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Convergent evolution of sperm gigantism and the developmental origins of sperm size variability in *Caenorhabditis* nematodes

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Sperm cells provide essential, if usually diminutive, ingredients to successful sexual reproduction. Despite this conserved function, sperm competition, and coevolution with female traits can drive spectacular morphological change in these cells. Here, we characterize four repeated instances of convergent evolution of sperm gigantism in *Caenorhabditis* nematodes using phylogenetic comparative methods on 26 species. Species at the extreme end of the 50-fold range of sperm-cell volumes across the genus have sperm capable of comprising up to 5% of egg-cell volume, representing severe attenuation of the magnitude of anisogamy. Furthermore, we uncover significant differences in mean and variance of sperm size among genotypes, between sexes, and within and between individuals of identical genotypes. We demonstrate that the developmental basis of sperm size variation, both within and between species, becomes established during an early stage of sperm development, that is at the formation of primary spermatocytes while subsequent meiotic divisions contribute little further sperm size variability. These findings provide first insights into the developmental determinants of inter- and intraspecific sperm size differences in *Caenorhabditis*. We hypothesize that life history and ecological differences among species favored the evolution of alternative sperm competition strategies toward either many smaller sperm or fewer larger sperm.

KEY WORDS: **Anisogamy, developmental evolution, sexual selection, sperm competition, sperm size.**

Postcopulatory sexual selection that manifests as sperm competition and cryptic female choice can drive rapid trait evolution in gametes and genitalia. Rapid evolution of such reproductive traits (Swanson and Vacquier 2002; Ellegren and Parsch 2007) is a hallmark of species differences and can accelerate the accumulation of reproductive isolation in species diversification (Arnqvist et al. 2000; Gavrilets 2000). Cases of exaggerated phenotypic evolution in sperm size, as for sperm gigantism in some *Drosophila* and other organisms (Bressac et al. 1994; Pitnick and Markow 1994; Bjork and Pitnick 2006; Pitnick et al. 2009b), provide some of the most dramatic examples in the evolution of form and function. Moreover, exaggerated male gamete formation as a consequence of sperm competition marks an evolutionary reversal in relative male investment away from extreme anisogamy. One perspective on this outcome holds that such increased male investment in offspring represents a means of paternal care via gametic provisioning (Bressac et al. 1994). Alternatively, incorporation of more male gamete cytoplasm into the zygote might set the stage for greater parental sexual conflict over offspring development (Patten et al. 2014; Ramm et al. 2014). Sperm evolution thus integrates the fundamental evolutionary processes that define intermale competition, intersexual conflict, and biological diversification all in the context of the exceptionally accessible cell biology and development of a single, discrete cell type.

Nematodes of the genus *Caenorhabditis* have emerged as key model organisms to study both mechanisms and the evolution

of sperm competition. In general, nematode spermatozoa usually lack cilia or flagella (Lee 2002), unlike the sperm cells of insects and mammals, so that changes in sperm size must be coupled to cell volume. In the androdioecious nematode *C. elegans* multiple lines of evidence implicate cell size as a key component of sperm competitive ability: (i) males make larger sperm than hermaphrodites, with male sperm consistently outcompeting hermaphrodite sperm (Ward and Carrel 1979; LaMunyon and Ward 1995), (ii) larger *C. elegans* male sperm are competitively superior and crawl faster (LaMunyon and Ward 1998), and (iii) experimentally enhanced male–male competition leads to the evolution of larger sperm (LaMunyon and Ward 2002; Palopoli et al. 2015; Poullet et al. 2016). *C. elegans* males (and hermaphrodites) have small sperm compared to related obligatorily outcrossing species (LaMunyon and Ward 1999; Baldi et al. 2011), which is thought to reflect part of a "selfing syndrome" in species like *C. elegans* that reproduce primarily by self-fertilization and so experience reduced sperm competition in nature (LaMunyon and Ward 1999; Cutter 2015). In addition, developmental biases due to an influence of the female-soma on spermatogenesis appear to act to reduce sperm size in hermaphrodites (Baldi et al. 2011). Interspecies matings also demonstrate the competitive superiority of larger sperm (Hill and L'Hernault 2001; Geldziler et al. 2006; Ting et al. 2014). Despite the competitive advantages to males of transferring large sperm to mates when under risk of sperm competition, sperm production rates are slower for *C. elegans* genotypes that make larger sperm (LaMunyon and Ward 1998; Murray et al. 2011). Such trade-offs might limit sperm size evolution, depending on the costs and benefits to sperm size versus number, in combination with mating rates and the probability of paternity in achieving fertilization success.

Despite the extensive theoretical, morphological, and experimental literature on sperm competition in sexual selection (Smith 1984; Birkhead and Moller 1998; Birkhead et al. 2009), the developmental origins of size variation in sperm traits, such as cell size or flagellum length, remain more elusive. Concerning *Caenorhabditis* nematodes, the genetic control of spermatogenesis is well-characterized in *C. elegans* (L'Hernault 2006; Geldziler et al. 2011; Ellis and Stanfield 2014), yet the developmental mechanisms underlying sperm size differences, for example between hermaphrodites and males, are not understood. During *C. elegans* spermatogenesis, the onset of meiosis involves the formation of primary spermatocytes, characterized by their separation from a syncytial germ cell progenitor pool, during which they increase substantially in cell size (Wolf et al. 1978; Ward et al. 1981; Shakes et al. 2009). Subsequently, the primary spermatocyte divides to form two secondary spermatocytes, which rapidly undergo the second meiotic division resulting in four haploid spermatids and an anucleate residual body (Ward et al. 1981). The secondary spermatocytes may either remain connected through cytoplasmic bridges or become separated so that each gives rise to two spermatids and a residual body (Ward et al. 1981). Meiotic divisions during *C. elegans* sperm development involve extensive cytokinesis and redistribution of cellular components to residual body and spermatids (Wolf et al. 1978; Ward et al. 1981; Huang et al. 2012). The rapid meiotic divisions in *C. elegans* go hand in hand with a corresponding cell size reduction from primary spermatocyte to spermatid (Ward et al. 1981). Male and hermaphrodite spermatogenesis in *C. elegans* appear equivalent, and it remains unclear how *C. elegans* spermatogenesis generates sperm of distinct size according to sex, genotype, individual, or how evolution has shaped male sperm development of male–female (i.e., dioecious/gonochoristic) *Caenorhabditis* species that display sperm size divergence (LaMunyon and Ward 1999; Geldziler et al. 2006; Baldi et al. 2011).

To address these questions surrounding sperm evolution and its developmental basis, we quantified sperm size variation across the *Caenorhabditis* phylogeny covering 26 species (Kiontke et al. 2011). In addition to the well-appreciated convergent evolution of sperm miniaturization in self-fertilizing species, we demonstrate convergent evolution of sperm gigantism in four independent lineages. The presence of gigantic *Caenorhabditis* sperm contributes to a 50-fold range of variation in sperm volume among species, with such sperm capable of comprising 5% of egg cell volume, in contrast to *C. elegans* hermaphrodite sperm that are just 0.2% the size of eggs. We further found substantial sperm size variability within species, between sexes of androdioecious species, as well as between and within individuals of the same genotype. We present experimental evidence implicating primary spermatocyte formation as the key stage establishing the developmental basis of both intra- and interspecific variation in sperm size, with little sperm size variability induced during subsequent cell divisions. Our findings provide first insights into the developmental determinants of sperm size variation and the results of our extended species survey are consistent with the notion that sexual selection has shaped the diversification of *Caenorhabditis* sperm size.

Materials and Methods

NEMATODE STRAINS AND CULTIVATION

Strains were maintained at a constant temperature of 20°C on 2.5% agar NGM (Nematode Growth Medium) plates seeded with the *E. coli* strain OP50 (Stiernagle 2006). A temperature of 20°C was chosen as it allows sustained growth of all *Caenorhabditis* species and isolates; note, however, that preferred thermal ranges of most species/isolates are unknown. In a few species, such as androdioecious species, temperatures optimal for reproduction may vary both between and within species (e.g., Kiontke et al. 2011; Gimond et al. 2013; Grimbert and Braendle 2014; Frézal and Félix 2015; Poullet et al. 2015). The following species

(strains) were used in this study: *C. angaria* (PS1010), *C. brenneri* (CB5161, JU1398, SB280), *C. briggsae* (AF16, ED3092, HK104, JU1341, QR24), *C. drosophilae* (DF5077), *C. elegans* (CB4856, JU258, LKC34, MY2, N2), *C. japonica* (DF5081), *C. plicata* (SB355), *C. remanei* (PB4641, SB146, VT733), *C.* sp. 1 (SB341), *C. doughertyi* (JU1333), *C. tropicalis* (JU1373, JU1630, JU1818, NIC58, QG131), *C. castelli* (JU1427), *C. virilis* (JU1528), *C. imperialis* (EG5716, NIC118), *C. kamaaina* (QG122), *C. wallacei* (JU1873), *C. nouraguensis* (JU1825), *C. macrosperma* (NIC293, NIC401, JU1857,) *C. yunquensis* (EG6142), *C.* sp. 2 (DF5070), *C. guadeloupensis* (NIC113), *C. sinica* (JU800), *C. portoensis* (EG4788), *C. afra* (JU1199), *C.* sp. 8 (DF5106, NIC184, QX1182), and *C. nigoni* (EG5268). *C. macrosperma* isolates NIC293 and NIC401 were isolated in French Guiana in 2013 (C.B. and A.D.C., unpubl. data). For detailed strain information, see references (Kiontke and Sudhaus 2006; Kiontke et al. 2011; Felix et al. 2013).

SPERM SIZE MEASUREMENTS

Males were isolated from strain cultures at the L4 stage and maintained on NGM plates containing males only, to obtain spermatid size measurements from synchronized and unmated males. After 24 hours at 20°C, when males had reached the adult stage, spermatids were obtained by needle dissection of males in sperm medium (50 mM HEPES pH7.8, 50 mM NaCl, 25 mM KCl, 5 mM $CaCl_2$, 1 mM $MgSO_4$, 1 mg/ml BSA) (Nelson and Ward 1980). Spermatids from multiple males were immediately imaged using DIC microscopy (60× or 63× objectives). Using ImageJ software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997–2014), we calculated length and width of each spermatid to obtain measures of cross-sectional area assuming an ellipse shape: π× (length/2) × (width/2) (measured at approximately 1000× magnification). Hermaphrodite spermatids were dissected from young unmated adults (mid L4 + 24 h), and imaging and spermatid size calculations were performed as described above. At this developmental stage, most hermaphrodite individuals contained both spermatids and activated sperm (spermatozoa), and the latter were thus not included for measurements.

BODY SIZE MEASUREMENTS

Estimates of body length and width were obtained by measuring adult males and females (hermaphrodites) at L4 + 24 h. Live individuals were on 4% agarose pads in M9 buffer, containing 100 mM sodium azide (Stiernagle 2006) and imaged using a 10× objective. Using ImageJ software, we measured the body midline (from mouth to tip of tail) to estimate length and we measured body width in the midsection of adult animals perpendicular to the anterior-posterior axis.

ANISOGAMY MEASUREMENTS

To estimate egg size we used embryo size measurements obtained by Farhadifar et al. (2015), with cross-sectional area calculated assuming an ellipse from length and width values. The index of anisogamy was calculated as egg volume divided by spermatid volume, where egg and spermatid volumes presume an ellipsoid cell shape: $(4/3) \times \pi \times (\text{length}/2) \times (\text{width}/2)^2$.

MATING EXPERIMENTS AND SPERM NUMBER MEASUREMENTS

To quantify numbers of transferred sperm after a single mating for three independent contrasts of species pairs differing in sperm size, we followed the phylogeny established by Kiontke et al. (2011) (Fig. 2F). For each strain, males and females were picked at the L4 stage and maintained on separate NGM plates for 36 h at 20°C. A single virgin female and five unmated males were then placed together on individual mating plates (*E. coli* OP50 lawn of 5 mm diameter) and observed at 80× magnification using a dissecting microscope. As soon as a single mating event had been completed, that is spicule insertion and ejaculation (visualized as sperm flow from the male *vas deferens* into the female uterus) and after which the male had left the female, the inseminated female was isolated and fixed in ice-cold Methanol. Females were then washed twice in M9 buffer and mounted in DAPI-containing Vectashield medium (Vector Laboratories, Inc., Burlingame, CA, USA). Imaging and sperm counts were performed as previously described (Poullet et al. 2015, 2016). In brief, images were taken at 40× magnification as Z-stacks covering the entire thickness of the animal using an epifluorescence microscope. We then manually counted sperm number (in uterus, spermatheca, proximal germline) by identifying condensed sperm nuclei of each focal plane using the ImageJ plugin Cell Counter (Rasband, W.S., Image J, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997–2014).

