Soil Biology and Biochemistry 177 (2023) 108891

Contents lists available at ScienceDirect

Soil Biology and Biochemistry

journal homepage: www.elsevier.com/locate/soilbio

Ecological stoichiometry drives the evolution of soil nematode life history traits

Xia Xue [a,b], Bishwo N. Adhikari [b,c], Becky A. Ball [d], John E. Barrett [e], Jinxin Miao [f], Ammon Perkes [b,1], Mac Martin [b,2], Breana L. Simmons [g], Diana H. Wall [h], Byron J. Adams [b,i,*]

[a] Henan Key Laboratory of Helicobacter Pylori & Microbiota and Gastrointestinal Cancer, Marshall Medical Research Center, The Fifth Affiliated Hospital of Zhengzhou University, Zhengzhou, 450002, China
[b] Department of Biology and Evolutionary Ecology Laboratories, Brigham Young University, Provo, UT, 84602, USA
[c] Plant Germplasm Quarantine Program, United States Department of Agriculture, Animal and Plant Health Inspection Service, Laurel, MD, 20708, USA
[d] School of Mathematical and Natural Sciences, Arizona State University at the West Campus, Glendale, AZ, 85306, USA
[e] Department of Biological Sciences, Virginia Tech, Blacksburg, VA, 24061, USA
[f] Academy of Chinese Medicine Science, Henan University of Chinese Medicine, Zhengzhou, 450000, China
[g] Department of Biology, East Georgia State College, Swainsboro, GA, 30401, USA
[h] Department of Biology and School of Global Environmental Sustainability, Colorado State University, Fort Collins, CO, 80523-1036, USA
[i] Monte L. Bean Life Science Museum, Provo, UT, 84602, USA

ARTICLE INFO

Keywords:
Elemental stoichiometry
Growth rate hypothesis
Life history theory
Molecular evolution
Nematoda
rRNA

ABSTRACT

Ecological stoichiometry is a useful theoretical framework for understanding the sources and controls on nutrient availability that structure the composition and diversity of biotic communities. One such relationship is that organismal development rate is positively linked to cellular Phosphorus (P). We hypothesized that P availability, relative to other nutrients, e.g., nitrogen and carbon, would drive the evolution of traits associated with organismal growth and development. We examined the effects of P availability both *in situ* and *in vitro,* on free-living soil nematodes. We found that P-deficient environments produce predictable changes in the ecology and evolution of important life history traits. Our results identify altered rRNA gene copy number and subsequent changes in gene expression and protein synthesis as mechanisms by which P-deficiency influences these traits. These findings have important implications for explaining soil ecological and evolutionary patterns across multiple levels of organization, including the structure and functioning of organisms, populations, communities, and ecosystems.

1. Introduction

All organisms are largely composed of the same elements (carbon, hydrogen, oxygen, nitrogen, phosphorus, and sulfur), but the relative proportions of these elements vary widely among species according to their evolutionary history and functional attributes. It has been proposed that this biochemical variation, known as organismal stoichiometry, is an outcome of elemental nutrient requirements for organismal growth and evolution (Sterner and Hessen, 1994; Elser et al., 2000b; Sterner and George, 2000; Zimmerman et al., 2013). The growth rate hypothesis (GRH) states that the production of ribosomal RNA, and thus organismal growth rate, is constrained by variation in the P-content of consumer taxa, which is directly coupled to bioavailable phosphorus (P) in the environment (Elser et al., 2000b, 2003, 2009). All evolutionary processes that result in changes in organismal growth rate may be manifested in changes in organismal C:N:P ratios, and a genetic basis for growth-related variation in P demand has also been proposed: increased growth rate and associated increases in transcriptional capacity for ribosomal production should be associated with changes in rDNA copy number (Elser et al., 2009). Furthermore, nutrient signals can impact

* Corresponding author. Department of Biology and Evolutionary Ecology Laboratories, Brigham Young University, Provo, UT, 84602, USA.
E-mail addresses: xue-xiasophia@hotmail.com (X. Xue), adhikaribn@gmail.com (B.N. Adhikari), Becky.Ball@asu.edu (B.A. Ball), jebarre@vt.edu (J.E. Barrett), miaojinxin2022@163.com (J. Miao), perkes.ammon@gmail.com (A. Perkes), macmartin23@gmail.com (M. Martin), bsimmons@ega.edu (B.L. Simmons), Diana.Wall@colostate.edu (D.H. Wall), bjadams@byu.edu (B.J. Adams).
[1] Current address: Animal Behavior Group, University of California-Davis, Davis, CA 95616, USA.
[2] Current address: Joint Base Elmendorf-Richardson Hospital, 955 Zeamer Ave, Anchorage, AK, 99506, USA.

https://doi.org/10.1016/j.soilbio.2022.108891
Received 27 July 2022; Received in revised form 9 November 2022; Accepted 15 November 2022
Available online 23 November 2022

ribosome maintenance by manipulating the cellular processes of ribosome assembly (Mathis et al., 2017). Prior studies have examined the evolutionary consequences of dietary P-deficiency at moderate to high levels and natural variation in rDNA copy number and gene expression in several organisms (Weider et al., 2005; Mulder and Elser, 2009). However, the evolutionary mechanisms that are implicated in driving these changes are still poorly understood (Elser et al., 2000a), in part because such approaches require coupled, long-term observations of natural and manipulative experiments (Mulder and Elser, 2009).

The McMurdo Dry Valleys (MDVs) of Antarctica host soil ecosystems that are among the coldest and driest on Earth (Bockheim and McLeod, 2008). In soils of the MDVs, organic matter, nutrients like nitrogen (N) and phosphorus (P) as well as biologically available C and liquid water are major limiting factors for life (Barrett et al., 2005). Bioavailable N and P differ significantly across landscape features in the MDVs, which are strongly influenced by atmospheric deposition, hydrology, and geological provenance, primarily till composition and age (Barrett et al., 2007; Bate et al., 2008). The MDVs are devoid of vascular plants, and thus nitrogen turnover is primarily performed by prokaryotes (Ortiz et al., 2020). Prior work has identified the response of microbial communities, soil respiration, and biogeochemical pools of nutrients to stoichiometric CNP ratios and water amendments (Ball et al., 2018; Aanderud et al., 2019) but the influences of such constraints on the biota of higher trophic levels have yet to be explored.

Nematodes are the most widely distributed and biologically diverse invertebrates in the MDVs (Freckman and Virginia, 1998; Adams et al., 2014). The structure and geographic distribution of Antarctic nematode communities is primarily driven by abiotic factors, especially soil geochemical properties like organic matter and salt concentration (Freckman and Virginia, 1997; Powers et al., 1998; Barrett et al., 2005; Bate et al., 2008). For example, *Plectus murrayi* is more likely to occur in soils with relatively higher soil moisture and contents of NH_4-N, NO_3-N, organic C, and organic C/organic N ratios than *Scottnema lindsayae* (Courtright et al., 2001; Adams et al., 2014). Population structure is also strongly influenced by climate and interannual variability (Ayres et al., 2010; Wlostowski et al., 2019; Nielsen et al., 2012; Andriuzzi et al., 2018).

