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THE AGING PROCESS OF THE NEMATODE *CAENORHABDITIS ELEGANS* IN BACTERIAL AND AXENIC CULTURE

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Croll, N. A., Smith, J. M., & Zuckerman, B. M. The aging process of the nematode *Caenorhabditis elegans* in bacterial and axenic culture. **Experimental Aging Research, 1977, 3 (3), 175-199.** While much is known of the morphological and some physiological changes which occur during the aging of *Caenorhabditis elegans*, little attempt has been made to measure the changes in behaviour. Wild type C. *elegans* (var. Bristol) were cultured axenically, individually observed each day for 15 minutes and their behavioural actions recorded on a multi-channel event recorder or on a video tape recorder of a closed circuit TV. Particular attention was paid to the rate of backwardly directed somatic waves, pharyngeal bulb pulsations, the interval between defecations and oviposition.

C. *elegans* lived significantly longer in axenic culture than in bacteria. A gradual linear decline occurred in the rate of backward waves between maturation (day 4) and death (day 20) for those worms in axenic culture. In striking contrast, the mean maximum rate of pharyngeal bulb pulsations maintained a plateau from day 4 to 18, while the mean interval between defecations doubled from 60 sec (days 4 to 8) to 120 sec (days 10-20). These results are discussed in the context of nematode coordination and the mechanisms of aging.

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There has been considerable recent research effort devoted to the metazoan nematode model, *Caenorhabditis elegans*. This has come from many branches of biology: genetics (Brenner, 1974), cytology (Hirsch, Oppenheim, & Klass, 1976) neuroanatomy (Ward, Thompson, White, & Brenner, 1975; Ware, Clark, Crossland, & Russell, 1975) gerontology (Zuckerman, 1976) and behaviour (Ward, 1973; Croll, 1975a; Dusenbery, 1974). The reason for the selection of *C. elegans* and its suitability for laboratory studies is based on its hermaphroditic nature, 4-5 day generation time, ease of mutagenesis, ease of culture, relatively simple organisation, constancy of certain tissues, noteably the nervous system and similar biological features (Brenner, 1974).

Gerontological studies on *C. elegans* and its close relative *C. briggsae* have proceeded entirely in axenic culture, as is the case with the other laboratory nematode models, *Turbatrix aceti, Panagrellus redivius* and *P silusiae* (Gershon, 1970; Westgarth-Taylor & Pasternak, 1973). Concurrently with these studies, research on behaviour, genetics and development have been conducted on *C. elegans* in cultures of the bacterium *Escherichia coli.*

Hirsch *et al.* (1976) have shown that the growth rate and fecundity of *C. elegans* is temperature dependent, between 16°C and 25.8°C. *C. briggsae* showed a progressive decline in the rate of movement with age (Zuckerman, Himmelhoch, Nelson, Epstein, & Kisiel, 1971) and the rate of vulval contractions of *C. elegans* in 5-hydroxytryptamine declined with age (Croll, 1975b).

We noted that *C. elegans* in our laboratories seemed to live longer in axenic culture than in bacterial culture. We, therefore, resolved to examine aging through an integration of some known biological parameters of aging (Zuckerman, 1976) with new behavioural measurements (Croll, 1976a).

Materials and Methods

C. elegans wild type (var Bristol), originally obtained from Dr. Ruth Pertel (NIH, Bethesda, Md., U. S. A.), was used throughout. It was cultured in lawns of *Escherichia coli* on 1.2% Czapek-

dox agar. The axenic medium comprised soy peptone 4 g, yeast extract 3 g and water 90 ml sterilized by autoclaving and liver extract sterilized by passage through Millipore filters 0.8, 0.6, 0.45 and 0.22μm successively. This medium is based on that of Sayre, Hansen, & Yarwood (1963). All cultures and behavioural observations were held at 20° $\pm$ 1°C.

Lengths were measured directly and volumetric calculations were derived from treating individuals as cylinders with the tail as a terminal cone (Sourcy, Kisiel, & Zuckerman, 1976).

