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RESEARCH ARTICLE

Experimental evolution of sperm count in protandrous self-fertilizing hermaphrodites

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SUMMARY

Sperm count evolution is driven by sexual selection, with an added role of selection on gamete resource allocation for hermaphrodite spermatogenesis. However, self-fertilization by hermaphrodites retards sexual selection and results in the evolution of reduced investment in sperm or pollen. In contrast to reproduction limited by female gametes (Bateman's Principle), self-fertilizing *Caenorhabditis elegans* hermaphrodites exhibit sperm-limited reproduction. *Caenorhabditis elegans* hermaphrodites are thought to experience a fitness trade-off between lifetime fecundity and generation time: longer sperm production decreases the risk of self-sperm depletion, but at the same time delays the onset of selfing and thus increases egg-to-egg generation time. Theory predicts that shorter larval development will favor lower sperm counts and longer development will favor more sperm. To investigate how developmental trajectories affect the evolution of sperm production, we performed experimental evolution by directly competing alleles controlling hermaphrodite sperm count, conducted under different environmental conditions that alter development time. Results are partially consistent with theory: rapid larval development generally favored alleles encoding production of few sperm. However, we identify some previously unrecognized simplifications of the theory and its application to our experimental system. In addition, we evaluated the generality of sperm limitation in *C. elegans*. Although optimal growth conditions yield sperm limitation, non-optimal conditions induce oocyte limitation, suggesting that this species might conform to Bateman's Principle under many natural settings. These findings demonstrate how developmental trajectories can shape the fitness landscape for the evolution of reproduction and sperm traits, even without sexual selection.

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/214/10/1740/DC1

Key words: *Caenorhabditis elegans*, sperm evolution, experimental evolution, hermaphrodite, sex allocation.

INTRODUCTION

The evolution of sperm production typically falls under the purview of theory on sexual selection (Birkhead and Moller, 1998; Simmons and Siva-Jothy, 1998). However, in the context of hermaphrodite reproduction, particularly in the absence of sexual selection, resource allocation helps explain relative investment in each gamete type (Charnov, 1982; Greeff and Michiels, 1999; Munday et al., 2006; Schärer, 2009). Specifically, sex-allocation theory applied to hermaphrodites predicts that self-fertilizing individuals will invest few resources in male function; the smallest number of sperm should be produced that can fertilize all ova (Charnov, 1982). In age-structured populations, an optimal brood size version of sex-allocation theory is most appropriate, because trade-offs between brood size and the time until the next episode of reproduction may favor intermediate brood sizes (Lack, 1947; Sibly and Calow, 1983; Charnov, 1988; Charlesworth, 1994). Such trade-offs have been observed in both gonochorists (Godfray, 1991) and hermaphrodites (Hodgkin and Barnes, 1991). Indeed, this is the case for the protandrous, self-fertilizing hermaphroditic nematode *Caenorhabditis elegans* (Maupas 1899), and related taxa with similar life history (Hodgkin and Barnes, 1991; Barker, 1992; Cutter, 2004). However, it is generally unknown to what extent the pace of juvenile development can affect the selective regime of reproductive traits in a population, outside of the framework of condition dependence and sexual selection (Rowe and Houle, 1996).

In *C. elegans*, the hermaphrodite ovotestes first produce ~280 sperm as last-stage larvae (L4) and young adults before switching irreversibly to oogenesis (Fig. 1) (Ward and Carrel, 1979). If a hermaphrodite receives additional sperm from a male, ovulation continues, allowing up to four times as many progeny compared with exclusive selfing (Kimble and Ward, 1988; Hodgkin and Barnes, 1991). Hermaphrodites are incapable of inseminating each other, however, analogous to complete 'pollen discounting' (Harder and Wilson, 1998). Under standard laboratory conditions, nearly 100% of all self-sperm fertilize an oocyte, making hermaphrodite self-fecundity sperm limited (Ward and Carrel, 1979). This contrasts with the widespread pattern in nature of female-gamete limitation, known as Bateman's Principle (Bateman, 1948). We expect *C. elegans* hermaphrodite sperm production to be determined almost entirely by selection on self-fitness because males, and mating events, are exceptionally rare in nature (reviewed in Cutter et al., 2009; Anderson et al., 2010). Therefore, extending the period of spermatogenesis to make more sperm might seem advantageous for hermaphrodite fitness. However, oogenesis and fertilization cannot begin until sperm production has ceased, so greater sperm production delays the onset of oogenesis and fertilization; when the duration of spermatogenesis increases, so does the egg-to-egg generation time (Hodgkin and Barnes, 1991). Thus, there is a trade-off between lifetime fecundity and generation time in *C. elegans* hermaphrodites (Hodgkin and Barnes, 1991).

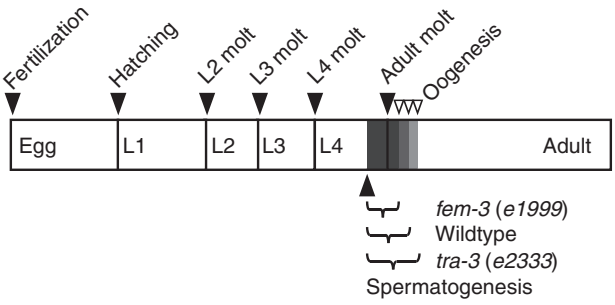

Fig. 1. *Caenorhabditis elegans* life stages from egg to larva (L1–L4) to adult with the approximate relative span of spermatogenesis of the alternative genotypes used in this study.

