Chapter 31

Assessing Aging and Senescent Decline in *Caenorhabditis elegans*: Cohort Survival Analysis

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Abstract

The nematode *Caenorhabditis elegans* provides a versatile and expedient platform for the genetic and molecular dissection of mechanisms underlying senescent decline and aging. Indeed, pioneering studies in this organism revealed the first genes and pathways directly influencing lifespan. In this chapter, we present routine, mainstream methods, developed for monitoring aging and senescent decline in *C. elegans*. These procedures allow the assessment of lifespan parameters on solid growth media that are typically used for rearing nematodes.

Key words: Aging, *Caenorhabditis elegans*, Lifespan, Longevity, Nematode, Senescent decline, Stochastic death, Survival analysis

1. Introduction

Aging and senescent decline are global phenomena that affect all living organisms. Senescence at the level of the whole organism can be broadly defined as the declining ability to maintain homeostasis and cope with inevitable spontaneous decay of cells, tissues organs, and biological systems, through time. Organismal senescence, or aging, involves all the obvious changes associated with old age that can be referred to as senescent decline. Although aging is largely driven by random and stochastic degenerative processes, it is extensively influenced by both genetic and environmental signals (1). The field of aging research has made significant progress and gained increasing attention during the last 50 years; however many mysteries still remain unresolved.

The roundworm *Caenorhabditis elegans*, a tiny (~1 mm total length), free-living, soil-dwelling nematode is one of the first and most widely used model organisms for aging research. Under standard laboratory cultivation conditions (20°C), animals complete a

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life cycle (egg to sexually mature adult) in 3.5 days. During this period, worms go through four successive larval stages (termed L1–L4), separated by molting events. After animals reach adulthood, they survive for a conveniently short period of about 2–3 weeks at 20°C. *C. elegans* is easily and inexpensively maintained in the laboratory, both on solid and in liquid media, feeding mainly on bacteria *E. coli* (monoxenic media), at temperatures that range from 15 to 25°C. Because *C. elegans* is a poikilotherm, its life cycle and lifespan are greatly influenced by the environmental temperature (2, 3). The predominant sexual form is the hermaphrodite but males also appear in populations at a low frequency (~0.1%), and can be used in genetic crosses. The reproductive period of a hermaphrodite lasts for the first 5 days of adulthood. One wild-type adult lays approximately 300 fertilized eggs within this period.

The body of *C. elegans* is transparent, permitting the detailed study of its embryonic and post-embryonic development. The complete cell lineage, from zygote to adult has been precisely mapped (4, 5). An adult hermaphrodite comprises 959 somatic cells, all of which are post-mitotic. The *C. elegans* germ line is an immortal lineage that gives rise to diploid germ cells, which in turn undergo meiotic division and differentiation, producing initially sperm and subsequently oocytes. By contrast, the soma of *C. elegans*, which is made up of 959 post-mitotic cells, experiences senescent decline, and gradually develops phenotypes that can be easily recognized as aging. Typical examples of such phenotypes are a decline and then pause of progeny production, progressively slower movement, darker hue due to accumulation of fat and the age pigment lipofuscin, reduced ultradian rhythms (feeding and defecation frequencies), and others. These easily discernible phenotypes, coupled with a relatively short lifespan and the capacity to establish genetically identical populations through clonal self-reproduction make *C. elegans* a particularly useful and convenient organism for the study of the biology of aging. Additional, relevant features, which benefit experimental efforts are the uncomplicated and flexible genetic manipulation (RNAi, introduction of transgenes, gene knock outs), the fully mapped nervous system and the extended arsenal of tools for in vivo imaging of cellular and developmental processes.

In this chapter, we describe a well-established method for acquiring and processing survival data for the nematode. We provide detailed information for cohort analysis of animals grown on solid growth media, including population synchronization techniques, censoring of death events, recording and analysis of survival data, and finally construction of survival curves. Alternative methods of acquiring survival data suitable for implementation in high-throughput and longitudinal studies are described in the accompanying chapter (see Chapter 32).

