established by adding 10 *fog-2* females and 20 PD4790 males, which were allowed to mate for 15 hr, so that all females were fertilized (confirmed by the presence of embryos in the uterus). Ten fertilized *fog-2* females were then randomly allocated to each new mating plate and allowed to mate with 20 males of each of the eight strains examined. Plates were kept under surveillance for single mating as described above. Upon completion of a mating event, both male and female were removed, and offspring production of the female was observed for the next 4 days. Total offspring were counted using a regular stereoscope and GFP-expressing offspring were counted with a fluorescence stereoscope.

Quantification of hermaphrodite self-sperm number

We quantified the number of self-sperm in synchronized young adult hermaphrodites, *i.e.*, adults containing one or two embryos in their uterus (Figure 5C). Animals were fixed overnight in ice-cold methanol (−20°), washed three times in 1× PBS containing 0.05% Tween and mounted in Vectashield (Vector Laboratories) supplemented with DAPI. Sperm images were acquired from adults containing oocytes to ensure that the sperm to oocyte transition had occurred. Imaging of the anterior spermatheca was performed with an Olympus BX61 microscope using a ×63 objective with epifluorescence. Z-sections (1 μm) of the entire spermatheca were taken and sperm number counted (cell counter plugin in ImageJ) (Poullet *et al.* 2015, 2016)

Body size measurements

Synchronized populations were used to isolate unmated males in the mid-L4 stage and were scored 24 hr later. Hermaphrodites were scored as early adults when they contained between one and two embryos in the uterus. Animals were then anesthetized in sodium azide on an agar pad and whole-animal images were captured immediately after under Nomarski optics (×20). Body length and width were measured with the ImageJ software and body volume was calculated as that of a cylinder ($\pi \times (\text{width}/2)^2 \times \text{length}$).

Primary spermatocyte measurements

Extruded gonads from unmated males at 24 hr post-L4 were obtained by dissection in levamisole-containing M9. Gonads were fixed in 4% paraformaldehyde for 10 min and permeabilized for 5 min in 1× PBS with 0.1% Triton X-100. Gonads were next stained for actin with phalloidin (1:500 dilution; Sigma-Aldrich, St. Louis, MO) overnight at 4° in a humidified chamber. Slides were mounted in Vectashield supplemented with DAPI (Vector Laboratories) and observed under an epifluorescence microscope. Primary spermatocyte area was measured by outlining cell boundaries using ImageJ software (Vielle *et al.* 2016).

RNA interference experiments

RNA interference (RNAi) by bacterial feeding for *C. elegans* (N2) and *C. plicata* (SB355) was performed as previously described (Timmons and Fire 1998; Kamath *et al.* 2003). Briefly, control RNAi (HT115) and *nurf-1* clone (provided by the Ahringer laboratory) were seeded on standard NGM with 50 μg/ml of ampicillin and 1 mM of IPTG and grown at room temperature for at least 24 hr before experiment. Worms were fed RNAi and control bacteria from the L1 stage and spermatid size was measured in the early adult stage (L4 + 24 hr).

Genome-wide association mapping

Genome-wide association mapping was performed using phenotype data from 97 *C. elegans* isotypes (Table S2). We used the *cegwas* R package for association mapping (Cook *et al.* 2017). This package uses the EMMA algorithm for performing association mapping and correcting for population structure (Kang *et al.* 2008), which is implemented by the *GWAS* function in the *rrBLUP* package (Endelman 2011). Specifically, the *GWAS* function in the *rrBLUP* package was called with the following command: *rrBLUP*::*GWAS(pheno = ph, geno = y, K = kin, min.MAF = 0.05, n.core = 1, P3D = FALSE, plot = FALSE)*. The kinship matrix used for association mapping was generated using a whole-genome high-quality single-nucleotide variant (SNV) set from CeNDR release 20160408 (Cook *et al.* 2016; Evans *et al.* 2017; Zdraljevic *et al.* 2017) and the *A.mat* function from the *rrBLUP* package. SNVs previously identified using Restriction site–associated DNA sequencing (Andersen *et al.* 2012) that had at least 5% minor allele frequency in this strain set were used for performing genome-wide association mappings. Burden test analyses were performed using RVtests (Zhan *et al.* 2016) and the variable-threshold method (Price *et al.* 2010). We called SNVs using bcftools (Li 2011) with settings previously described (Cook *et al.* 2016, 2017; Zhan *et al.* 2016). We next performed imputation using BEAGLE v4.1 (Cook *et al.* 2017) with *window* set to 8000, *overlap* set to 3000, and *ne* set to 17,500. Within RVtests, we set the minor allele frequency range from 0.003 to 0.05 for burden testing.

Statistical analyses

Statistical tests were performed using R, JMP, or SPSS. Data for parametric tests were transformed where necessary to meet the assumptions of ANOVA procedures (homogeneity of variances and normal distributions of residuals); all size data were log-transformed. For *post hoc* comparisons, Tukey's honestly significant difference procedure was used. For data, where ANOVA assumptions could not be met, we used nonparametric tests (*e.g.*, Kruskal–Wallis).

Broad-sense heritability (H^2) was estimated using the *lmer* function in the lme4 package (Bates *et al.* 2015) with the linear mixed model (phenotype $\sim 1 + (1|\text{strain})$. H^2 was then calculated as the fraction of the total variance explained by the random component (strain) of the mixed model.

Data availability

All raw data are provided in Additional File 1. The authors affirm that all data necessary for confirming the conclusions of the article are present within the article, figures, and tables. Supplemental material available at FigShare: https://doi.org/10.25386/genetics.9389255.

Results

Natural variation in C. elegans male sperm size

We quantified male sperm size variation of a worldwide collection of 97 *C. elegans* strains (Andersen *et al.* 2012), including two related laboratory strains (N2 and LSJ1), using measures of spermatid cross-sectional area. Average male sperm size, ranging from 15 to 27 μm^2, varied significantly across strains (Figure 1 and Table S2). 90% of strains exhibited a male sperm size between 20 and 25 μm^2, and we detected only two significant outliers: the wild strain JU561 (France) and the laboratory-adapted strain LSJ1 (McGrath *et al.* 2011) with the smallest male sperm size (Figure 1, A and B). As found previously for *C. elegans* and other *Caenorhabditis* species (Vielle *et al.* 2016), we also detected high levels of interindividual and intraindividual variation in male sperm size for most strains (Figure 1, A and C).

Coefficients of variation (CV), *i.e.*, the ratio of the SD to the mean, in sperm characters have been predicted and shown to be lower in species or genotypes experiencing higher levels of sperm competition (Gomendio *et al.* 2006; Calhim *et al.* 2007; Immler *et al.* 2008; Kleven *et al.* 2008; Fitzpatrick and Baer 2011). Therefore, we tested whether *C. elegans*