Hodgkin and Barnes observed that populations of genetic mutant strains of *C. elegans* that were either more or less fecund than wildtype, by virtue of differences in the timing of the spermatogenesis–oogenesis switch, exhibited slower exploitation of a food resource than the wildtype reference strain (Hodgkin and Barnes, 1991). From these non-competitive assays of reproductive rates, they concluded that intermediate self-sperm counts maximize fitness. Following on this work, Barker, and later Cutter, provided theoretical treatments of the evolution of sperm production in *C. elegans* (Barker, 1992; Cutter, 2004). Barker's model described the survival probability and costs associated with the time spent producing gametes and how these costs relate to the trade-off between brood size and generation time (Barker, 1992). Cutter expanded on this framework and predicted that optimal sperm number for hermaphrodites (i.e. the number of sperm that maximizes the intrinsic rate of population growth) would increase with faster gamete production rates or longer larval development, assuming independence between these factors (Cutter, 2004). Faster gamete production for a given duration of larval development is selectively equivalent to slower gamete production for a more rapid larval phase. Specifically, maximum population growth rate is shown to be:

$$r_{\max} = \frac{1}{n^*} e \ln\left[\frac{(1-p)+\frac{s}{e}}{1-p}\right] - u \,, \qquad (1)$$

where $r_{\max}$ is the maximum growth rate, n^* is the equilibrium number of sperm, u is the mortality rate, e is the egg-laying rate, s is the sperm-production rate and p is the fraction of precociously made sperm [note that eqn 3 in Cutter (Cutter, 2004) has a typographical error; the above equation is correct]. Although the model decouples rates of gamete production from development through the juvenile period, it is unknown empirically what occurs in practice.

Caenorhabditis elegans has proved an important model system for understanding the mechanisms of sperm-trait evolution since the pioneering experimental and comparative studies by LaMunyon and Ward on sperm size and competitive ability (LaMunyon and Ward, 1995; LaMunyon and Ward, 1998; LaMunyon and Ward, 1999; LaMunyon and Ward, 2002) (see also Geldziler et al., 2006). Much of this work emphasizes sperm competition in relation to males, in one instance applying one of the first explicit regimes of experimental evolution with this organism (LaMunyon and Ward, 2002; LaMunyon et al., 2007). To obtain a deeper understanding of the forces governing sperm evolution, we explore the evolution of self-fecundity in *C. elegans* hermaphrodites in the absence of sperm competition and test, using an experimental evolution approach based on defined genetic variants, whether the duration of larval development acts as a selective agent on sperm count. We also evaluate the appropriateness of two model assumptions for sperm count evolution in this system: that sperm limitation is ubiquitous and that rates of ontogenetic development and sperm production are decoupled.

MATERIALS AND METHODS

Strains, culturing and maintenance

We obtained strains CB4419 *tra-3* (*e2333*) and PD4790 (*mls12*) from the *Caenorhabditis* Genetics Center (CGC) and strain CB4886 *fem-3* (*e1999*) was a gift of Jonathan Hodgkin (University of Oxford, Oxford, UK). Each strain shares the same genetic background. The *fem-3* and *tra-3* genes both are integral downstream components of the sex-determination pathway in *C. elegans* (Hodgkin, 1987). These strains all derive from the canonical strain N2 and differ in the number of sperm produced: PD4790 (*mls12*) contains a pharyngially expressed green fluorescent protein (GFP) transgene marker and produces a wildtype number of sperm, *tra-3* (*e2333*) produces more sperm than wildtype and *fem-3* (*e1999*) produces fewer sperm than wildtype (Fig. 1) (Hodgkin and Barnes, 1991). We cultured and maintained the nematodes in Petri plates on NGM-Lite agar seeded with *Escherichia coli* strain OP50 (Stiernagle, 2005). For the experimental evolution assay, we spread *E. coli* OP50 into lawns on NGM-Lite agar in plates measuring 6 cm in diameter (liquid culturing described below).

Temperature dependence of hermaphrodite fecundity

Previous research demonstrated that *fem-3* (*e1999*) and *tra-3* (*e2333*) exhibit lower and higher fecundity, respectively, than wildtype when reared at 20°C (Hodgkin and Barnes, 1991). To confirm consistency across rearing temperatures, we quantified lifetime fecundity at three temperatures (15, 20 and 25°C). First, strains were age-synchronized using the sodium hypochlorite method (Stiernagle, 2005) and reared at one of the three temperatures. We then isolated 15 single L4 hermaphrodites from each strain–temperature combination and transferred them daily until eggs were no longer laid for 2 days in a row or until unfertilized eggs were laid. Progeny grew to late larval or early adult stage, at which point we counted and tallied them to determine lifetime fecundity. We maintained parental hermaphrodites and their progeny at the same growth temperature. To determine an effect of strain and temperature on lifetime fecundity we used an ANOVA in JMP 8 (SAS Institute, Cary, NC, USA) with the factors temperature, strain and temperature × strain interaction. We also performed *post hoc* tests [Tukey's honestly significant difference (HSD)] to compare pairwise differences.