In this study, we used environmental data, samples, cultures, and archived specimens associated with the McMurdo Long-Term Ecological Research program to explore the validity and mechanisms of the GRH. Accordingly, we examined populations of two species of nematode worms, *P. murrayi* and *S. lindsayae,* from naturally occurring P-rich and P-poor environments, and laboratory cultures of *P. murrayi* and *Caenorhabditis elegans* reared on P-rich and P-poor artificial media for over 345 and 30 generations, respectively. We set out to test the explanatory power of the GRH, specifically, whether P-availability can drive the evolution of ecologically relevant genomic architecture, gene expression, and ultimately, life history traits. We quantified the amount of elemental P-content (PO_4^-) in individual nematodes (somatic P-content), rDNA copy number, and rates of rRNA transcription in the context of P-availability, soil CNP ratios, and organismal life history traits (growth rate, body size at maturity, reproductive output).

2. Materials and methods

2.1. Study site

Soil samples were collected during the 2008–2009 austral summer from two glacial till sequences, the Ross Sea till and Taylor II till, occurring in the Lake Fryxell and Lake Bonney basins, respectively, of Taylor Valley (Fig. 1A). The Ross Sea till formed as a terminal moraine of the Western Antarctic Ice sheet when it impounded a glacial lake (Lake Washburn) at the mouth of the Taylor Valley during the last glacial maximum (12–24 KYA) (Bockheim and McLeod, 2008). The Taylor II till formed during an expansion phase of the Taylor Glacier, a glacial tongue of the East Antarctic ice sheet 113–120 KYA in western portions of Taylor Valley (Bockheim and McLeod, 2008). These specific locations were chosen because they represent a distinct contrast in soil properties, especially nitrate and P content (Barrett et al., 2007), but with similar elevation (Fryxell site = 20 m ASL; Bonney site = 60 m ASL) and climate regime (degree days above freezing, air temperature, soil temperature at 0, 5, and 10 cm, wind speed, wind direction, relative humidity, PAR) (Obryk et al., 2020). Precipitation occurs as snow, most of which sublimates prior to melting (Obryk et al., 2020), thus limiting infiltration into soils. Variation in N content is mainly driven by differences in depositional history associated with proximity to the McMurdo Sound

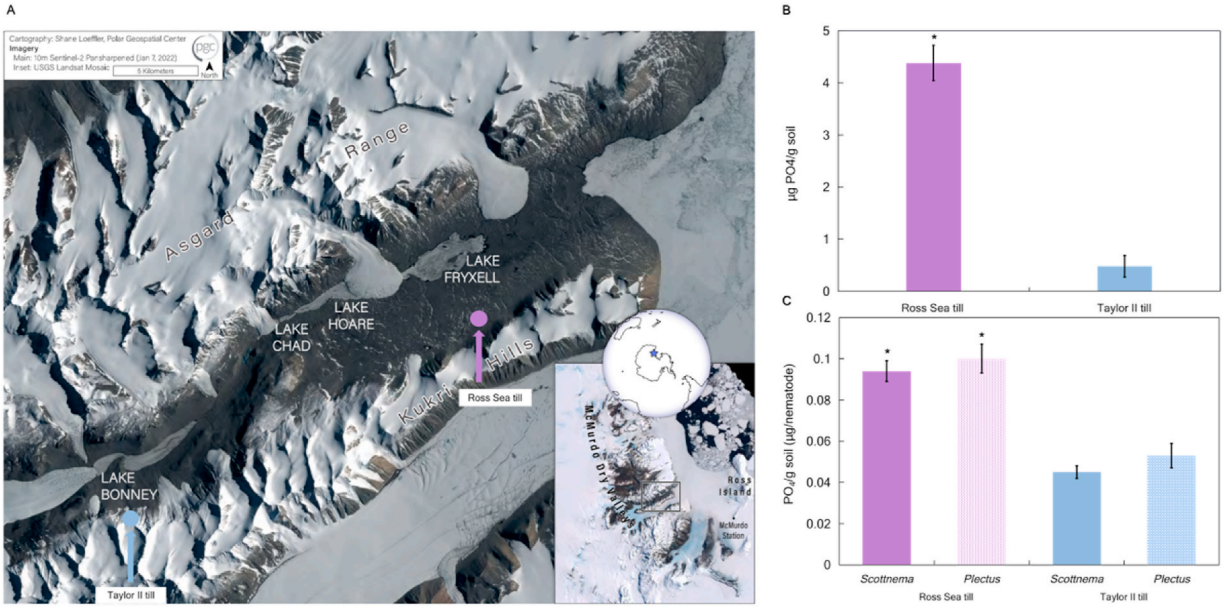


Fig. 1. Geographic differences in bioavailable soil P and nematode somatic P-content. A: Location of study area in Taylor Valley, Antarctica. Filled circles denote sampling sites; B: Ortho-phosphate (PO_4^{2-}) content of Ross Sea till and Taylor II till soils. Bars indicate mean ± SE of samples pooled from each of the bulk samples from the two sites. Asterisks indicate statistical significance ($P < 0.05$; N = 3); C: PO_4^- content of *P. murrayi* and *S. lindsayae* from Ross Sea till soil and Taylor II till soil. Bars indicate mean ± SE, three replicates each; asterisks indicate statistical significance ($P < 0.05$; N = 6).

and local hydrology, which influences the accumulation or flushing of soluble N species in the surface soil (Lyons et al., 1998; Barrett et al., 2006, 2007). In contrast, differences in soil P are due largely to the clast composition of the tills and greater incidence of apatite bearing rocks in the Ross till (Bate et al., 2008). Thus, spatial variation in P content is more predictable than N, and is highly correlated with habitat suitability (Wall and Viginia, 1999) and ecological processes, such as soil respiration and extracellular enzyme activity (Parsons et al., 2004; Zeglin et al., 2009).

2.2. Soil sampling and determination of soil carbon, nitrogen and phosphorus

Six soil samples from the Ross-sea till site and seven samples from the Taylor-till site were sampled to 10 cm depth using a clean plastic scoop. The samples were chosen from proximate areas that would broadly represent the variation among soils within the same drift sheet (till type). Each sample was placed in a WhirlPak® bag and transported in a cooler via helicopter to nearby McMurdo Station, Antarctica. Soils were sub-sampled within 48 h of collection under a laminar flow hood in the Crary Laboratory at McMurdo Station, Antarctica, to provide material for invertebrate and chemical analyses.