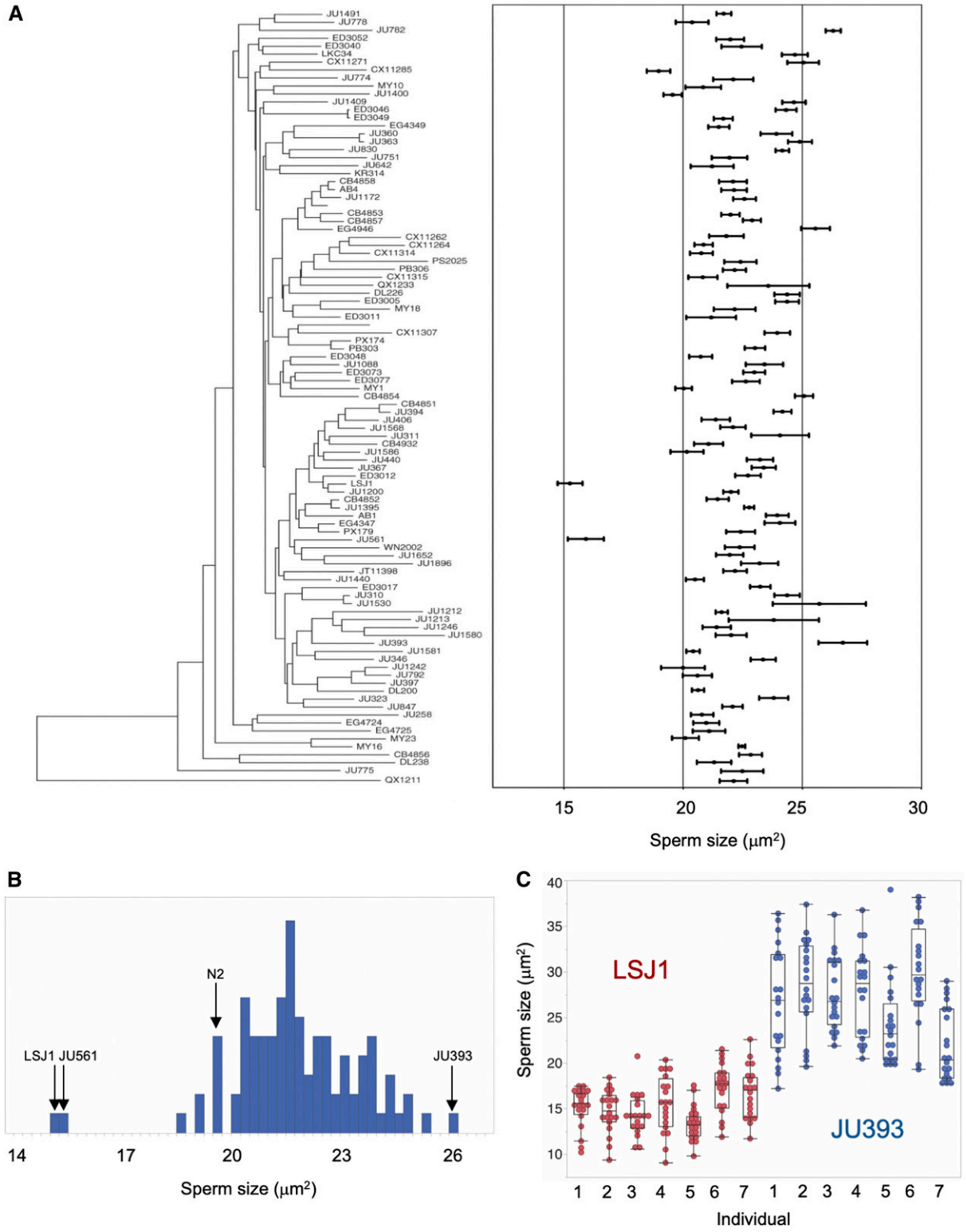

Figure 1 Natural variation in *C. elegans* male sperm size. (A) Quantification of male spermatid cross-sectional area (mean ± SEM) in 97 *C. elegans* strains, arranged with respect to the neighbor-joining tree of Andersen *et al.* (2012) based on genome-wide SNP data. There is significant genetic (and interindividual) variation in sperm size (ANOVA, effect *strain*: $F_{96,\ 13539} = 24.75$, $P < 0.0001$; effect *individual(strain)*: $F_{580,\ 13539} = 3.08$, $P < 0.0001$). Twenty spermatids from each of seven individuals were measured per strain ($N = 140$) with the exception of strains JU397 and KR413 for which 20 spermatids from each of six individuals ($N = 120$) were measured (Table S2). (B) Histogram of sperm size across strains (least-squares mean estimates) shows the significant outlier trait values for the two strains (JU561 and LSJ1) with smallest male sperm. (C) Illustration of inter- and intraindividual variation in male sperm size for strains with smallest (LSJ1) *vs.* largest male sperm size (JU393) ($N = 20$ sperm per individual).

strains with larger male sperm showed reduced variability. However, we did not detect a negative correlation between mean and CV of within-individual ($\rho_{Pearson} = 0.14$, $P = 0.17$, $N = 97$) or between-individual ($\rho_{Pearson} = -0.09$, $P = 0.36$, $N = 97$) male sperm size (Table S2), as expected under such a scenario.

Natural variation in C. elegans male sperm size: testing for covariation with male reproductive performance, hermaphrodite sperm size, and body size

Several studies have shown that sperm size may explain differences in male reproductive success and competitive ability among *C. elegans* wild isolates (LaMunyon and Ward 1998; Wegewitz *et al.* 2008; Murray *et al.* 2011). Using assays similar to those described in these previous studies, we tested whether the observed natural variation in *C. elegans* male sperm size correlates with variation in male mating ability, fertilization success, and male competitive ability, using eight strains with divergent male sperm size (Figure 2, A–G and Figure S1). Although we found significant heritable variation in all measured phenotypes, *C. elegans* male sperm size did not significantly correlate with any of them. In addition, the polymorphic plugging phenotype of males, *i.e.*, the deposition of a gelatinous plug on the vulva after copulation, likely representing a trait of male competitive ability as it affects mating ability of subsequent males (Barker 1994; Hodgkin and Doniach 1997; Palopoli *et al.* 2008), did not depend on sperm size as the average male sperm size did not differ between plugging *vs.* nonplugging strains (ANOVA, $F_{1,96} = 0.09$, $P = 0.77$) (Table S3).

These results imply that additional sperm characteristics or other morphological and behavioral traits need to be considered to account for natural variation in male competitive ability and overall male reproductive performance. Consistent with this idea, we found that sperm transfer during a single mating (ejaculate size) (Figure S1, E–H) rather than sperm size shows a strong positive correlation with male fertility when mated to hermaphrodites (Figure 2H).

An unresolved question is whether genetic mechanisms regulating *C. elegans* sperm size are shared between the sexes. Previously, a weak positive correlation between the average sperm size of hermaphrodites and males was only found in *C. tropicalis* but not in *C. elegans* or *C. briggsae* (Vielle *et al.* 2016). This analysis was based on a small set of strains ($n = 5$), so we revisited this question using 12 strains differing in male sperm size. In agreement with previous reports (Baldi *et al.* 2011; Vielle *et al.* 2016), hermaphrodite sperm showed significant genetic variation and were substantially smaller than male sperm in all strains (Figure 3A and Table S4). Again, as in Vielle *et al.* (2016), there was no significant cross-sexual correlation in sperm size (Figure 3B). Given the presence of significant natural variation in *C. elegans* hermaphrodite sperm size, we tested whether this variation correlates with differential sperm production, which could be indicative of a potential trade-off between hermaphrodite sperm size and sperm number. Across eight strains hermaphrodite sperm production differed significantly (ANOVA, $F_{7,184} = 5.37$, $P < 0.0001$) (Table S5), but we found no correlation between hermaphrodite sperm size and number (Figure 3C).

Finally, we tested whether natural variation in *C. elegans* sperm size may reflect fixed allometric relationships between body and cell size. Such positive correlations between sperm size (length or cell size) and animal body size or mass have been frequently observed in diverse invertebrate taxa (Pitnick *et al.* 2009). Size of amoeboid sperm of nematodes, including *Caenorhabditis*, partly correlates with male body size across species (LaMunyon and Ward 1999; Vielle *et al.* 2016). In contrast, whether intraspecific variation in body size is linked to variation in sperm size in *C. elegans* had so far not been evaluated. Measuring early adult body size of hermaphrodites and males in a subset of strains, we found significant variation across strains and sex (Table S6); however, we did not detect any positive correlation between average sperm size and body size (length) in either sex (Figure S2, A and B). In males only, sperm size was correlated with body width ($F_{1,10} = 16.86$, $R^2 = 0.65$, $P = 0.0027$) (Figure S2, C and D). A larger male body width could potentially allow for larger gonad width, thus allowing production of larger spermatocytes. On the other hand, male body width may also simply increase as consequence of storing of larger sperm. Further experiments are thus required to consolidate the evidence for a positive scaling relationship between male body size and sperm size.

Genome-wide association mapping of male sperm size

The significant variation in male sperm size enabled the mapping of genomic regions that could underlie this variation using genome-wide association studies, as has been performed successfully for a variety of traits using this *C. elegans* isolate panel (Andersen *et al.* 2012; Ghosh *et al.* 2012; Ashe *et al.* 2013). Broad-sense heritability was low (~14%) for both sperm size cross-sectional area and diameter, and we found no significant genomic regions for these two traits and additional sperm size traits, including CV measurements (Figure 4). This result suggests that many loci could regulate differences in sperm size. Additionally, we used rare-variant based burden testing (Price *et al.* 2010; Bates *et al.* 2015; Zhan *et al.* 2016) to look for association of genes affected by deleterious rare variants with sperm area and diameter. As with marker-based association testing, we did not identify any significant genomic regions (data not shown).

Because specific natural niches could drive mating preferences, we investigated any effect of geography (*e.g.*, latitude/longitude of strain origin) (Table S1, CeNDR: https://www.elegansvariation.org) on average male sperm size but found no such relationships (Spearman rank correlations, all $P > 0.05$).

