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Resolving an adaptive conundrum: reproduction in *Caenorhabditis elegans* is *not* sperm-limited when food is scarce

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ABSTRACT

Question: Has the less expensive gamete (sperm) really been selected as the limiting factor in a nematode's reproduction, as laboratory studies have implied?

Hypothesis: Reproductive output of the self-fertilizing hermaphroditic nematode *Caenorhabditis elegans* is oocyte-limited in the worm's natural environment: soil.

Organism: Sperm depletion and laying of unfertilized oocytes occurs in well-fed laboratory cultures of the self-fertilizing hermaphroditic nematode, *Caenorhabditis elegans*. This phenomenon has been much discussed as a possible contradiction to the usual expectation of selection for maximum fertility.

Methods: *Caenorhabditis elegans* were maintained in a variety of food regimes, not just the *ad libitum Escherichia coli* typical of laboratory cultures. Sperm production, oocyte production and body size were measured for nematodes cultured on agar with feeding treatments of serially diluted bacteria suspensions, before and during gamete formation. Body sizes were also measured for worms cultured in soil or compost microcosms.

Results: Reproduction was sperm-limited only for nematodes in the highest food treatments (10^6 or more *E. coli* cells per day); lower food treatments produced smaller worms with considerable excess sperm. *Caenorhabditis elegans* grown in natural soil or compost microcosms achieved the same sizes as those in treatments that showed oocyte limitation, not the large sizes of those typical of *C. elegans* in treatments that exhibited sperm limitation. Enriching soil or compost by adding *E. coli* produced appreciably larger *C. elegans*, but only if numbers of other invertebrates – potential predators/competitors of *C. elegans* – were minimized. Even with increased nutrients and reduced numbers of other invertebrates, only a few *C. elegans* in natural soil or compost were large enough to be consistent with sperm limitation.

Keywords: diet restriction, hermaphrodite, oocyte limitation, phenotypic plasticity, soil, sperm limitation, streptomycin.

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INTRODUCTION

The nematode *Caenorhabditis elegans* occurs mainly as self-fertilizing hermaphrodites that produce male and female gametes during different stages of the life cycle; there are no females and males are rare, at least in the laboratory. Sperm are produced by hermaphrodites only during the fourth larval stage and oocytes are produced during subsequent adult stages, so *C. elegans* that have moulted to the adult stage have no possibility of returning to sperm production (Nigon and Dougherty, 1949; Kimble and Ward, 1988). *Caenorhabditis elegans* adults fed on *Escherichia coli ad libitum* run out of sperm on about the sixth day of age (in the absence of rare males), and must lay unfertilized oocytes thereafter (Maupas, 1900). Since an oocyte of *C. elegans* is roughly 500 times larger in volume than a sperm (Hodgkin and Barnes, 1991), foregoing production of a single oocyte in favour of sperm production would have a large pay-off in reproductive fitness.

How can it be adaptive for sperm, rather than eggs, to limit reproduction in this self-fertilizing hermaphrodite? At least four adaptive hypotheses (reviewed in Barker, 1992) have been advanced to resolve the conundrum of cheap sperm, rather than expensive oocytes, limiting reproductive capability in *C. elegans*. Maupas (1900) hypothesized that sperm shortage was a sign that the nematodes are still in transition from an outcrossing to a selfing life-history strategy. However, *all* hermaphroditic rhabditid nematodes studied to date show sperm shortage, and it is unlikely that all are currently in transition (Ward and Carrel, 1979).

Barker (1992) proposed, and then rejected, the hypothesis that sperm limitation is favoured because the life span of *C. elegans* might be shorter in the wild, with sperm shortage appearing only in cosseted laboratory populations. Many *C. elegans* individuals live in the laboratory for weeks after reproduction has ceased (Gems and Riddle, 2000), but with increasing evidence of senescence (i.e. Klass, 1977). Wild nematode life spans are unknown. Ward and Carrel (1979) hypothesized that if males are available as a source of additional sperm, extra oocytes can be used to produce outcrossed offspring. However, outcrossing occurs rarely in the laboratory, and may be infrequent in the wild. Moreover, males do not depend on depletion of hermaphrodite sperm to achieve fertilization, as male sperm outcompetes hermaphrodite sperm (Ward and Carrel, 1979).

