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GETTING BETTER WITH AGE

Applications of Cold Temperature Stress to Age Fractionate *Caenorhabditis elegans*: A Simple Inexpensive Technique

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The nematode *Caenorhabditis elegans*'s (CE) successful use in studies of aging is well documented. Cold temperature stress of mixed populations of CE provides a rapid inexpensive means of obtaining three life stage–specific cohorts. Cohorts are obtained in quantities that allow acquisition of replicate metabolite profiles of changes associated with development, aging, and senescence. The fractionation technique is effective with monoxenic and axenic CE cultures. Cohort Y contains 100% young worms, and Cohort A contains 75% adult worms. Cohort M, prereproductive and reproductive, contains some A and Y due to continuous egg laying and hatch. Principal component analysis of normalized data from metabolite profiles obtained using high-performance liquid chromatography electrochemical analysis clearly separates Cohort Y from Cohort A and monoxenic from axenic cultured worms. Access to replicate quantities of age-defined worms will aid studies of alterations in homeostatic controls associated with aging and senescence.

Key Words: Fractionation technique—*Caenorhabditis elegans*—Metabolite profiles—Aging.

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EMPLOYING the nematode *Caenorhabditis elegans* (CE) as a model system for the exploration of numerous fundamental biologic processes is well documented and has proven particularly successful (1–4). A short life cycle, optical transparency, fully sequenced genome, amenability to genetic manipulation, and extensive sequence homology with particular sets of mammalian genes contribute to CE's utility as a biologic model (2,3,5,6). The organism is now used as a model system in a wide arena of biologic research (4,7–9) and has found considerable use in studies on aging (10–15).

Research across a variety of biologic systems adds support and substance to the hypothesis that irreversible alterations in homeostatic controls serve as precipitating events, leading to the onset of aging and death (16–18). Metabolic profiling experiments with CE as the model system are a means of probing Shock's (19) hypothesis on aging that the onset of the senescent phenotype results from breakdown in control systems. The metabolic profiles obtained define, display, and track alterations, either static or dynamic, in the presence and quantity of ~2,000 electrochemically active metabolites from cellular systems amenable to this analytic approach (Figure 3) (20,21).

The ESA CoulArray analytical instrumentation responds to electrochemically active metabolites (13). This allows recording of alteration in analytically stable metabolite profiles (in particular pathways involved in oxidative stress and the neurotransmitter pathways) in response to physiological state change. The small-molecule biochemical metabolite patterns serve, in turn, as significant indicators of cellular status and functional change (20,22–25).

In order to assess the effects of aging or other stressors on CE at different stages of development, it is necessary to fractionate or separate the population into select age cohorts: larval stages and prereproductive and reproductive adults through postreproductive and senescent individuals. Such a separation allows study of age-dependent alterations in metabolic profiles associated with overt system responses and phenotypic display at discrete stages of the organism's life span.

Several techniques for acquiring age-synchronous CE have been developed over the years. The basic method of handpicking CE to obtain age-synchronous cultures was used in genetic and phenotypic studies (26–29), and filtration devices were used to separate fertile adults from their progeny (30,31). Filtration coupled with differential sedimentation (or settling) allowed separation of large populations of adult worms from newly hatched larvae (32,33). Initiating culture from an isolate of eggs is used to generate age-synchronous populations of larval CE (27,34–36).

Temperature-sensitive mutants and DNA synthesis inhibitors have allowed production of synchronous cultures of nonreproductive aging adults (26,27,33,35,37). An automated high-throughput system, the "Copas Biosort," is available that can sort large mixed populations of N2 worms in a liquid culture into select life stages, larval through adult. This platform is a specialized flow cytometer that analyzes and sorts CE by size and fluorescence parameters (38–43).

Protocols are available that produce amounts of CE in age-defined cohorts sufficient for biochemical analysis, but ready access to late-stage and senescent adults proves problematic as a result of the stress factors associated with the protocols employed (3,12,31–33,38,44). All the techniques referenced earlier allow production of separate age fractionations of CE. The method reported here provides advantages in comparison to each. Handpicking (26–29) is effective in providing access to individuals at any particular life stage sought from monoxenic cultures but is labor intensive, time consuming, and not particularly convenient for obtaining large numbers of any particular life stage. Individuals obtained through use of DNA inhibitors, or temperature-sensitive mutants (12,26,27,33,35,37), provide synchronous populations, requiring interventions that alter access to the processes of aging and senescence associated with reproduction. Filtration and sedimentation techniques (32,33) for obtaining age-fractionated populations require repeated filtrations or sedimentations, often taking substantial amounts of time, to affect the level of separation sought. These techniques can prove detrimental to senescent CE given their sensitivity to mechanical stress and osmotic shock. The commercially available Copas Biosort (38–43) is expensive but can provide large quantities of worms from mixed populations, from eggs to adults. The instrument is most effective in separation of synchronous population of CE ranging from L1 to robust adult. The instrument's function is slowed when conducting separation of select life stages from mixed populations of CE, and particularly, large individuals can clog the system. The value of isolating large numbers of nematodes at specific life stages is exemplified in a recent publication where the nature and behavioral effects of select metabolites were determined. Here, large numbers of nematodes (eggs to adults) were obtained from monoxenic liquid culture (45).