PRIMARY SPERMATOCYTE MEASUREMENTS

Because individual primary spermatocytes are difficult to isolate and not easily staged using DIC microscopy, we estimated cell size of undissected male primary spermatocytes in the karyosome stage at the end of meiotic prophase, when DNA content is highly condensed (Shakes et al. 2009), using DAPI to stain nuclei and Phalloidin to visualize cell outlines (Fig. 3B). Males were isolated from strain cultures at the L4 stage and maintained on NGM plates containing males only, to obtain spermatid size measurements from synchronized and unmated males. After 24 hours at 20°C, male gonads were extruded in M9 buffer supplemented with levamisole using syringe needles. Extruded gonads were fixed in 4% paraformaldehyde for 10 minutes followed by a 5 min PBS-Triton X-100 (0.1%) wash at room temperature. Extruded gonads were

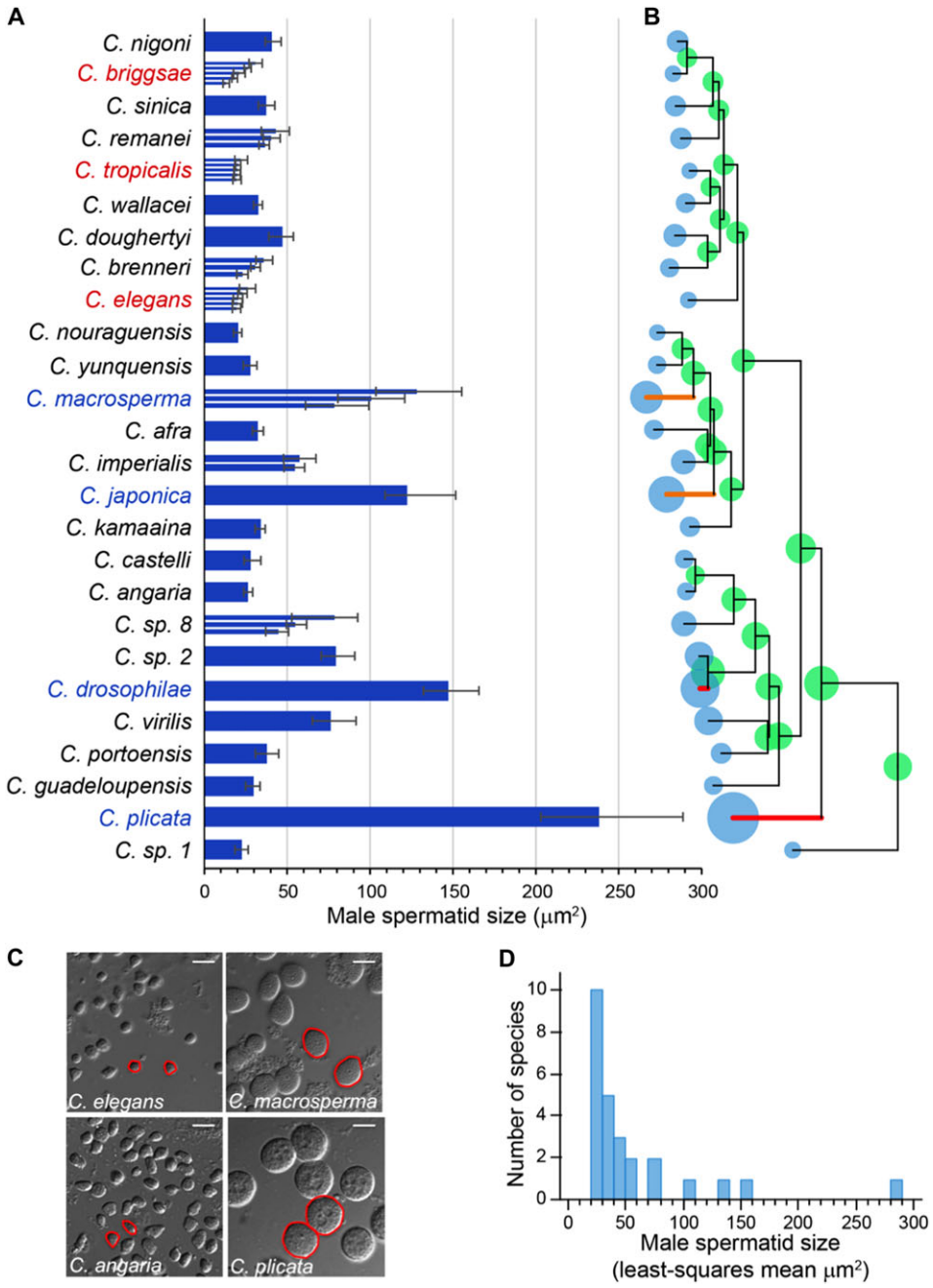

Figure 1. Species differences in *Caenorhabditis* spermatid size show convergent evolution of sperm gigantism. (A) The 26 species show substantial variation in male spermatid cross-sectional area (ANOVA, effect *species*: $F_{25,\ 13736} = 3406.40$, $P < 0.0001$; per strain: 85–653 spermatids from 4–11 individuals; median ± interquartile range of pooled measurements shown to illustrate range of variation). Narrow bars within a species indicate medians of different wild isolate genotypes. For details of sample sizes and complete statistical results, see Tables S1 and S2. (B) Mapping of extant and ancestral spermatid size on the *Caenorhabditis* phylogeny identifies four evolutionary transitions to gigantic sperm. Lineages highlighted red (*C. plicata*, *C. drosophilae*) and orange (*C. macrosperma*, *C. japonica*) indicate the two convergent sperm size regimes identified by SURFACE under a Hansen model of the Ornstein–Uhlenbeck process of stabilizing selection in trait evolution (Ingram and Mahler 2013). The area of circles at each node is proportional to the species least-squares mean spermatid cross-sectional area (blue), with ancestral states (green) inferred from a Brownian motion model of trait evolution in GEIGER (Harmon et al. 2008). Metrics of phylogenetic signal for sperm size indicate strong dependence of trait values on phylogenetic relationships of the taxa (Pagel's $\lambda = 0.96$, Blomberg's $K = 0.48$). Phylogeny and branch lengths from (Kiontke et al. 2011). (C) DIC images of representative spermatid cells for four species of *Caenorhabditis* illustrate the sperm gigantism of *C. plicata* (SB355) and *C. macrosperma* (JU1857) relative to *C. elegans* (N2) and *C. angaria* (PS1010). Scale bars: 15μm. (D) A histogram of sperm size across species (least-squares mean estimates) reveals the outlier trait values for the four species with sperm gigantism.

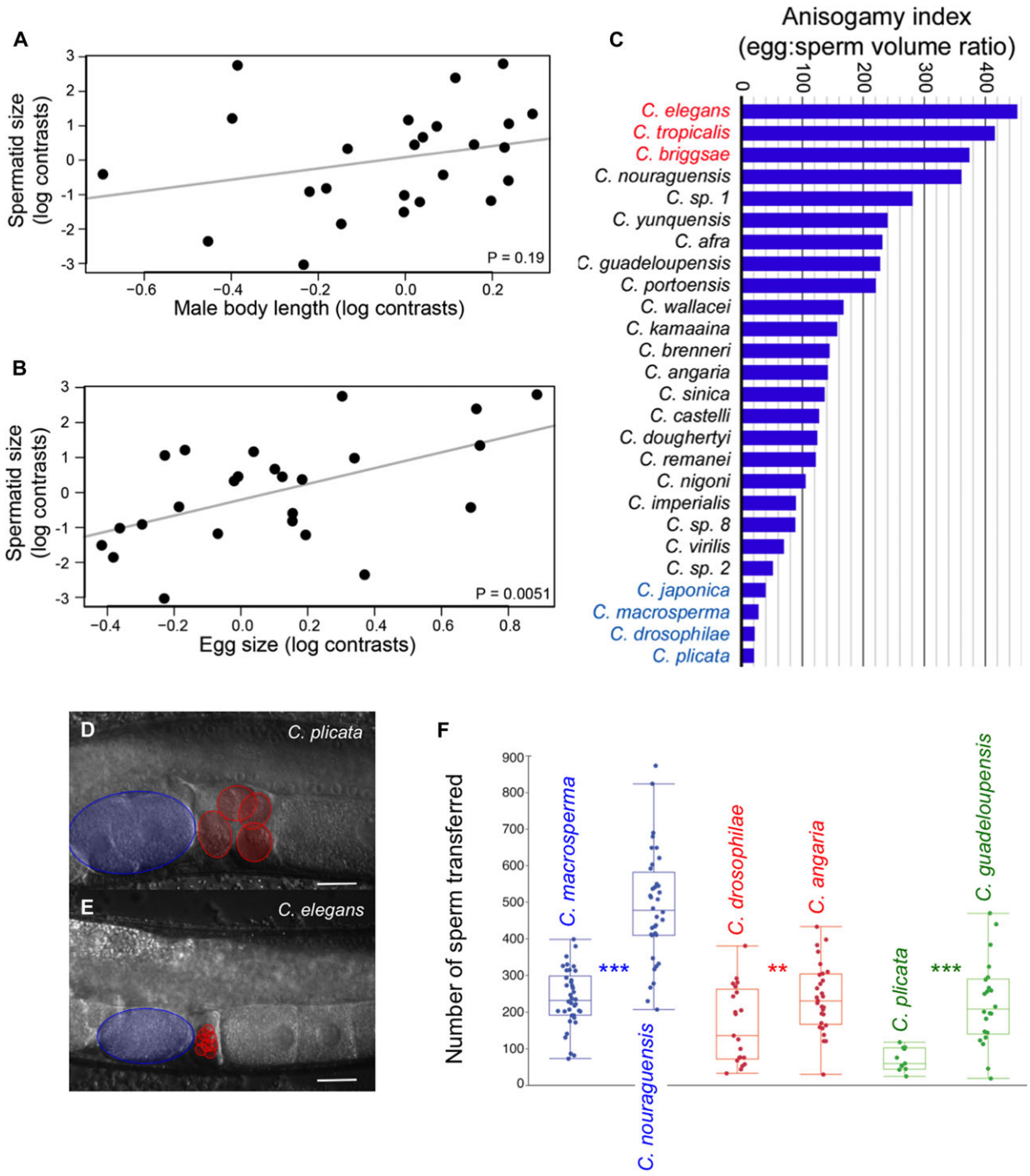

Figure 2. Allometry, anisogamy, and trade-offs in sperm size evolution. (A) Species with larger males do not make larger sperm, as indicated in the nonsignificant slope in this plot of phylogenetic independent contrasts (PIC) of sperm cross-sectional area and male length (log-transformed) ($F_{1,23} = 1.821$, $P = 0.19$). (B) PIC plot of spermatid size versus egg (embryo) size (log-transformed) shows a significant positive correlation ($F_{1,23} = 9.58$, $P = 0.0051$); embryo size data from Farhadifar et al. (2015). (C) Despite variation in egg size and its correlation with male sperm size, an index of anisogamy (embryo volume/spermatid volume) differs drastically across species, with sperm size being responsible for most of the heterogeneity. (D, E) DIC images of the reproductive tract in an inseminated female of *C. plicata* (SB355) and a hermaphrodite *C. elegans* (N2) with self-sperm, illustrating the extreme species divergence in anisogamy. Spermatozoa in the spermatheca are outlined in red, and an early embryo in the uterus is outlined in blue. Scale bars: 20 μm. (F) In three contrasts of species with "standard" versus "gigantic" sperm, the species with larger sperm transfers significantly fewer of them per mating (*C. nouraguensis* vs. *C. macrosperma*: $F_{1,72} = 82.66$, $P < 0.0001$; *C. angaria* vs. *C. drosophilae*: $F_{1,48} = 8.16$, $P = 0.0063$; *C. guadeloupensis* vs. *C. plicata*: $F_{1,31} = 21.71$, $P < 0.0001$).