Hodgkin and Barnes (1991) proposed that the sperm shortage is the result of selection pressure to produce offspring at the earliest possible time. A model by Barker (1992) confirmed this possibility. Our studies of life-history responses of *C. elegans* to variation in food availability have led us to a broader hypothesis: oocyte production is unusually high in well-fed laboratory cultures, but the reproductive capability of *C. elegans* is limited by sperm production in its natural soil habitat only when environmental conditions are especially favourable. Environmental conditions that might affect growth and reproduction include the amount and distribution of available nutrients (Sohlenius, 1973), and competition or predation (Beckman *et al.*, 1997).

Caenorhabditis elegans are typically grown in the laboratory with *ad libitum* food – growing cultures of *E. coli* on the surface of an agar plate. We examined *C. elegans* growth, and production of sperm and oocytes, in a series of food availability treatments, and discovered size differences among treatments associated with sperm and oocyte production. We reared *C. elegans* in soil microcosms, using growth rate to determine what food availabilities the worms encounter in natural conditions. Finally, we manipulated nutrient content and presence of competitors and predators in soil microcosms to determine how these factors affect *C. elegans* growth.

METHODS

Food availability experiment

To determine how sperm and oocyte production each vary with food availability, we manipulated levels of streptomycin-sensitive *E. coli* provided as food in laboratory cultures of *C. elegans*. (We use gamete 'production' to include both the formation of gametes in the gonads, and survival through the reproductive phase of the life cycle.) We used streptomycin (0.2 g $\cdot$ l^{-1}), which does not affect worm growth and which inhibits the initiation of protein synthesis at the bacterial ribosome (Madigan *et al.*, 2003), to stop the growth of streptomycin-sensitive *E. coli* (without killing the cells) – and so maintain constant, limited levels of food availability.

Caenorhabditis elegans (wild-type strain N2) were grown on 6 cm petri dishes with one of five food treatments: with 10^4, 10^5, 10^6, 10^7 streptomycin-treated *E. coli* cells added daily to fresh agar (no other nutrients), or with *ad libitum E. coli*, growing on standard NGM nutrient agar (Sulston and Hodgkin, 1988). In the *ad libitum* treatments, *E. coli* was grown over a large area of each plate. For the other treatments, serial dilutions were applied in three large drops (20 μl total); each dilution provided *C. elegans* with 10 times the number of cells per linear distance grazed than the next lower dilution. Each food treatment was performed in two different ways: (1) nematodes were maintained in the food treatment from egg throughout life; and (2) nematodes were maintained in *ad libitum E. coli* from egg to the second larval stage, and then moved to the food treatment at the third larval stage (age = 43 h). For each of these two treatment schedules, nematodes experience the food treatment for the entire duration of gamete formation, since sperm development takes place between roughly 50 and 61 h of age, and oocyte development occurs between roughly 62 and 144 h of age in *ad libitum E. coli* food (Cassada and Russell, 1975).

Finding the numbers of oocytes and sperm produced was simple for the *ad libitum* treatment: we took the number of offspring produced by each *C. elegans* to be the number of sperm produced; to determine the number of oocytes produced, we simply added the number of infertile oocytes found to the number of offspring produced. No unhatched eggs – recognizable by their hard shells – were seen in the experimental cultures. Determining sperm and oocyte numbers is more complex when sperm are present in excess of oocytes, so for each of the 10^4 through 10^7 treatments *C. elegans* were divided into two groups. We counted the total oocytes produced over the entire life cycle in one group of *C. elegans*, and calculated sperm production of each worm in the other. To calculate the number of sperm produced in these treatments, *C. elegans* from each treatment were returned to *ad libitum E. coli* once they began to lay eggs until only unfertilized oocytes were laid; the number of stored sperm equalled the total number of offspring produced – as in the *ad libitum* treatment. Egg-laying commenced in most treatments at roughly 62 h of age, but began somewhat later in third-larval-transfer 10^4 and 10^5 worms.