An inexpensive technique that allows separation of mixed life stage populations of CE into discrete life stage cohorts and provides ready access to late-stage and senescent adults has been developed and is described in this article. This technique provides age-fractionated populations of CE in less than 3 hours in quantities sufficient to obtain multiple replicates of the metabolic profiles sought. Life stage samples acquired using this technique are derived from mixed populations containing all stages of growth and development, cultured either axenically or monoxenically. Axenic cultured CE populations avoid the issue in metabolic profiling studies of having to sort analyte responses specific to the nematode from those arising from the organism (*Escherichia coli*) utilized as the nutrient source (46,47). Cohorts of CE derived using this technique can be used in experiments to monitor metabolic profiles of CE's normal stages of development, aging, and senescence and specific life stage responses to acute or chronic exposure to pharmaceuticals, environmental contaminants, reactive oxygen species inhibitors, and other potential modulators of cellular homeostatic controls.

The dependence of CE's recovery from the stress of long-term exposure to cold (4°C for 24 hours) was first observed in a population of monoxenic cultured nematodes that had been harvested, washed with M9 buffer, and stored in 5 mL of 0.03% saline. On warming to room temperature, it was found that larval stages (L1–L3) regained motility well before adults, and postreproductive (including senescent) individuals regained motility last. In prior age fractionation studies with the nematode *Panagrellus redivivus*, it was found that the density of individuals increased with age (48). This was found to be the case for CE as well (31,32,49). This difference in response to cold, recovery of motility on return of temperature to normal values of active culture, an inherent response of the worm, is used here as the discriminator that provides age separation of individuals within a normal population. Altered behavior(s) of CE in response(s) to temperature shifts and the mechanisms driving such responses remain active areas of investigation (10,50,51). It is noteworthy that these behaviors are age specific and thus likely determined by status of and alterations in homeostatic controls specific to the particular stage of development of the worm recovering from exposure to cold.

Materials and Methods

Nematode Strains

N2 (Bristol) axenic strain was obtained from Zuckerman (1989), whereas the N2 (Bristol) monoxenic strain was obtained from Caenorhabditis Genetics Center (Minneapolis, MN) in December 2008. Monoxenic strains were maintained in nematode growth medium (NGM) agar plates (100-mm-diameter Petri dishes) under standard conditions in an incubator (Precision Low Temperature Incubator 815) maintained at 21°C for 7 days (2,52). Axenic CE were grown in125-mL flasks (Pyrex) in sterile liquid medium of Bacto-Soytone (BD Scientific, Sparks, MD), Bacto Yeast Extract (BD Scientific), and heated liver extract, maintained at 21°C for 14 days, a modified version of the axenic culture technique (53).

Harvesting CE Cultures

Two-week-old axenic CE cultures (three flasks, 125 mL Pyrex) were transferred into three 15-mL polypropylene Falcon tubes and washed twice with 10 mL aliquot of 0.03% sterile saline solution, followed by sterile distilled water. The majority of the liquid was separated from the CE by centrifugation (Allegra 6R centrifuge; Beckman Coulter,

Fullerton, CA) at 2290 rcf for 10 minutes at 21°C temperature. The supernatant was removed and discarded from each wash. The pellet (~500 μL [~120,000 CE], with about 250 μL of supernatant) contained the CE.

One-week-old monoxenic CE cultures were harvested by washing three NGM agar plates (100-mm-diameter Petri dishes) twice. Five milliliters of 0.03% sterile saline was added to each plate to detach the worms from the surface. The resulting aqueous layer containing the worms was removed and washed on a nylon filter (pore size 10 μm; Spectrum Labs, Rancho Dominguez, CA) seated on a vacuum filter system (Nalgene, Rochester, NY). Worms on the nylon filter seated on the vacuum were washed using 10 mL of 0.03% saline to get rid of *E coli* (OP50). As much OP50 is removed at this point as is possible to avoid complicating the analysis of the CE metabolic profiles obtained. The procedure employed is effective in removing most of the OP50 from the harvested worms and avoids clogging the electrochemical detectors of the ESA CoulArray high-performance liquid chromatography (HPLC) instrument used in these studies.

The nylon filter containing CE was placed on the wall of the 50-mL beaker (Pyrex). The worms were washed from the filter into the beaker using 10 mL of 0.03% saline and placed in 15-mL polypropylene Falcon tubes. These tubes were then centrifuged at 2290 rcf, for 10 minutes, at 21°C. The supernatant was removed and discarded. One final washing was repeated with 10 mL sterile distilled water. The pellet (~300 μL [~92,000 CE], with about 250 μL of supernatant) was the harvested mixed population of monoxenic CE. This procedure of washing the worms on the nylon filters also can be applied to the harvesting of the axenic worms.

Age Fractionation

Nematodes harvested from monoxenic or axenic cultures of CE were subjected to cold temperature stress. Age fractions that contain senescent and egg-laying adult, length more than 1,000 μm, are referred to as Cohort A; fractions of CE, early L4 and reproductive L4 with length between 500 and 1,000 μm, are referred to as Cohort M, and early CE larval stages L1–L3 length less than 500 μm are referred to as Cohort Y. Measures of autofluorescence associated with presence of age pigments for each of these cohorts correlated well with the size class discriminator as an indicator of physiological status with respect to aging and senescence.