Laboratory-derived strains LSJ1 and LSJ2 exhibit strongly reduced male and hermaphrodite sperm size

Across all 97 *C. elegans* strains measured, LSJ1 exhibited the smallest male sperm size (Figure 1A). LSJ1 is a

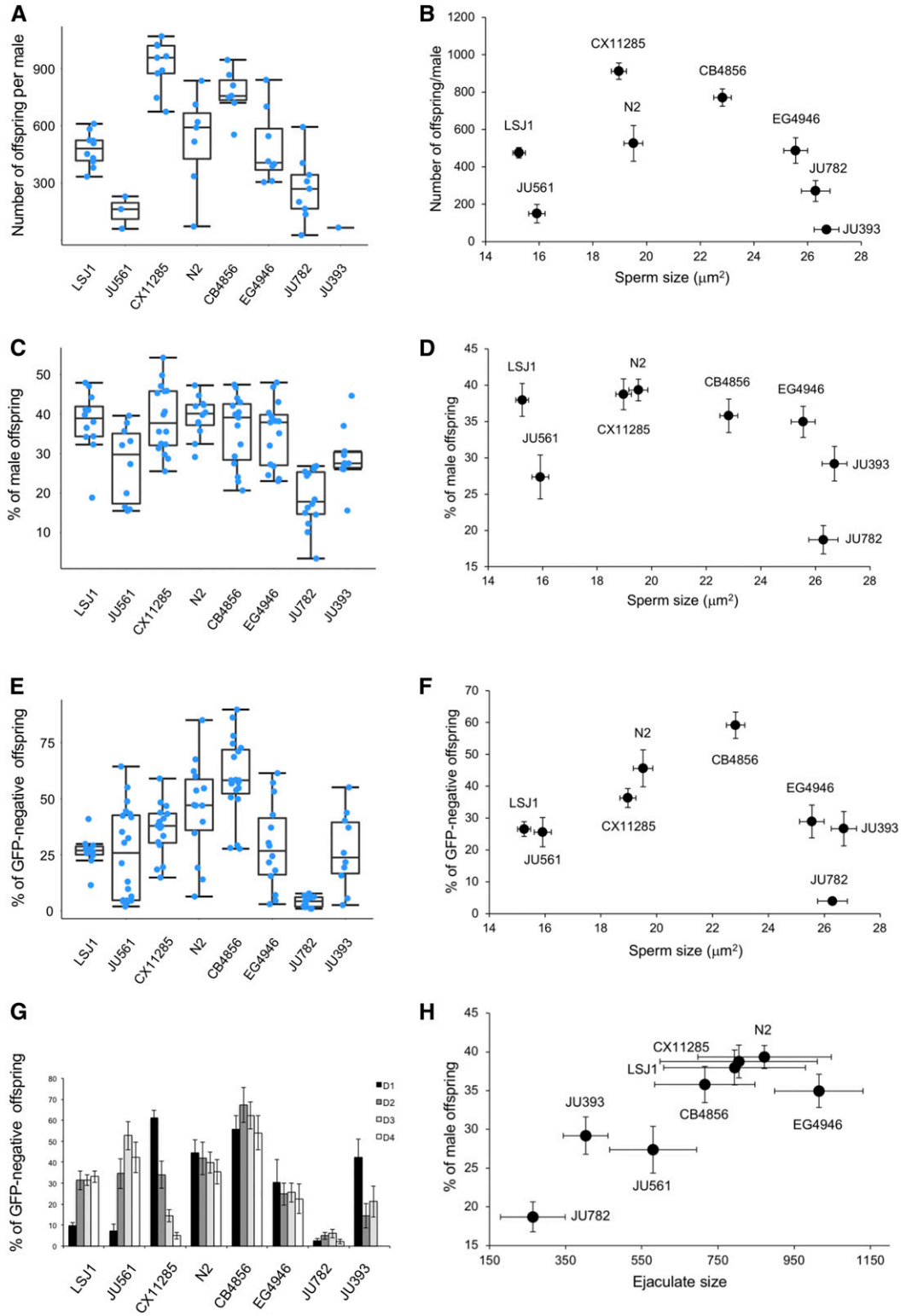

Figure 2 Covariation of male sperm size with male reproductive performance. Male reproductive performance in eight *C. elegans* strains with different average male sperm size. Strains with smallest (LSJ1) to largest (JU393) male sperm are arranged from left to right. (A) Significant strain variation in the number of offspring sired by a single male during 8 hr of mating with up to 10 fog-2 females (Kruskal–Wallis, $\chi^2 = 37.78$, df = 7, $P < 0.0001$) and (B) absence of correlation with average male sperm size ($\rho_{Spearman} = -0.29$, $P = 0.49$). (C) Significant strain variation in male fertilization success when competing with hermaphrodite self-sperm (strain CB4856) (ANOVA, $F_{7,103} = 11.20$, $P < 0.0001$) and (D) absence of correlation between male fertilization success and male sperm size ($\rho_{Spearman} = -0.45$, $P = 0.45$). (E) Significant strain variation in male-male competitive ability (in fertilization success) sperm (ANOVA, $F_{7,113} = 14{,}66$, $P < 0.0001$). Male competitive ability of a given strain (*vs.* the GFP-positive strain PD4790) quantified by the proportion of GFP-negative offspring produced over 4 days after mating. (F) No correlation between competitive ability and male sperm size