Soil microcosms

To determine what resource regimens *C. elegans* encounters in nature, and how these compare with the experimental food treatments, we needed to somehow integrate all the complex factors influencing food availability in soils (wild *C. elegans* live in water films in soils): the nutritional regimes deriving from bacteria that vary in nutritional content and

in community composition, the effects of co-exploitation of resources by competitors, the interference with foraging behaviour posed by both competitors and predators, and so on. We decided to have the *C. elegans* do this integration for us, using the growth rate of the worms (length at about age 120 h) as a vicariant for the overall resource state.

We added water (~3 ml) and around 40 third-stage *C. elegans* larvae, previously washed to remove most *E. coli* from their cuticles, to 5 g of fresh soil in 6 cm glass petrie dishes, and maintained the culture at 20°C for 5 days (the age when worms achieved maximum size under these conditions). The 5-day-old adult worms were recovered by washing the soil (we found them in water at the base of the soil or after washing the soil into a larger dish), photographed under constant magnification, and then measured by summing short linear measurements along standard positions on each worm photograph, using MOCHA 1.2.10 software. Statistical analyses were performed using SPSS 11.5, Statistica 2.0 or a custom Matlab program (The Mathworks, Inc.).

We needed worms for these experiments that could be easily distinguished from free-living *C. elegans* and from any other morphologically similar nematodes that live in soil. We used the CGC PD4792 *C. elegans* strain, which was specifically produced to be identical to the wild type (N2) except for a marker of green fluorescent protein (GFP) expressed in the pharyngeal muscle (by Kelly Liu, donated to the CGC by D. Riddle, University of Missouri, and not at the acceptance of this manuscript mentioned in a journal article). We have found no differences in any life-history features between the CGC PD4792 and N2 strains of *C. elegans*.

A variety of soils, which might contain varying concentrations of nutrients (decomposing organic material), of numbers of competitors or predators, and of other environmental effects on growth, were tested: (1) soil removed from under leaf litter in an oak/pitch pine forest in southeastern Massachusetts (containing many potential predators and competitors); (2) faunally reduced forest soil (dried for a month, removing many organisms, rehydrated just before testing); (3) compost; (4) potting soil (sterile, no predators or competitors); (5) enriched forest soil (10^9 *E. coli* cells added); (6) enriched faunally reduced forest soil; (7) enriched compost; and (8) enriched potting soil. Physical factors such as temperature, moisture and pH were held constant. The forest soil had not received direct fertilizer or pesticide applications for at least 30 years and the compost had never been treated with pesticides or fertilizers.

The carbon content of the soils was estimated by drying the soil to a constant weight (35°C), then removing the carbon by heating to 450°C for 20 h. About half of the lost weight is carbon (Finkl, 1979). By this method, forest soil and potting soil each contained about 12% carbon – that is, approximately 0.4 of the carbon in the average compost sample (31% carbon).

RESULTS

Food availability experiment

Slowed maturation was the initial, strong response of *C. elegans* to food shortage early in life. When grown from eggs in the 10^4 and 10^5 treatments, *C. elegans* rarely if ever matured and, compared with the third-larval-transfers, from-egg worms in the 10^6 *E. coli* treatment took 1.5 days longer to lay their first eggs, and those in the 10^7 treatment took 1.1 days longer (*t*-test, $P < 0.0001$). No significant differences in gamete production were

found between worms moved as eggs or as third-larval-transfers into the 10^6 or 10^7 treatments. Hence, for the 10^6 and 10^7 treatments, from-egg results were pooled with third-larval-transfer results in the analysis of this experiment.