To the washed CE pellet obtained from the harvesting process, sterile distilled water (18.2 M Ω) was added to a total volume of 2 mL. The worms were gently swirled and then placed in an ice bath (containing ice and salt, temperature −2°C) for 5 minutes. The temperature inside the test tube dropped to 4°C. The oldest CE (senescent) settled to the base of the test tube, followed by adult CE (egg laying), mid-aged CE (L3, L4, and young adults), and finally the young CE (L1, L2, and early L3). The test tube contents were then allowed to warm at room temperature for 0.5–1 minute. Cohort Y became active first and moved into the supernatant, followed by Cohort M. The supernatant containing Cohorts Y and M was removed and retained from the test tubes, without disturbing the Cohort A pellet that settles at the bottom of the test tube. Two milliliters of distilled water was added to Cohort A pellet. The cooling (7 minutes) and warming (0.5–1 minute) processes were repeated twice with Cohort A. The supernatant (containing Cohorts Y and M) was removed in each instance following the cold and warming treatment.

The retained supernatant containing Cohorts Y and M was cooled for 10 minutes (where the temperature inside the test tube dropped to 2°C), and the warming process (1–1.5 minutes) was performed. At this point, the supernatant removed contained Cohort Y, and the pellet at the bottom of the tube was Cohort M. This process was repeated at least once to obtain fewer spills (of Cohorts Y and A) in Cohort M.

Counts for each cohort (based on length) were achieved using a calibrated compound microscope (CK; Olympus, Tokyo, Japan). Several 20 μL samples from each age-fractionated cohort were obtained for nematode counts. Based on the quantity of worms in each age-separated cohort, the samples were diluted prior to counting. The counting was done using a calibrated stage micrometer and light microscope, with an ocular unit (×4). The pellets containing the age fractions, A, M, and Y, were transferred into 1.5-mL Eppendorf tubes (polypropylene). Excess water from the worm pellets was removed after centrifugation (9800 rcf for 15 minutes at room temperature). Approximately 250 μL of water was left with the pellet (volume of the pellet varied with the cohort).

Photographs

Photographs were taken of the age fractions using a Leica M2 IGFA (number 10445930) with Nikon Imaging Software (NIS) elements (D2.20). Each sample was diluted with water (as needed), and 100 μL volume was placed onto a depression slide. Before the photographs were taken, the slides were placed in the refrigerator, at 4°C, for 3 minutes (to slow down the motility of the CE, resulting in better photographs). An ocular of ×40 and exposure time of 1 millisecond were used. Subsequently, the contrast and darkness of the photographs were evaluated and modified using Microsoft Office Picture Manager, 2006.

Fluorescence Microscopy

A sample of cold (2°C) Cohort A (age-fractionated axenic CE; 15–20 CE in 10 μL sterile water) was placed in 150 μL sterile water on a Fisherbrand hanging drop slide with one concavity (Fisher catalog number 12-560A) and covered with a matching transparent glass coverslip. The slide was immediately visualized for epi-illumination

fluorescence using a Nikon Eclipse 90i microscope. Autofluorescence of lipofuscin and other age pigments was measured using the DAPI filter (UV-2E/C, excitation range of 340–380, with mean 355 nm, and emission range 435–485, with mean 461 nm). A second sample was prepared and the process was repeated to obtain epifluorescence for a total of 25 axenic adult CE from Cohort A. The process was repeated with Cohorts M and Y of axenic CE cultures and Cohorts A, M, and Y of monoxenic CE cultures to measure epifluorescence. The region of interest (ROI) tool in NIS elements (D3.0) was used to calculate the mean intensity of epifluorescence in individual worms showing fluorescing pigmentation. Numbers of individuals within the set of nematodes analyzed from each cohort reflected the percentage distribution of "aged" individuals as determined by size and length.

Sample Preparation

Each pellet of life stage–fractionated nematodes, Cohorts Y, M, and A, was prepared for HPLC analysis. Before freezing the samples at −20°C, a polar solvent, mobile phase A (MPA; see HPLC technique section) with a pH of 3.0, was used to precipitate the soluble protein by addition of 500 μL MPA to each pellet, and the samples were frozen at −20°C for 1 hour. The frozen samples in the Eppendorf tubes were removed from −20°C and placed in an ice bath. The CE samples were homogenized using a sonicator/cell disrupter (Fisher Scientific 60 Sonic Dismembrator) with a microtip at Level 7 (700 W) for 30 seconds. The samples were chilled in an ice bath before and after ultrasonication. The microtip was washed with 50% methanol between samples to prevent cross-contamination. The sonicated samples were centrifuged for 15 minutes at 9800 rcf using a centrifuge (Eppendorf Centrifuge 5415 C; Brinkmann, Westbury, NY), and 200 μL of the supernatant was placed in a small syringe fitted with 0.22-μm filter (Cameo 3N Syringe; filter, nylon, 0.22 μm; Micron Separations Inc., Westborough, MA) and filtered into a polypropylene HPLC vial (0.25-mL vials; Sun Inc., Rockwood, TN) and appropriately labeled.