A subsample of the less than 2 mm size fraction of each soil sample was ground in a ball mill. Approximately half of the ground material was acidified with 50% HCl to remove carbonates. Approximately 75 mg of acidified and un-acidified soil were analyzed with a Carlo Erba 1500 elemental analyzer in the Crary Analytical Lab to determine total carbon and nitrogen, and organic carbon content. Carbonate content of the soil was calculated as the difference between total carbon and organic carbon by comparing results from the acidified and un-acidified samples.

Inorganic nitrogen (ammonium and nitrite + nitrate) was measured on 2 M KCl extracts of freshly collected soils on a Lachat FIA in the Crary Analytical Lab using standard methods (e.g., Barrett et al., 2002).

Ortho-phosphate (PO_4^{2-}) content of the soil was measured by extracting 10 g of soil in 50 ml of 0.5M $NaHCO_3$ at pH 8.5. Extracts were shaken for 90 min at 170 rpm, then decanted into centrifuge tubes and spun at 27,216×*g* for 10 min. Supernatant was poured into Nalgene bottles and acidified to approximately pH 2. The acidified filtrate was stored at −20 °C, shipped to Dartmouth College, and analyzed on a Lachat QuikChem 8500 (Lachat Instruments)(Ball et al., 2018).

2.3. Nematode extraction from soil

Field collected soils were sub-sampled within 48 h of collection under a laminar flow hood in at McMurdo Station. Nematodes were extracted using a sugar-density centrifugation technique modified for Antarctic soils (Freckman and Virginia, 1997). Two endemic nematode species from Antarctica were used in this study: *P. murrayi* and *S. lindsayae*. For laboratory experiments we also employed an animal model organism, the N2 strain of *C. elegans* (Félix and Braendle, 2010).

2.4. Determination of somatic phosphorus content

Nematodes were identified based on morphology under light microscopy and picked into RNAlater® (Ambion Inc.) solution. Nematodes preserved in RNAlater® were shipped to Brigham Young University, Provo for RNA extraction and further analyses. Nematode samples preserved in RNAlater were washed twice with a 5% PBS (Phosphate-Buffered Saline) solution to prevent cells and tissues from rupturing and reduce the number of exogenous microbes. Samples were shipped to ALS Laboratory Group (Salt Lake City, UT, USA) for total phosphorus analysis. To test if preservation in PBS had any effect on the amount of phosphorus contained in the body of individual nematodes, or somatic P-content, samples containing 10, 50, and 100 *Heterorhabditis bacteriophora* nematodes stored in distilled water and PBS for two weeks were analyzed and compared for somatic P-content (Fig. S1). For *S. lindsayae* and *P. murrayi*, six bulk samples containing 25 nematodes each were used to measure the somatic P-content of individual nematodes. Measurement of nematode somatic total P was done using inductively coupled plasma mass spectrometry (ICP-MS, ALS Laboratory Group, Salt Lake City, UT, USA) with external calibration set according to the manufacturer's standard protocol.

2.5. Nematode body size and mass

In 2002 the climate regime of MCM soil ecosystems dramatically shifted from a decadal cooling trend (Doran et al., 2002) to a warmer, wetter, climate with greater interannual variability (Gooseff et al., 2017). To account for potential effects of this climate shift on habitat suitability and nematode growth and development, we measured adult *S. lindsayae* of each sex from each till type (N = 118 (1999), 94 (2004) for Ross Sea till and N = 46 (1999), 54 (2004) for Taylor II till) sampled from 6 different soil monitoring plots per till type during the 1999/2000 and 2004/2005 seasons. All nematode samples were preserved in 10% hot formalin (~60 °C). Animals were photographed using SPOT 3.0 imaging software for microscope digital cameras (SPOT Imaging, Diagnostics Instruments). Morphological features were measured (lengths from tail to mouth, widths from just behind the basal pharyngeal bulb) using Carnoy 2.0 digital image analysis program (Biovolution). Body mass calculations were made using the formula of Andrassy (1956) as directed by Freckman (1982).

2.6. Experimental evolution: culturing conditions

Cultures of *P. murrayi* were established according to Adhikari et al. (2010). *C. elegans* strain N2 (CGC, University of Minnesota) and *P. murrayi* were reared on media of two different P concentrations for 46 months which is 23–31 generations for *P. murrayi* and approximately 345 generations for *C. elegans*. Nematodes were grown on phosphorous sand agar media, which includes 15g Agar, 965 mL H_2O, 20 ml BMB (Bold Modified Basal) for both P-poor and P-rich plates, and 1.033 mg K_2HPO_4 for P-poor and 10.33 mg K_2HPO_4 for P-rich. We did not adjust for differences in potassium concentration, and thus K also varied proportionally with P in the two treatments. The pH was adjusted 7.0, H_2O was added to 1.0 L, and the mixture was autoclaved for 20 min at 120 °C. Sterile builder's sand was poured onto cooled 60-mm plates and stored at 4 °C. The plates were then inoculated with 30 μL of stock OP-50 bacteria and incubated at 37 °C for 2 days. Nematodes were transferred onto the plates and incubated at 27 °C for 1 week, followed by incubation at 15 °C for 3 weeks. The process was then repeated for each successive generation (Adhikari et al., 2010). For all subsequent comparative tests, populations were reared on common plates of P-rich and P-poor nematode growing media (NGM)(Chaudhuri et al., 2011) for at least two generations prior to testing.

2.7. Life history trait observations

P. murrayi and *C. elegans* were reared on P-poor and P-rich plates (50 replicates per treatment) as described above. Ten cultured plates with healthy populations were randomly selected for further observation. For each of the 10 cultured nematode populations, 20 pregnant females were individually picked onto fresh petri dishes and seeded with OP50 *Escherichia coli* as a food source. All plates were kept at 15 °C and checked daily to record eggs laying. After hatching, juveniles were individually transferred to new plates and observed three times a day. The hatching day was recorded as day 0 and the development stage and duration was defined by their body volume based on de Tomasel et al. (2013). The duration of each stage and total egg abundance of each culture plate was observed and recorded. Photographs were taken daily, and juvenile body sizes were captured using a CKX 41 Olympus inverted microscope. Body size, including body length and volume, was measured and calculated by the modified Andrássy formula (de Tomasel

et al., 2013) in MatLab (MATLAB 6.1, 2000).

2.8. RNA extraction and real-time PCR

Nematodes stored in RNAlater solution were washed twice with a 5% solution of phosphate buffer saline (PBS) before RNA extraction. Total RNA for Quantitative real-time PCR (qRT-PCR) was extracted using Trizol reagent (Molecular Research Center Inc.). Three replications of each sample were used for RNA extraction, yielding three independent RNA extracts for each bulk sample. Nematodes were directly homogenized in liquid nitrogen, mixed with Trizol Reagent, and the suspension was exposed to three freeze-thaw cycles using liquid nitrogen and 37 °C water bath. The suspension was ground using mortar and pestle, vortexed, and phase separated using chloroform. After centrifugation (15 min, 12000 g, 4 °C), the aqueous phase containing RNA was separated from the other phases, which were stored for DNA preparation (see below). The colorless upper aqueous phase was transferred into fresh vials to precipitate the RNA by addition of 100 ml isopropyl alcohol. The samples were incubated for 10 min and centrifuged (20 min, 12000 g, 4 °C). The RNA precipitates were then washed twice with 75% ethanol, air-dried, eluted in nuclease-free water, and quantified and quality-checked via spectrophotometer (A260/A280 $>$ 1.9; NanoDrop ND-1000, NanoDrop Technologies, Thermo Fisher Scientific Inc.) and agarose gel electrophoresis.