Rate of hermaphrodite sperm production

We sampled age-synchronized hermaphrodites from *tra-3* (*e2333*), *fem-3* (*e1999*) and wildtype individuals grown in four replicates at 15, 20 and 25°C to determine an effect of rearing temperature on the rate of sperm production. Worms were sampled at regular intervals from L4 through early young adult (intervals of 6 h at 15°C, 4 h at 20°C and 2 h at 25°C). We prepared, fixed and DAPI DNA-stained worms on glass slides with cold (–20°C) methanol (Sulston and Hodgkin, 1988) to view under fluorescent microscopy and capture digital images of sperm nuclei, as described previously (Cutter, 2004). We counted the number of sperm in both gonad arms for ~10 worms per strain per temperature and regressed sperm counts on age. The least-squares regression slope for the first three time points represents the rate of sperm production for a given strain at

a given temperature (Cutter, 2004). We excluded the fourth time point from the rate estimates because oocyte production had begun in some instances, indicating that sperm production had ceased and some sperm had been used for fertilization of oocytes. Sperm counts at the fourth time point, however, were used as an estimate of the total number of sperm produced to compare with the number of offspring produced. These estimates of total sperm count will be slightly underestimated to the extent that a small number of sperm had been used for fertilization during the preceding interval.

To test for independence of the effects of temperature on sperm production and larval development, we compared our sperm production values with previously reported values of hermaphrodite lifespan and embryonic development (Klass, 1977; Wong et al., 1995; Lee and Kenyon, 2009). Specifically, we contrasted the wildtype ratio of developmental rates (embryonic, total lifespan or sperm) at low- (15°C) to moderate-temperature rearing (20°C; $R_{15/20}$) to the ratio of developmental rates at moderate to high temperature rearing (25°C; $R_{20/25}$). If $R_{15/20}=R_{20/25}$, then we would conclude that the proportional change in development rates per °C is equivalent for cool and warm changes in temperature. Because we are unable to compare these metrics statistically, we present the patterns and their implications in the Discussion.

Experimental evolution of sperm number

We used experimental evolution in *C. elegans* to test the hypothesis that a temperature-induced change in the duration of larval development affects the number of sperm that maximizes hermaphrodite fitness. We tested whether genotypes producing more or fewer sperm increased in frequency in a temperature-dependent manner. We first grew age-synchronized populations of each strain in isolation at 15, 20 and 25°C. We then initiated populations with a 1:1 ratio of two component genotypes: 20 L4 wildtype hermaphrodites (containing the GFP marker) and either 20 *tra-3* (*e2333*) or 20 *fem-3* (*e1999*) hermaphrodite individuals ('solid media' plates). We transferred an unbiased sample of genotypes to a fresh plate every week for 10 weeks by washing experimental plates with M9 buffer and aliquotting 0.5 ml to the new plates (containing 500–700 individuals). Simultaneously with each transfer, we age-synchronized a separate 1 ml aliquot of the population *via* bleaching (Stiernagle, 2005) and scored ~200 individuals to estimate the proportion of each strain (GFP or non-GFP) in the population. We performed eight replicates for each strain combination [*tra-3* (*e2333*) *vs* wildtype and *fem-3* (*e1999*) *vs* wildtype] at each temperature (20 and 25°C; six replicates at 15°C). No males were observed among the individuals counted to determine allele frequencies (males can arise spontaneously by X-chromosome non-disjunction during hermaphrodite self-fertilization). Thus, both the potential for male–male and male–hermaphrodite sexual selection and for recombination of genotypes is negligible. The consistency of solid and liquid media results (see below; mating is precluded in shaking liquid culture) supports the lack of an influence of rare males on the outcome of experimental evolution.

We repeated this experiment by rearing the worms in liquid culture ('liquid media'), rather than on solid agar medium to test for robustness to the specifics of environmental growth regime and because larger populations could be maintained in liquid which might accelerate allele frequency changes. Four replicate worm populations per treatment were maintained for 7 weeks in shaking flasks at 100 rpm with 50 ml NGM-Lite liquid media inoculated with *E. coli* strain OP50. Starting populations contained 400 individuals (200 from each strain; same genotype combinations as for the solid-media experiment). We transferred aliquots of 2 ml (~500–800 individuals) weekly to fresh NGM-Lite liquid media, as well as aliquots for genotype quantification as above. Because of the smaller number of replicates and shorter length of the experiment in liquid culture, we focus our discussion on the solid-media experiment results; with one exception, the results of solid and liquid media experiments were qualitatively consistent.