HPLC Technique

Coulometric array detection instrumentation (CoulArray) has found considerable use and application in biochemical analytical procedures (54). HPLC coupled with coulometric electrochemical detection is particularly effective for differential detection and quantification of small molecules based on their oxidation–reduction potentials (13,20). This platform has found extensive application in the study of neurotransmitters and other metabolites derived from the tryptophan and tyrosine biochemical pathways. The CoulArray (CoulArray Multi-Channel ECD system; ESA, Inc, Chelmsford, MA) HPLC system comprised two ESA solvent delivery systems (pump Model 582), ESA autosampler (Model 540), Waters Micro-Bondapak C_{18} 3.9 × 150 mm Column, ESA 16-channel detectors (four cells), and CoulArray for Windows (Version 2.0) software. A step gradient flow method was run for 140 minutes between solvents MPA and mobile phase B (MPB). The column was kept at 26°C, and the 16 electrodes were set at increments of 60 mV from 0 to 900 mV for the channels.

The system allows detection of 5 pg of most redox-active substances (55). Two mobile phases, MPA (80 mM lithium hydroxide and 0.15 mM lauryl sulfate lithium salt [HPLC grade; Sigma, St. Louis, MO]), an aqueous polar phase (18.2 M Ω water) with a pH of 3.0, and MPB (80 mM lithium hydroxide, 1.5 mM lauryl sulfate lithium salt, and 60% methanol), a methanol–water phase with a pH of 4.0, are employed in the HPLC separation schema. Both phases, MPA and MPB, use a lithium buffer stock made by dissolving lithium hydroxide monohydrate (3.5 M, HPLC grade; Sigma) with 85% HPLC-grade phosphoric acid (Fisher Scientific, Fair Lawn, NJ) and water (18.2 M Ω).

Standards consisting of known compounds (intermediates of purine, tyrosine, and tryptophan catabolic pathways) in cocktails or as individual compounds were run at the beginning, during, and at the end of each sample batch. A majority of the individual standards (HPLC grade) were obtained from Sigma. They were dissolved in water and/or 0.1% formic acid, stored, and frozen in smaller subsets of 1 mL, at −80°C. Standard concentrations as individuals and in cocktails were not greater than 1 μg/mL.

Sample Analysis

Samples (CE cell lysate) were assayed for total protein content using the Bradford protein assay procedure (PI23236; Bio-Rad, Hercules, CA). The amount of soluble protein measured in CE samples was used to normalize the chromatographic data, obtained from CoulArray HPLC analysis. The CoulArray-generated chromatograms are a representation of the electrochemical signals of the redox-active compounds (peaks), separated by HPLC. The chromatograms were converted into text files using a module of the CoulArray software (Data Module Version 2.0), a Perl script, and CEAS_511 software provided by ESA. Each chromatogram was converted into a one-dimensional matrix using a script written in Python (Version 2.4). The chromatographs of interest were normalized (using corresponding Bradford protein assays) and merged into one file using the Python software (56). This data set was then imported into Array Studio software (Version 3.6; Omicsoft Corporation, Research Triangle Park, NC), where principal component analysis (PCA) was performed to extract relevant information from the data sets or sample types.

The text files generated from the CoulArray chromatographic data (from Cohorts Y, M, and A) were used to generate PCA and line graphs. Data from two different cohorts (eg, cohorts Y and A) were subjected to the subtraction algorithm written in Python. These data demonstrated

differences in the age-fractionated cohorts using the subtraction algorithm (56). The peaks from the second cohort (eg, Cohort A) were aligned to the peaks of the first cohort (eg, Cohort Y), and the data were normalized against soluble protein data for the respective cohorts. Differences were obtained and displayed by subtracting Cohort Y data from Cohort A data.

Statistics

Metabolic profile analysis of each age fraction obtained was performed in triplicate, and *p* values obtained from the statistical analysis of the resulting data sets were less than 0.05. The Student *t* test (unequal variances) was used to calculate the significance of xanthine:uric acid ratios for the *p* values of the respective cohorts. In applying PCA to the data, the *p* value was set to less than 0.05.

Results and Discussion

Nematode Cultures

The axenic CE population peaked at 14 days, whereas the monoxenic CE population peaked at 7 days (57). The overall development rate and population profile at the point of maximum population density reached were different for axenic and monoxenic CE populations.

Axenic cultures contained relatively more L3 and early reproductive L4 (size 500–1,000) CE than did monoxenic cultured populations (average of the three populations from Table 1: Y was 77.5 ± 2.1% [STDEVP], M was 6.0 ± 0.7% [STDEVP], and A was 16.5 ± 1.9% [STDEVP]), whereas in monoxenic, there were fewer individuals within this set (average of the three populations from Table 2: Y was 77.9 ± 1.0% [STDEVP], M was 3.2 ± 0.3% [STDEVP], and A was 18.9 ± 0.8% [STDEVP]). Eggs of axenic CE are laid in clumps (Figure 1B, axenic-2), whereas those of monoxenic worms are found singly on the agar plates (Figure 1B, monoxenic-1).

Age Fractionation

The age fractionation technique works effectively for axenic and monoxenic cultures of CE. Losses incurred during the procedure were less than 5% of the nematodes present in the mixed population (eg, total number of CE in axenic culture [Table 1, Population 1] after harvest—118,980 CE, and total number of CE in axenic culture after age fractionation—115,520 CE) obtained on culture and harvest. Tables 1 and 2 show the results obtained on fractionation of the mixed populations from both monoxenic and axenic cultured CE into the three defined life stage cohorts. With the axenic nematodes, most of the eggs in clumps were found with the adults after age fractionation (Figure 1B, axenic-2). Most of the eggs from the monoxenic CE were found as individuals with the young nematodes (Figure 1B, monoxenic-1).