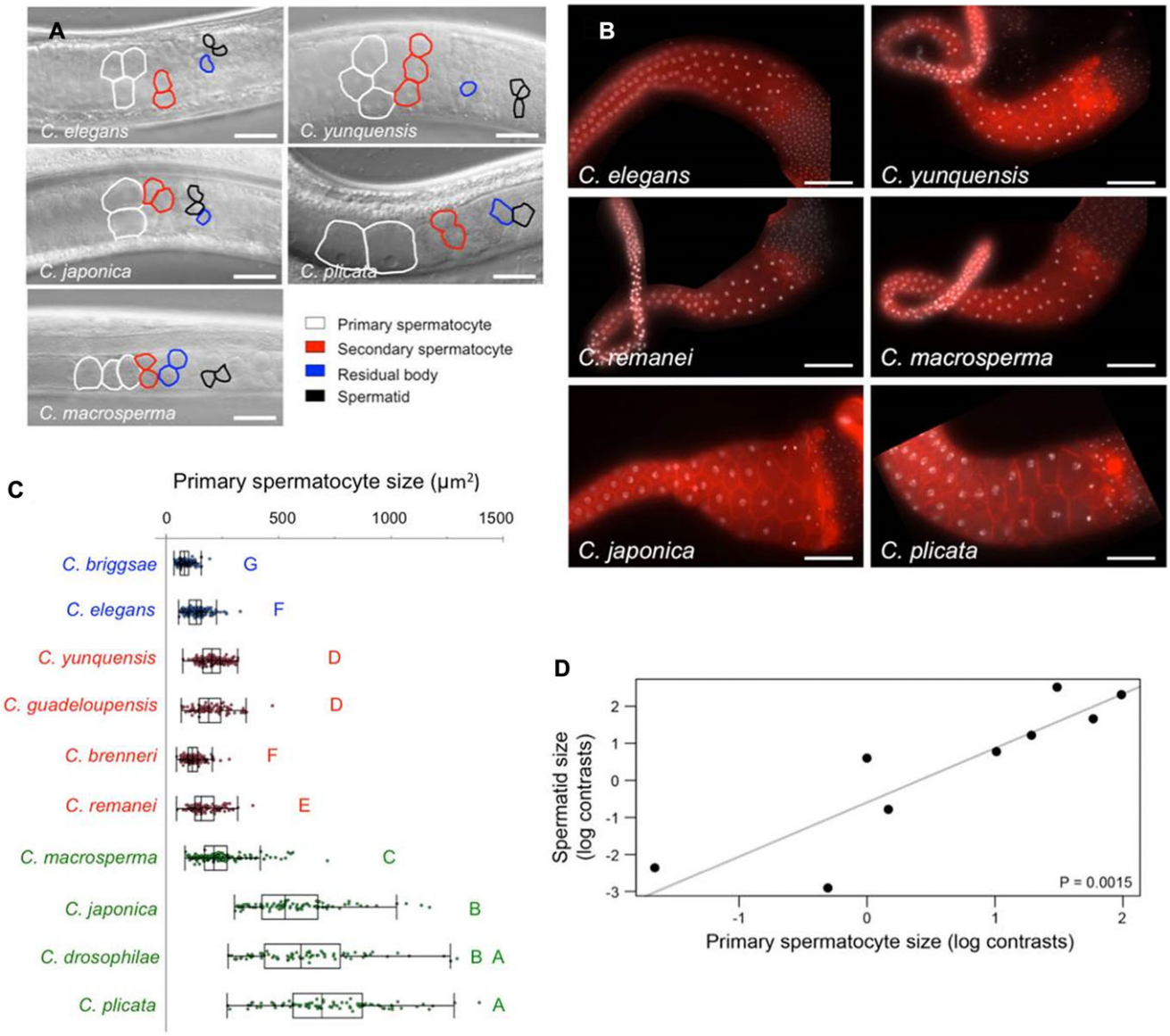

Figure 3. **Developmental origin of sperm size differences among species. (A) DIC images of representative male gonads for two species with "small" sperm sizes (*C. elegans, C. yunquensis*) and three species with "gigantic" sperm (*C. japonica, C. macrosperma, C. plicata*), visualizing the transition from primary spermatocytes to spermatids. Scale bars: 20 μm. (B) Phalloidin (red) and DAPI (white) stainings of dissected male gonads of select species with divergent sperm size. Scale bars: 40 μm. (C) Quantification of male primary spermatocyte size in ten *Caenorhabditis* species with divergent sperm size, arranged by increasing spermatid size from top to bottom. Androdioecious species with the smallest male sperm (blue), dioecious species with "standard" sperm size (red) and "gigantic" sperm (green). Primary spermatocyte size shows significant variation among species (ANOVA, effect *species*: $F_{9,1101} = 603.88$, $P < 0.0001$; for sample sizes and complete statistical results, see Tables S5 and S6). Values labeled with different letters indicate significant differences (Tukey's HSD, $P < 0.05$). (D) The size of spermatids and primary spermatocytes correlates strongly across examined species (10 species; phylogenetic independent contrast on log-transformed values; $r^2_{adj} = 0.76$, $F_{1,7} = 25.65$, $P = 0.0015$).**

stained for actin using Phalloidin (1:500 dilution, Sigma-Aldrich) overnight at 4°C in a humidified chamber. Gonads were washed in PBS and mounted in Vectashield mounting medium supplemented with DAPI. Images of the germline section containing primary spermatocytes were obtained using an epifluorescence microscope (40× objective). Size measurements were restricted to cells where the polygonal cell outline was completely visible and where DNA was highly condensed. Measurements of spermatocyte area were obtained by delineating the circumference of cells using ImageJ software.

Measurements of primary spermatocyte size were obtained from the following strains:

C. elegans: N2, *C. guadeloupensis:* NIC113, *C. remanei*: PB4641, *C. yunquensis*: EG6142, *C. brenneri*: CB5161, *C. japonica*: DF5081, *C. drosophilae*: DF5077, *C. plicata:* SB355, and *C. macrosperma*: JU1857.

SPERM ACTIVATION ASSAYS AND MEASUREMENTS OF SPERMATOZOA

In vitro sperm activation assays in *C. elegans* N2 and *C. macrosperma* JU1857 (Fig. 6B–D) were performed by dissecting male spermatids in sperm medium supplemented with Pronase E (Sigma-Aldrich), as previously described (Ward et al. 1983; Singaravelu et al. 2011). Images of spermatozoa were taken within15–20 minutes after dissection, using DIC microscopy (60× or 63× objective). Size estimates (area) were obtained my measuring the circumferences of cell body and pseudopod of each spermatozoon using ImageJ software; total spermatozoon size was calculated as the sum of cell body plus pseudopod.

MEASUREMENTS OF *C. macrosperma* SPERMATOZOA AFTER MATING

To test for sperm size differences between males, females (0 h after mating), and females (24 h after mating) (Fig. 6E), we isolated *C. macrosperma* JU1857 males and females at the L4 stage, and kept them on separate plates to prevent mating. After 24 hours, 10 males and 5 virgin females were transferred to each of several mating plates (*E. coli* OP50 lawn of 5 mm diameter). After 5 hours, both males and females were dissected in sperm medium to obtain spermatids and spermatozoa, respectively. Spermatids dissected from males were activated using Pronase and measured after 15–20 minutes. Sizes of spermatid and spermatozoon (cell body and pseudopod) were measured as described above. Additional females from the same experiment were maintained without males for another 24 hours prior to dissection of spermatozoa. Size measurements were done in the same fashion as outlined above.

PHYLOGENETIC ANALYSIS

We performed phylogenetic independent contrasts (PIC) on species mean trait values (log-transformed) for the *Caenorhabditis* phylogeny and branch lengths from (Kiontke et al. 2011), as implemented in the R package APE (Paradis et al. 2004). Body size (male and female length and width) and egg (embryo cross-sectional area) measurements used species mean values from measurements described above. For sperm size, we used least-squares mean estimates of spermatid cross-sectional area for each species from a generalized linear model that incorporated variation within species in our spermatid size measurements owing to strain and individual. PIC analyses were performed on log-transformed trait values to eliminate scale-dependence. We also applied SURFACE (Ingram and Mahler 2013) to test for the number of independent and convergent shifts in sperm size and embryo size along the phylogeny, which uses the Akaike Information Criterion (AIC) for model selection of the number of phenotypic regimes under Ornstein-Uhlenbeck process using a Hansen model of trait evolution along the phylogeny. To complement AIC, we also conducted 500 phylogenetic trait simulations in SURFACE to derive a *P*-value for the inferred number of regime transitions (*c*) for sperm size. For graphical mapping of ancestral state inference, however, we applied GEIGER (Harmon et al. 2008), which uses a Brownian motion model of trait change on the phylogeny. Metrics of phylogenetic signal (Pagel's λ, Blomberg's k) were calculated in R using the GEIGER and PICANTE packages (Webb et al. 2008). All R scripts are publicly available at http://github.com/cutterlab.

Results

REPEATED EVOLUTION OF EXTREMELY LARGE MALE SPERM IN DIOECIOUS CAENORHABDITIS SPECIES

We quantified male sperm size across the *Caenorhabditis* phylogeny from measures of spermatid cross-sectional area, demonstrating substantial disparity in sperm size among 26 species that include representatives from all major subgroups in the genus (Kiontke et al. 2011; Felix et al. 2014) (Fig. 1–D). Average sperm size varies >13-fold in cross-sectional area of male spermatids (i.e., >50-fold in spermatid volume), ranging from the tiny 20 μm^2 sperm of the androdioecious *C. tropicalis* to the gigantic 281 μm^2 sperm of the dioecious *C. plicata* (Fig. 1, Tables S1 and S2). Defining sperm gigantism heuristically as an average sperm size >100 μm^2, we identified four such species in our survey: *C. plicata, C. drosophilae, C. japonica,* and *C. macrosperma*, with no instances of sperm gigantism among the set of species from the *Elegans* group analyzed here (Fig. 1A and B). Applying a multipeak Ornstein–Uhlenbeck process of phenotypic evolution along the phylogeny (Ingram and Mahler 2013), we find statistical support for four independent transitions in sperm size in these species toward evolutionary convergence of giant sperm ($c = 4$ size shifts to two convergent states, $P = 0.041$; Figs. 1B and S1). Our survey of all 26 species is consistent with previous observations that, on average, dioecious species make substantially larger sperm than androdioecious species (LaMunyon and Ward 1999; Baldi et al. 2011). However, sperm size of several dioecious species (*C. nouraguensis, C. yunquensis, C. angaria, C.* sp. 1, *C. castelli*) falls within a similar size range (20–30 μm^2) as the small male sperm of androdioecious species (Fig. 1A).

ALLOMETRY, ANISOGAMY, AND TRADE-OFFS IN SPERM SIZE EVOLUTION

Macroevolutionary patterns of sperm size disparity could simply reflect a consistent allometric scaling of animal and cell size. However, when we test for coevolution of sperm and male body

length (Table S3, Fig. S2) using phylogenetic corrections, we find no relationship (log-transformed values; $F_{1,23} = 1.821$, $P = 0.19$) (Fig. 2A). Measures of egg (embryo) size also differ among species (Farhadifar et al. 2015), although, in contrast to sperm, the range of variation is less than threefold (embryo longitudinal area from 859 μm^2 for *C. angaria* to 2474 μm^2 for *C. plicata*) (Fig. S2 and S3). Sperm size and egg size do correlate positively (Fig. 2B), and yet substantial residual variation remains in sperm size among species. Consequently, the evolution of male sperm size yields striking changes in the magnitude of anisogamy among species (Fig. 2C), ranging from an egg: sperm volumetric ratio of 453:1 in *C. elegans* to 20:1 in *C. plicata* (Fig. 2C–E). These findings are consistent with previous conclusions for *Caenorhabditis* that sexual selection by sperm competition is the key driver in the evolution of male sperm size.

Previous work within species of *Caenorhabditis* has demonstrated fertilization advantages to large compared to small sperm (LaMunyon and Ward 1998), even leading to the evolution of larger sperm under experimentally elevated polygamous mating conditions in *C. elegans* (LaMunyon and Ward 2002; Palopoli et al. 2015). However, if males transferring large sperm are constrained in the number that they can transfer to a female, then such a trade-off could limit the evolution of ever-larger sperm. The evolution of fewer sperm per ejaculate should occur only with greater assurance of paternity, for example less polygamy and weaker intermale sperm competition (Parker and Begon 1993). To test for a possible fitness trade-off for males having gigantic sperm, we quantified the number of transferred sperm following a single mating for three contrasts of species pairs differing in sperm size. Consistent with the possibility of a size-number trade-off, in each case, the species with larger sperm transferred fewer of them per copulation (Fig. 2F). Given the large fraction of the body cavity comprised of gonad tissue, male width also may correspond to investment in testis; for example, increased width of the vas deferens may allow passage of larger sperm and/or increased width of the distal germline may permit growth of larger spermatocytes. Consistent with these possibilities, spermatid diameter can be >25% of the width of male worms in species with sperm gigantism and we indeed observed a significant positive correlation of spermatid size and male body width (phylogenetic contrasts of log-transformed values; $F_{1,23} = 19.21$, $P = 0.00022$).

DEVELOPMENTAL ORIGIN OF SPERM SIZE DIFFERENCES AMONG SPECIES

Given the enormous disparity in sperm size across *Caenorhabditis* species, what developmental underpinnings at the cellular level might account for the origins of large versus small sperm? In particular, to what extent do initial steps of spermatogenesis, that is the formation of primary spermatocytes, diverge to generate sperm size variation? To address this question, we examined spermatogenesis in male gonads of ten species with divergent sperm size (Fig. 3). Species producing large spermatids also display large primary spermatocytes in live animals (Fig. 3A). We then quantified primary spermatocyte cell size in the karyosome stage at the end of meiotic prophase from dissected germlines of young adult males, when chromosomes aggregate into a highly condensed mass (Shakes et al. 2009) (Fig. 3B). Species variation in primary spermatocyte size variation mirrors spermatid size variation (Fig. 3C), and the size distribution of primary spermatocytes strongly predicts spermatid size (phylogenetic contrasts on log-transformed values; $F_{1,7} = 25.65$, $r^2_{adj} = 0.76$, $P = 0.0015$) (Fig. 3D), indicating that male spermatid size is largely determined by the initial size of primary spermatocytes. We conclude that *Caenorhabditis* sperm size determination occurs during an early stage of sperm development, prior to the detachment of primary spermatocytes from the rachis (Shakes et al. 2009), that is prior to meiotic divisions.