Fecundity measurements were made by individually placing worms into vials. After the first eggs were observed, the nematodes were then transferred to fresh vials every day. The number of progeny in the vials was counted after a further two days allowing eggs to hatch and give rise to larvae. This method counted only fertile eggs. The degree of fertility of the eggs was monitored during the 15-minute periods used for behavioural studies.

Survival or longevity was followed by carefully selecting a minimum of 50 individual worms and placing them in a vial of either *E. coli* or axenic culture. Every individual was transferred to fresh cultures throughout its reproductive period to avoid it becoming confused with its progeny. These individuals were observed daily for any sign of movement until 50% were dead. A few individuals were kept to establish the maximum longevity.

Behavioural analyses were made after day 3 or 4 once the adult was formed. Backward waves, defecations, and bulb pulsations were all recorded for a 15 min period for each individual every two days. The movements were either recorded directly on a multi-channel event recorder, or filmed on a Shibaden/Sony closed circuit television and analysed on a video tape recorder. During this period of observation, worms foraged, fed or swam freely, usually completing portions of all actions of their behavioural repertoire. If there were no movements, but the worms were considered to be alive, they were included with a zero value in the calculation of means. Because an individual might feed for

eight min then forage for four min, then remain inactive for the remainder, the maximum rates for backward waves and bulb pulsations during the observation period were considered to be more meaningful. Once calculated, further results are expressed as means of the maximum rate. The rate of backward waves in bacterial films and in axenic culture are not comparable because of the very considerable differences in the physical conditions of purchase, viscosity and density (Croll, 1976b).

As individuals died, so the number on which the means were based became smaller. It would not have been reliable to keep the numbers of survivors higher by selecting 'long-livers' because this would have biased the results. The results, therefore, represent the mean rates of survivors up to a point where no further members of the experimental group were alive.

Where behavioural data from bacterial and axenic cultures were compared (Figures 3 & 4), the individuals used were all the progeny of axenic adults. The eggs were laid during the same period, and divided into bacterial and axenic groups. All of the handling and storage of both groups were identical.

Results

Size of worms

C. elegans continued to increase in length throughout its adult life. While there was no significant difference in the overall length of individuals cultured from bacteria or axenically, those cultured on *E. coli* had a significantly greater volume (Table 1). The width of worms at the oesophago-intestinal junction was significantly ($P = 0.02$) greater in worms from bacterial culture.

Fecundity

Reproduction began slightly earlier in axenic culture (day 3) than on bacteria (day 4), but the actual hour was not calculated. Table 2 shows typical fecundity data based on 100 individuals, 50 in each medium. No consistent difference was found between the

Table 1
Growth of *C. elegans* in axenic and in bacterial culture at 20° C[a]

	Axenic culture				*E. coli*			
Day	Length overall	Width at oesoph-intest.	Width at anus	Volume* ($\times 10^5 \mu^3$)	Length overall	Width at oesoph-intest.	Width at anus	Volume ($\times 10^5 \mu^3$)
	μm	μm	μm		μm	μm	μm	
3	792.2	27.0**	20.5	4.18**	914	44 5**	22.8	13.44**
5	1226.5	35.8**	25.3	11.58**	1301.5	51.35**	27.5	28.02**
7	1250.0	39.0**	26.3	14.21**	1345.0	54.9**	30.75	31.39**
14	1454.0	37.8**	26.6	18.96**	1455.0	59.6**	29.20	35.76**

* $\pi r^2 1 + 1 / 3\pi r^2 1$; the first r is derived from width at the base of the oesophagus and 1 is the length from head to anus. The second r is the width at the anus and 1 is the distance from the anus to tail length.

** Pairs of data on the same horizontal line significantly different at the 0.001 level. (Differ at 0.02% level)

[a]Based on measurements of 50 nematodes in each group.

Table 2
The mean number of larvae developing per day per individual from *C. elegans* in axenic culture and bacterial culture[1].

Day	Axenic culture	*E. coli* culture
3	33.5	0
4	35.4	33.4
5	26.6	29.4
6	27.5	23.8
7	22.8	19.4
8	15.9	12.0
9	7.1	6.2
10	0.9	3.2
11	0.0	0.8
12	0.0	0.0
	Total 169.5	128.2

[1]Based on measurements of 40 nematodes from axenic culture and 40 nematodes from bacterial culture.