Twenty microliter samples from the age-fractionated cohorts were obtained for nematode counts. The samples were diluted prior to counting based on the quantity of worms in each cohort. Replicate counts of two or three were performed. Counting was performed using a calibrated stage micrometer and a light microscope, with an ocular unit (×4). Classification of CE was based on length measurements, as described in Age Fractionation section. Each cohort population density was calculated by multiplying actual counts with the respective dilution factor. The data obtained are presented in Tables 1 and 2 (cohort vs size) for each of the three populations.

A survey of the literature indicates that this age fractionation technique enhances prospects for obtaining senescent CE from mixed populations of worms for use in metabolic profiling or other biochemical analysis. The conditions used to affect age separation, the age-dependent response of CE to recovery from exposure to cold, avoid mechanical and osmotic stresses that often result in lysis of fragile senescent adults (32). In order to study the aging process, data from senescent individuals are critically needed. This separation technique provides select age cohorts from mixed populations in amounts needed for metabolic profiling studies. Cohort Y resulted in 100% young, whereas Cohort A resulted in more than 74.8% adults (including senescent adults). Spills in Cohort M reported in Tables 1 and 2 result from eggs being continuously laid and hatched and the worms tangling with each other when the nematode population becomes very dense. Size is characteristic of age. The percentage contamination of young CE in Cohort A was less than 15%. Cohort Y is free of individuals from Cohort A. Thus, if there is a component in Cohort A that is absent in Cohort Y, contamination by small numbers of young is inconsequential. The contamination level was determined by numbers of young relative to numbers of adults based on size. In addition, the cellular mass per individual between young and adult individuals is significant by at least a factor of 3. The levels of unique components in Cohort Y (young) allow one to calculate the percentage of the signal due to young present in Cohort A.

Lipofuscin and other age-related compounds have been shown to accumulate with age in CE (58,59). Axenic Cohort A (adult) epifluorescence values (intensity per pixel per individual- pixels/nematode) ranged from 1,051 to 3,450 (with a mean of 2,512) in 25 live CE and 912 to 2,116 (with a mean of 1,414) in 22 live monoxenic adult CE Cohort A. Axenic Cohort M contained very few epifluorescent individuals, concordant with the measure of a maximum of 3.5% spill of adults in this cohort having size measures more than 1,000 μm. Individuals within Cohort M showing fluorescence were late-stage reproductive individuals containing six or fewer eggs, with epifluorescence values ranging from 923 to 1,029 (with an average of 1,020). Epifluorescence values in Cohort M from monoxenic adult CE ranged from 651 to 840 (with a mean of 773) in Cohort M.

Table 1. Age Fractionation Population and Percentage Data for Three 14-Day Axenic Populations

Size	Young (<500 μm)	Mid-aged (500–1,000 μm)	Adults (>1,000 μm)	Description
Cohort Y				
Population 1	75,120	0	0	100% Y
Population 2	61,460	0	0	100% Y
Population 3	83,200	0	0	100% Y
Cohort M				
Population 1	10,620	4,940	540	66.0% Y, 30.7% M, 3.3% A
Population 2	12,000	5,850	650	64.9% Y, 31.6% M, 3.5% A
Population 3	11,000	5,400	300	65.9% Y, 32.3% M, 1.8% A
Cohort A				
Population 1	3,200	1,100	20,000	13.2% Y, 4.5% M, 82.3% A
Population 2	2,500	1,200	17,500	11.8% Y, 5.7% M, 82.5% A
Population 3	2,300	1,600	16,400	11.3% Y, 7.9% M, 80.8% A

Note: Three cohorts were collected, counted, and identified as Cohort Y (young), Cohort M (mid-aged), or Cohort A (adult).

None of the other life stages in Cohort M showed measurable epifluorescence. This is reflected in the values for Cohort Y, where epifluorescence values were below detection limits of the instrumentation (measured through ROI tool in NIS elements, D3.0 software).

The analysis indicates that adults in Cohort A were largely senescing and senescent worms. The presence of a subset of early-stage progeny (no more than 15% of the numbers present in axenic CE Cohort A) reflects the viability and presence of some late-stage reproductive adults. The intensity of epifluorescence illumination and the size of monoxenic CE Cohort A were much lower than those of the axenic CE Cohort A. The longevity of CE, which is greater in axenic culture when compared with monoxenic culture, could account for this difference (60). No measurable epifluorescence illumination was detected in Y Cohorts of axenic or monoxenic CE.

The time involved for harvest of the nematodes (any population size) is less than 2 hours and for age fractionation is less than 1 hour. This procedure provides cohorts for studies of stress-mediated responses, both acute and chronic, as well as dose–response studies, for example, heavy metals, pharmaceuticals, environmental toxicants.

Photographs

The photographs in Figure 1 show the CE obtained from the age fractionation cohort referenced here as Cohort Y, which comprised L1, L2, and very few L3 (Figure 1C). A mixed assemblage of life stages (L3, L4, and young adults) are found in the life stage fraction referenced as Cohort M (Figure 1D). The third age fraction, life stage group, referenced here as Cohort A is composed primarily of adults (Figure 1E). The population observed in Cohort M is not as dense as that seen in Cohorts Y and A because the initial populations prior to age fractionation of both the axenic and the monoxenic cultures had fewer individuals in this life stage segment, Cohort M: L3, L4, and young adults (~6.0% for axenic and 3.2% for monoxenic) to young (~77.5% for axenic and 77.9% for monoxenic) or adults (~16.5% for axenic and 18.9% for monoxenic).