EXTENSIVE GENETIC VARIATION FOR SPERM SIZE WITHIN SPECIES AND BETWEEN THE SEXES

In addition to assessing sperm size differences among species, we next quantified sperm size variation of multiple wild isolates (genotypes) in dioecious and androdioecious species. Measuring male sperm size for isolates of dioecious species, we find substantial heritable variation within all four species examined (Fig. 4A). For the three androdioecious species, we measured sperm sizes separately for males and hermaphrodites from each of five distinct wild isolates per species, also revealing extensive genetic variation in sperm size for both sexes (Fig. 4B). Hermaphrodite sperm are consistently smaller than male sperm in all species including *C. tropicalis* (Fig. 4B), consistent with previous studies of *C. elegans* and *C. briggsae* (LaMunyon and Ward 1999; Hill and L'Hernault 2001). Hermaphrodite sperm of *C. briggsae* are smallest, and *C. tropicalis* largest, both in absolute size and relative to conspecific males (Fig. 4B). We also observed a weak-positive correlation between the average sperm size of males and hermaphrodites in *C. tropicalis*, but not in *C. elegans* or *C. briggsae* (Fig. 4C). The absence of consistent or strong intersexual correlations of sperm sizes suggests that developmental genetic control of sperm size regulation need not be shared between sexes of androdioecious species despite their superficially equivalent spermatogenic developmental programs.

We also found that, in androdioecious species, differences in sperm size between sexes are correlated with corresponding differences in primary spermatocyte size. This observation is indicated clearly in the comparison of *C. briggsae* isolates AF16 and HK104, for which males exhibit significantly larger primary spermatocytes than hermaphrodites (Fig. 4D). HK104 males also displayed significantly larger primary spermatocytes compared to AF16 males (Fig. 4D), consistent with male sperm size differences

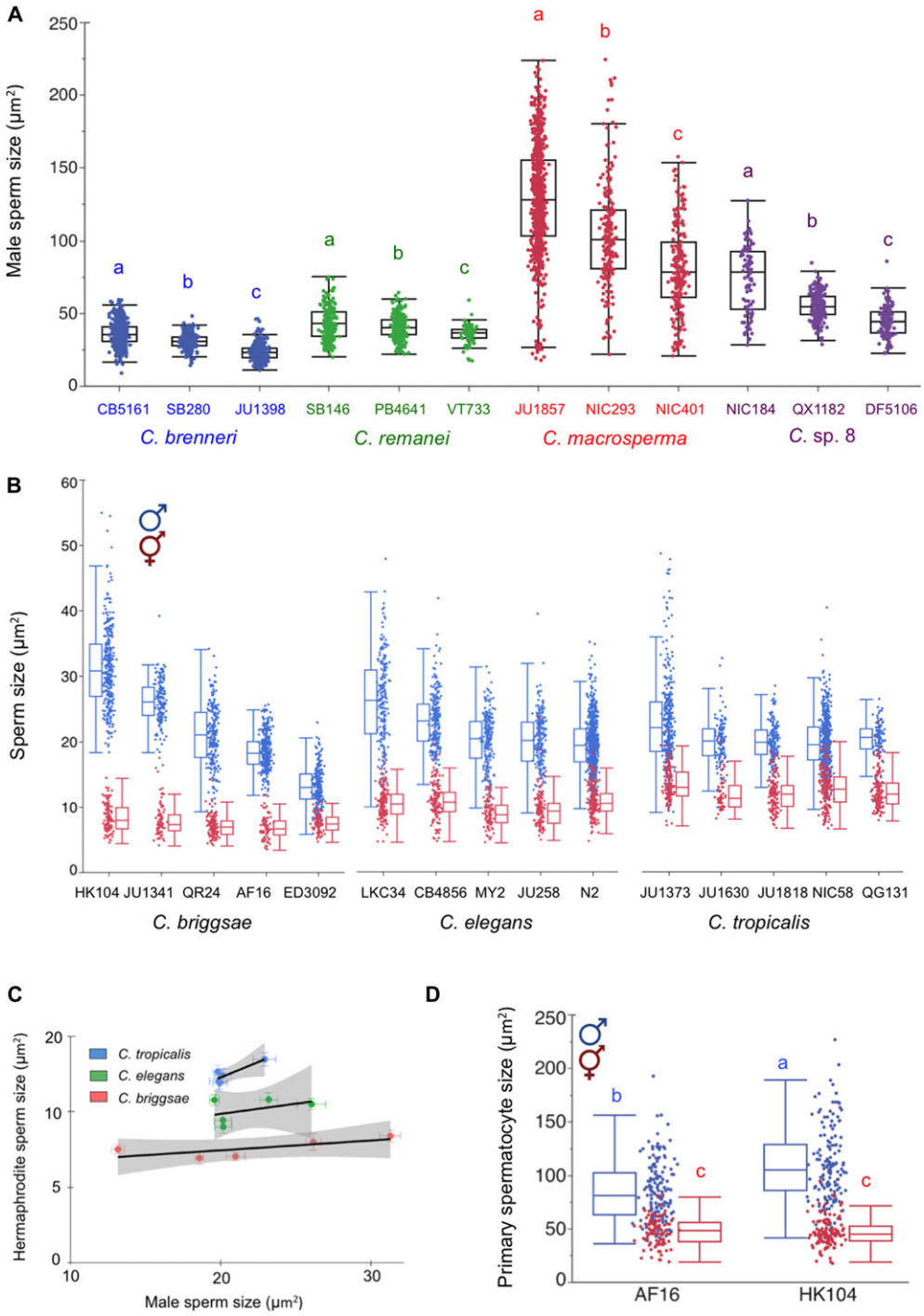

Figure 4. Genetic variation for male sperm size and sperm size differences between males and hermaphrodites. (A) Male sperm size shows extensive genetic variation within each of four dioecious *Caenorhabditis* species (ANOVAs performed separately for each species, *C. brenneri*: $F_{2,1211} = 506.88$, $P < 0.0001$; *C. remanei*: $F_{2,599} = 27.47$, $P < 0.0001$; *C. macrosperma*: $F_{2,1026} = 160.08$, $P < 0.0001$; *C.* sp. 8: $F_{2,450} = 147.55$, $P < 0.0001$; for complete statistical results, see Table S7). Values labeled with different letters indicate significant differences among isolates within a species (Tukey's HSD, $P < 0.05$). (B) Distributions of sperm size for hermaphrodites and males of five distinct wild isolates of each of the three androdioecious species. In each species, there is significant genetic variation for both male and hermaphrodite sperm size, and average male sperm size is always greater than hermaphrodite sperm size (for data and complete statistical tests, see Tables S8 and S9). (C) Male and hermaphrodite mean sperm sizes are significantly correlated across isolates for *C. tropicalis* ($F_{1,3} = 15.62$, $R^2 = 0.83$, $P = 0.028$) but not *C. elegans* ($F_{1,3} = 1.20$, $R^2 = 0.29$, $P = 0.35$) and *C. briggsae* ($F_{1,3} = 1.42$, $R^2 = 0.32$, $P = 0.32$). (D) Primary spermatocyte size variation of hermaphrodites and males in *C. briggsae* isolates AF16 and HK104. Values labeled with different letters indicate significant differences (Tukey's HSD, $P < 0.05$; for complete statistical results, see Table S10).

between these isolates (Fig. 4B). These results indicate that, as for species differences, sperm size differences among genotypes and between sexes can be explained by corresponding size differences in primary spermatocytes.

PRONOUNCED INTRA- AND INTERINDIVIDUAL SPERM SIZE VARIABILITY ACROSS SPECIES

In addition to the substantial differences in average male sperm size among genotypes and species, we also observed considerable sperm size variability between and within single animals for all isolates examined (e.g., Fig. 4A and B). Theory predicts that species with stronger sperm competition ought to exhibit lower coefficients of variation (CV; ratio of standard deviation to mean) in sperm traits (Gomendio et al. 2006; Immler et al. 2008; Pitnick et al. 2009bb; Fitzpatrick and Baer 2011). Therefore we tested for reduced CV in species with larger spermatids, which might be expected if sperm size provides the principal indicator of the intensity of sperm competition in a species (LaMunyon and Ward 1999). However, we find no evidence of disproportionately lower within-male sperm size variability for species with larger sperm (phylogenetic contrasts on log-transformed values; $F_{1,23} = 0.15$, $P = 0.70$) (Fig. 5A), with the between-male CV and the phylogenetically uncorrected analysis actually showing a trend of higher CV in species with larger sperm (between-male PIC $F_{1,23} = 4.47$, $P = 0.045$; Fig. S4). These observations suggest that the net strength of selection on *Caenorhabditis* sperm size might be similar among species, with equally strong stabilizing selection favoring different optimal sperm sizes in different species.

A nested analysis of variance to partition variation among sources of male sperm size variation across the 26 *Caenorhabditis* species indicates that 5% of the total variation can be attributed to interindividual differences, and 13% to intraindividual differences (Table S2). Moreover, comparison of intra- versus interindividual variation in male sperm size suggests that intraindividual sperm size variation exceeds interindividual variation in 42 of 47 isolates (21 of 26 species) analyzed (Table S11). Two species with stark differences in average male sperm size, *C. elegans* (N2) versus *C. macrosperma* (JU1857), illustrate such pervasive sperm size variability within and between individuals irrespective of species mean sperm size (Fig. 5B and C). In both species, differences among individuals of a single genotype are significant for both mean and variance of sperm size (Fig. 5D and E). Intraindividual variation also is pronounced for *C. elegans* and *C. macrosperma*. For example, a single *C. macrosperma* individual may produce sperm that vary more than tenfold in cross-sectional area, from approximately 20 μm^2 to over 200 μm^2 (mean 127.1 $\pm$ 0.6 μm^2) (Fig. 5E). As we observed for inter- and intraspecific variability in average sperm size, primary spermatocyte size shows similarly high variability within and between individuals (Fig. 3C), consistent with the interpretation that sperm size variation originates primarily from size variation in primary spermatocytes (Fig. 3D).

While variation in sperm size, whether due to genetic or environmental sources, appears to be explained predominantly by size variation in primary spermatocytes, additional intraindividual variation could potentially be introduced later in spermatogenesis. For example, size modification of spermatids derived from a single spermatocyte through asymmetric divisions during meiosis I and II could contribute to downstream heterogeneity in sperm size. To test this possibility, we examined meiotic division stages of male spermatogenesis in *C. elegans* N2 and *C. macrosperma* JU1857 (Fig. 5F). Labeling with DAPI and antibodies for Actin and MSP (Major Sperm Protein) suggests evolutionarily conserved processes of sperm maturation, characterized by MSP-positive sperm cells and the exclusion of actin into the residual body (Miller et al. 2001; Huang et al. 2012) (Fig. S5). Quantifying size variation of spermatids derived from individual spermatocytes using DIC microscopy, we observed no evidence of consistent asymmetric meiotic divisions: spermatids budding off the same residual body are similar in size (Fig. 5G and H). However, we cannot exclude that additional size variation of spermatids is occasionally introduced due to asymmetric resorption of residual body contents, as previously reported in *C. elegans* (Ward et al. 1981). Nevertheless, residual body size correlates positively with average spermatid size (Fig. 5G and H), lending further support to the conclusion that it is size variation of primary spermatocytes that provides the dominant source of size variation among spermatids (Fig. 3D).

In *C. briggsae*, and perhaps other species, sperm bearing the X chromosome enjoy a fertilization advantage over nullo-X sperm (LaMunyon and Ward 1997; Bundus et al. 2015). Differential sperm sizes owing to asymmetric meiotic cell division provides one possible mechanism for manifesting such an advantage, as occurs in a species of *Rhabditis* nematodes (Shakes et al. 2011). However, we detected no strong signal of bimodality in male sperm size distributions of individuals for *C. briggsae* or other species (Figs. 5D, E, and S6), arguing against an obvious size difference between X versus nullo-X sperm and being consistent with our previous results that sperm size determination occurs early, that is prior to the formation of haploid spermatids.