Reverse transcription (RT) was performed with 1 μg of total RNA extracted from pooled samples of nematodes. The RT of polyadenylated mRNA to cDNA was done using the ImPromp-II™ reverse transcriptase (Promega Corporation) and a random hexamer primer. Total RNA was incubated with 20 pmol random hexamer primer at 70 °C for 5 min and then quickly chilled on ice. The reverse transcription mixture (20 μl) was mixed with RNA template and incubated at 25 °C for 5 min to encourage annealing and the first strand was extended for 60 min at 42 °C. The cDNA was precipitated in 100% ethanol and washed twice with 75% ethanol, air-dried, and dissolved in DEPC-treated water.

Quantitative real-time PCR was performed with LightCycler 480 SYBER Green I Mastermix (three replicate samples for each extraction) and gene specific primers in a Light Cycler 480 RT-PCR system (Roche Applied Science) equipped with LightCycler 480 software with the following program: 3 min at 95 °C; 45 cycles of 30 s at 94 °C, 30 s at 58 °C and 1 min at 72 °C followed by a standard melt curve. The RT-PCR reaction had a final volume of 10 μl including SYBR Green Mastermix (Roche Applied Science), and template DNA. Negative control reactions containing water in place of cDNA were included in each batch of PCR reactions to identify potential contamination. We used nematode β-actin, β-tubulin and GAPDH genes as internal controls for natural populations of *S. lindsayae* and *P. murrayi* and β-actin for laboratory populations of *P. murrayi* and *C. elegans*.

2.9. qPCR and transcriptomic analysis

Absolute quantification method was used for rDNA gene copy number determinations. To determine the copy number of 18S rDNA, a single-copy reference gene ribosomal protein L3 (*rpl-3*) was used. Since the *rpl-3* gene is highly conserved in nematodes and has been shown to be present as a single copy in many nematodes, we selected this gene to use as the single-copy reference probe in the qRT-PCRs. Confirmation was done by using relative quantification (ratios of one gene to another) to determine the number of copies present per genome. Quantification standards were run in conjunction with each set of samples after primers and probes for the *rpl-3* and 18S rDNA genes were optimized for PCR amplification efficiency and relative efficiency of target and reference gene. Six serial 1:2 dilutions (20, 10, 5, 2, 1.25, and 0.625 ng/l) of genomic DNA were used to generate standard curves of *CT* (threshold cycle) value against the DNA concentration on each PCR plate for the *rpl-3* and 18S rDNA genes. Each experiment was performed three separate times from one DNA preparation and run in duplicate. *CT* values were determined and then converted into template quantity.

Absolute quantification requires that absolute quantities of standards be determined by some independent means first. Accordingly, nematode DNA was used to make absolute standards. Concentration and DNA quality were measured by determining the A260 and by gel electrophoresis and converted to the number of copies by use of the molecular weight of the DNA. The equation $CT{=}m$ (log quantity) $+ b$ from the equation for a line ($y = mx + b$) was constructed by plotting the standard curve of log quantity versus its corresponding *CT* value. After the creation of standard curves, the copy number of each gene was determined by DNA quantification. PCR cycle numbers were plotted against the value of fluorescence signal, and then threshold values were plotted against the copy number of the template DNA used to generate standard curves. Comparative copy numbers were determined using the relative quantification (ΔΔCT) $2^{-\Delta\Delta CT}$ method. The 18S rDNA copy numbers were determined by the absolute quantitation method, by which total copies were calculated using the following equation: total 18S rDNA copies $= 10([CT - b]/m)$. The number of 18S rDNA copies per genome was then determined by the following equation: 18S rDNA copies per genome = (total copies of 18S rDNA)/(total copies of *rpl-3*). Copy number was calculated as the ratio of template quantity for 18S rDNA to the template quantity for *rpl-3*.

Total RNAseq data of *P. murrayi* and *C. elegans* were obtained by Illumina HiSeq2500 (Xue et al., 2021b). Assembled transcripts were mapped to the reference genome and transcriptome of nematode *C. elegans* from Wormbase. Raw reads from *P. murrayi* and *C. elegans* were assembled using Trinity (Haas et al., 2013). The reads were aligned and mapped using the single-end default parameter in Bowtie (V-2.2.5) (Langmead and Salzberg, 2012). Open reading frames were predicted by Transdecoder in Trinity. The transcriptomes of *P. murrayi* and *C. elegans* were sorted and prepared for NCBI transcriptome shotgun assembly submission. Blobtools (Laetsch, 2017) was used for contamination and low quality scaffolds screening and contaminated sequences were trimmed and excluded.

Cufflinks (V 2.2.1) (Trapnell et al., 2012) was used to compare the expression pattern between P-rich and P-poor environment for both *C. elegans* and *P. murrayi*. Differentially expressed genes were identified and a matrix of normalized FPKM (fragments per kilobase of exon model per million reads mapped) values were calculated using TMM (Trimmed Mean of M-values) normalization due to the different library size across both datasets. Although the raw fragment counts are used for differential expression analysis, the normalized FPKM values are used below to examine profiles of expression across different samples and are shown in heatmaps and related expression plots.

2.10. Statistical analyses

We performed two-sample *t*-test and ANOVA using SAS 9.1 (SAS Institute Inc.) and R (3.6.2), respectively, to test for variation in soil P-content, relative expression level, rDNA copy number, nematode body P-content and nematode body mass. Total mass calculations were analyzed as a mixed model in SAS 9.1 and calculated by determining the average individual dry weight from individual lengths and widths, correcting for water content as per Andrassy (1956). For qRT-PCR experiments, changes in target gene expression were calculated using equation $2^{-\Delta\Delta CT}$ (Livak and Schmittgen, 2001). Expression level changes (fold change) of the rRNA gene were normalized to single-copy nuclear gene expression (β-actin, β-tubulin and GAPDH). The fold change in expression of rRNA in samples from Taylor II till is set as equal to one. An *F*-test at a significance level of $P < 0.05$ was used to compare the ratio of the mean gene expression of F6 samples with that of Taylor II till. β-actin, β-tubulin and GAPDH were also used as internal controls to minimize genomic template contamination bias and correct for the variations among samples (Vandesompele et al., 2002). The differences between each group in life history observation were analyzed by *t*-test ($P < 0.05$) in R (RStudio Team, 2015)(Horton and Kleinman, 2015).