The eggs from monoxenic worms were found singly on the agar plates, whereas in the axenic media, they were found in gel-like clumps (Figure 1B). The egg preparation technique [a modified version from Fabian and Johnson (33)] required twice the number of monoxenic adults to obtain the same number of axenic (17,000 adults resulted in ~72,000 eggs) and monoxenic (17,000 adults resulted in ~35,000 eggs) eggs. The differences in the population density and egg laying may be due to the difference in the medium and nutrient for culture: liquid axenic culture media for the axenic worms and NGM agar plates with *E coli* for the monoxenic worms.

Table 2. Age Fractionation Population and Percentage Data for Three 7-Day Monoxenic Populations

Size	Young (<500 μm)	Mid-aged (500–1,000 μm)	Adults (>1,000 μm)	Description
Cohort Y				
Population 1	55,220	0	0	100% Y
Population 2	51,350	0	0	100% Y
Population 3	50,600	0	0	100% Y
Cohort M				
Population 1	11,500	2,540	340	80.0% Y, 17.7% M, 2.3% A
Population 2	12,600	2,850	250	80.3% Y, 18.1% M, 1.6% A
Population 3	12,100	2,400	300	81.8% Y, 16.2% M, 2.0% A
Cohort A				
Population 1	5,200	150	16,000	24.4% Y, 0.7% M, 74.9% A
Population 2	4,900	450	16,900	22.0% Y, 2.0% M, 76.0% A
Population 3	5,300	350	17,000	23.4% Y, 1.5% M, 75.1% A

Note: Three cohorts were collected, counted, and identified as Cohort Y (young), Cohort M (mid-aged), or Cohort A (adult).

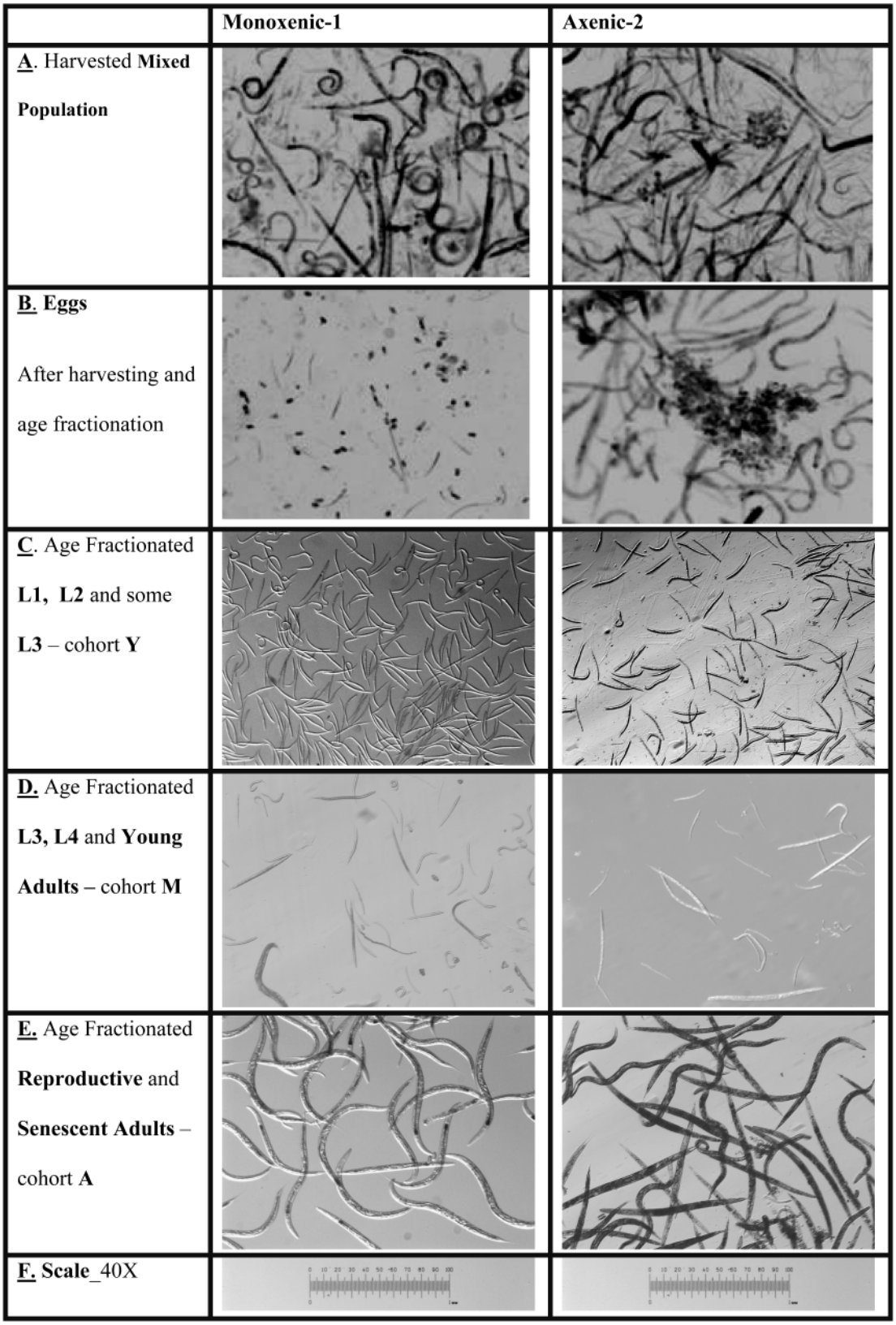

Figure 1. Photographs of harvested and age-fractionated *Caenorhabditis elegans* cohorts from both monoxenic(1) and axenic(2) populations. (A) Harvested mixed populations. (B) Eggs. (C) Cohort Y. (D) Cohort M. (E) Cohort A. (F) Scale. These representations were done at ×40. Photographs were taken using the Leica Microscope. Alterations to the contrast and lighting were applied.