Table 3
Survival of *C. elegans* in axenic culture and in *E. coli* culture at 20°C, mean from 4 experiments[1].

Axenic culture		*E. coli* culture	
50% survival	Nematodes	50% survival	Nematodes
17.6 days	60	12.3	60

[1]Differ at 0.1% significance level.

total progeny from either culture. Figure 1 is based on those eggs which were laid during the 15 min observation periods of the behavioural records. Fertile eggs made up all of those laid on day 4, but only about one-half of those passed on day 6. During day 6 and even more so on day 8 most fertile eggs contained fully formed larvae. All those eggs observed on days 10 and 12 in axenic culture were infertile.

Longevity

Those individuals in axenic culture lived significantly longer ($P = 0.1$) than those in bacteria (Table 3). Some individuals in axenic culture lived for periods in excess of 40 days.

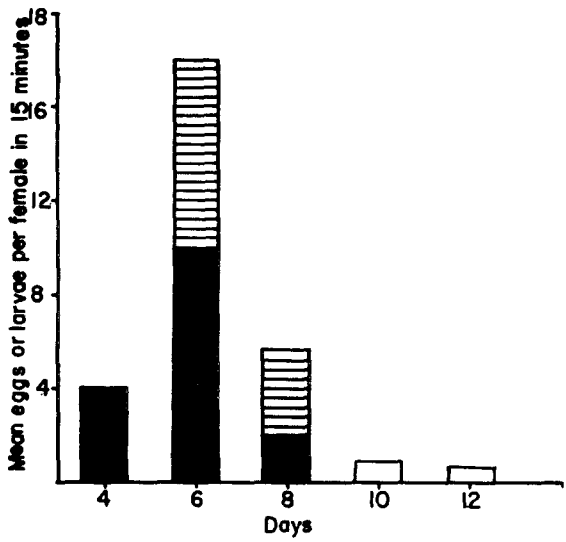

Fig. 1. Mean eggs laid per individual by *C. elegans* during a daily 15 minute observation period. Black: fertile; cross-hatched: larvae in eggs; and open: infertile eggs.

Behavioural analyses

Because of the relative periods of time spent feeding during observations, it may be seen that maximum rates are more meaningful than rates per 15 min period (Figure 2).

The mean rate of backward waves involving all or most of the somatic musculature declined almost linearly between day 4 and 20 for worms in axenic culture (Figure 3). The variance was relatively small in the younger individuals but, as with other behavioural parameters, it increased with age.

The mean maximum rate of pharyngeal bulb pulsations provides the most reliable method of measuring the rate of ingestion (Croll, 1975). It was significantly different in the two cultures only on day 6 and up to day 10 the results were almost identical.

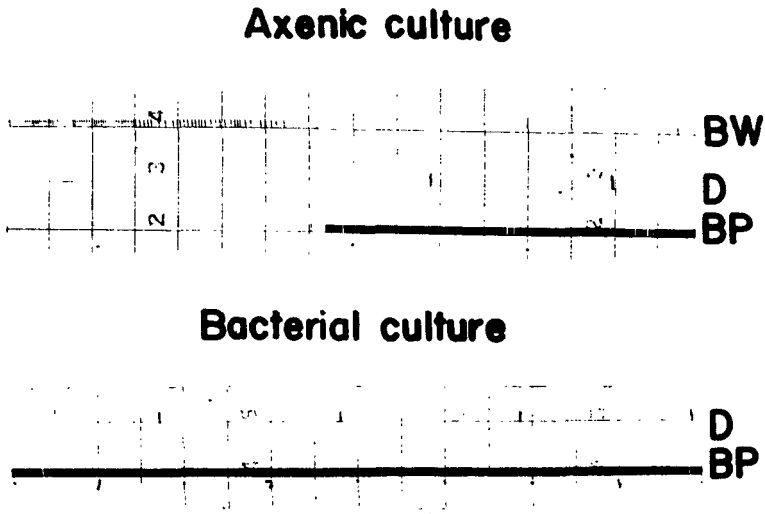

Fig. 2. Recording of the behavioural events of 6-day-old *C. elegans* in bacteria and axenic culture from which numerical date is taken. BW: backward waves; D: defecation; BP: bulb pulsations. Horizontal bars represent 15 sec intervals.