Hochberg correction was applied on each test (Dagum and Dagum, 2001).

3. Results

3.1. Geographic variation in soil nitrogen and phosphorus pools and somatic P-content

The two sites (Fig. 1A) exhibited significant differences in soil nitrate and P (PO_4^-) content ($P < 0.05$). Soils from the Ross Sea till had twice as much organic C and eight-fold higher bioavailable P-content (4.36 ± 1.91 μg/g) relative to soils on Taylor II till in the Lake Bonney basin (0.55 ± 0.12 μg/g) (Fig. 1B), while soils from the Lake Bonney basin had ~10X the availability of inorganic N, mainly as nitrate (Table 1). These differences in soil P-content and especially the N:P ratios are reflected in the somatic P-content of nematodes extracted from the two tills. Individual *P. murrayi* extracted from Ross Sea till soils had 1.13 times higher somatic P-content than those from Taylor II ($P < 0.05$) (Fig. 1C). No significant difference in somatic P-content was observed among *P. murrayi* and *S. lindsayae* from within a particular site ($P > 0.05$). Overall, nematodes from the Ross Sea drift had higher somatic P-content (2.31–2.54%) compared to those from Taylor II (1.01–1.22%; $P > 0.05$).

3.2. P-availability and nematode body mass

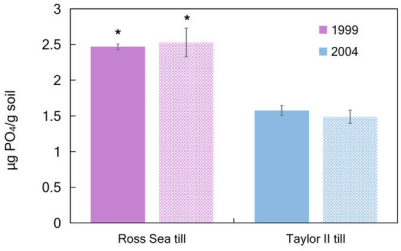
The average body size of individual adult nematodes from Ross Sea till and Taylor II till was inversely proportional to soil phosphate concentration (Fig. 2). That is, nematodes living in higher P-content Ross Sea till soils have shorter body length than those living in low P-content Taylor II till soils, regardless of differences in interannual and decadal differences in habitat suitability ($P<0.05$). Nematode mass followed a similar pattern for animals collected during a cool, dry climate regime (1999; $P < 0.05$) but these differences were not significant for animals collected following a climate shift to a warmer, wetter regime (2004; $P > 0.05$).

3.3. Ribosomal RNA expression level and gene copy number (field tests)

S. lindsayae and *P. murrayi* collected from the same soil till sequence (Ross Sea till) exhibited little intra- or inter-specific variation in rRNA gene expression ($P < 0.05$). However, *S. lindsayae* extracted from Ross Sea tills exhibited 5.42 ± 0.41-fold greater rRNA expression on average relative to conspecific nematodes collected from Taylor II tills. Similarly, rRNA expression levels in *P. murrayi* were 5.93 ± 0.18 times higher in Ross Sea tills than in Taylor II. Additionally, both *S. lindsayae* and *P. murrayi* from Ross Sea till had 2.45- and 2.71-times higher rDNA copy numbers, respectively, compared to *S. lindsayae* and *P. murrayi* extracted from Taylor II ($P < 0.05$) (Fig. S2). There was a strong interaction between nematode species and sampling sites ($P < 0.05$).

Table 1
Average (+/1 standard deviation) organic carbon, total nitrogen, Total P (HCl-soluble phosphorus measured as P), inorganic nitrogen (KCl-extractable ammonium and nitrate), and inorganic P ($NaHCO_3$-extractable phosphate), as well as CNP and inorganic NP ratios.

	Organic C	Total N	Total P	C:N:P	Inorganic N	Inorganic P	N:P
	g/kg soil				mg/kg soil		
Ross Sea Till	0.46 ± 0.14	0.04 ± 0.01	0.68 ± 0.06	23/2/34	0.19 ± 0.10	4.36 ± 1.91	1/23
Taylor II Till	0.17 ± 0.02	0.02 ± 0.01	0.28 ± 0.02	17/2/28	0.45 ± 0.08	0.55 ± 0.12	9/11



	Ross Sea till (118+94)			Taylor II till (46+54)		
Year	Length (μm)	Diameter (μm)	Biomass (μg)	Length (μm)	Diameter (μm)	Biomass (μg)
1999	620.56±3.48	33.96±0.23	0.11±0.002	666.94±5.30	36.01±0.27	0.13±0.002
2004	617.29±4.68	31.86±0.24	0.09±0.002	633.27±5.66	31.69±0.30	0.11±0.002

Fig. 2. Differences in nematode somatic P-content between Ross Sea and Taylor II till. Figure shows percent of phosphorus content from soils of different provenance. Table shows differences in size and mass of the nematodes extracted from the two soil provenances (tills). Each bar represents the mean ± S.E. Asterisks indicate a significant difference between the two sampling sites ($P < 0.05$).

3.4. Ribosomal RNA expression level and gene copy number (experimental evolution)

C. elegans, a well-studied free-living, bacterivorous nematode was selected as an ecological analog of *P. murrayi*. Since *P. murrayi* reared in the laboratory has a much longer generation time than *C. elegans* (6–8 weeks versus 72–96 h at 15 °C, respectively), it should take much longer for *P. murrayi* to evolve a similar response to changes in P availability. After approximately 345 generations, the expression of the 18S ribosomal DNA tandem array in *C. elegans* reared in a P-poor environment decreased to become 13.92 times lower than populations of *C. elegans* reared in a P-rich environment ($P < 0.05$). Similarly, after approximately 30 generations under P-poor conditions, rDNA gene expression in *P. murrayi* also decreased, although not as dramatically, to 0.24 times lower than populations reared in P-rich conditions ($P > 0.05$). Importantly, the expression level of β-actin (a single-copy housekeeping gene) remained unchanged in both *C. elegans* and *P. murrayi* (Fig. S3).

3.5. Transcriptomic comparison of lab-cultured P. murrayi and C. elegans

Ribosomal RNA-associated genes from *P. murrayi* and *C. elegans* reared under different P-availability are more highly expressed in nematodes from P-rich conditions (Fig. 3A & B, Supplementary Table 1), consistent with the qPCR results. Furthermore, *C. elegans* reared on P-rich media evolved higher expression levels of genes associated with development and reproduction (Fig. 3C, Supplementary Table 2). For example, expression of *daf-18* and *daf-10*, both implicated in growth and development, was significantly elevated in *C. elegans* reared on P-rich compared to P-poor media. Evolved differences for the same suite of growth and development genes in *P. murrayi* are not statistically significant (yet).