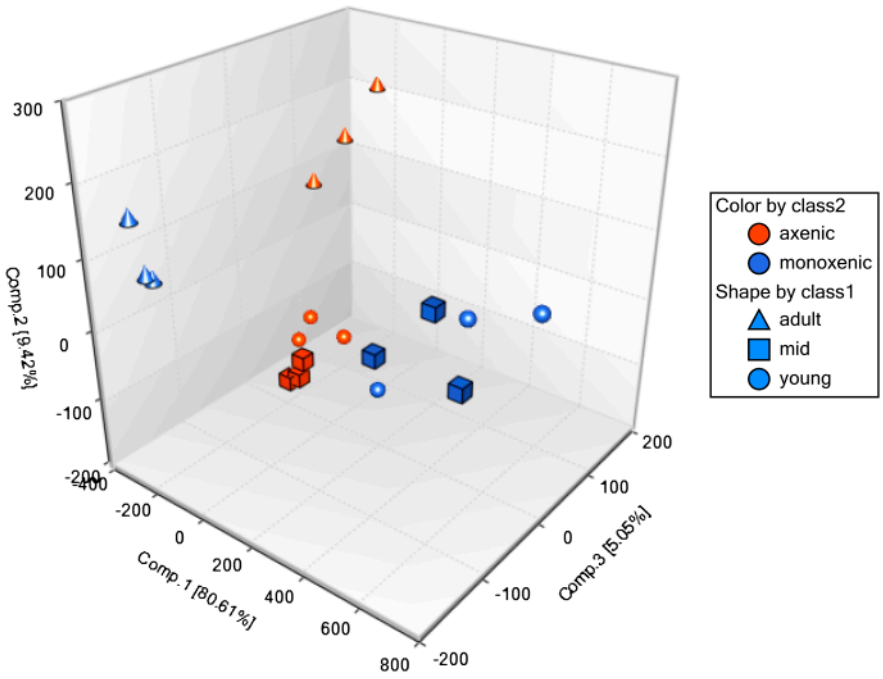

Figure 2. Principal component analysis of normalized chromatographic data obtained from age-separated *Caenorhabditis elegans* cohorts. Royal blue represents monoxenic *C elegans*. Orange red represents axenic *C elegans*. Cohorts Y, M, and A are represented as spheres, cubes, and cones, respectively.

Analysis of Chromatograms From the Age-Separated Cohorts

PCA is a standard tool used in modern data analysis. It is a nonparametric method for extracting relevant information from complex data sets. In Figure 2, Components 1–3 captured 95.08% of the metabolic differences in the data. The combination of the three components (1–3) separated Cohort A from Cohorts M and Y, the axenic Cohort A from monoxenic Cohort A, and axenic Cohorts M and Y from monoxenic Cohorts M and Y. The variances could be due to differences in peak intensities and/or the presence or absence of peaks in the different cohorts. Because Cohorts Y and M have a lot of young CE (Tables 1 and 2), this could explain why their chromatographic data are found close to each other (Figure 2). These chromatographic profiles provide signature patterns that reflect differences in biochemical metabolite functions that are inherent to CE at each life stage. An example of the complexity of electrochemical signals (chromatographs) from an axenic Cohort A is represented in Figure 3. In addition, differences in the same cohorts from axenic and monoxenic cultures could be due to the nutritional difference in the CE diet and their type of movement (in the liquid or solid medium used for culture). Nuclear magnetic resonance spectroscopy has

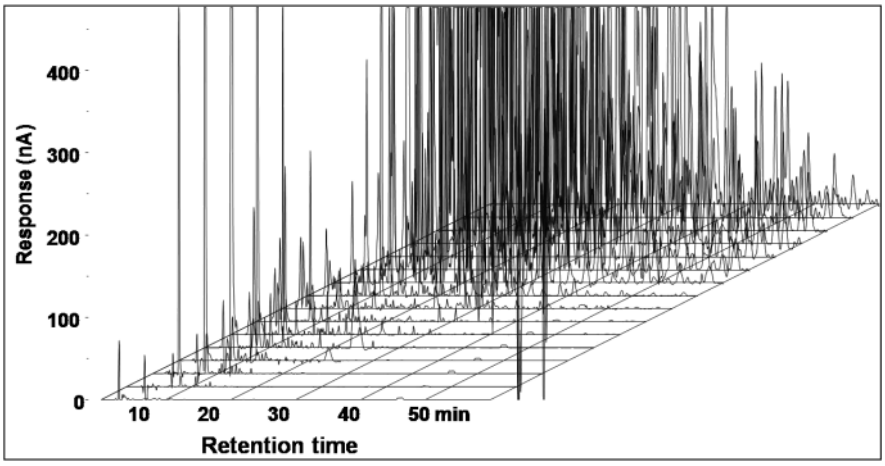

Figure 3. Chromatographic data obtained from a Cohort A sample, obtained from CoulArray high-performance liquid chromatography analysis. This figure displays the complexity of the data; ~2,000 electrochemically active metabolites from cellular systems are amenable to detection using this analytic approach.