Two striking findings emerge from the analysis of these results (Fig. 1, food treatment data). First, both sperm and oocyte production are higher with greater food availability, but the positive relationship between food availability and gamete production is stronger for oocytes than for sperm. Analysis of variance ($P \leq 0.001$) divides the five food treatments into three sperm production groups: sperm numbers were not significantly different between the 10^4 and 10^5 treatments or between the 10^6 and 10^7 treatments, but both groups were different from each other and from the *ad libitum* treatment ($10^4 = 10^5 < 10^6 = 10^7 <$ *ad libitum*). Analysis of variance divides the five treatments into three oocyte production groups: $10^4 = 10^5 < 10^6 < 10^7 =$ *ad libitum*. Worms fed *ad libitum* produced three times as many sperm as those in the 10^4 cells per day treatment, but oocyte production increased by more than 30 times over the same range of food availability.

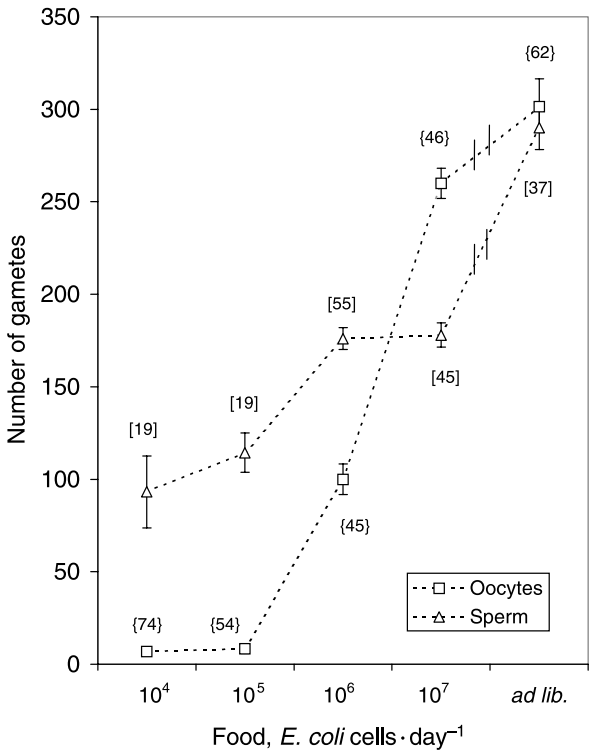

Fig. 1. Lifetime total fertilized and unfertilized oocytes and total sperm produced by hermaphrodite *C. elegans*, provisioned with different amounts of *E. coli* food from day 2 through its remaining life span. Means and 95% confidence intervals were bootstrapped using 2000 resampled populations via a custom Matlab program (The Mathworks, Inc.). Confidence interval bars do not appear where they are smaller than the symbols. Number of worms (n) is shown in parentheses beside each point. Unfertilized oocytes were laid by many 10^7 and *ad libitum* nematodes, and by a very few nematodes in the 10^6 treatment.

Second, sperm production is sufficient for fertilization of all oocytes produced except at high levels of food availability. At food availabilities of 10^6 *E. coli* cells per day or less, we found that significantly fewer oocytes than sperm were produced (independent *t*-tests for 10^6, 10^5 and 10^4, $P \leq 0.0001$). Very few *C. elegans* fed 10^6 *E. coli* per day or less produced unfertilized eggs (4 of 54 in 10^6, and none in 10^5 or less), but 43 of 46 *C. elegans* cultured at 10^7 and 31 of 64 at *ad libitum* food densities produced some unfertilized eggs, with an average of 31 ± 37 unfertilized eggs per worm. Sperm-limitation occurs only if food is plentiful.

Soil microcosms

Recovery of fluorescent worms from our soil microcosms averaged about 9%, with a wide range (0 to 30%, $n = 120$). Predation and starvation in natural soils may have reduced recovery rates: recovery was higher in the faunally reduced microcosms ($P = 0.006$) and in the enriched microcosms ($P = 0.007$). As expected, soils containing less carbon than compost, such as potting soil (40% less carbon), returned not only fewer but also shorter worms (approximately 20% shorter in potting soil).