3.6. Life history evolution in the laboratory

P. murrayi and *C. elegans* reared in P-poor media had delayed egg-laying and an extended reproductive period compared to those reared in P-rich media (Fig. 4). Nematodes living in P-rich media had earlier, and shorter, peak reproductive rates ($P < 0.05$ for *C. elegans*), though not significantly for *P. murrayi* ($P > 0.05$). Total number of eggs produced by *C. elegans* and *P. murrayi* reared in P-rich and P-poor media was measured at 345 and 37 generations, respectively. Evolved differences in the number of offspring were not observed ($P > 0.05$ for both *C. elegans* and *P. murrayi*). The average total number of eggs was 122.6 ± 14.5, 136.2 ± 15.9, for *C. elegans* in P-rich and P-poor media, and 19.33 ± 0.24 and 19.8 ± 0.27 for *P. murrayi* in P-rich and P-poor media,

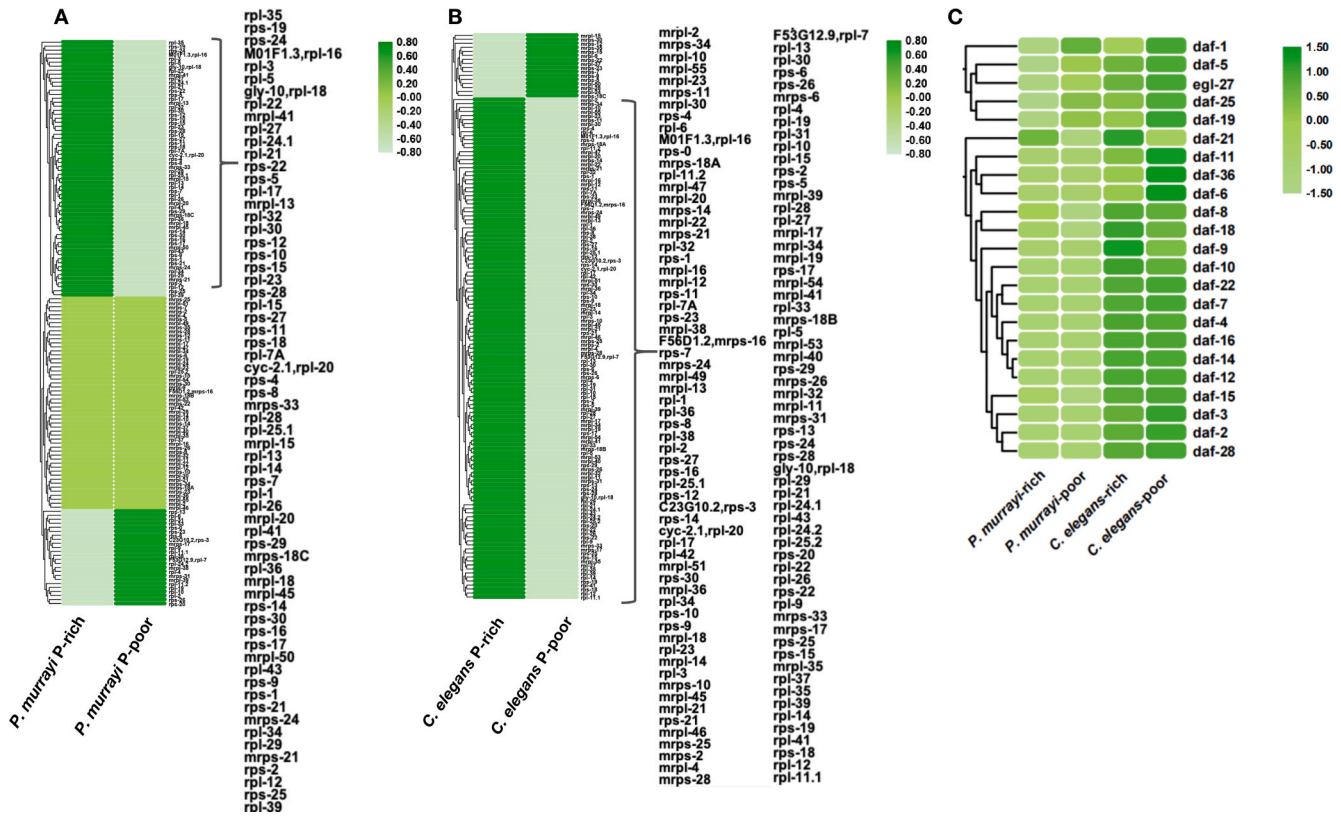


Fig. 3. Differential expression of genes associated with ribosome assembly and function, growth, and development in *P. murrayi* reared on P-rich and P-poor environments (45 and 33 generations, respectively). A: Expression heatmap of 142 rRNA-associated genes in P-rich and P-poor-reared *P. murrayi*, respectively. Genes up-regulated in P-rich-reared *P. murrayi* are enlarged; B: Expression heatmap of 142 rRNA-associated genes in P-rich and P-poor-reared *C. elegans, respectively*. Genes up-regulated in P-rich-reared *C. elegans* are enlarged; C: Expression heatmap of genes associated with growth and development in *P. murrayi* and *C. elegans* from P-rich and P-poor rearing conditions. Color gradient from dark to light green indicates decreasing levels of gene expression. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

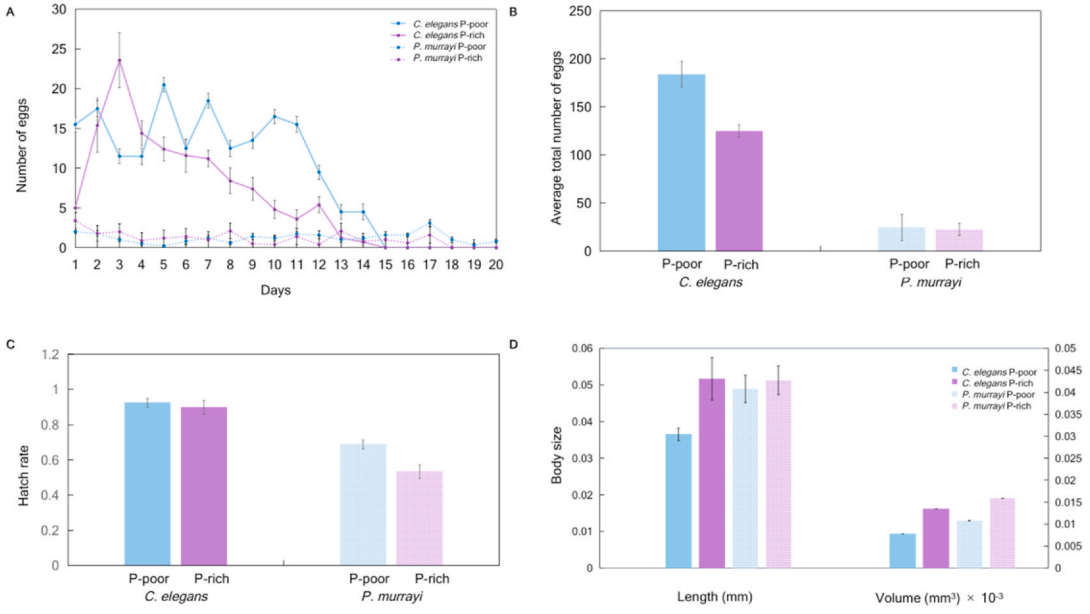


Fig. 4. Differences in *C. elegans* and *P. murrayi* fecundity after rearing in P-poor and P-rich media for 345 and 37 generations, respectively**. A.** Comparative timing of egg-laying. **B.** Average total number of eggs per individual. C. Comparisons of hatch rate. D. Body size comparisons. Bars show standard error.

respectively.