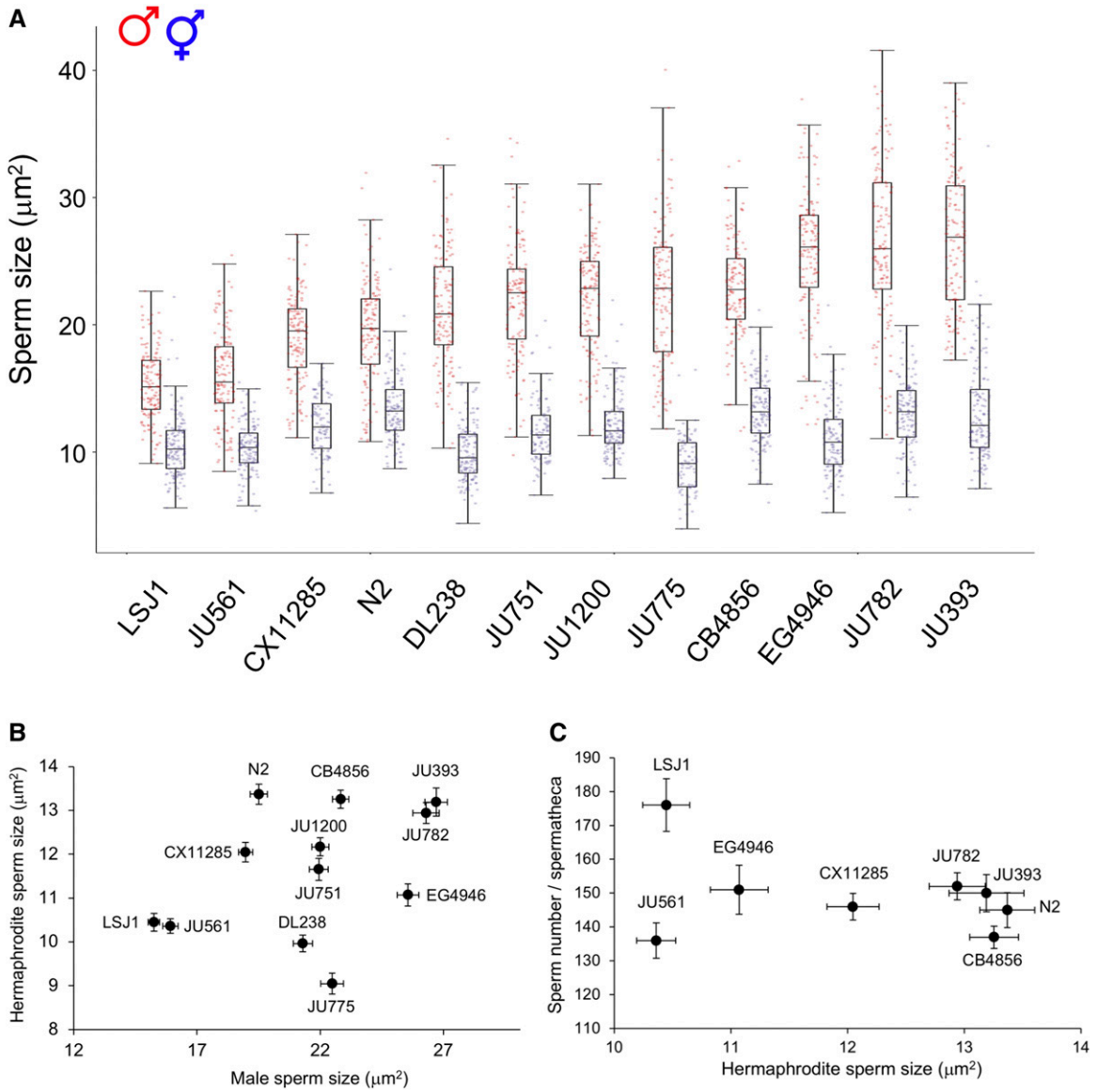

Figure 3 No correlation of *C. elegans* male and hermaphrodite sperm size across strains. (A) Hermaphrodite sperm size across 12 strains. Strains with smallest (LSJ1) to largest (JU393) male sperm are arranged from left to right. Effect of strain genotype and sex on *C. elegans* sperm size (ANOVA, effect *sex*: $F_{1,3207} = 6267.55$, $P < 0.0001$; effect *strain:* $F_{11,\ 3207} = 107.14$, $P < 0.0001$; interaction *sex* × *strain*: $F_{11,\ 3207} = 50.90$, $P < 0.0001$). For each strain, 87–152 hermaphrodite spermatids from 7 to 13 individuals were measured (male sperm size data are the same as shown in Figure 1). (B) Absence of significant correlation between male and hermaphrodite sperm size across 12 strains, inferred from least-squares regression of strain mean values ($F_{1,\ 11} = 1.38$, $R^2 = 0.11$, $P = 0.27$). (C) No correlation between average hermaphrodite sperm size and sperm number across eight strains ($F_{1,\ 7} = 0.59$, $R^2 = 0.10$, $P = 0.43$). Hermaphrodite self-sperm number were established by measuring all sperm contained within a single spermatheca of 12–31 individuals per strain.

laboratory-derived strain and shares a common ancestor with the reference strain N2 (Sterken *et al.* 2015) (Figure 5A), which shows a much larger male sperm size (Figure 1A and Figure 5B). The two lineages diverged between 1957 and 1958. N2 was then maintained on agar plates seeded with *E. coli* for ~15 years, while LSJ1 was maintained in axenic liquid culture for close to 40 years before cultures were cryopreserved. LSJ2 is a derivative of LSJ1 that was kept in liquid culture for another 14 years prior to freezing (McGrath *et al.* 2009, 2011; Sterken *et al.* 2015; Large *et al.* 2017). We therefore also measured LSJ2, which displayed a similarly small sperm size as LSJ1 (Figure 5B). In contrast, the N2-derived

($\rho_{Spearman} = 0.00$, $P = 1$). (G) Details of time course of progeny production across the 4 days after mating event (same data as in E). (H) Ejaculate size, as measured by the number of sperm deposited by one male in a single mating (same data as in Figure S1E), and male fertilization success (C) show a significant correlation ($\rho_{Spearman} = 0.74$, $P = 0.036$). Sample sizes: n = 10–20 per strain per experiment. Box and whiskers plots: boxes are delimited by the data's first and third quartiles, broken by a band at the median, and flanked by whiskers of which length is equal to 1.5 × the interquartile range.

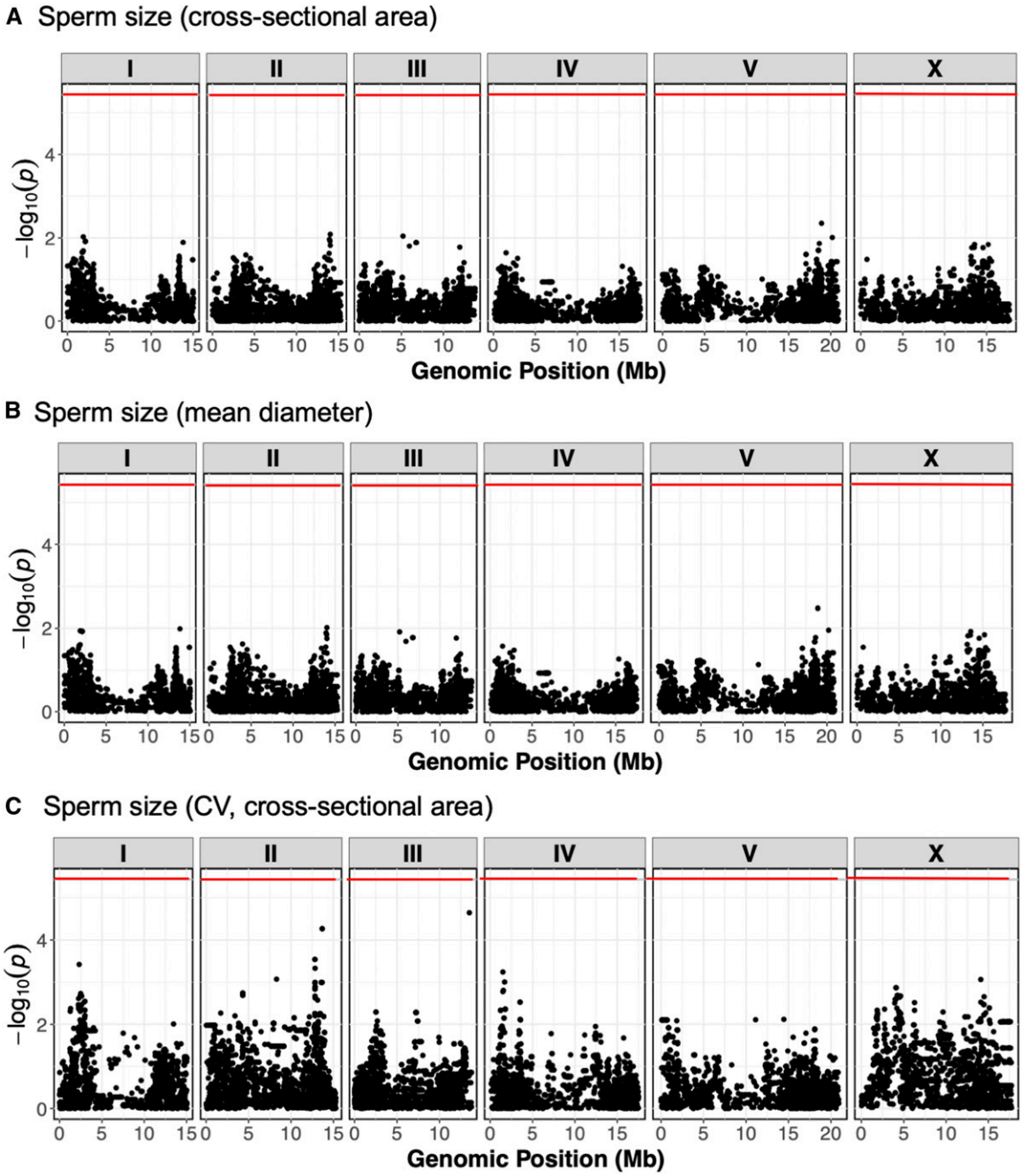

Figure 4 Genome-wide association mapping for *C. elegans* male sperm size. Manhattan plots of single-marker based GWA mappings show no significant genomic regions for least-squares mean estimates (LSM) of (A) sperm cross-sectional area, (B) sperm mean diameter and (C) coefficient of variation (CV) (sperm cross-sectional area). Each dot represents an SNV that is present in at least 5% of the assayed population. The genomic location of each SNV is plotted on the *x*-axis, and the statistical significance is plotted on the *y*-axis. The Bonferroni-corrected significance threshold is shown as a red horizontal line.

strain CC1, grown in liquid axenic medium for only 4 years, did not differ from N2 in male sperm size (Figure 5B). Given the common, inbred, and likely isogenic "Bristol ancestor" of LSJ and N2-CC1 lineages, these results suggest that the evolution of reduced male sperm size in the LSJ lineage occurred due to *de novo* mutations before 1995. Of note, the LSJ1 and LSJ2 strains also showed significantly reduced hermaphrodite sperm size relative to N2 (and CC1) (Figure 5C).