Do *C. elegans* in soil environments experience low to moderate food levels (oocyte limitation) or high food levels (sperm limitation)? We compared body lengths of *C. elegans* for all experimental treatments (serial dilution treatments and soil microcosms), and found that there were differences among treatments (analysis of variance: $P < 0.0001$; Fig. 2). Notably, the plain, fresh forest soil worm lengths were grouped in the same Tukey subset with worm lengths from the 10^4 and 10^5 serial dilutions. The plain compost worm lengths, though highly variable, were similar to or slightly longer than the forest worm lengths. By contrast, worms in the 10^7 and *ad libitum* treatments – those treatments with sperm-limited *C. elegans* – formed a distinct Tukey subset, longer than any soil treatments.

To investigate further the reasons for length differences, we looked more closely at our forest soil, which in its natural state teemed with arthropods and with nematodes as long as or longer than *C. elegans*. Forest soil that had been dried, then rehydrated a month later, did not contain the fresh soil's numerous large potential predators and competitors; only a few organisms, smaller than *C. elegans*, were present. Worm lengths from plain, undried forest soil grouped in the same Tukey subset with 10^4 and 10^5 serial dilutions – and separately from the 10^7 and *ad libitum* treatments – whether enriched with *E. coli* (length 0.63 ± 0.11 mm; mean ± standard deviation) or not (length 0.59 ± 0.14 mm; many of the recovered worms had not matured). Similarly, worm lengths of both unenriched and enriched compost grouped separately from worm lengths in the 10^7 and *ad libitum* treatments. However, worms from faunally reduced soil (previously dried) were significantly longer (0.76 ± 0.06 mm) than the fresh-soil worms, and worms from faunally reduced soil enriched by *E. coli* were substantially longer (1.08 ± 0.08 mm) than worms from all the other microcosms and statistically longer than all the microcosms except enriched compost. Like the faunally reduced forest soil microcosms, potting soil microcosms were free of *C. elegans*-sized competitors and predators and also produced larger worms if enriched by *E. coli*. Thus in the presence of added nutrients, worms grew substantially longer in the absence of competition or predation (faunally reduced forest soil, potting soil) but not if competitors or predators were present (fresh forest soil, compost).

Overall, very few of the worms in these soil microcosms are getting large enough to be likely candidates for sperm limitation. Only 23 of 187 worms in these soil microcosms

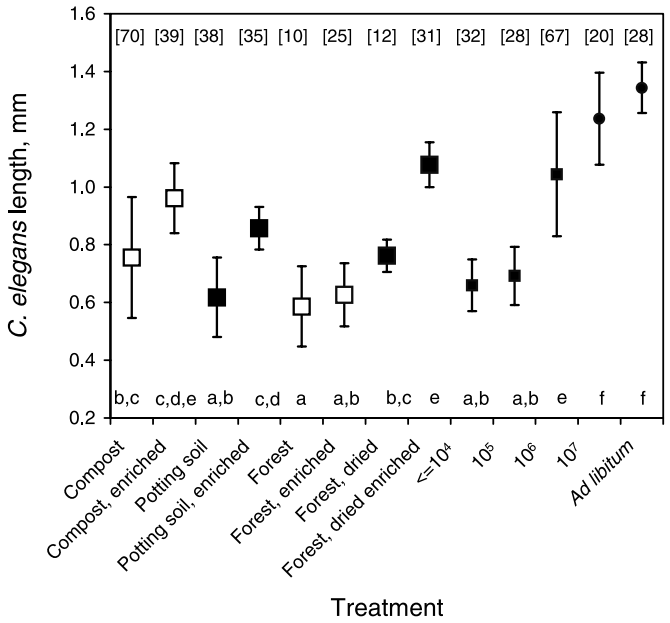

Fig. 2. Lengths of adult *C. elegans* cultured in agar and soil treatments. 10^9 *E. coli* cells were added to 'enriched' cultures; 'faunally reduced' treatments were dried to remove potential predators and competitors. Large symbols, soil treatments; small symbols, agar; squares, expected oocyte limitation; circles, expected sperm limitation; open symbols, high competition/predation; closed symbols, low competition/predation. Error bars represent 1 standard deviation. Number of worms (*n*) is shown in parentheses above each point. Results of analysis of variance ($P < 0.000$): treatment allocation into Tukey subsets a, b, c, d, e and f are noted below each treatment symbol.

achieved sizes that are associated with sperm limitation, assuming a cut-off for sperm limitation at the size of the *very* smallest worms to produce unfertilized eggs (1.08 mm; one standard deviation below the mean size of worms that produced unfertilized eggs in the 10^7 food treatment), and 11 of these individuals were in the enriched, faunally reduced forest microcosm.