Analysis of experimental evolution data

To assess the changes in frequency of mutant strains through time, we used a repeated-measures general linear model to account for the partial dependence of measurements on previous time points (SAS PROC MIXED) (Neter et al., 1996). We included temperature and strain (*fem-3* or *tra-3*) as categorical independent variables, and time as a continuous variable. Analyses initially assessed the effects of all factors and their interactions. We characterized the covariances between responses by individual trials because the analysis involved repeated measurements of the same trial over time (Jennrich and Schluchter, 1986). Denominator degrees of freedom for *F*-tests were calculated using Kenward and Roger's (Kenward and Roger, 1997) approximation, which can result in fractional degrees of freedom. We interpret significant interactions with contrasts, and used a strict Bonferonni correction to account for multiple tests. Of most relevance is the time × strain × temperature interaction, which will reflect whether strains differ in how allele frequencies change over time as a function of the temperature treatment. Finally, we used a random regression mixed model analysis to investigate the proportion of mutant *versus* wildtype individuals in each temperature treatment over time. This approach fits individual intercepts and slopes for each replicate trial, accounting for other model effects.

We also inferred the strength of selection ($2N_es$, where N_e is the effective population size and s is the selection coefficient) in the experimental evolution lines (Bollback et al., 2008). This method uses a diffusion process to describe allele frequency change in time series data for two alleles. To calibrate the time series for maximum likelihood estimation of selection coefficients, we assumed 3.5 generations per weekly transfer for all 25°C experiments, two generations per transfer for 20°C experiments and 1.17 generations per transfer for 15°C experiments. For each replicate, we computed the likelihood surface for $1\leq N_e\leq 700$ (spacing interval=10) and $-12\leq 2N_es\leq 20$ (spacing interval=0.1). We then summed the negative log-likelihood ($-\ln L$) values across replicates for equivalent parameter combinations to generate a composite likelihood surface for each treatment, from which we inferred the parameter estimates corresponding to the maximum. This approach is useful for quantifying the magnitude of selection in population genetic terms, but the repeated-measures general linear model described above provides a more powerful method for hypothesis testing.

RESULTS

Genetic and environmental effects on hermaphrodite self-fecundity

The fecundity pattern of sperm-mutant strains was largely consistent across temperatures (Hodgkin and Barnes, 1991) (Fig. 2). Specifically, *fem-3* (*e1999*) self-fecundity was lower than wildtype and *tra-3* (*e2333*) self-fecundity was higher than wildtype ($F_{2,133}=51.78$, $P<0.0001$; supplementary material Tables S1, S2). Each strain has highest lifetime fecundity at 20°C, with reduced brood sizes at 15 and 25°C ($F_{2,133}=11.22$, $P<0.0001$; supplementary material Tables S1, S2). These results confirm the expected influence on self-fecundity of these genotypes (Fig. 1) (Hodgkin and Barnes, 1991) and of temperature for all three strains (Klass, 1977; Harvey and Viney, 2007).

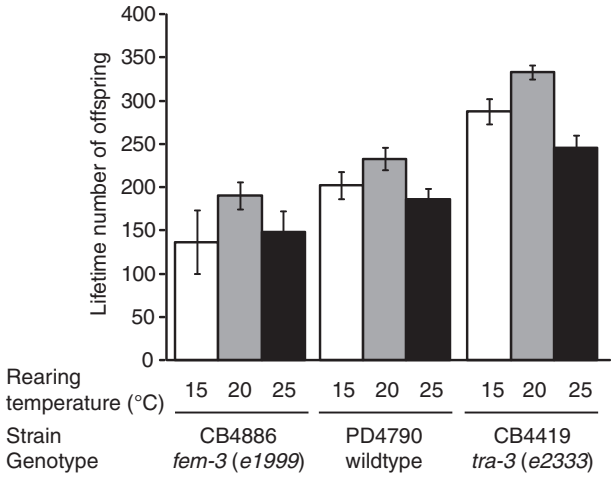

Fig. 2. Mean lifetime self-fecundity for three strain genotypes of *C. elegans* hermaphrodites reared at 15, 20 and 25°C. Error bars represent ±s.e.m. (N=15 for all except N=16 for wildtype at 25°C).

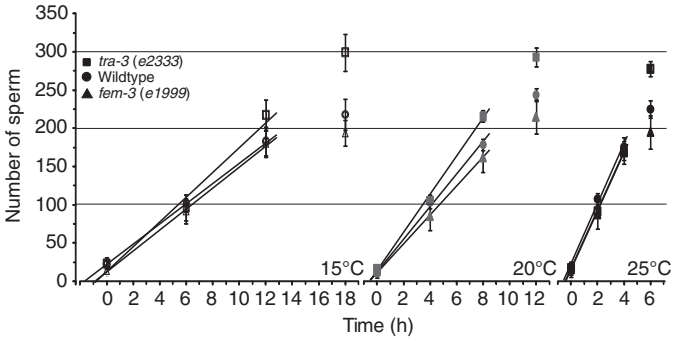

Fig. 3. Mean increase in sperm number over time in three N2-derived strains [triangles, *fem-3* (*e1999*); squares, *tra-3* (*e2333*); circles, wildtype using GFP marker] of *C. elegans* hermaphrodites at three rearing temperatures (15, 20 and 25°C). The slope of the linear regression corresponds to the estimated rate of sperm production (first three time points) and the final time point provides an estimate of total sperm count (Table 1). Error bars represent ±s.e.m.