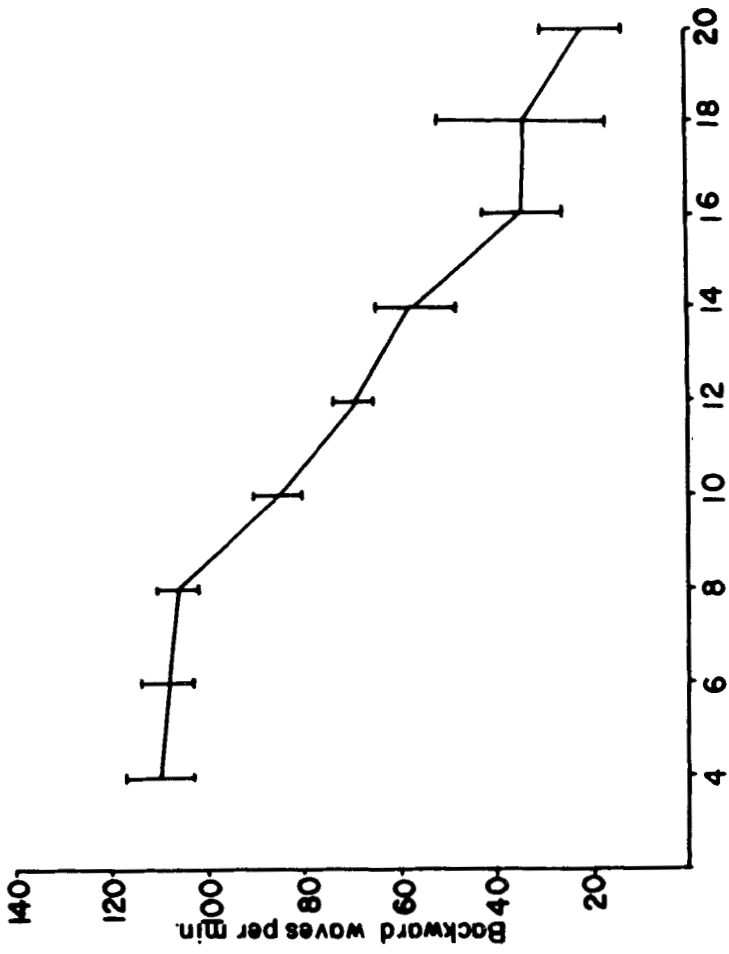

Fig. 3. Mean maximum backward waves per minute of *C. elegans* in axenic culture at 20° during aging. (Vertical bars, ±S.E.).