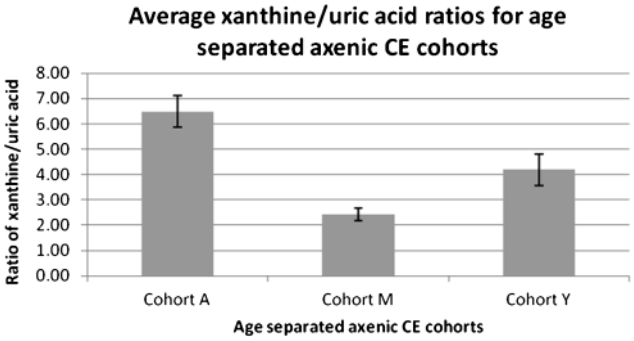

Figure 4. Bar graph of the average ratios of xanthine:uric acid for each axenic *Caenorhabditis elegans* cohort. There were three separate populations, with the number of worms in each cohort listed in Table 1. Student's *t* test for unequal variances was performed, resulting in $p < 0.05$ significance, for each combination. The error bars are the standard deviations of the xanthine:uric acid ratios.

revealed metabolic differences due to altered physiological processes in L1–L4 larval stages in N2 CE (61). The chromatographic data obtained from the CoulArray HPLC (instrument) include the biogenic amines and other electrochemically active metabolites. The biogenic amines measured include most of the neurotransmitters, such as dopamine and serotonin, which play key roles in aging and behavior (62–67). The amino acids tryptophan and tyrosine and several purines and their derivatives were also identified by this technique. Significant variation with age is found in the levels of two of the purine catabolic pathway analytes measured: free xanthine and uric acid (Figure 4). The xanthine:uric acid ratios for the various cohorts are displayed in Figure 4. The ratio of analytes displays the metabolic flux in the pathway. In this case, the ratio of xanthine:uric acid for Cohort M was the smallest when compared with those for Cohorts Y and A, with *p* values less than 0.005 (using the Student *t* test). Purine metabolites have multiple critical functions in a wide array of physiological processes: cellular growth and signaling, energy metabolism, neuromodulation, and neurotransmission (68).

Figure 5 presents an example of differences between Cohorts A and Y, using a subtraction algorithm, for a portion of the data. Both qualitative and quantitative information on the analytes detected can be obtained using this approach. Unique peaks were found in Cohorts A and Y (Figure 5).

Conclusions

The age fractionation technique reported here provides a simple rapid method for obtaining separate select life stages (age cohorts) of the nematode CE in amounts sufficient for a variety of replicate biochemical analysis. The technique does not require the use of aggressive or toxic solvents. This is of particular value in studies of senescence, and the factors that precede the onset of systems decline. Responses of CE's life stages on exposure to cold are sufficiently different to allow their separation. Costs associated with this protocol and equipment necessary for age fractionation are minimal. One can separate four mixed populations (ranging from 1×10^5 to 2.7×10^6 axenic CE) into three separate and distinct life stage cohorts in 3 hours or less. The separation schema developed uses a minimally invasive protocol (room temperature to 2°C and return to room temperature). As such, metabolite profiles obtained from each life stage reflect specific inherent differences in the biochemical processes associated with each. Metabolic profile differences in particular sets of metabolic pathways known to be associated with higher levels of homeostatic controls, tryptophan, tyrosine, and purine metabolites are of particular value in examining aspects of control system function and aging. Stress levels are lower than those generated in other large-scale age fractionation procedures, enhancing the prospects for obtaining significant amounts of late-stage senescent individuals.

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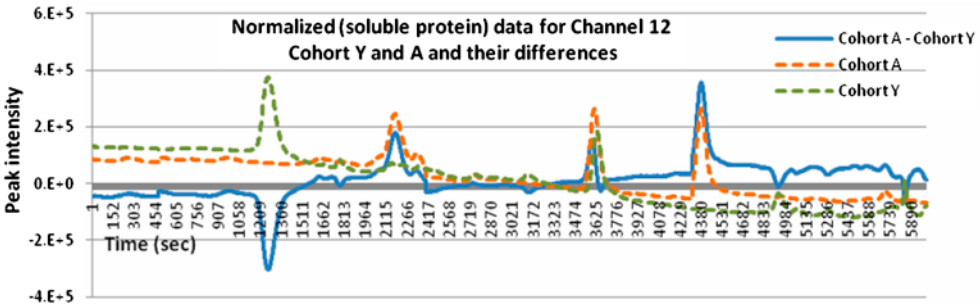

Figure 5. A line graph of the chromatographic data at 720 mV (Channel 12), between 60 and 110 minutes, displaying the difference between Cohorts A and Y after the peaks were aligned against each other and normalized against soluble protein using a subtraction algorithm. The difference (blue line) can be positive and/or negative depending on the presence and/or absence of peaks and their concentrations in the cohorts being compared.

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