Genetic and environmental effects on hermaphrodite sperm production

Higher rearing temperatures significantly hastened rates of sperm production (age × temperature interaction, $F_{1,26}$=93.17, P<0.0001), but the three strains did not differ significantly from each other in their rates of sperm production (strain × age × temperature interaction, $F_{2,26}$=0.19, P=0.83) (Tables 1 and 2, Fig. 3, supplementary material Table S3). Hermaphrodites produced sperm at rates of ~0.24, ~0.39 and ~0.68 sperm min^{-1} when reared at 15, 20 and 25°C, respectively (Table 1). These estimates are fully consistent with estimates for the N2 genetic background (0.39 sperm min^{-1} at 20°C) (Cutter, 2004).

Using the fourth and final time point to measure total sperm production, the three genotypes differed from each other in total sperm count ($F_{2,112}$=28.6, P<0.0001) but temperature did not significantly affect total sperm count ($F_{2,112}$=0.41, P=0.67). Thus, the detrimental effect of high and low temperature on hermaphrodite self-fecundity is not a consequence of sperm limitation resulting from different total numbers of sperm produced by the ovotestes of worms reared at different temperatures.

Experimental evolution of sperm number

Our experimental evolution yielded significantly different patterns of allele frequency change among the three rearing temperatures (Table 3, Fig. 4, supplementary material Table S4 and Fig. S1). Focusing on the solid-media experiments for which we had greatest experimental replication, we found that: (1) when reared at 25°C, inducing rapid larval development, the 'high sperm count' *tra-3* (*e2333*) allele declined in frequency and the 'low sperm count' *fem-3* (*e1999*) allele increased in frequency, in accord with theoretical predictions (Cutter, 2004); (2) when reared at 20°C, the *tra-3* (*e2333*) allele frequency remained roughly constant and the *fem-3* (*e1999*) allele declined in frequency; and (3) when reared at 15°C, inducing slow larval development, the proportion of 'high sperm count' *tra-3* (*e2333*) alleles increased over time, as expected by theory. And yet, the 'low sperm count' *fem-3* (*e1999*) allele frequency also increased over time at 15°C, indicating a contrasting pattern of sperm count evolution for the *tra-3* and *fem-3* treatments conducted at 15°C.

Note that our repeated-measures analysis properly accounts for the partial dependence of allele frequencies on previous generations. However, the temperature treatments differed in the number of generations that elapsed by the end of the experiment. Consequently, statistical tests among treatments are conservative with respect to detecting differences among them. Nevertheless, we observed consistent qualitative patterns among temperature treatments for solid agar media and liquid culture experiments, with one exception (*fem-3* 15°C *cf.* 25°C), confirming the general robustness of these patterns to the specifics of rearing environment (Fig. 4, supplementary material Fig. S1). The maximum likelihood estimates

Table 1. Summary of rate of hermaphrodite sperm production for three strains of *C. elegans* at three developmental temperatures

Temperature (°C)	Strain	Rate of sperm production (sperm min^{-1})	Total sperm count*
15	*tra-3* (*e2333*)	0.26±0.02	299±24.11
	fem-3 (*e1999*)	0.23±0.06	194±11.99
	Wildtype	0.22±0.04	218±10.01
20	*tra-3* (*e2333*)	0.42±0.01	293±16.99
	fem-3 (*e1999*)	0.37±0.01	215±13.33
	Wildtype	0.38±0.01	244±9.45
25	*tra-3* (*e2333*)	0.72±0.02	278±20.43
	fem-3 (*e1999*)	0.65±0.04	195±8.62
	Wildtype	0.66±0.07	225±11.54

*Mean number of sperm observed at the final (fourth) time point (shown in Fig. 3).
Values are means ± s.e.m.

Table 2. Comparison of rates of sperm production, lifespan and developmental growth in *C. elegans*

	Sperm production (sperm min^{-1})[a]	Lifespan (days)[b]	Lifespan (days)[c]	Embryonic development (h)[d]
Rearing temperature (°C)				
15	4.55	26.1	–	23.6
16	–	–	23.0	–
20	2.63	19.3	14.5	13.3
25	1.52	15.2	–	10.3
25.5	–	–	8.9	–
Developmental rate ratio				
$R_{15/20}$	1.73	1.35	1.59	1.77
$R_{20/25}$	1.74	1.27	1.63	1.29

[a]Present study.
[b]Lee and Kenyon, 2009.
[c]Klass, 1977.
[d]Wong et al., 1995.

of selection coefficients also are qualitatively consistent with the statistical results derived from the general linear model (Table 4), and the likelihood analysis does incorporate the numbers of elapsed generations in each treatment.

DISCUSSION

We used experimental evolution to investigate the potential influence of temperature-induced change in the duration of larval development as a factor contributing to different fitness optima in the number of sperm produced by self-fertilizing protandrous hermaphrodites. We demonstrated that manipulating the duration of *C. elegans* larval development *via* temperature significantly alters brood size, the rate of sperm production and the number of sperm that maximizes hermaphrodite fitness. Our results reveal new insights into the reproductive biology of *C. elegans* and imply that existing models of the evolution of hermaphrodite sperm number may be incomplete and that alternative experimental approaches may be required to test fully some predictions from theory. More generally, we demonstrate that developmental trajectories have the potential to profoundly impact the fitness landscape underlying the evolution of reproductive and sperm traits, even in the absence of sexual selection and sperm competition.