SIZE CHANGES DURING THE TRANSITION FROM SPERMATID TO SPERMATOZOON

During the process of sperm activation, termed spermiogenesis, sperm cells undergo extensive morphological changes, marked by growth of a single pseudopod that is required for motility and which thus represents a presumed key element of sperm competitive ability (LaMunyon and Ward 1998; Singson et al. 1999; Hansen et al. 2015). We therefore asked whether spermatozoon morphology might differ between *Caenorhabditis* species with

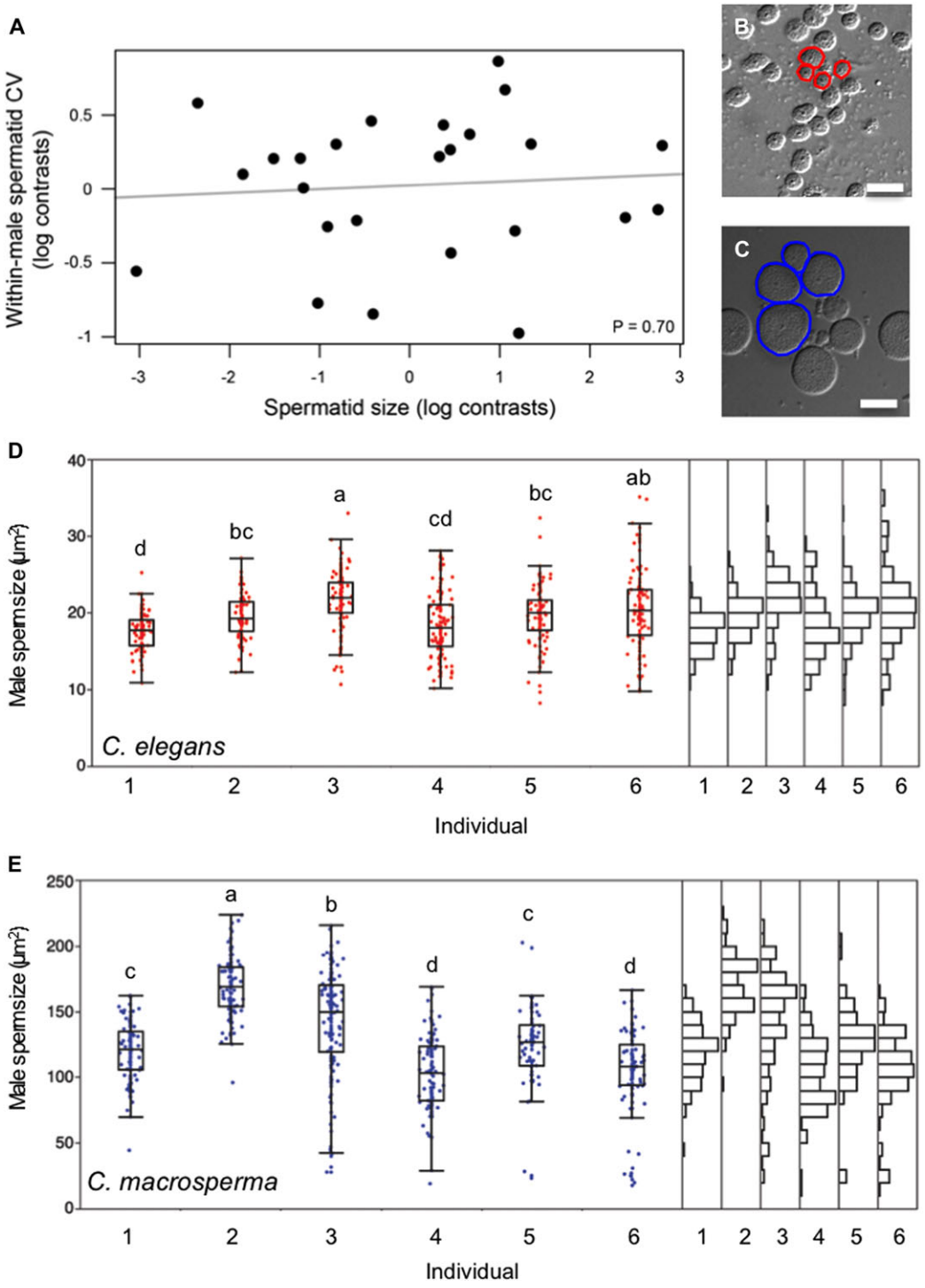

Figure 5. Intra- and interindividual variation in male sperm size. (A) PIC plot of mean sperm size versus mean within-male CV of sperm size indicates no association ($F_{1,23} = 0.15$, $P = 0.70$). (B, C) DIC images illustrating spermatid size variation within individual males of (B) *C. elegans* N2 and (C) *C. macrosperma* JU1857. Scale bars: 10 μm. (D, E) Inter- and intraindividual variation in male sperm size in (D) *C. elegans* N2 (N = 94–121 sperm per individual) and (E) *C. macrosperma* JU1857 (N = 74–138 sperm per individual). In both species, individuals show significant differences in average sperm size (*C. elegans* N2: $F_{5,647} = 14.73$, $P < 0.0001$; *C. macrosperma* JU1857: $F_{5,646} = 76.64$, $P < 0.0001$) and variance of sperm size (Levene's Test, *C. elegans* N2: $F_{5,647} = 9.29$, $P < 0.0001$; *C. macrosperma* JU1857: $F_{5,646} = 10.43$, $P < 0.0001$). Values labeled with different letters indicate significant differences in mean sperm size (Tukey's HSD, $P < 0.05$). (F) DIC images of spermatocyte divisions in *C. elegans* N2 and *C. macrosperma* JU1857. Scale bars: 10 μm. (G, H) Relationship between size of residual body and spermatid size (N = 3–4/spermatocyte) originating from the same primary spermatocyte (mean ± SEM) in (G) *C. elegans* N2 ($F_{1,11} = 6.16$, $R^2 = 0.36$, $P = 0.0305$) and (H) *C. macrosperma* JU1857 ($F_{1,19} = 12.95$, $R^2 = 0.41$, $P = 0.0019$).

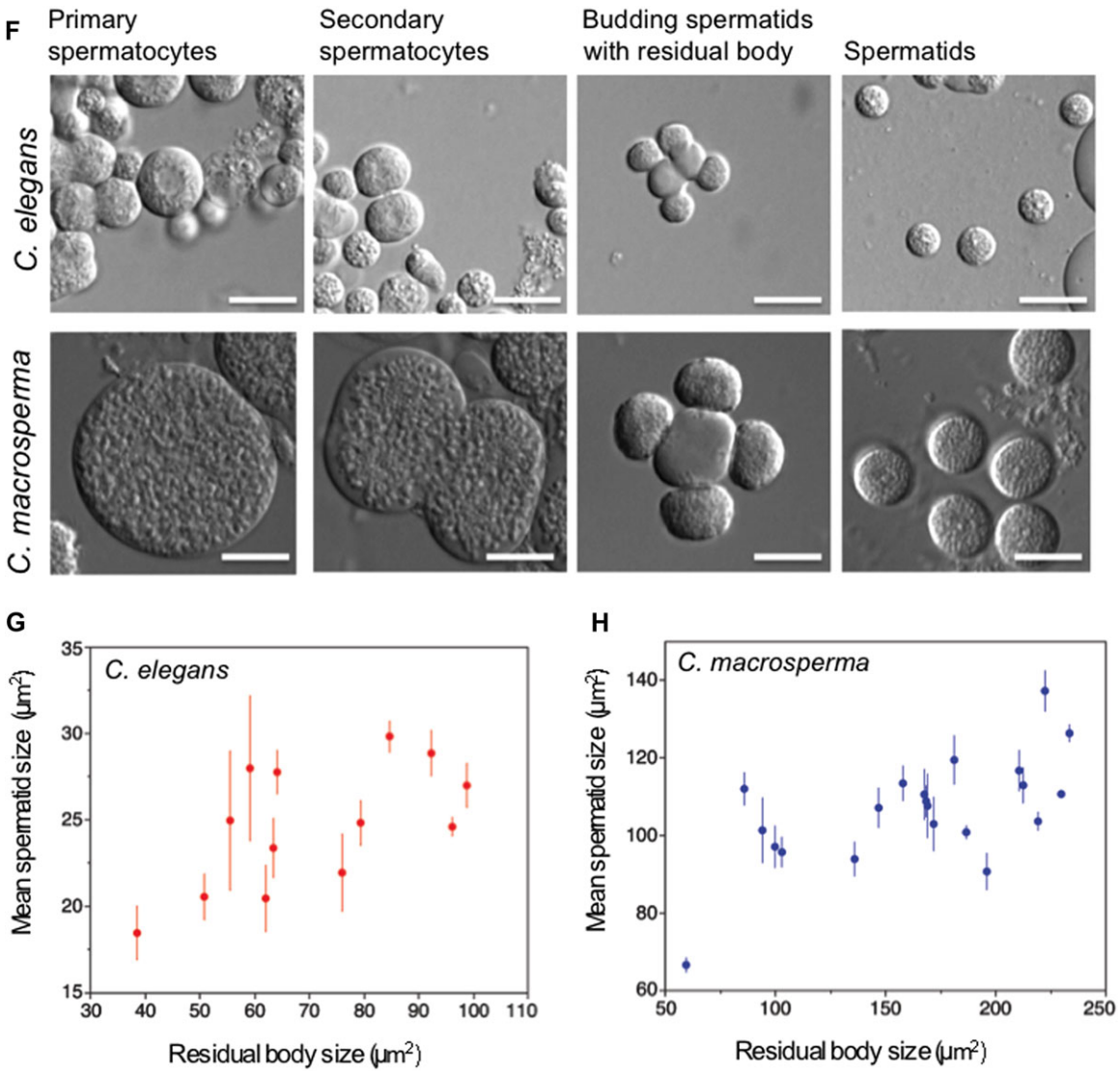

Figure 5. Continued

divergent spermatid sizes. All species examined displayed a spermatozoon phenotype similar to *C. elegans*, characterized by the polarization of the sperm cell into a cell body retaining the nucleus and other membranous cell components (L'Hernault 2006; Ellis and Stanfield 2014) and the formation of a single pseudopod of variable shape (Fig. 6A). The one exception was the spermatozoon morphology in *C. plicata* that, although clearly polarized, appear to retain a spherical shape without stereotyped pseudopod formation (Fig. 6A). However, this atypical sperm morphology for *C. plicata* could be caused by special sensitivity to the nonphysiological conditions of the sperm medium, resulting in pseudopod retraction as observed for spermatozoa of other species after prolonged exposure to sperm medium (data not shown).

To test whether species differences in spermatid size affect spermatozoon morphology, we focused again on *C. elegans* N2 and *C. macrosperma* JU1857. In both species, in vitro sperm activation rapidly induces polarization of sperm cells, which progressively form a mature spermatozoon with a hemispherical cell body containing granular cell components and a single pseudopod (Fig. 6B). Spermatid size differences between *C. elegans* N2 and *C. macrosperma* JU1857 translate into corresponding differences in size of cell body and pseudopod, and thus final size of spermatozoa (Fig. 6C and D), suggesting that the entire size range of spermatids activate successfully into mature spermatozoa. Moreover, we observed no difference between the species in the size ratio of pseudopod to cell body (ANOVA, $F_{1,298} = 0.23$, $P = 0.63$), suggesting that relative sizing of cell body versus pseudopod formation is maintained irrespective of spermatid size. These measurements, although limited to analysis with DIC microscopy, further suggest that the cell body size of spermatozoa becomes substantially reduced, by a factor of two on average, relative to the spermatid size (Fig. 6C and D).