Reduced male sperm size of LSJ strains is caused by genetic variation in nurf-1

Whole-genome short-read sequencing identified 188 and 94 new mutations fixed in the LSJ2 and N2 lineages,

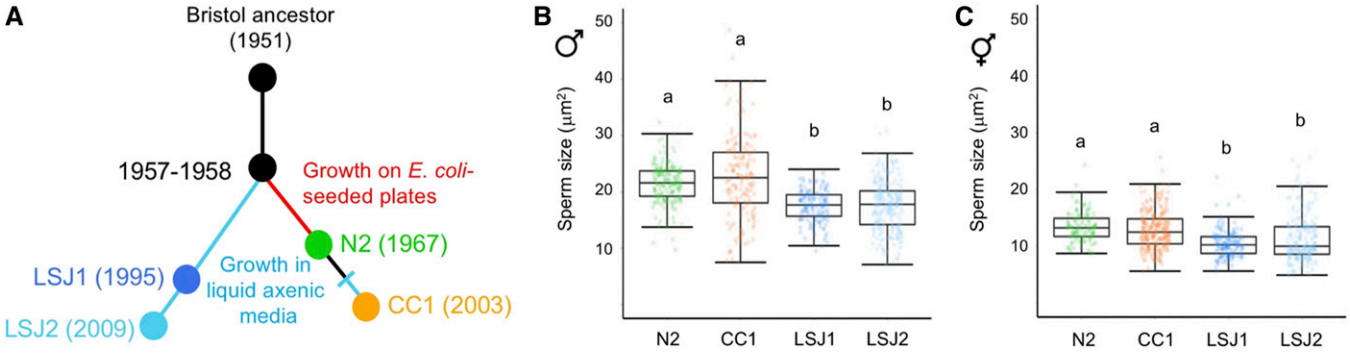

Figure 5 LSJ1 and LSJ2 strains exhibit strongly reduced male and hermaphrodite sperm size. (A) Laboratory evolution of LSJ and N2 lineages in the laboratory, after isolation of the common ancestral strain "Bristol," derived from a single hermaphrodite individual, in 1951. LSJ1 and LSJ2 were cultivated in axenic liquid medium and N2 was cultivated on agar plates [after McGrath *et al.* (2011)]. (B) Male sperm size: LSJ1 and LSJ2 exhibit significantly reduced male sperm size compared to N2 and CC1 (ANOVA, effect *strain*: $F_{3,\ 658} = 65.62$, $P < 0.0001$). (C) Hermaphrodite sperm size: LSJ1 and LSJ2 exhibit significantly reduced hermaphrodite sperm size compared to N2 and CC1 (ANOVA, effect *strain*: $F_{3,\ 666} = 40.95$, $P < 0.0001$). For male sperm measurements, 135–225 sperm were analyzed from 9 to 15 individuals of each strain. For hermaphrodite sperm measurements, 123–248 sperm were analyzed from 9 to 19 individuals of each strain. Values with the same letter are not significantly different from each other (Tukey's honestly significant difference, $P < 0.05$). Box and whiskers plots: boxes are delimited by the data's first and third quartiles, broken by a band at the median, and flanked by whiskers of which length is equal to 1.5 × the interquartile range.

respectively (McGrath *et al.* 2011). These mutations include a 60 bp deletion in the 3′ end of *nurf-1*, which encodes the ortholog of the BPTF subunit of the NURF chromatin remodeling complex (Andersen *et al.* 2006; McGrath *et al.* 2011; Large *et al.* 2016). This variant is predicted to replace the last 16 amino acids of the protein with 11 novel residues and is known to underlie multiple life history differences between N2 and LSJ2, including reproductive timing, progeny production, growth rate, life span, and Dauer formation (Large *et al.* 2016, 2017). Moreover, the NURF complex had previously been shown to function in the *C. briggsae* sperm-oocyte decision (Chen *et al.* 2014) as well as *Drosophila* spermatogenesis (Kwon *et al.* 2009). We thus reasoned that the *nurf-1* deletion specific to the LSJ lineage provides a good candidate explaining reduced male (and hermaphrodite) sperm size. This hypothesis was supported by the observation that RNAi knockdown of *nurf-1* resulted in a significant reduction of male sperm size in the N2 strain (Figure S3). To further test our hypothesis, we first examined the introgression line CX13248 (*kyIR87*) containing the LSJ2 region surrounding *nurf-1* in an N2-like background (CX12311, which is of N2 genotype, except for introgressed *npr-1* and *glb-5* alleles from the strain CB4856) (McGrath *et al.* 2011). The *kyIR87* introgression contains the 60 bp deletion along with LSJ2 alleles of eight additional variants, including an SNV in the intron of *nurf-1* that was fixed in the N2 lineage (Large *et al.* 2016). Consistent with our hypothesis, sperm size of the CX13248 strain containing the *nurf-1* deletion was significantly smaller compared to CX12311, both in males (Figure 6A) and hermaphrodites (Figure 6B). In addition, sperm size of the *nurf-1(n4295)* deletion mutant (N2 background) (Andersen *et al.* 2006) was also strongly reduced in both sexes (Figure 6, C and D); therefore, strains containing two different deletions in the 3′ coding region of *nurf-1* result in reduced *C. elegans* sperm size. Specifically, the 60 bp *nurf-1* deletion of LSJ strains covers the 3′ coding region (plus stop codon and 8 bp of the 3′ UTR region), and the *nurf-1*(*n4295*) deletion spans 1078 bp of the 3′ coding region (Large *et al.* 2016). Interestingly, the 60 bp and *n4295* deletions differentially affect NURF-1.B and NURF-1.D, two of the four main isoforms of NURF-1, described to play antagonistic roles in the regulation of gametogenesis: while NURF-1.B is involved in sperm production, NURF-1.D is necessary for the switch to oogenesis (Xu *et al.* 2019). The *nurf-1*(*n4295*) mutation affects three exons found only in the 3′-terminal region of the *nurf-1.d* transcript. In contrast, the 60 bp deletion covers not only the very end of the 3′ coding region, but also the STOP codon and 8 bp of the 3′ UTR, present in both *nurf-1.b* and *nurf-1.d* transcripts (Xu *et al.* 2019). Our finding that sperm size is reduced in both *n4295* mutant and LSJ strains thus suggests that NURF-D or both NURF-D and NURF-B affect sperm size determination.

To test whether *nurf-1* is the causal gene underlying reduced sperm size in the LSJ lineage, we performed a quantitative complementation test (Long *et al.* 1996) taking advantage of the two *nurf-1* deletion alleles present in LSJ1 and *nurf-1(n4295)* (Figure 6E). The recessive phenotype caused by either *nurf-1* deletion was confirmed by the large, N2-like sperm size in F1 males derived from crosses between N2 and LSJ1 and between N2 and *nurf-1(n4295)* (Figure 6E). However, F1 males derived from bidirectional crosses between LSJ1 and *nurf-1(n4295)* exhibited small sperm size, comparable to parental strains (Figure 6E). We conclude that variation in the gene *nurf-1* underlies the evolution of reduced male sperm size in LSJ strains.

Collectively, our experiments suggest that the 60 bp deletion in *nurf-1* is the causal variant responsible for the decreased sperm size in males and hermaphrodites in the LSJ lineage. First, quantitative complementation indicates *nurf-1* to be the causal gene. Second, the LSJ1/LSJ2 strains are