Genetic and environmental influences on fecundity and sperm production

We first determined that the phenotypic effects on self-fecundity of laboratory mutants (Hodgkin and Barnes, 1991) are indeed due to differences in sperm counts and confirmed that they extend across a broad range of rearing temperatures. Despite different total sperm counts for *tra-3* (*e2333*), *fem-3* (*e1999*) and individuals wildtype at *tra-3* and *fem-3*, these genotypes do not produce sperm at different rates. Consequently, it is unlikely that sperm sizes differ appreciably as a pleiotropic effect of the number of sperm produced, because larger sperm are produced at a slower rate (LaMunyon and Ward, 1998; Murray et al., 2011). In addition, we recovered patterns of fecundity across rearing temperatures within each genotype that recapitulate observations for wildtype strains (Klass, 1977; Harvey and Viney, 2007).

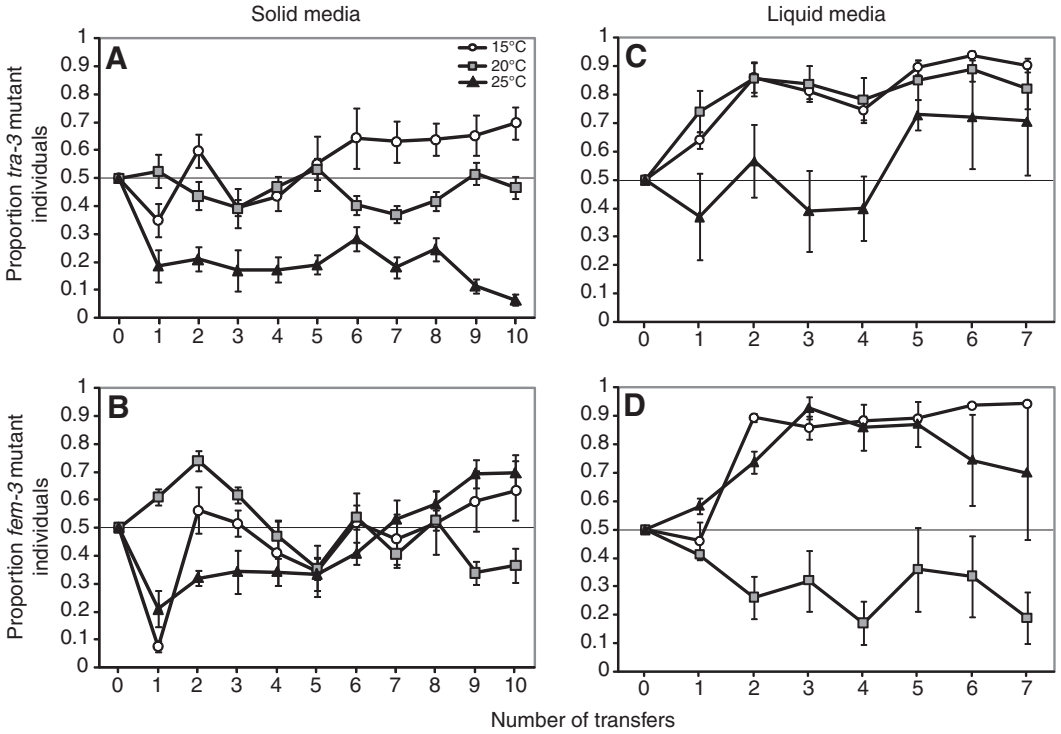

Fig. 4. Mean mutant-allele frequency in experimental evolution trials conducted on solid (left panels) and liquid media (right panels). The *tra-3* (*e2333*) mutant allele causes higher sperm production than wildtype; the *fem-3* (*e1999*) mutant allele causes lower sperm production than wildtype. Populations were transferred weekly, so treatments experienced ~35 generations, ~20 generations and ~12 generations at 25, 20 and 15°C, respectively, on solid media. *N*=8 per treatment (*N*=6 for 15°C) for the solid-media experiment; *N*=4 per treatment for the liquid-media experiment. Error bars represent ±s.e.m.