Nematodes reared in P-poor media produced more eggs than those raised in P-rich media, but these differences are not statistically significant. The average egg size of *C. elegans* reared in P-rich and P-poor media was $1.6x10^{-5}$ mm^3 and $0.93x10^{-6}$ mm^3, respectively ($P<0.05$). Average egg size of *P. murrayi* reared in P-rich and P-poor media was

$1.59x10^{-5}$ mm^3 and $1.08x10^{-5}$ mm^3, respectively ($P < 0.05$). Average hatch rates for *C. elegans* reared in P-rich and P-poor media were 93.71%, 91.70%, respectively and not significantly different from one another ($P > 0.05$). Average hatch rate of *P. murrayi* reared in P-rich media was significantly higher than it in P-poor media ($P < 0.05$) which was 70.93% and 57.30%, respectively.

The adult body volumes of both *C. elegans* and *P. murrayi* reared in P-rich conditions were smaller than those reared in P-poor conditions, although not significantly ($P > 0.05$ for *C. elegans* and *P. murrayi*), while body length displayed the opposite trend ($P < 0.05$ for *C. elegans* and *P. murrayi*) (Fig. 5A–B). For both species, populations reared on P-poor media showed delayed maturity compared to those reared in P-rich media ($P < 0.05$ for *C. elegans* and *P. murrayi*).

By tracking the developmental period of these four groups, our results show that nematodes reared in P-rich media mature earlier than those reared in P-poor media. They also evolved significant differences in the timing of some life history traits (but not all; Fig. 6). In *C. elegans*, the time required to develop from egg to young adult and from egg to death are different between P-rich and P-poor growth conditions. *C. elegans* reared in P-rich media exhibit increased longevity and reach the young adult stage earlier than those grown in P-poor media. In *P. murrayi*, the duration time from egg to young adult, egg to adult and egg to egg laying differ, but not significantly ($P > 0.05$). However, it still took less time for *P. murrayi* growing in P-rich media to reach those stages than those grown in P-poor media.

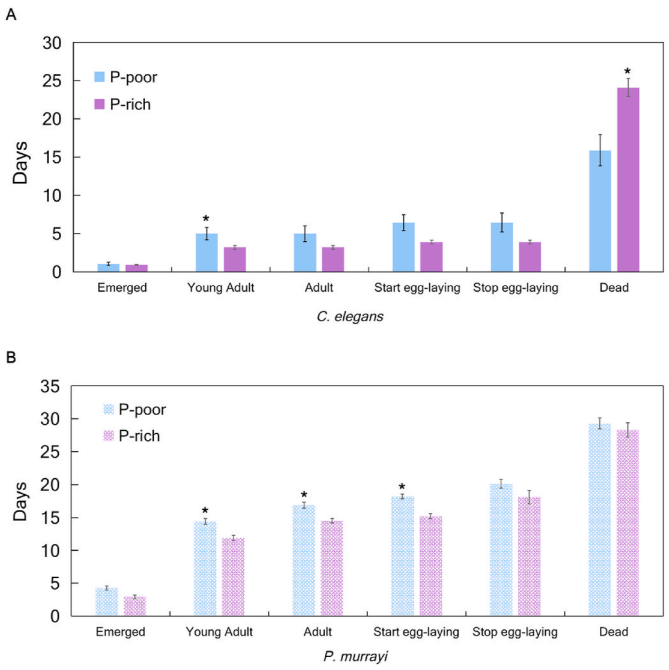


Fig. 6. Comparison of life history stages of *C. elegans* and *P. murrayi* reared in P-rich and P-poor medium. A: Life history stages in *C. elegans* reared in P-rich and P-poor medium. Time to young adult and death dates are significantly different at $P < 0.05$; B: Life history stages in *P. murrayi* reared in P-rich and P-poor medium. Time to young adult, adult and start of egg laying dates are significantly different at $P < 0.05$. (For *P. murrayi*, young adult refers to J4 stage, for *C. elegans*, young adult refers to L4 stage). The bars show standard error. Asterix indicates significant difference ($P < 0.05$).

4. Discussion

Organisms have evolved a variety of responses to variation in environmental stoichiometry, especially P deficiency, which can be observed at multiple levels of organization. While we know that certain species perform better under high P conditions (Jeyasingh and Weider, 2005), we know little about the genetic variation in natural populations necessary for growth performance under contrasting P regimes. In this study, we found significant variation in gene-expression in two Antarctic nematode species in situ and two lab-cultured species under contrasting P-supply environments. This variation evidently gave rise to differences in the growth rate and body size of the two species, indicating that P availability can strongly impact gene expression and ontogenetic traits. Nematodes extracted from low N:P, P-rich soils grew faster, reached maturity and started laying eggs earlier than those from soils deficient in P with higher N:P ratios. Although such variation in growth rate and body size could be driven by factors other than those considered in this study, such as observed differences in microclimate, the patterns of nematode P-content, body size, rRNA gene copy number and gene expression of both the free-living and lab-reared *P. murrayi*, and the lab-reared *C. elegans,* are consistent with the predictions of the GRH (Elser et al., 2000b).

Differences in P-content observed between the Ross Sea and Taylor II tills may be explained by the differences in in situ physical, chemical and biological processes. Previous work has demonstrated that soils collected from Ross Sea tills had both higher total P and soluble phosphate content (Blecker et al., 2006; Bate et al., 2008). Based on our findings, it appears that landscape legacies responsible for variation in soil P-content contribute to standing genetic variation in P acquisition and/or P-use efficiency (PUE) in nematode populations. Intuitively, our observation that soils with high bioavailable P also have higher organic C points to the role that P availability plays in energetic support for increased trophic complexity.