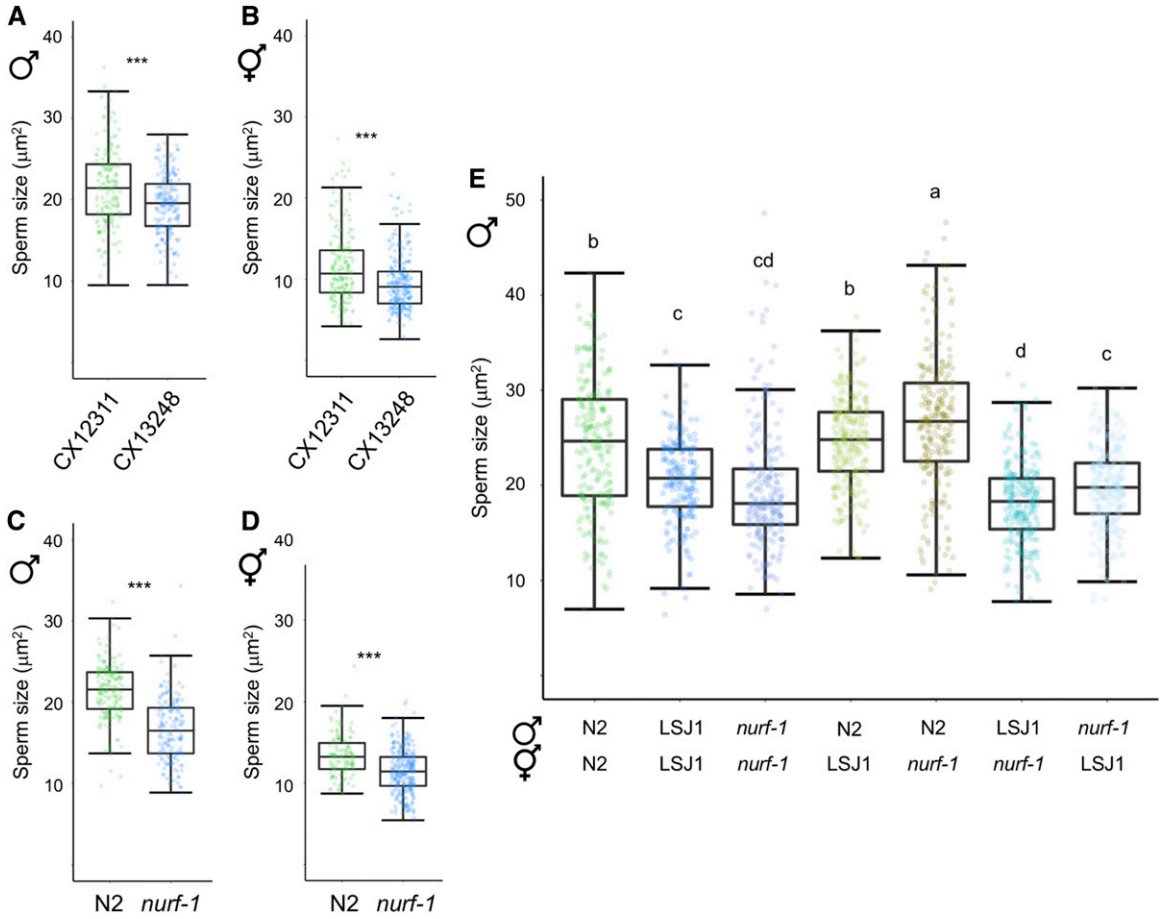

Figure 6 Reduced male sperm size of LSJ strains is caused by variation in *nurf-1*. (A and B) A near-isogenic line (CX13248) with the LS2 genomic region containing the *nurf-1* deletion exhibits reduced sperm size relative to the N2-like parent CX12311 in (A) males (ANOVA, $F_{1,\ 336} = 23.11$, $P < 0.0001$) and (B) hermaphrodites (ANOVA, $F_{1,\ 420} = 30.35$, $P < 0.0001$). (C and D) Sperm size of the deletion mutant *nurf-1(n4295)* is reduced in (C) males (ANOVA, $F_{1,\ 322} = 184.30$, $P < 0.0001$) and (D) hermaphrodites (ANOVA, $F_{1,\ 383} = 61.64$, $P < 0.0001$). (E) Quantitative complementation tests using the strains N2, LSJ1, and *nurf-1(n4295)* (ANOVA, effect *strain*: $F_{6,\ 1502} = 97.09$, $P < 0.0001$). Values with the same letter are not significantly different from each other (Tukey's honestly significant difference, $P < 0.05$). Box and whiskers plots: boxes are delimited by the data's first and third quartiles, broken by a band at the median, and flanked by whiskers of which length is equal to 1.5 × the interquartile range (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

outliers with regards to sperm size, suggesting that a mutation occurred in this lineage, like the 60 bp deletion, to reduce sperm size. Finally, the *n4295* allele, which phenocopies the LSJ strains, is genetically similar to the 60 bp deletion, affecting the C terminus of the protein. However, the intron SNV in *nurf-1*, derived in the N2 lineage, cannot be completely ruled out to explain observed sperm size differences.

Mutants of different NURF-complex components exhibit reduced sperm and spermatocyte size

Observed sperm size reduction caused by the two independent *nurf-1* deletions implies a potential role of the NURF chromatin remodeling complex (Figure 7A) in *C. elegans* sperm size determination. We therefore tested whether mutants of another complex member, the ATPase component ISW-1 (Figure 7B) (Tsukiyama *et al.* 1995; Andersen *et al.* 2006) show altered sperm size. Indeed, males of two independent deletion mutants of *isw-1* (Andersen *et al.* 2006) exhibited strongly reduced male sperm size, and even smaller than in *nurf-1(n4295)* (Figure 7B). (In addition, males heterozygous for *pyp-1(n4599)*, another member of the NURF complex, also made smaller sperm; data not shown.) Using the *isw-1(n3297)* allele (Andersen *et al.* 2006), we also found that hermaphrodite sperm size was significantly reduced compared to the N2 wild-type (Figure S4). Overall germline structure and organization of strains with small sperm size (including LSJ1 and LSJ2) appeared intact, except for a fraction of *isw-1* mutant individuals that displayed severe errors, such as displacement of spermatids into the distal region. Given that *Caenorhabditis* sperm size differences (between species, genotypes within species, and sexes within species) are developmentally established at the primary spermatocyte stage (Vielle *et al.* 2016), we measured primary male spermatocyte size in *isw-1(n3297)* animals with intact germline structure: primary spermatocyte size was on average significantly smaller than in the wild-type N2 strain (Figure 7, C–E). Size variation of *C. elegans* sperm observed in NURF-complex mutants was thus introduced prior to, or at, the primary

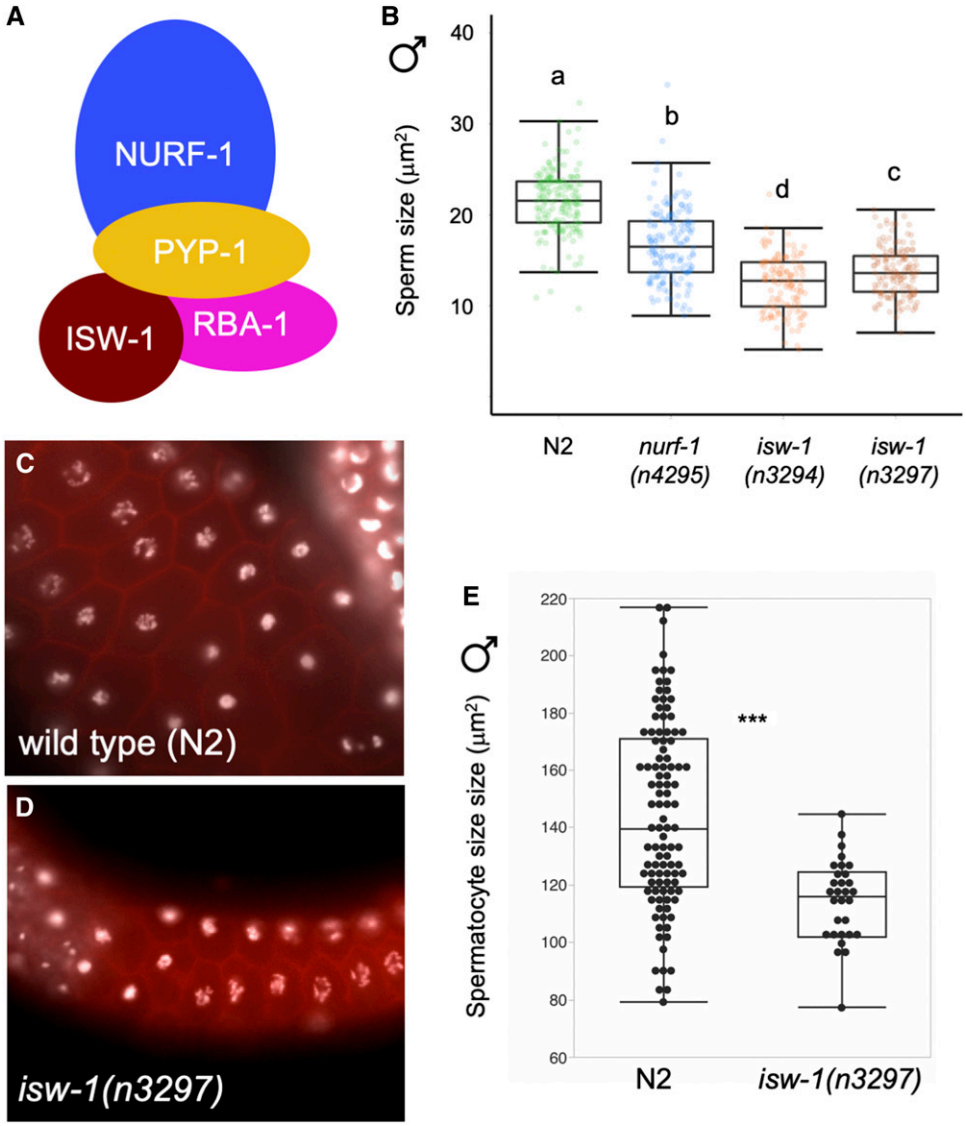

Figure 7 Mutants of the NURF complex exhibit reduced size of both male sperm and primary spermatocytes. (A) Illustration of NURF-complex components. Adapted from Alkhatib and Landry (2011). (B) *nurf-1* and *isw-1* mutants display reduced male sperm size (ANOVA, effect *strain:* $F_{4,\ 728} = 192.23$, $P < 0.0001$). (C and D) Microscopy images of primary spermatocytes in (C) wild type (N2) and (D) *isw-1(n3297)* (DAPI: white, Phalloidin: red). (E) Primary spermatocytes (area measurements) of the mutant *isw-1(n3297)* are significantly smaller than in the wild type (N2) (ANOVA, effect *strain*: $F_{1,\ 119} = 83.47$, $P < 0.0001$). Values with the same letter are not significantly different from each other (Tukey's honestly significant difference, $P < 0.05$). Box and whiskers plots: boxes are delimited by the data's first and third quartiles, broken by a band at the median, and flanked by whiskers of which length is equal to 1.5 × the interquartile range. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$)