Table 3. Summary of general linear model of the influences of rearing temperature (15, 20 and 25°C), competition strain genotype [*tra-3* (*e2333*) and *fem-3* (*e1999*)] and time on the proportion of mutant alleles in the population

	Solid media			Liquid media		
Source of variation	d.f.	*F*	*P*	d.f.	*F*	*P*
Strain	1,38	0.02	0.89	1,14	1.01	0.33
Temperature	2,38	68.46	<0.0001	2,14	1.73	0.20
Time	1,38	3.86	0.057	1,14	0.77	0.62
Strain × Temperature	2,38	3.72	0.0335	2,14	1.17	0.29
Time × Strain	1,38	4.25	0.046	1,14	0.06	0.056
Time × Temperature	2,38	18.86	<0.0001	2,14	1.10	0.41
Time × Strain × Temperature	2,38	17.08	<0.0001	2,14	2.45	0.076

Surprisingly, we found that hermaphrodites reared at high and low temperatures produced more sperm than the number of offspring produced in fecundity trials: more sperm were produced than were used to fertilize oocytes. This contrasts with Ward and Carrel's classic result that, under optimal growing conditions of 20°C, hermaphrodites exhibit sperm-limited fecundity by using self-sperm with near-perfect efficiency (Ward and Carrel, 1979). Moreover, hermaphrodites of a given strain appear to produce roughly equal numbers of sperm regardless of the temperature regime (Fig. 3). These results mimic a similar recent observation in *C. briggsae*, for which a role of defective sperm fertility was implicated as a main cause of temperature-dependent variation in fecundity, rather than sperm abundance *per se* (Prasad et al., 2011); experiments in *C. elegans* by Harvey and Viney also are consistent with this (Harvey and Viney, 2007). When *C. elegans* are grown in soil and compost microcosms, hermaphrodite fecundity also is not sperm limited (Goranson et al., 2005). These observations together suggest that sperm limitation may be the rule only under benign conditions that are optimal for growth, whereas oocyte limitation may prevail under stressful conditions. This violates the implicit assumption of hermaphrodite sperm limitation under all growth conditions in models of *C. elegans* sperm production (Barker, 1992; Cutter, 2004) and, consequently, we might not expect the model predictions to hold exactly in our experimental evolution system. Moreover, this finding demonstrates that Bateman's Principle – the limitation of reproduction by female gametes (Bateman, 1948) – is context dependent in *C. elegans*.

An outstanding question about the *fem-3* and *tra-3* mutant strains is how, exactly, they differ in resource allocation for sperm and oocytes. The *tra-3* hermaphrodites invest more in sperm than do *fem-3* or wildtype animals, and yet they also produce more oocytes when self-fertilizing (i.e. higher brood size), implying that *tra-3* hermaphrodites also have a greater net investment in oocytes than the other genotypes. This is consistent with the observation of a lack of a trade-off between male and female function in many hermaphroditic organisms (Schärer, 2009). Future tests of sex allocation theory in *C. elegans* will benefit from exploring the potential for a trade-off between sperm count and maximum fecundity when provided with *ad libidum* sperm *via* mating, or for trade-offs between sperm count and non-reproductive life-history traits (e.g. lifespan) (Schärer, 2009).

Another model assumption is that the rate of sperm production need not scale directly with the duration of larval development time (Cutter, 2004). To gauge whether the temperature-induced change in rates of sperm production mirrors temperature-induced change in rates of larval development, we contrasted the ratio of developmental rates (embryonic, total lifespan or sperm) at low- (15°C) to moderate-temperature rearing (20°C; $R_{15/20}$) to the ratio of developmental rates at moderate to high temperature rearing (25°C; $R_{20/25}$) (Klass, 1977; Wong et al., 1995; Lee and Kenyon, 2009) (Table 2). Both lifespan and sperm development showed a constant rate of change with temperature (i.e. $R_{15/20} \approx R_{20/25}$; Table 2). However, embryonic development showed a diminishing returns response with temperature, with a more drastic change between 15 and 20°C ($R_{15/20} \approx 1.8$) than between 20 and 25°C ($R_{20/25} \approx 1.3$). This mirrors classic observations (Hirsh et al., 1976), which indicated a similar disparity, and is also consistent with how temperature exerts a non-linear saturating effect on the physical growth rate of the length of the embryo following the comma stage (Rabin and Podbilewicz, 2000). Thus, sperm production (and lifespan) may be partially

Table 4. Maximum likelihood estimates of the strength of selection in each experimental evolution treatment

Rearing conditions				Maximum likelihood estimates			
Media	Temperature (°C)	Experimental locus	Replicates	*s*	N_e	$2N_es$	$\Sigma(-\ln L)$
Solid	15	*tra-3*	6	0.015	20	0.6	–321.23
Solid	20	*tra-3*	8	–0.004	50	–0.4	–427.53
Solid	25	*tra-3*	8	–0.007	90	–1.2	–398.70
Solid	15	*fem-3*	6	0.015	10	0.3	–381.15
Solid	20	*fem-3*	8	–0.006	50	–0.6	–424.12
Solid	25	*fem-3*	8	0.007	50	0.7	–441.23
Liquid	15	*tra-3*	4	0.075	30	4.5	–152.34*
Liquid	20	*tra-3*	4	0.024	50	2.4	–159.90
Liquid	25	*tra-3*	4	0.008	30	0.5	–181.29
Liquid	15	*fem-3*	4	0.090	20	3.6	–163.17*
Liquid	20	*fem-3*	4	–0.020	30	–1.2	–151.21
Liquid	25	*fem-3*	4	0.008	80	1.3	–146.98

$2N_es$, strength of selection; $-\ln L$, negative log likelihood; *s*, selection coefficient; N_e, effective population size.
*Significantly different from $2N_es=0$ (non-overlapping $2\ln L$ interval).

independent of developmental rate, as assumed theoretically (Cutter, 2004). However, Hekimi and colleagues have argued that lifespan and developmental rate are linearly related (Lakowski and Hekimi, 1996; Hekimi et al., 2001), suggesting that further work is required to solve this issue.