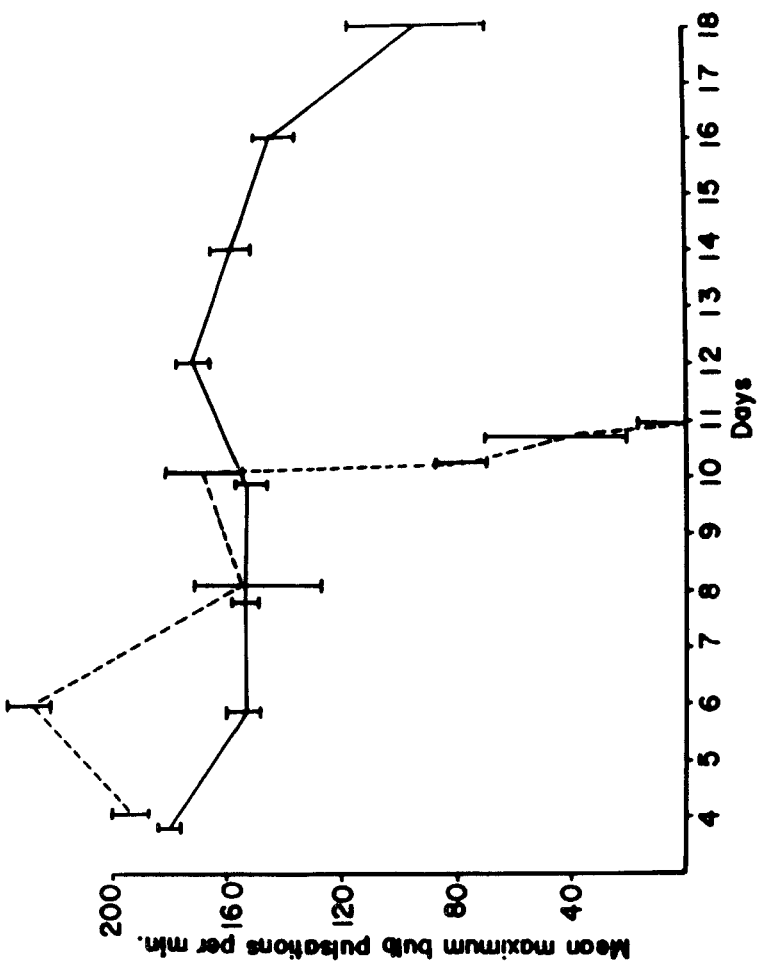

Fig. 4. Mean maximum pharyngeal bulb pulsations per min for *C. elegans* in bacterial (broken) and axenic (continuous) culture. (Vertical bars, ±S.E.).

Because it appeared that individuals on day 11 in bacteria were moribund, they were recorded at 4 hr intervals on this day (Figure 4). A rapid decline in the mean rate of pharyngeal bulb pulsations was seen at the end of which the individuals were dead. Those worms in axenic culture lived and maintained a plateau in the mean rate of pulsations until day 16.

The mean period between defecations is not directly related to the feeding rate (Croll, 1975a), but is believed to be under spontaneous endogenous control and is typical of the feeding phase. From day 4 to day 8 the mean period between defecations (about 70 sec) did not differ significantly ($P = 0.10$) either between days or between bacterial and axenic culture (Figure 5). After day 10 those worms from bacteria were very slow or dead. A new plateau was seen from day 9 to day 18, the new mean interval had almost exactly doubled to about 130 sec (Figure 5). This very big rate change was not related to a significant change in the mean rate of backward waves or to a change in the mean maximum rate of pharyngeal bulb pulsations.

Discussion

The volume attained on bacteria was significantly greater than that for *C. elegans* in axenic culture and the nematodes lived significantly longer in axenic culture than in bacterial culture. A longevity in bacteria of 12.3 days at 20°C was comparable to that reported by Hirsch *et al.* (1976) of 14.5 $\pm$ 2 days at 20°C. We cannot find sufficient reason to support experimentally the findings of Tilby & Moses (1975). Even though their experiments were conducted at 23°C and the medium and aeration conditions were somewhat different, there is no ready explanation for the differences (which Tilby & Moses (1975) point out themselves).

Tilby & Moses (1975) found that growth occurred in the adult stage in axenic culture and they asked if the same might occur in bacteria. We found that it did (Table 1).

This is only the third time that behavioural parameters have been used in aging (Zuckerman, *et al.*, 1971; Croll, 1975a being the