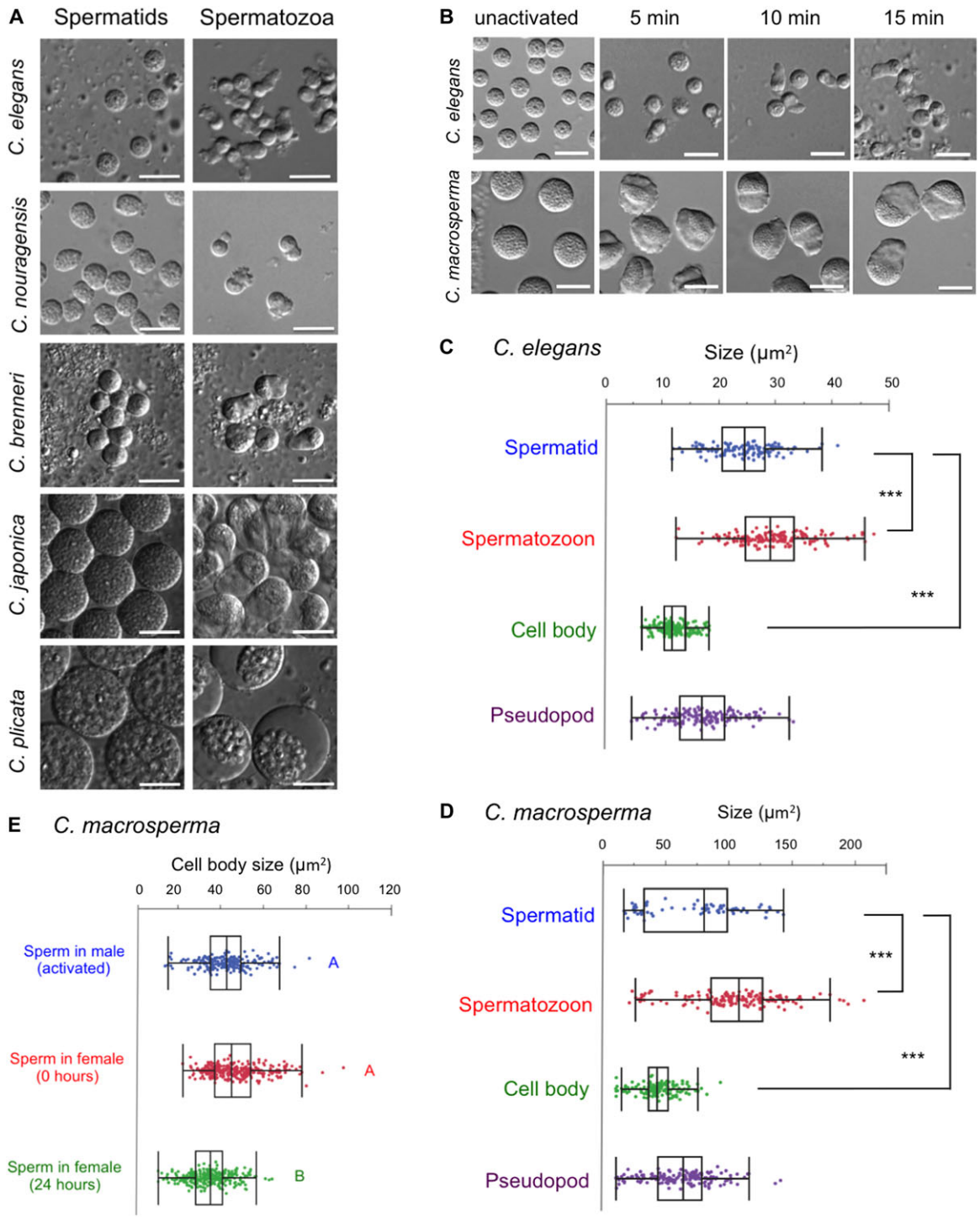

Figure 6. Size variation in spermatozoa. (A) DIC images of spermatids and spermatozoa (obtained from inseminated females) in *Caenorhabditis* species with divergent sperm size. Scale bars: 10 μm. (B) DIC images taken during time course of Pronase in vitro sperm activation for *C. elegans* N2 and *C. macrosperma* JU1857. Scale bars: 10 μm. (C, D) Size comparison of spermatids, spermatozoa (sum of cell body and pseudopod sizes), spermatozoon cell body, and pseudopod for *C. elegans* N2 and *C. macrosperma* JU1857 (D). Spermatozoon cross-sectional area is larger than for spermatids (ANOVA, *C. elegans* N2: $F_{1,263} = 64.42$, $P < 0.0001$; *C. macrosperma* JU1857: $F_{1,208} = 32.08$, $P < 0.0001$) and spermatozoon cell body size is strongly reduced relative to spermatid size (ANOVA, *C. elegans* N2: $F_{1,263} = 654.48$, $P < 0.0001$; *C. macrosperma* JU1857: $F_{1,208} = 73.84$, $P < 0.0001$). (E) Size distributions of spermatozoa (inferred from measurements of cell body size) in males (after in vitro activation) and in mated females (0 h vs. 24 h after mating) in *C. macrosperma* JU1857. Average sperm size is significantly smaller in females 24 h after mating compared to females immediately after mating (and activated sperm from males) (ANOVA, $F_{2,726} = 88.83$, $P < 0.0001$). Values labeled with different letters indicate significant differences (Tukey's HSD, $P < 0.05$).

The above data indicate that *Caenorhabditis* females inseminated by a single or multiple males will contain a pool of available spermatozoa that varies substantially in size. Under size-dependent sperm competition, the sperm size distribution in a female's reproductive tract should thus shift over time as larger sperm take precedence in fertilization and leave smaller remaining sperm at later time points. Consistent with this prediction, we found in *C. macrosperma* that females retain significantly smaller complements of sperm 24 hours after mating compared to the sperm size distribution found in their reproductive tracts immediately following mating or derived directly from males (ANOVA, $F_{2,726} = 88.83$, $P < 0.0001$) (Fig. 6E).

Discussion

CONVERGENT EVOLUTION OF SPERM SIZE GIGANTISM IN *Caenorhabditis* NEMATODES

Our phylogenetic analyses of sperm size evolution, including many novel *Caenorhabditis* species, uncover surprisingly high disparity in male sperm size among dioecious species that reveal multiple independent origins of sperm gigantism. The recurrent evolution of exceptionally large sperm has been reported for diverse taxonomic groups, including invertebrates (e.g., insects and molluscs) and vertebrates (e.g., mammals and birds) (Bressac et al. 1994; Pitnick and Markow 1994; Bjork and Pitnick 2006; Joly et al. 2008; Pitnick et al. 2009b); however, in contrast to *Caenorhabditis* nematodes, most of these taxa possess flagellate sperm. While our observations are consistent with the idea that *Caenorhabditis* sperm size divergence reflects the product of sexual selection by sperm competition, it will be critical to determine how characters of the female reproductive morphology associate with observed sperm size variation to evaluate the significance of coevolutionary processes between sperm size and female reproductive tract (Birkhead et al. 2009).

Moreover, how specific ecological or life-history factors of different *Caenorhabditis* species might have shaped such differential investment for many small versus fewer large sperm remains unresolved, given our limited but expanding knowledge of *Caenorhabditis* natural history (Félix and Braendle 2010; Cutter 2015; Frezal and Felix 2015). Notably, however, two of the four species with gigantic sperm display specialized life histories: *C. japonica* and *C. drosophilae* have dispersal associations with specific phoretic host insects (Kiontke and Sudhaus 2006; Yoshiga et al. 2013; Li et al. 2014). The two other species with gigantic sperm (*C. macrosperma* and *C. plicata*) also potentially represent "specialists" given that *C. macrosperma* displays a very localized geographic distribution (Felix et al. 2013) and that *C. plicata* was isolated only once and is the only *Caenorhabditis* in lab culture that was isolated from carrion (Kiontke and Sudhaus 2006). This limited evidence suggests that specialist life histories might be predisposed to conditions favorable to the evolution of extremely large male sperm. The life cycle in nature of *C. japonica* is known in most detail, for which reproduction takes place in isolated populations among the tens of founding individuals (Yoshiga et al. 2013). Because male reproductive success will be determined by the product of the number of mates and the number of fertilizations per mate, the relative importance of sperm size versus number in male fitness will depend on whether mate number or fertilization success per mate most constrains male fitness. We hypothesize that, in such tiny mating groups, male reproductive success might be constrained less by number of mates than by successful fertilization of their multiply inseminated mates, thus putting a fitness premium on the competitiveness of individual sperm and favoring the evolution of exaggerated sperm size. In contrast, we hypothesize that for most *Caenorhabditis*, a larger number of mates inseminated provides a greater relative benefit to male fitness and thus favors the evolution of especially vigorous mating ability and rapidly produced, numerous sperm at the expense of sperm being individually modest in size.

Of note, for consistency, we carried out all sperm size measurements at a single temperature (20°C), which might differ from the optimal growth temperatures among different species and isolates. It remains possible that genotype-dependent differences in thermal optima could contribute to some of the size variation observed among species and isolates, although we know of no evidence for or against this idea in *Caenorhabditis*. In general, plasticity of *Caenorhabditis* sperm size across different environments remains to be tested, though it has been observed only rarely in other systems (Pitnick et al. 2009b), such as dung flies and bruchid beetles that have flagellate sperm (Blanckenhorn and Hellriegel 2002; Vasudeva et al. 2014).

EVOLUTION OF REDUCED SPERM SIZE IN ANDRODIOECIOUS SPECIES

Male sperm size evolution in androdioecious species provides conditions of especially weak selection on male–male sperm competition, owing to male rarity in populations (Barrière and Félix 2005; Anderson et al. 2010; Frezal and Felix 2015). Thus, in addition to relaxed selection on male reproductive performance in general (Cutter 2008; Thomas et al. 2012), the convergent evolution of miniature sperm in androdioecious species likely results from a combination of: (i) selection favoring small sperm under low sperm-competition risk conditions (both sexes), (ii) selection for enhanced hermaphrodite self-fertilizing reproductive success that disfavors resource allocation to sperm (hermaphrodites only), (iii) developmental biases causing sperm size reduction because of the somatic-sex developmental environment (hermaphrodites only) (Baldi et al. 2011). Interestingly, our finding of limited evidence for correlated sperm size between hermaphrodites and

males of a given species suggests a genetic decoupling of sperm size determination between the sexes in this sexually dimorphic trait. Despite the special selective forces on male reproductive traits in androdioecious species, we also found that males of some dioecious *Caenorhabditis* have similarly miniature sperm (*C. nouraguensis, C. yunquensis, C. angaria, C.* sp. 1, *C. castelli*). As a consequence, future research on sperm size evolution in this group should aim to understand the factors that drive sperm miniaturization as well as gigantism. It remains unknown whether these factors might be more likely to involve mating-group size dynamics that maximize sperm count rather than size or an increased role of seminal fluid components in sperm competition, as analysis of *comp-1* mutants in *C. elegans* has made it clear that sperm size is not the sole determinant of sperm competitive ability (Hansen et al. 2015).

CONSISTENTLY HIGH LEVELS OF INTRA- AND INTERINDIVIDUAL SPERM SIZE VARIABILITY

For all *Caenorhabditis* species examined, we find previously underappreciated high levels of intraspecific sperm size variability that includes pronounced inter- and intraindividual sperm size heterogeneity. Significant variation in sperm morphometric traits between and within individuals is also common in diverse taxa that produce flagellate sperm (Ward 1998; Morrow and Gage 2001). However, in contrast to our observations for *Caenorhabditis,* within-individual sperm size variances in these other taxa were generally low and smaller than size variance calculated among different individuals (Ward 1998; Morrow and Gage 2001; Pitnick et al. 2003; Pattarini et al. 2006). In general, the evolutionary significance and developmental origins of inter- and intraindividual sperm trait variance are largely unknown, perhaps with the exception of some insect species that display pronounced sperm heteromorphism associated with functional differentiation of distinct sperm forms (Cook and Wedell 1999; Sahara and Kawamura 2002; Holman and Snook 2006). While our data provide no obvious signature of discrete size classes of sperm with different functions, it remains to be determined whether sperm across the entire size range of an individual are all fully functional, and whether the behavior of an individual sperm depends on its size. The presence of consistently high intraindividual sperm size variance across all *Caenorhabditis* species and genotypes–-largely irrespective of mean sperm size–-suggests several potential underlying causes. For example, (i) the maintenance of low sperm size variance may be costly, for example because increased precision would come at the cost of reduced sperm production speed (Parker and Begon 1993; Gomendio et al. 2006), (ii) increased sperm size variance reflects an adaptive strategy to maximize both mean size and number of sperm produced, or (iii) the developmental architecture of spermatogenesis constrains the precision with which sperm trait size can be achieved. Experimentally evaluating the relative contributions of such adaptive and nonadaptive forces in the determination of sperm size variability should thus be a key priority for future research.

EVOLUTION OF SPERM TRAIT CORRELATIONS, TRADE-OFFS, AND SIGNIFICANCE OF SPERM GIGANTISM

In this study, we have focused on size in sperm evolution. However, male allocation of ejaculate expenditure per mating comprises not only sperm size, but also the number of sperm and the amount and composition of the nonsperm seminal fluid (Perry et al. 2013). In *Caenorhabditis*, sperm size and number trade off such that genotypes that produce larger sperm also make them at a slower rate (LaMunyon and Ward 1998; Murray et al. 2011) and, as we show here, transfer fewer per mating (Fig. 2F). Despite the greater competitive ability of larger sperm within a reproductive tract, it remains unresolved what, mechanistically, is most critical for securing the competitive advantage: is it the greater speed conferred by a larger pseudopod (LaMunyon and Ward 1998; Singson et al. 1999; Hansen et al. 2015), or might it be better adhesion capability to the interior walls of the uterus and spermathecae, or a greater capacity to dislodge smaller sperm from the best locations? Resolving these possibilities would shed more light on the details of sperm competition traits most subject to selection, and the molecules underpinning them.

Caenorhabditis males, however, also transfer a largely unknown mixture of seminal fluid components and deposit a copulatory plug upon mating. The plug is comprised primarily of the mucin protein PLG-1 with possible functions including mate guarding and sperm retention (Barker 1994; Palopoli et al. 2008; Timmermeyer et al. 2010; Smith and Stanfield 2011; Hansen et al. 2015). It remains to be discovered how these nonsperm components of the ejaculate affect fertilization success and whether they might also contribute a source of trade-offs in reproductive resource allocation (e.g., plug size, seminal fluid quantity, or complexity) for overall ejaculate expenditure.