spermatocyte stage, as observed for sperm size variation occurring within and between different *Caenorhabditis* species (Vielle *et al.* 2016). Taken together, these data suggest that the NURF chromatin remodeling complex likely acts, directly or indirectly, in *C. elegans* sperm size determination. Furthermore, the role of *nurf-1* in sperm size determination seems to be evolutionarily conserved because *nurf-1* RNAi also reduced sperm size of a gonochoristic *Caenorhabditis* species, *C. plicata*, which normally exhibits substantially larger sperm (Vielle *et al.* 2016) (Figure S5).

Male sperm size is reduced independently of body size in NURF-complex mutants

An earlier study has shown that the 60 bp deletion in *nurf-1* is responsible for reduced hermaphrodite body length in LSJ2, and similarly, the deletion allele *nurf-1(n4295)* was shown to exhibit a significantly reduced hermaphrodite body length relative to N2 (Large *et al.* 2016). We therefore hypothesized that perturbing activity of the NURF chromatin complex may cause systemic size reduction of diverse tissues and organs, including spermatids. Inconsistent with this hypothesis, we found male body size of *nurf-1(n4295)* with small sperm size to be significantly larger, rather than smaller than in the wild-type N2 strain (Figure 8); in addition, the *isw-1(n3294)* mutant with very small sperm had the same male body size as N2 (Figure 8). Male sperm size reduction in NURF mutants thus occurs independently of body size, suggesting that reduced male sperm size of LSJ strains is not necessarily a pleiotropic consequence of reduction in male body size.

Discussion

Our survey of intraspecific variation in *C. elegans* male sperm size uncovered significant heritable variation for this trait, generally thought to associate with sperm competitive ability. Yet, examining a subset of strains with divergent male sperm size, we did not detect a strong effect of male sperm size on

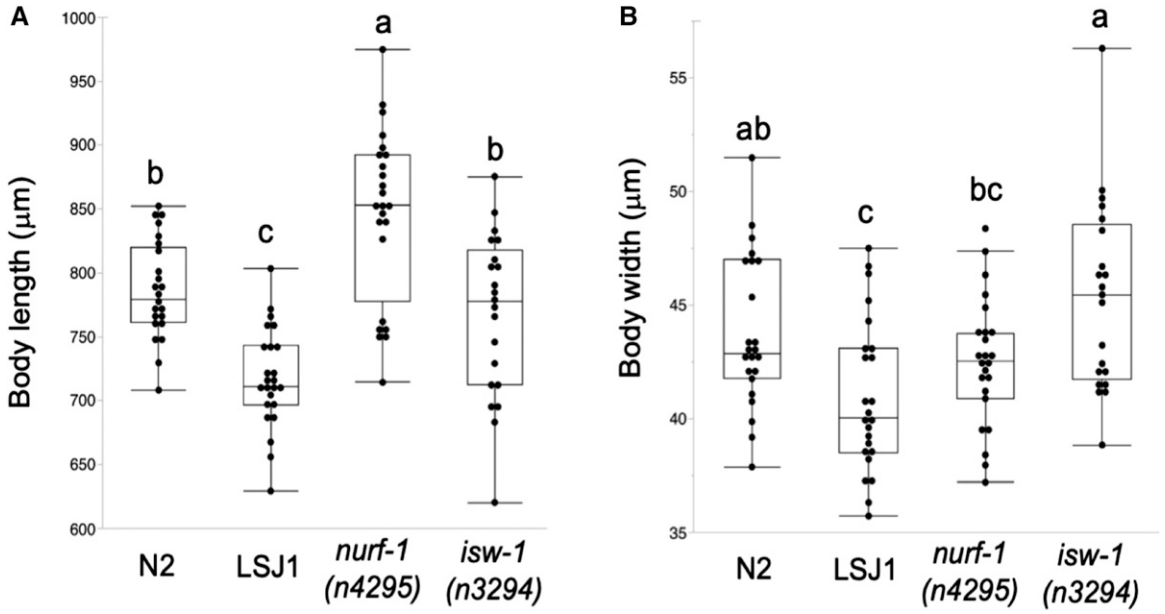

Figure 8 Male sperm size reduction in NURF mutants occurs independently of body size. Significant differences in body size of strains N2, LSJ1, *nurf-1(n4295)*, and *isw-1(n3294)*. (A) Body length (ANOVA, $F_{3, 91} = 23.36$, $P < 0.0001$). (B) Body width (ANOVA, $F_{3, 91} = 6.83$, $P = 0.0003$). Values with the same letter are not significantly different from each other (Tukey's honestly significant difference, $P < 0.05$).

competitive ability and reproductive performance. The second part of our study focused on the genetic basis underlying the evolutionary reduction in sperm size of laboratory-adapted strains, LSJ1 and LSJ2, which exhibit the smallest sperm size among all strains examined. This analysis, by means of mutants and quantitative complementation tests, shows that genetic changes in the nucleosome remodeling factor *nurf-1* underlie the evolution of small male sperm size in the LSJ lineage. These and additional experimental results suggest that the NURF chromatin remodeling complex acts in *C. elegans* sperm size regulation.

Natural variation in C. elegans sperm size

Although we observed significant natural genetic variation in *C. elegans* male sperm size, the vast majority of isolates show a relatively narrow average sperm size range, from 20 to 25 μm^2, and the only two significant outliers were the strains JU561 and LSJ1, with reduced male sperm size ($\sim$15 μm^2) (Figure 1). Performing a genome-wide association study to detect potential genetic loci explaining variation in male sperm size did not yield any quantitative trait loci, likely due to low statistical power and/or complex genetic trait architecture. As previously observed for some *C. elegans* isolates and other *Caenorhabditis* species (Vielle *et al.* 2016), male sperm size showed extreme variability within single individuals, and to a lesser extent, among populations of genetically identical individuals (Figure 1). Such within-genotype variability is often observed for sperm size traits, such as sperm length in taxa with flagellate sperm (Ward 1998; Morrow and Gage 2001b; Miller *et al.* 2003; Joly *et al.* 2004). Possibly, such high sperm size variance could reflect a means to maximize both average size and number of sperm produced, or developmentally decreasing sperm size variance may come at the cost of reduced sperm production speed (Parker and Begon 1993; Gomendio *et al.* 2006; Vielle *et al.* 2016).

Unlike a previous study (Murray *et al.* 2011), we did not find any correlations between male sperm size and multiple traits related to male reproductive performance and competitive ability. This mixed evidence suggests that male sperm size is not a reliable predictor and sole key determinant of male reproductive success, in line with studies reporting diverse genetic factors affecting *Caenorhabditis* sperm competitive ability independently of sperm size (Thomas *et al.* 2012; Ting *et al.* 2014, 2018; Fierst *et al.* 2015; Hansen *et al.* 2015; Yin *et al.* 2018; Yin and Haag 2019). Moreover, many other traits that we did not measure here, including male mating behavior or hermaphrodite receptivity, will contribute to overall male reproductive success. We also found that a rarely measured trait—the number of sperm transferred per mating (ejaculate size)—was strongly correlated with the number of male offspring sired across different strains (Figure 2H). Although we did not detect a potential trade-off between male sperm size and ejaculate size (Figure S1F), an earlier study (Murray *et al.* 2011) did report that the rate of sperm production was reduced in *C. elegans* strains with larger male sperm. Trade-offs between male sperm size and sperm number could thus potentially shape and limit the extent of sperm size evolution. Importantly, the observed absence of a tight relationship between male sperm size and competitive ability might also represent a signature of reduced male conflict and sperm competition, in line with reports of low rates of outcrossing in *C. elegans* wild populations (Barrière and Félix 2005, 2007; Félix and Braendle 2010) and reduced maintenance of (male) mating function

(Chasnov and Chow 2002; Teotónio *et al.* 2006; Thomas *et al.* 2012; Chasnov 2013; Yin *et al.* 2018). Nevertheless, *C. elegans* male sperm seem to maintain the evolutionary potential to respond to changes in the extent of male mating competition: several experimental evolution studies have shown that male sperm size may rapidly evolve toward increased size in response to increased male-male competition (LaMunyon and Ward 2002; Palopoli *et al.* 2015; Poullet *et al.* 2016).