Experimental evolution of sperm number

We constructed populations composed of genetically identical individuals, excepting a bi-allelic polymorphism for a gene that affects sperm production (at either *fem-3* or *tra-3*) and a linked visible GFP transgenic marker. We then measured the change in allele frequency at these loci over time for two strain combinations to determine whether differences in larval development time, mediated through rearing temperature, induce differential selection for alternative alleles controlling sperm number in *C. elegans* hermaphrodites. Specifically, we allowed the 'low sperm count' *fem-3* (*e1999*) allele to compete against the wildtype *fem-3* allele or we allowed the 'high sperm count' *tra-3* (*e2333*) allele to compete against the wildtype *tra-3* allele. Previous theory predicted that, all else being equal, more rapid larval development would favor the evolution of lower sperm counts and that longer larval development time would favor the evolution of higher sperm counts (Cutter, 2004).

When larval development was slow, induced by low rearing temperature, we observed an increase in frequency over time for the *tra-3* (*e2333*) allele, which confers high sperm count. In contrast, this allele declined in frequency when populations were reared under high temperature conditions that induced rapid development. These results are consistent with the theoretical prediction that increased duration of larval development favors the evolution of more sperm in protandrous selfing hermaphrodites and that decreased duration of larval development favors the evolution of fewer sperm (Cutter, 2004). However, the same overall pattern did not hold for experiments conducted with *fem-3* (*e1999*), an allele that confers low sperm count relative to the wildtype allele. Instead, we found that the *fem-3* (*e1999*) allele increased in frequency under conditions of both long and short larval development – and declined in frequency at an intermediate rearing temperature and duration of larval development – whereas theory would predict a possible evolutionary advantage of the *e1999* allele only with rapid larval development. Thus, the dynamics of *fem-3* (*e1999*) in the 15°C treatment is the principal outlier with respect to theoretical predictions. This pattern was consistent in both solid- and liquid-media experiments, so we find it unlikely that stronger drift or fewer generations in the *fem-3* experiments conducted at 15°C could have contributed substantially to the pattern that ran counter to theoretical predictions.

The *fem-3* (*e1999*) allele might exert previously undescribed temperature-dependent pleiotropic fitness effects, but we could only speculate as to how context-dependent pleiotropy might manifest. Similarly, the integrated GFP transgene in strain PD4790 might have previously undocumented temperature-dependent detrimental effects on fitness – although we demonstrate that previously published reports for the standard lab strain N2 have equivalent rates of sperm production as PD4790 and that sperm counts and fecundity are only slightly lower for PD4790. Regardless, our direct competition of alternative alleles under differing environmental conditions illustrates how non-competitive fitness measures of individual genotypes in isolation in a single environment, as quantified previously (Hodgkin and Barnes, 1991), might not manifest as predicted in a competitive context or in alternative environments. Other recent *C. elegans* experimental evolution studies have similarly demonstrated this competition effect in challenging or stressful environments (Morran et al., 2009a; Morran et al., 2009b; Wegewitz et al., 2009).

Further work with GFP markers introgressed into the sperm-number mutant strains would allow for the two sperm mutant alleles *tra-3* (*e2333*) and *fem-3* (*e1999*) to compete against one another directly. This might reveal a more extreme result, such as allele fixation, over the same time period. Other genetic manipulations of sperm count, such as alleles of *fbf-1* that increase sperm count (Crittenden et al., 2002), would provide a third genetic replication. Our environmental manipulation of the speed of development was a compromise for experimental ease (Reznick, 1992). Generating double-mutants of sperm-production alleles with genes that affect the pace of development could permit a direct genetic test of how these factors would evolve in combination, without performing environmental manipulations. For example, mutant alleles of *gro-1* and *clk-1* cause *C. elegans* to develop more slowly. However, these developmental mutants also have a broad array of known pleiotropic affects, such as slowing various behaviors and reducing brood size (Wong et al., 1995; Lemieux et al., 2001), that might compromise the conceptual advantages of genetic over environmental manipulation (Reznick, 1992).

Our experimental evolution trials determined that a shorter duration of larval development induced by high growth temperatures consistently favored the evolution of alleles encoding fewer sperm, in accord with theory. This illustrates the potential importance of developmental life-history traits in the evolution of sperm and reproductive traits, even without the operation of sexual selection. However, some experimental evolution treatments did not conform to theoretical predictions, suggesting that existing theory on the evolution of sperm count in this system is incomplete and/or that alternative experimental approaches are required to fully test theoretical predictions. We also demonstrated that hermaphrodite fecundity is not sperm limited when individuals are reared at high and low temperatures, consistent with findings in *C. briggsae* (Prasad et al., 2011) and for other non-optimal growth conditions (Goranson et al., 2005). This suggests that environmental and ecological stress may generally lead to oocyte-limited reproduction in this organism, and conformity with Bateman's Principle (Bateman, 1948), rather than the sperm limitation observed under benign conditions.

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