DISCUSSION

Finding additional variation in the reaction norm of gamete production, we have shown that self-fertilizing *Caenorhabditis elegans* hermaphrodites cultured on two-dimensional agar plates covered with a film of bacteria grow large and may have sperm-limited reproduction, but with reductions in the density of bacteria they achieve smaller sizes and are oocyte-limited. The *C. elegans* in our highest food availability treatments generally produced unfertilized eggs. We do not see a difference between the considerable unfertilized egg productions of worms of the 10^7 and *ad libitum* treatments, except as a result of lower 10^7 treatment sperm production. Even our well-fed worms do not always produce the 100 or so unfertilized eggs noted in the literature (Klass, 1977).

Compared with the typical agar plate, nematode growth rates are likely to be low and highly variable in three-dimensionally patchy soil environments. Adding 10^9 *E. coli* cells to

the 5 g soil did not produce the large increases in worm size associated with similar additions of bacteria to the two-dimensional, competitor-free agar plates. Sohlenius (1973) obtained a similar result with the nematode *Acrobeloides nanus*: individuals grown in soil were about 70% as long as well-fed laboratory individuals. The *E. coli* on flat agar can be more readily harvested than bacteria coating the vastly larger and more three-dimensional surface area of soil.

The many organisms that are found in forest soil – other (sometimes very large) nematodes, arthropods, rotifers, paramecia, etc. – may compete against or prey on *C. elegans*. More *C. elegans* were recovered from those soil microcosms from which most of these other organisms had been removed, and sizeable increases in worm length resulted from food augmentation only in these faunally reduced situations. Reduced recovery of the experimental worms is likely to be the result of predation, and failure to grow larger with augmented food availability could be a direct result of competition for nutrients. Alternatively, predators could be responsible for the poor response to food augmentation as well as the low recovery – decreasing worm length by culling large individuals or through trait-mediated indirect interactions involving predator avoidance (i.e. worms hiding instead of foraging). Whatever the factors affecting growth of *C. elegans* in soil microcosms, the dramatic length increase after both feeding and removal of other organisms suggests that only fortunate worms in unusual conditions may increase reproduction to the point of sperm-limitation.

That the response to increases in food availability is greater for oocyte production than for sperm production is to be expected from the reproductive chronology of *C. elegans*. Sperm production takes place only in worms aged about 50–61 h, so any increases are based on a short exposure to plenty, whereas increases in oocyte production may be based on a much longer exposure to high food availability, since oocyte production continues from roughly age 62 to 144 h. The greater responsiveness of oocyte production makes adaptive sense if the expensive eggs typically limit reproduction. The apparent strategy is to produce early in life an amount of sperm that will be ample for even bountiful environments with high (more than 10 times higher than what we found in natural soil) food levels. The expenditure on this ample number of male gametes is quite small. Commitment of energy to oocytes occurs much later in life, when the amount and timing of production of these expensive gametes can be adjusted to the environmental availability of food.

Increased understanding of the scope of the reaction norms for *C. elegans* growth and reproduction places the basic conundrum of sperm supply limiting reproduction in a self-fertilizing hermaphrodite in context. At one end of the norm, in *C. elegans* with very abundant food, reproduction is limited by sperm as found by Hodgkin and Barnes (1991) and Barker (1992). Reacting to a food bonanza in this way reduces the number of *C. elegans* offspring, but may allow them to be produced at the earliest possible time. We have followed the norm to the opposite end: with low food availability, reproduction is limited by oocytes. Under natural soil conditions (moderate but potentially variable food availability), *Caenorhabditis elegans* fertility is usually limited by lack of nutrients to produce oocytes, rather than by lack of sperm.

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