A consequence of sperm gigantism in *Caenorhabditis*, in the absence of correspondingly large oocytes, is the drastic reduction in the magnitude of anisogamy. We estimate that 3–5% of the volume of the zygote will derive from the sperm in such species, in contrast to $<0.5\%$ for the sperm contribution to the zygote for species at the other extreme that have minute sperm. How efficient will the maternally provisioned proteasome and RNA degradation machinery be in the face of such a large influx of cytoplasmic material, given that dogma holds that sperm contribute only the haploid complement of chromosomes and centrioles to the zygote? Might changes in sperm-mediated anisogamy resulting from male–male sperm competition produce an arena for novel selective pressures? Two hypotheses immediately spring from the consequences of a large cytoplasmic input from

extremely large sperm. First, could the sperm's cytoplasm provide a resource, provisioning "nutrients" to the developing zygote? For example, a variety of insect males deliver seminal "nuptial gifts" to their mates that can act as a food or water resource that fosters female reproduction (Bressac et al. 1994; Gwynne 2008), so it is conceivable that exceptionally large sperm might represent a more direct route for a paternal energetic contribution to their offspring. Second, might the sperm pack a suite of molecular and cellular components that engage in parental sexual conflict over the control of gene regulation in early zygotic development? In *Caenorhabditis*, such sexual conflict might be mediated by maternal and paternal contributions of small RNAs (Sarkies and Miska 2014) or protein products, with paternal-effect examples including PEEL-1, which may act in a dose-dependent fashion depending on sperm size (Seidel et al. 2011). Sperm size might also influence the potential for meiotic drive (Brandvain and Coop 2015). The significance of these mechanisms in mediating potential sexual conflict, and whether they can be linked to differences in sperm size, remains to be addressed. In addition, because *Caenorhabditis* offspring production usually is not viviparous, models of parental conflict from mammals and plants largely do not apply (Patten et al. 2014). However, sexual antagonism in the control over sexual development in the embryo provides an arena for paternal interests to manifest the intersexual conflict (Day and Bonduriansky 2004; Patten et al. 2014).

DEVELOPMENTAL ORIGINS OF *Caenorhabditis* SPERM SIZE VARIATION

The developmental mechanisms regulating sperm size, in both flagellate and aflagellate taxa, are generally poorly understood. Our study shows that *Caenorhabditis* sperm size is largely determined during early spermatogenesis, and specifically implicates heterogeneity in the size of primary spermatocytes as establishing the developmental basis of variation in sperm size. By contrast, subsequent cell divisions introduce little sperm size variability, as would result if the birth of spermatids during meiosis II cell divisions were asymmetric. The key role of primary spermatocyte size in controlling subsequent sperm size holds true for comparisons between species, between genotypes within species, and even within individuals and between the sexes for those androdioecious species in which both males and hermaphrodites produce sperm. These results suggest that *Caenorhabditis* sperm size is established at the diploid stage, similar to previous studies, which did not find any evidence for haploid determination of sperm length in flies (Pitnick et al. 2009a).

Why does *Caenorhabditis* sperm size determination occur so early during development and what is it that sets the size of primary spermatocytes? The beginning of the growth phase of spermatocytes coincides with the transition from pachytene into diplotene stages of meiosis (Shakes et al. 2009) (Fig. 3B). Growth of spermatocytes seems to occur throughout the progression through this condensation zone until entry into metaphase when spermatocytes detach from the rachis, the gonad core with shared cytoplasm, and start to divide (Shakes et al. 2009). The beginning of the growth phase occurs roughly at the same stage (diakinesis) in *C. elegans* oocytes although meiotic progression of sperm cells is considerably (2–3×) faster than for oocytes (Jaramillo-Lambert et al. 2007), consistent with gamete size differences. Further mechanisms of growth control for spermatocytes and oocytes are distinct, at least in part: streaming of cytoplasmic material from the gonad syncytium into growing oocytes, that is a likely provisioning mechanism, does not occur during spermatogenesis (Wolke et al. 2007). In addition, "physiological" germ cell apoptosis—thought to generate resources for developing oocytes—does not occur during spermatogenesis of *C. elegans* males or hermaphrodites (Gumienny et al. 1999). Overall, then, early size determination of *Caenorhabditis* can be explained by the limited growth phase corresponding to the time of primary spermatocyte formation and when spermatocytes are still connected to the gonad rachis, potentially providing nutrients. After detachment from the rachis, spermatocyte growth may thus become impossible, and/or meiotic divisions occur too rapidly to allow for significant cell growth.

Given the extreme size disparity of spermatocytes and oocytes in *C. elegans*, spermatocyte growth may not require oocyte-like mechanisms of gamete provisioning. However, this situation may not hold for those *Caenorhabditis* species that produce much larger sperm. It remains to be tested whether spermatogenesis in large-sperm species might have coopted oocyte-like mechanisms to nurture sperm growth. Fundamental mechanisms regulating cell size, and thus also spermatocyte size, include growth rate and timing of cell cycle progression (Ginzberg et al. 2015); for example, if spermatocyte growth rate remains constant, simply delaying entry into metaphase may lead to increased cell size. Consistent with this scenario, sperm production rates are slower for *C. elegans* genotypes that make larger sperm (LaMunyon and Ward 1998; Murray et al. 2011). Further experimental analysis may permit the disentangling of the interplay among the diversity of potential extracellular factors influencing spermatocyte growth with cell-autonomous mechanisms involved in *Caenorhabditis* sperm size regulation.

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C.B. and A.D.C. Performed the experiments: A.V., N.C., C.G., and N.P. Analyzed the data: C.B., A.D.C., A.V., and J.C.G. Wrote the paper: C.B. and A.D.C.

COMPETING INTERESTS

The authors declare no competing interests.

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LITERATURE CITED

Anderson, J. L., L. T. Morran, and P. C. Phillips. 2010. Outcrossing and the maintenance of males within *C*. elegans populations. J. Hered. 101 (Suppl. 1):S62–S74.

Arnqvist, G., M. Edvardsson, U. Friberg, and T. Nilsson. 2000. Sexual conflict promotes speciation in insects. Proc. Natl. Acad. Sci. USA 97:10460–10464.

Baldi, C., J. Viviano, and R. E. Ellis. 2011. A bias caused by ectopic development produces sexually dimorphic sperm in nematodes. Curr. Biol. 21:1416–1420.

Barker, D. M. 1994. Copulatory plugs and paternity assurance in the nematode *Caenorhabditis elegans*. Anim. Behav. 48:147–156.

Barrière, A., and M.-A. Félix. 2005. High local genetic diversity and low outcrossing rate in *Caenorhabditis elegans* natural populations. Curr. Biol. 15:1176–1184.

Birkhead, T. R., D. J. Hosken, and S. Pitnick. 2009. Sperm biology: an evolutionary perspective. Academic Press (Elsevier), Boston.

Birkhead, T. R., and A. P. Moller. 1998. Sperm competition and sexual selection. Academic Press, New York.

Bjork, A., and S. Pitnick. 2006. Intensity of sexual selection along the anisogamy-isogamy continuum. Nature 441:742–745.

Blanckenhorn, W. U., and B. Hellriegel. 2002. Against Bergmann's rule: fly sperm size increases with temperature. Ecol. Lett. 5:7–10.

Brandvain, Y., and G. Coop. 2015. Sperm should evolve to make female meiosis fair. Evolution 69:1004–1014.

Bressac, C., A. Fleury, and D. Lachaise. 1994. Another way of being anisogamous in *Drosophila* subgenus species: giant sperm, one-to-one gamete ratio, and high zygote provisioning. Proc. Natl. Acad. Sci. USA 91:10399–10402.

Bundus, J. D., R. Alaei, and A. D. Cutter. 2015. Gametic selection, developmental trajectories, and extrinsic heterogeneity in Haldane's rule. Evolution 69:2005–2017.

Cook, P. A., and N. Wedell. 1999. Nonfertile sperm delay female remating. Nature 397:486–486.

Cutter, A. D. 2008. Reproductive evolution: symptom of a selfing syndrome. Curr. Biol. 18:R1056–R1058.

———. 2015. *Caenorhabditis* evolution in the wild. Bioessays 37:983–995.

Day, T., and R. Bonduriansky. 2004. Intralocus sexual conflict can drive the evolution of genomic imprinting. Genetics 167:1537–1546.

Ellegren, H., and J. Parsch. 2007. The evolution of sex-biased genes and sex-biased gene expression. Nat. Rev. Genet. 8:689–698.

Ellis, R. E., and G. M. Stanfield. 2014. The regulation of spermatogenesis and sperm function in nematodes. Semin. Cell Dev. Biol. 29:17–30.

Farhadifar, R., C. F. Baer, A.-C. Valfort, E. C. Andersen, T. Müller-Reichert, M. Delattre, and D. J. Needleman. 2015. Scaling, selection, and evolutionary dynamics of the mitotic spindle. Curr. Biol. 25:732–740.

Félix, M.-A., and C. Braendle. 2010. The natural history of *Caenorhabditis elegans*. Curr. Biol. 20:R965–R969.

Felix, M. A., C. Braendle, and A. D. Cutter. 2014. A streamlined system for species diagnosis in *Caenorhabditis* (Nematoda: Rhabditidae) with name designations for 15 distinct biological species. PLoS ONE 9:e94723.

Felix, M. A., R. Jovelin, C. Ferrari, S. Han, Y. R. Cho, E. C. Andersen, A. D. Cutter, and C. Braendle. 2013. Species richness, distribution and genetic diversity of *Caenorhabditis* nematodes in a remote tropical rainforest. BMC Evol. Biol. 13:10.

Fitzpatrick, J. L., and B. Baer. 2011. Polyandry reduces sperm length variation in social insects. Evolution 65:3006–3012.

Frezal, L., and M. A. Felix. 2015. *C. elegans* outside the Petri dish. Elife 4 doi: 10.7554/eLife.05849.

Gavrilets, S. 2000. Rapid evolution of reproductive barriers driven by sexual conflict. Nature 403:886–889.

Geldziler, B., I. Chatterjee, P. Kadandale, E. Putiri, R. Patel, and A. Singson. 2006. A comparative study of sperm morphology, cytology and activation in *Caenorhabditis elegans*, *Caenorhabditis remanei* and *Caenorhabditis briggsae*. Dev. Genes Evol. 216:198–208.

Geldziler, B. D., M. R. Marcello, D. C. Shakes, and A. Singson. 2011. The genetics and cell biology of fertilization. Methods Cell Biol. 106:343–375.

Gimond, C., Jovelin, R., Han, S., Ferrari, C., Cutter, A. D., and Braendle, C. 2013. Outbreeding depression with low genetic variation in selfing *Caenorhabditis* nematodes. Evolution 67:3087–3101.

Ginzberg, M. B., R. Kafri, and M. Kirschner. 2015. On being the right (cell) size. Science 348:1245075–1245075.

Gomendio, M., J. Martin-Coello, C. Crespo, C. Magana, and E. R. S. Roldan. 2006. Sperm competition enhances functional capacity of mammalian spermatozoa. Proc. Natl. Acad. Sci. USA 103:15113–15117.

Grimbert, S., and C. Braendle. 2014. Cryptic genetic variation uncovers evolution of environmentally sensitive parameters in *Caenorhabditis* vulval development. Evol. Dev. 16:278–291.

Gumienny, T. L., E. Lambie, E. Hartwieg, H. R. Horvitz, and M. O. Hengartner. 1999. Genetic control of programmed cell death in the *Caenorhabditis elegans* hermaphrodite germline. Development 126:1011–1022.

Gwynne, D. T. 2008. Sexual conflict over nuptial gifts in insects. Annu. Rev. Entomol. 53:83–101.

Hansen, J. M., D. R. Chavez, and G. M. Stanfield. 2015. COMP-1 promotes competitive advantage of nematode sperm. Elife 4 doi: 10.7554/eLife.05423.

Harmon, L. J., J. T. Weir, C. D. Brock, R. E. Glor, and W. Challenger. 2008. GEIGER: investigating evolutionary radiations. Bioinformatics 24:129–131.

Hill, K. L., and S. W. L'Hernault. 2001. Analyses of reproductive interactions that occur after heterospecific matings within the genus *Caenorhabditis*. Dev. Biol. 232:105–114.

Holman, L., and R. R. Snook. 2006. Spermicide, cryptic female choice and the evolution of sperm form and function. J. Evol. Biol. 19:1660–1670.

Huang, J., H. Wang, Y. Chen, X. Wang, and H. Zhang. 2012. Residual body removal during spermatogenesis in C. *elegans* requires genes that mediate cell corpse clearance. Development 139:4613–4622.

Immler, S., S. Calhim, and T. R. Birkhead. 2008. Increased postcopulatory sexual selection reduces the intramale variation in sperm design. Evolution 62:1538–1543.

Ingram, T., and D. L. Mahler. 2013. SURFACE: detecting convergent evolution from comparative data by fitting Ornstein-Uhlenbeck models with stepwise Akaike Information Criterion. Meth. Ecol. Evol. 4:416–425.