Our observation that nematodes from P-rich soils have significantly higher rDNA copy number and body mass indicates strong coupling

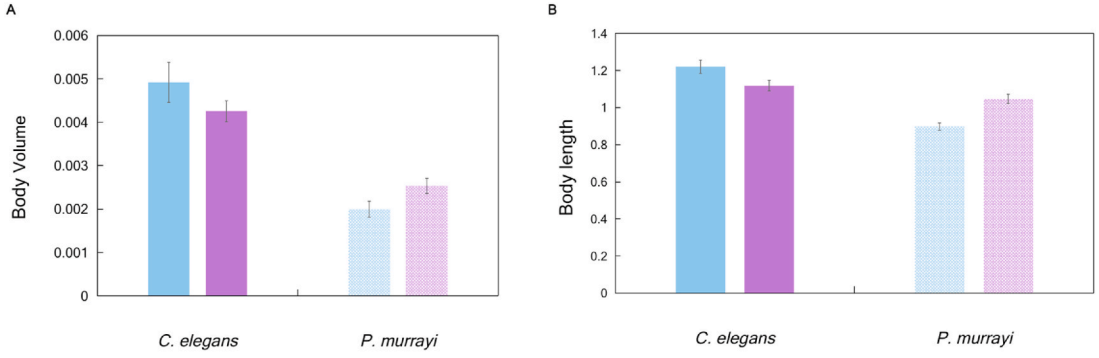


Fig. 5. Evolved differences in body size in response to P-availability. A: *C. elegans* in P-poor conditions have greater body volume than those in P-rich ($P > 0.05$) whereas the body volume of *P. murrayi* in P-poor conditions is less than those reared in P-rich conditions ($P > 0.05$); B: Body lengths of *C. elegans* in P-poor conditions are longer than those cultured in P-rich media ($P < 0.05$) while the body lengths of *P. murrayi* reared under P-poor conditions are longer than those reared in P-rich media ($P < 0.05$). Bars indicate standard error.

between soil P-content and rDNA gene copy number. The association of P-rich rRNA and growth rate has drawn attention to the role of rRNA genes in supporting elevated production of rRNA for rapid growth (Elser et al., 2000b). For example, variations in rDNA genotype, i.e. rDNA copy number (White and McLaren 2000) have been implicated as being responsible for differences in rRNA synthetic capacity and concomitant variation in P-content and growth rate. Our results suggest that rDNA copy number has evolved with nematode biomass and soil P-content and is significantly correlated with growth rate (as indicated by rRNA expression). Additionally, our findings provide further evidence that rRNA gene copy number is connected not only to growth, but to the elemental composition of living biomass. Our findings are consistent with the fundamental premise of the GRH that there may be an intrinsic link between the stoichiometric P demands of organisms and critical molecular structures (i.e., nucleic acids such as RNA) that ultimately affect key organismal processes, such as growth rate.

Our laboratory observations revealed a significant increase in rRNA gene expression in *C. elegans* grown in P-rich medium compared to those in P-poor conditions, whereas the rRNA gene expression levels of *P. murrayi* reared in the laboratory with P-rich and P-poor condition (Fig. 4A) are similar (Fig. 4B). However, the observed changes in life history traits of *P. murrayi* correlate to differences in stoichiometric P constraints, demonstrating that *P. murrayi* growth rate evolves in response to bioavailable P. Since *P. murrayi* reared in the laboratory has a much longer generation time (6–8 weeks under 15 °C) than *C. elegans* (72–96 h under 15 °C), it likely will take much longer for *P. murrayi* to evolve heritable changes in growth and development we observed in C. *elegans*.

We observed a strong coupling of P availability with rRNA gene expression in both natural and laboratory-evolved populations of nematodes. Nematodes from P-rich conditions had higher somatic P-content, indicating the possibility that observed changes in body phosphorus content are partially caused by increases in rRNA expression, resulting in increased ribosome activity and growth rate. The role of P as potentially limiting gene expression and protein synthesis has been proposed in many studies and for several P-rich and fast-growing organisms. This is because RNA constitutes a major fraction of somatic P, suggesting a close association between bioavailable P, somatic P-content, and growth rate (Andersen and Hessen, 1991; Sterner and Hessen, 1994; Elser et al., 1996). Several studies have shown that rRNA is a particularly attractive proxy for growth rate because the efficiency of ribosomal protein synthesis is thought to vary relatively little; therefore, cellular ribosome (or rRNA) content can be expected to reflect cellular protein synthesis rate (Dennis and Bremer, 2008).

We found that nematodes raised in P-poor conditions evolve extended reproductive periods than those from P-rich conditions, while the total number of eggs produced by those groups is not significantly different. Accordingly, we suggest that P resource availability can impact the evolution of reproductive traits for both *C. elegans* and *P. murrayi* by slowing development and extending reproduction when P-content is insufficient. Moreover, nutrient availability can also drive microbial community structure and composition, which is important when considering nematode development and reproduction (Shapira, 2017). Some studies suggest that life span should decrease in response to allocating more energy and resources to reproduction (Flatt, 2011; Boonekamp et al., 2014), although extending this principle more broadly should be viewed with caution (Maklakov and Immler 2016). Development and duration of juvenile growth are important drivers of differences in life span. Lind et al. (2017) demonstrated a strong developmental response to stress selection and that growth rate can have a significant effect on longevity. Our field and experimental observations demonstrate that soil P availability can play an important role in the evolution of traits associated with development and reproduction.

Prior work has shown that N can be an important regulator of bioavailable P, and thus it would be expected that growth rates could be positively correlated with N:P ratios (Hessen et al., 2007; Elser et al., 2003). Perhaps as an even stronger inference of bioavailable P as a driver of life history evolution, our field observations revealed patterns of growth and development that are consistent with the GRH even when N is far in excess and P is extremely limited. That is, the strong responses to P limitation that we observed occur not where N is co-limiting, but where N is greatly in excess.

Much additional work is needed to clarify the potential associations among growth, body size, rDNA evolution, bioavailable P, and elemental stoichiometry in general. Additional taxa from diverse and more complex environments need to be examined to assess the generality of our findings, and more sophisticated field and laboratory work is also needed to unravel the specific mechanisms by which P and elemental stoichiometric ratios influence the evolution of rRNA gene copy number and expression. Still, our work points to a compelling example of how soil P availability can influence organismal evolution and points to its broader influence on community and ecosystem structure and functioning, including the organization of trophic interactions and nutrient cycling over evolutionary time.

Statement of authorship

XX, BNA and BJA designed this study. BJA, BNA and XX collected the soil samples. BNA prepared and conducted the field tests. XX, AP and MM carried out the experimental evolution tests. JEB and BAB conducted the soil chemistry analyses. BLS conducted the measurements of the field-collected nematodes. XX, BNA and JXM carried out data analysis. XX, BNA and BJA drafted the initial manuscript. All authors contributed to the interpretation of the results and manuscript drafts. All authors read and approved the final manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

All data are available from EDI. Sequence data are at NCBI PRJNA437987 for transcriptomes; raw reads are at Genbank under SSR15015645-15015648 (BioSample: SAMN19967347-19967350) (Xue et al., 2021a).

Acknowledgments

This material is based on work supported by National Science Foundation grants OPP-1637708, 1115245, and 0423595 for Long Term Ecological Research. Computational analyses were supported by the Natural Science Foundation of China (project 42207342). Andrew R. Thompson provided helpful comments on drafts of this manuscript. Brigham Young University and Zhengzhou University offices of computing provided essential support for transcriptome analyses. The constructive criticisms of Dr. N. Wrage-Mönnig and two anonymous reviewers substantially improved this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.soilbio.2022.108891.

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