We also aimed to assess to what extent *C. elegans* sperm size differs between sexes, given that little is known about natural genetic variation in hermaphrodite sperm size and whether male and hermaphrodite sperm size may correlate. Specifically, a significant correlation between male and hermaphrodite sperm size could be indicative of shared genetic regulation of sperm size across sexes. Our analyses of intraspecific variation in *C. elegans* hermaphrodite sperm size confirmed previous reports (Ward and Carrel 1979; LaMunyon and Ward 1995, 1998; Vielle *et al.* 2016) that for any given strain, hermaphrodite sperm are always significantly smaller than male sperm (Figure 3A). As for male sperm size, we detected substantial levels of heritable variation in hermaphrodite sperm size. Although the two strains with smallest male sperm (LSJ1 and JU561) also had the smallest hermaphrodite sperm, male and hermaphrodite sperm size did not correlate across examined strains (Figure 3B). The *C. elegans* sperm size dimorphism is thought to reflect differential selection on conflicting sex-specific size optima: larger male sperm to increase sperm competitive ability *vs.* smaller hermaphrodite sperm to accelerate sperm production to allow for a rapid switch to oogenesis, which is critical for early maturity and reproduction (Hodgkin and Barnes 1991; Cutter 2004); *C. elegans* hermaphrodites are protandrous with initial production of sperm, stored in the spermathecae, before switching to oocyte production. Therefore, the sequential spermatogenesis-oogenesis switch in *C. elegans* causes a trade-off between maximal sperm number (*i.e.*, potential number of self-progeny) and minimal age at maturity (*i.e.*, generation time). Consequently, evolution of small hermaphrodite sperm size results from selection for rapid self-sperm production, consistent with the fact that hermaphrodite sperm are always drastically smaller than male sperm (Figure 3A). In the same fashion, evolution of increased hermaphrodite sperm production may lead to smaller self-sperm. However, we did not find evidence for a trade-off between hermaphrodite sperm size and number across a set of *C. elegans* strains differing in sperm size (Figure 3C). Importantly, the evolution of small hermaphrodite sperm in androdioecious species seems not only to result from selection for improved self-fertilization but also from direct developmental effects, when spermatogenesis takes place in a female soma, that reduce hermaphrodite sperm size (Baldi *et al.* 2011). Disentangling these different evolutionary and developmental mechanisms in shaping *C. elegans* sperm size dimorphism therefore remains a major challenge.

Genetic variation in nurf-1 explains the evolution of small sperm and suggests a role for the NURF chromatin remodeling complex in C. elegans sperm size determination

We uncovered strong differences in male sperm size between two very closely related *C. elegans* laboratory lineages, N2 *vs.* LSJ1/LSJ2. Given their recent evolutionary divergence, and thus close genetic similarity (McGrath *et al.* 2011), we successfully applied a candidate approach to demonstrate that variation in the gene *nurf-1*, encoding a subunit of the NURF chromatin remodeling complex, explains reduced LSJ sperm size. The observation that evolutionary reduction of *C. elegans* male sperm size is caused by variation in *nurf-1* suggests that this gene acts in *C. elegans* sperm size determination. Our analysis of multiple mutants in NURF-complex genes, all of which displayed reduced sperm size, not only in males but also in hermaphrodites, confirms this idea. As a subunit of the NURF chromatin remodeling complex, NURF-1 is a BPTF ortholog, involved in histone modification on nucleosomes and remodeling of nucleosomes through recruitment of ISWI, to modulate transcription (Badenhorst *et al.* 2002; Andersen *et al.* 2006; Wysocka *et al.* 2006; Ruthenburg *et al.* 2011). Members of the *C. elegans* NURF chromatin remodeling complex are expressed in diverse tissues and organs, including the developing germline (Reece-Hoyes *et al.* 2007; Feng *et al.* 2012; Craig *et al.* 2013). Consistent with these expression patterns, *nurf-1* mutations affect diverse phenotypes in *C. elegans*, ranging from vulval development (Andersen *et al.* 2006) to multiple life history traits (Large *et al.* 2016), and most relevantly in the context of our findings, a very recent study showed that *nurf-1* plays specific roles in spermatogenesis and the sperm-oocyte decision (Xu *et al.* 2019). Together with our findings that *nurf-1* variants of LSJ strains and mutants of NURF-complex genes (*nurf-1*, *isw-1*, *pyp-1*) reduce sperm size, this experimental evidence thus clearly points to a role of the NURF chromatin remodeling complex in regulating *C. elegans* sperm size.

The evolution of *nurf-1* function in the LSJ lineage underlies laboratory adaptation to an axenic liquid medium (Large *et al.* 2016, 2017). Specifically, a key causal molecular variant underlying improved LSJ hermaphrodite reproduction in this environment (relative to N2) is the 60 bp *nurf-1* deletion (Large *et al.* 2016), which we identified here as the (very likely) causal variant responsible for reduced male sperm size (Figure 6E). This deletion has been shown to have highly pleiotropic effects on hermaphrodite life history traits, including reproduction, growth rate, life span, and Dauer formation (Large *et al.* 2016). Given this demonstration that this *nurf-1* variation specific to the LSJ lineage confers improved fitness of hermaphrodites, the evolution of reduced male sperm size in the LSJ lineage likely reflects a pleiotropic consequence stemming from selection on hermaphrodite function. Very possibly, the *nurf-1* variation explaining reduced male sperm size is also responsible for the observed reduction of hermaphrodite sperm size in LSJ strains (Figure 5C); however, this remains to be experimentally confirmed. If true,

evolution of reduced hermaphrodite sperm size in LSJ strains could thus be more directly linked to reported evolutionary changes of the LSJ lineage in both gametogenesis (Xu *et al.* 2019) and reproductive traits (Large *et al.* 2016). For example, production of smaller hermaphrodite sperm may go in hand with observed increases in sperm production (Figure 3C), which in turn, could explain observed increases in self-fertility (Large *et al.* 2016). More specifically, given that the different *nurf-1* deletions occurring in LSJ strains and in the *nurf-1(n4295)* mutant differentially affect B and D isoforms (Xu *et al.* 2019), our findings that hermaphrodite sperm size is reduced in both *n4295* mutant and LSJ strains (Figure 5C and Figure 6D) suggests that either NURF-D or both NURF-D and NURF-B affect sperm size determination. This could occur through a direct effect of NURF-1.D on genes regulating sperm size or as an indirect consequence of NURF-1.D acting on the timing of the sperm-oocyte decision. In the latter scenario, sperm size reduction may result as a by-product of increased sperm production as observed for LSJ hermaphrodites (Figure 3C).

C. elegans strains LSJ1 and LSJ2 were derived from the ancestral N2 strain by growing them in liquid culture for over 40 years (Large *et al.* 2016, 2017). Given that *C. elegans* males are generally presumed to be incapable of mating in liquid culture, laboratory evolution of the LSJ lineage likely occurred in the complete absence of outcrossing. Our finding of reduced male sperm size in the LSJ lineage thus illustrates how selection for improved *C. elegans* hermaphrodite function (Large *et al.* 2016) can affect a (likely unselected) male trait through a specific genetic variant. Consistent with the presumed absence of outcrossing over many hundreds of generations in liquid culture, LSJ1 showed significantly reduced male-male competitive ability relative to N2 (Figure 2, E–G). On the other hand, we found that LSJ1 male sperm function and mating ability with hermaphrodites remained largely preserved (Figure 2, A–D and Figure S1). These observations exemplify how essential male functions in *C. elegans* can be maintained despite absent or very rare outcrossing, *i.e.*, strongly relaxed selection.

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