Jaramillo-Lambert, A., M. Ellefson, A. M. Villeneuve, and J. Engebrecht. 2007. Differential timing of S phases, X chromosome replication, and meiotic prophase in the C. *elegans* germ line. Dev. Biol. 308:206–221.

Joly, D., N. Luck, and B. Dejonghe. 2008. Adaptation to long sperm in *Drosophila*: correlated development of the sperm roller and sperm packaging. J. Exp. Zool. B. Mol. Dev. Evol. 310:167–178.

Kiontke, K., M.-A. Felix, M. Ailion, M. Rockman, C. Braendle, J.-B. Penigault, and D. Fitch. 2011. A phylogeny and molecular barcodes for *Caenorhabditis*, with numerous new species from rotting fruits. BMC Evol. Biol. 11:339.

Kiontke, K., and W. Sudhaus. 2006. Ecology of *Caenorhabditis* species. WormBook:1–14.

L'Hernault, S. W. 2006. Spermatogenesis. WormBook:1–14.

LaMunyon, C. W., and S. Ward. 1995. Sperm precedence in a hermaphroditic nematode (*Caenorhabditis elegans*) is due to competitive superiority of male sperm. Experientia 51:817–823.

———. 1997. Increased competitiveness of nematode sperm bearing the male X chromosome. Proc. Natl. Acad. Sci. USA 94:185–189.

———. 1998. Larger sperm outcompete smaller sperm in the nematode *Caenorhabditis elegans*. Proc. R. Soc. 265:1997–2002.

———. 1999. Evolution of sperm size in nematodes: sperm competition favours larger sperm. Proc. R. Soc. 266:263–267.

———. 2002. Evolution of larger sperm in response to experimentally increased sperm competition in *Caenorhabditis elegans*. Proc. R. Soc. 269:1125–1128.

Lee, D. L. 2002. The biology of nematodes. CRC Press, Boca Raton.

Li, S., R. Jovelin, T. Yoshiga, R. Tanaka, and A. D. Cutter. 2014. Specialist versus generalist life histories and nucleotide diversity in *Caenorhabditis* nematodes. Proc. Biol. Sci. 281:20132858.

Miller, M. A., V. Q. Nguyen, M. H. Lee, M. Kosinski, T. Schedl, R. M. Caprioli, and D. Greenstein. 2001. A sperm cytoskeletal protein that signals oocyte meiotic maturation and ovulation. Science 291:2144–2147.

Morrow, E. H., and M. J. G. Gage. 2001. Consistent significant variation between individual males in spermatozoal morphometry. J. Zool. 254:147–153.

Murray, R. L., J. L. Kozlowska, and A. D. Cutter. 2011. Heritable determinants of male fertilization success in the nematode *Caenorhabditis elegans*. BMC Evol. Biol. 11:99.

Nelson, G. A., and S. Ward. 1980. Vesicle fusion, pseudopod extension and amoeboid motility are induced in nematode spermatids by the ionophore monensin. Cell 19:457–464.

Palopoli, M. F., C. Peden, C. Woo, K. Akiha, M. Ary, L. Cruze, J. L. Anderson, and P. C. Phillips. 2015. Natural and experimental evolution of sexual conflict within *Caenorhabditis* nematodes. BMC Evol. Biol. 15:93.

Palopoli, M. F., M. V. Rockman, A. Tinmaung, C. Ramsay, S. Curwen, A. Aduna, J. Laurita, and L. Kruglyak. 2008. Molecular basis of the copulatory plug polymorphism in *Caenorhabditis elegans*. Nature 454:1019–1022.

Paradis, E., J. Claude, and K. Strimmer. 2004. APE: analyses of phylogenetics and evolution in R language. Bioinformatics 20:289–290.

Parker, G. A., and M. E. Begon. 1993. Sperm competition games: sperm size and number under gametic control. Proc. R. Soc. 253:255–262.

Pattarini, J. M., W. T. Starmer, A. Bjork, and S. Pitnick. 2006. Mechanisms underlying the sperm quality advantage in *Drosophila melanogaster*. Evolution 60:2064–2080.

Patten, M. M., L. Ross, J. P. Curley, D. C. Queller, R. Bonduriansky, and J. B. Wolf. 2014. The evolution of genomic imprinting: theories, predictions and empirical tests. Heredity 113:119–128.

Perry, J. C., L. Sirot, and S. Wigby. 2013. The seminal symphony: how to compose an ejaculate. Trends Ecol. Evol. 28:414–422.

Pitnick, S., R. Dobler, and D. J. Hosken. 2009a. Sperm length is not influenced by haploid gene expression in the flies *Drosophila* melanogaster and *Scathophaga stercoraria*. Proc. Biol. Sci. 276:4029–4034.

Pitnick, S., D. J. Hosken, and T. R. Birkhead. 2009b. Sperm morphological diversity. Pp. 69–149 *in* T. R. Birkhead, D. J. Hosken, and S. Pitnick, eds. Sperm biology: An evolutionary perspective. Academic Press (Elsevier), Boston.

Pitnick, S., and T. A. Markow. 1994. Large-male advantages associated with costs of sperm production in *Drosophila hydei*, a species with giant sperm. Proc. Natl. Acad. Sci. USA 91:9277–9281.

Pitnick, S., G. T. Miller, K. Schneider, and T. A. Markow. 2003. Ejaculate-female coevolution in *Drosophila* mojavensis. Proc. Biol. Sci. 270:1507–1512.

Poullet, N., A. Vielle, C. Gimond, C. Ferrari, and C. Braendle. 2015. Evolutionarily divergent thermal sensitivity of germline development and fertility in hermaphroditic *Caenorhabditis* nematodes. Evol. Dev. 17:380–397.

Poullet, N., A.Vielle, C. Gimond, S. Carvalho, H. Teotonio, and C. Braendle. 2016. Complex heterochrony underlies the evolution of hermaphrodite sex allocation in experimental *C. elegans* populations. Evolution. In press.

Ramm, S. A., L. Schärer, J. Ehmcke, and J. Wistuba. 2014. Sperm competition and the evolution of spermatogenesis. Mol. Hum. Reprod. 20:1169–1179.

Sahara, K., and N. Kawamura. 2002. Double copulation of a female with sterile diploid and polyploid males recovers fertility in *Bombyx mori*. Zygote 10:23–29.

Sarkies, P., and E. A. Miska. 2014. Small RNAs break out: the molecular cell biology of mobile small RNAs. Nat. Rev. Mol. Cell Biol. 15:525–535.

Seidel, H. S., M. Ailion, J. Li, A. Van Oudenaarden, M. V. Rockman, and L. Kruglyak. 2011. A novel sperm-delivered toxin causes late-stage embryo lethality and transmission ratio distortion in *C. elegans*. PLoS Biol. 9:e1001115.

Shakes, D. C., B. J. Neva, H. Huynh, J. Chaudhuri, and A. Pires-Dasilva. 2011. Asymmetric spermatocyte division as a mechanism for controlling sex ratios. Nat. Commun. 2:157.

Shakes, D. C., J.-C. Wu, P. L. Sadler, K. Laprade, L. L. Moore, A. Noritake, and D. S. Chu. 2009. Spermatogenesis-specific features of the meiotic program in *Caenorhabditis elegans*. PLoS Genet. 5:e1000611.

Singaravelu, G., I. Chatterjee, M. R. Marcello, and A. Singson. 2011. Isolation and in vitro activation of *Caenorhabditis elegans* sperm. J. Vis. Exp. 47 doi: 10.3791/2336.

Singson, A., K. L. Hill, and S. W. L'Hernault. 1999. Sperm competition in the absence of fertilization in *Caenorhabditis elegans*. Genetics 152:201–208.

Smith, J. R., and G. M. Stanfield. 2011. TRY-5 is a sperm-activating protease in *Caenorhabditis elegans* seminal fluid. PLoS Genet. 7.

Smith, R. L. 1984. Sperm competition and the evolution of animal mating systems. Academic Press, Orlando.

Stiernagle, T. 2006. Maintenance of *C. elegans*. WormBook:1–11.

Swanson, W. J., and V. D. Vacquier. 2002. The rapid evolution of reproductive proteins. Nat. Rev. Genet. 3:137–144.

Thomas, C. G., G. C. Woodruff, and E. S. Haag. 2012. Causes and consequences of the evolution of reproductive mode in *Caenorhabditis* nematodes. Trends Genet. 28:213–220.

Timmermeyer, N., T. Gerlach, C. Guempel, J. Knoche, J. F. Pfann, D. Schliessmann, and N. K. Michiels. 2010. The function of copulatory plugs in *Caenorhabditis* remanei: hints for female benefits. Front Zool 7:28.

Ting, J. J., G. C. Woodruff, G. Leung, N.-R. Shin, A. D. Cutter, and E. S. Haag. 2014. Intense sperm-mediated sexual conflict promotes gametic isolation in *Caenorhabditis* nematodes. PLoS Biol. 12:e1001915.

Vasudeva, R., D. C. Deeming, and P. E. Eady. 2014. Developmental temperature affects the expression of ejaculatory traits and the outcome of sperm competition in *Callosobruchus maculatus*. J. Evol. Biol. 27:1811–1818.

Ward, P. I. 1998. Intraspecific variation in sperm size characters. Heredity 80 (Pt 6):655–659.

Ward, S., Y. Argon, and G. A. Nelson. 1981. Sperm morphogenesis in wild-type and fertilization-defective mutants of *Caenorhabditis elegans*. J. Cell Biol. 91:26–44.

Ward, S., and J. S. Carrel. 1979. Fertilization and sperm competition in the nematode *Caenorhabditis elegans*. Dev. Biol. 73:304–321.

Ward, S., E. Hogan, and G. A. Nelson. 1983. The initiation of spermiogenesis in the nematode *Caenorhabditis elegans*. Dev. Biol. 98:70–79.

Webb, C. O., D. D. Ackerly, and S. W. Kembel. 2008. Phylocom: software for the analysis of phylogenetic community structure and trait evolution. Bioinformatics 24:2098–2100.

Wolf, N., D. Hirsh, and J. R. McIntosh. 1978. Spermatogenesis in males of the free-living nematode, *Caenorhabditis elegans*. J. Ultrastruct. Res. 63:155–169.

Wolke, U., E. A. Jezuit, and J. R. Priess. 2007. Actin-dependent cytoplasmic streaming in *C*. elegans oogenesis. Development 134:2227–2236.

Yoshiga, T., Y. Ishikawa, R. Tanaka, M. Hironaka, and E. Okumura. 2013. Species-specific and female host-biased ectophoresy in the roundworm *Caenorhabditis* japonica. Naturwissenschaften 100:205–208.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Table S1. Male spermatid size variation in *Caenorhabditis* nematodes (cross-sectional area in μm2).
Table S2. Analysis of variance results for male spermatid size (log-transformed data).
Table S3. Body size variation (at L4 stage + 24h) of (A) females/hermaphrodites and (B) males in the 26 *Caenorhabditis* species.
Table S4. Analysis of variance for (A) male body length, (B) male body width, (C) female/hermaphrodite body length, (D) female/hermaphrodite body width (log-transformed data).
Table S5. Primary spermatocyte size variation in *Caenorhabditis* nematodes (crosssectional area in μm2).
Table S6. Analysis of variance results for male primary spermatocyte size (logtransformed data).
Table S7. Analysis of variance results for male spermatid size (log-transformed data) in dioecious species: (A) *C. brenneri*, (B) *C. macrosperma*, (C) *C. remanei,* (D) *C.* sp. 8.
Table S8. Hermaphrodite spermatid size variation in androdioecious *Caenorhabditis* species (cross-sectional area in μm2).
Table S9. Analysis of variance results for male and hermaphrodite spermatid size in androdiecious species, (A) *C. briggsae*, (B) *C. elegans*, (C) *C. tropicalis* (log-transformed data).
Table S10. Analysis of variance results for male and hermaphrodite spermatocyte size in *C.*
Table S11. Coefficients of variation (CV) for within-individual and between-individual male spermatid size (SD/mean x 100).
Figure S1. SURFACE analysis of sperm size evolution along the *Caenorhabditis* phylogeny.
Figure S2. Brownian motion inference of ancestral states for male body length (A), female/hermaphrodite body length (B), spermatid cross-sectional area (C), and embryo longitudinal cross-sectional area (D).
Figure S3. Phylogenetic independent contrasts show no significant correlation across species between female/hermaphrodite body length and the cross-sectional area of embryos analysis of sperm size evolution along the *Caenorhabditis* phylogeny (log-transformed species mean values; $F_{1,23}=2.53$, $P=0.126$).
Figure S4. Trait bi-plots uncorrected for phylogenetic relatedness.
Figure S5. Meiotic division stages of male spermatogenesis in *C.*
Figure S6 (below). Individual sperm size distributions, ordered alphabetically by species/strain names.