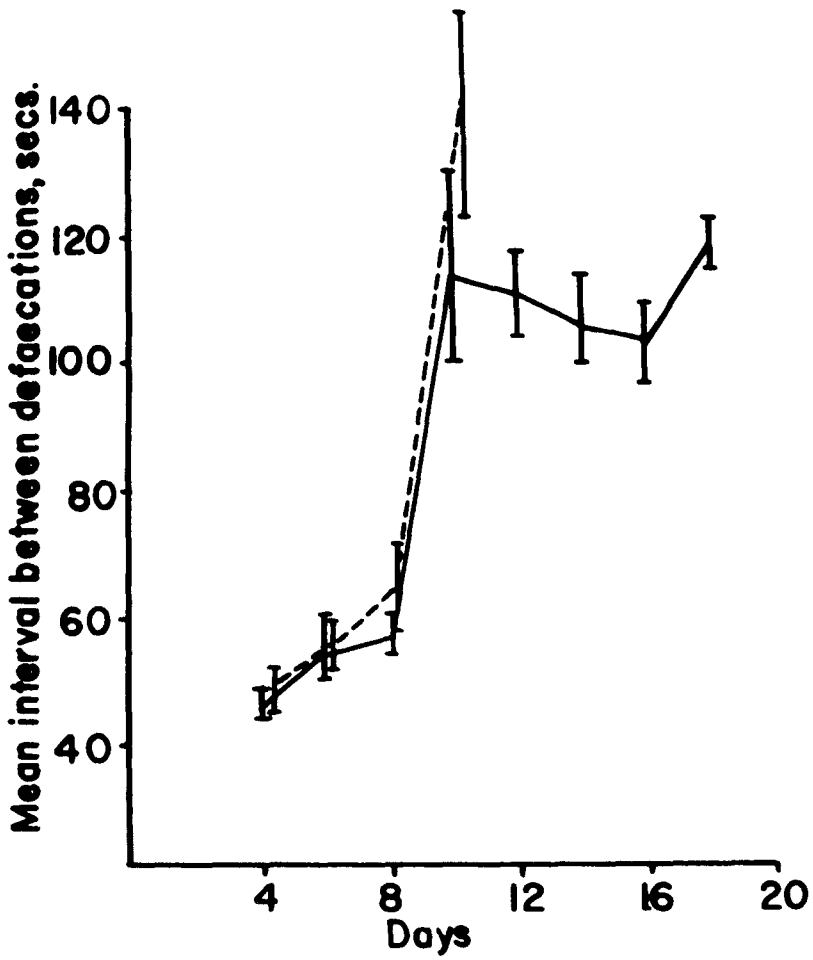

Fig. 5. Mean interval between defecations for *C. elegans* in bacterial (broken) and axenic (continuous) culture. (Vertical bars, ±S.E.).

earlier ones). There is evidence that acetylcholine and acetylcholine esterase is functional in sensory physiology (Pertel, Paran & Mattern, 1976) and in the somatic musculature (Sulston, Dew & Brenner, 1975) of *C. elegans*. More recently the biogenic amines have been implicated in feeding, oviposition and possibly in copulation of *C. elegans* (Croll, 1975a; Sulston, *et al.*, 1975). It is premature to fully interpret the current results in terms of neurotransmitters. Nevertheless, the decline in the rate of somatic contractions was linear from day 4 to 20 (Figure 3) but there was no comparable decline in the mean feeding rate (Figure 4) or the interval between defecations (Figure 5). This may indicate basic differences in the coordinating mechanisms of these activities (Croll, 1976a). It definitely shows that all behavioural activities do not decline during aging at the same rate.

One of the most interesting observations is the rapid doubling of the mean interval between defecations which occurred between days 8 and 10 in both bacteria and axenic culture (Figure 5). The main behavioural correlation of this change is that oviposition ceases at this time. There was not a comparable decrease in pharyngeal bulb pulsations so it may be due to reduced cuticular rigidity or to the greater volume of the intestine as it expands with age or to the absence of a full female reproduction tract.

The data presented in Figures 4 and 5 may give insight into the basis of the longevity differences in axenic and bacterial culture. It is clear that until day 10, there is no real difference between the two cultures. Then within 24-hr, there is a rapid decline which leads to the death of those in bacteria. Aging in bacteria does not, therefore, occur at a constantly more rapid rate, but is due to a sudden decline. This was monitored in both the mean pharyngeal bulb pulsations and the interval between defecations. These changes occurred after the completion of oviposition and so had no effect on population growth. We believe that the nature of death itself may explain this striking difference. *C. elegans* living in bacteria become opaque, possibly indicating that they are rapidly destroyed by their food and bacterial surroundings. Perhaps the bacteria and their cytolytic secretions are destroying the cells of *C. elegans* and thereby killing them. Possibly, Metchnikoff

(as quoted by De Kruif, 1926) recognized a partial truth when he stated:

> "Auto-intoxication, poisoning from the wild, putrefying bacilli in our large intestines — that is surely a cause of the hardening of the arteries, that is what helps us grow old too soon."

Worms that die in culture remain ghost-like for many days after death. This observation gives prominence to the significant role of the environment in determining the longevity of *C. elegans* and suggests that differences, other than genetic and nutritional, account for longevity in axenic and bacterial culture.

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