2. Materials

- Wormpick: Cut 3 cm of platinum wire (90% platinum, 10% iridium wire, 0.010 inches diameter; e.g., Tritech Research, Los Angeles, CA) and flatten one end using pincers or a light hammer. Break off the thin part of a glass Pasteur pipette. Melt the glass at the site of breakage on a Bunsen burner and attach the sharp end of the platinum wire. When using the wormpick always sterilize the tip over a flame.
- Nematode Growth Medium Agar (NGM) plates: For 1 L, add 3 g NaCl, 2.5 g Bacto-Peptone, 0.2 g streptomycin, 17 g agar, double distilled (dd) H_2O up to 975 mL. Add a magnetic stirrer in the medium and autoclave it. Let it cool down (with stirring) to approximately 55°C and then add 25 mL Phosphate Buffer 1 M pH 6.0 (sterile), 1 mL $CaCl_2$ 1 M (sterile), 1 mL $MgSO_4$ 1 M (sterile), 1 mL cholesterol 5 mg/mL (sterile) and 1 mL nystatin 10 mg/mL (sterile). Pour 11.5 mL of NGM agar in each plate.
- Cholesterol stock 5 mg/mL: For 100 mL, add 0.5 g cholesterol in 100 mL of absolute ethanol. Stir to dissolve. Store at 4°C (see Note 1).
- Nystatin suspension 10 mg/mL: For 50 mL, add 0.5 g nystatin, in 70% ethanol in dd H_2O. This is going to be a suspension, so it needs shaking prior to use (see Note 2).
- Streptomycin stock 100 mg/mL: For 10 mL, add 1 g of streptomycin in 10 mL of dd H_2O. Shake until the streptomycin is dissolved. Filer-sterilize the solution and store at 4°C.
- Phosphate buffer 1 M: For 1 L, add 102.2 g KH_2PO_4, 57.06 g K_2HPO_4, adjust the volume to 1 L with dd H_2O and autoclave.
- Luria Bertani (LB) medium and agar plates containing streptomycin: For 1 L, add 10 g Bacto-tryptone, 5 g yeast extract, 10 g NaCl. Adjust pH to 7.0 with NaOH, adjust volume to 1 L with dd H_2O and autoclave. For LB agar plates add 20 g of agar to the previous mixture and a magnetic stirrer prior to autoclaving. After autoclaving let the mixture cool down to 55°C (with stirring when it is LB agar) add 1 mL of streptomycin stock 100 mg/mL and poor into petri dishes (10 mm diameter).
- Petri dishes (60 mm × 15 mm).
- Peristaltic pump.
- Dissecting microscope.
- Incubator for stable temperature.
- *E. coli* strain OP50-1.

3. Methods

3.1. Preparation of NGM Plates with Bacterial Lawn (Seeded Plates)

1. Streak the bacterial *E. coli* strain OP50-1 on LB agar plates containing the antibiotic streptomycin (6).
2. Incubate for 16–18 h at 37°C.
3. Pick a single colony and inoculate 50 mL of LB for ~4–6 h at 37°C.
4. Disperse 100–200 μL of OP50-1 culture on a medium plate and spread it with the tip of a glass pipette or with a glass rod in order to form a circle in the center of the plate surface (see Note 3).
5. Incubate the plates at room temperature overnight to allow the bacterial lawn to grow before transferring worms to the plate.

3.2. Choosing Parental Worms

1. Add five (5) L4 larvae on a freshly seeded NGM plate. Prepare five (5) such plates.
2. Incubate the plates at 20°C (see Note 4).
3. Four days later the plates contain mixed worm population from which we can start the lifespan experiment (Fig. 1, step 1) (see Note 5).

3.3. Synchronizing Populations

There are several ways of synchronizing populations to be used in the assay. The most common are described in the following paragraphs, and summarized in Fig. 1 (step 2: a, b, c).

3.3.1. Egg Preparation from Gravid Adults

1. Starting from a plate with a well-fed, mixed population (prepared in Subheading 3.2) that contains plenty of gravid adult worms, embryos are gathered by dissolving the bodies of embryo-bearing, gravid adult worms in sodium hypochlorite solution (see Note 6).
2. Disperse the egg suspension using a glass pipette in 2–3 seeded plates, and allow eggs to hatch for 3 days in a 20°C incubator.
3. By the end of the third day, randomly pick worms (L4 larvae) from this synchronous population and transfer them to new plates (10–15 worms per plate), to begin the lifespan experiment.

Depending on whether or not post-embryonic development is included in the lifespan assay, either the day of hatching or the first day of adulthood may be set as day 1 of experiment (Fig. 1, step 2a).

3.3.2. Egg-Laying for a Short Period of Time

An alternative way of acquiring synchronized embryos is to allow gravid adults to lay their eggs for a short period of time (a few hours) on freshly seeded plates (Fig. 1, step 2b).

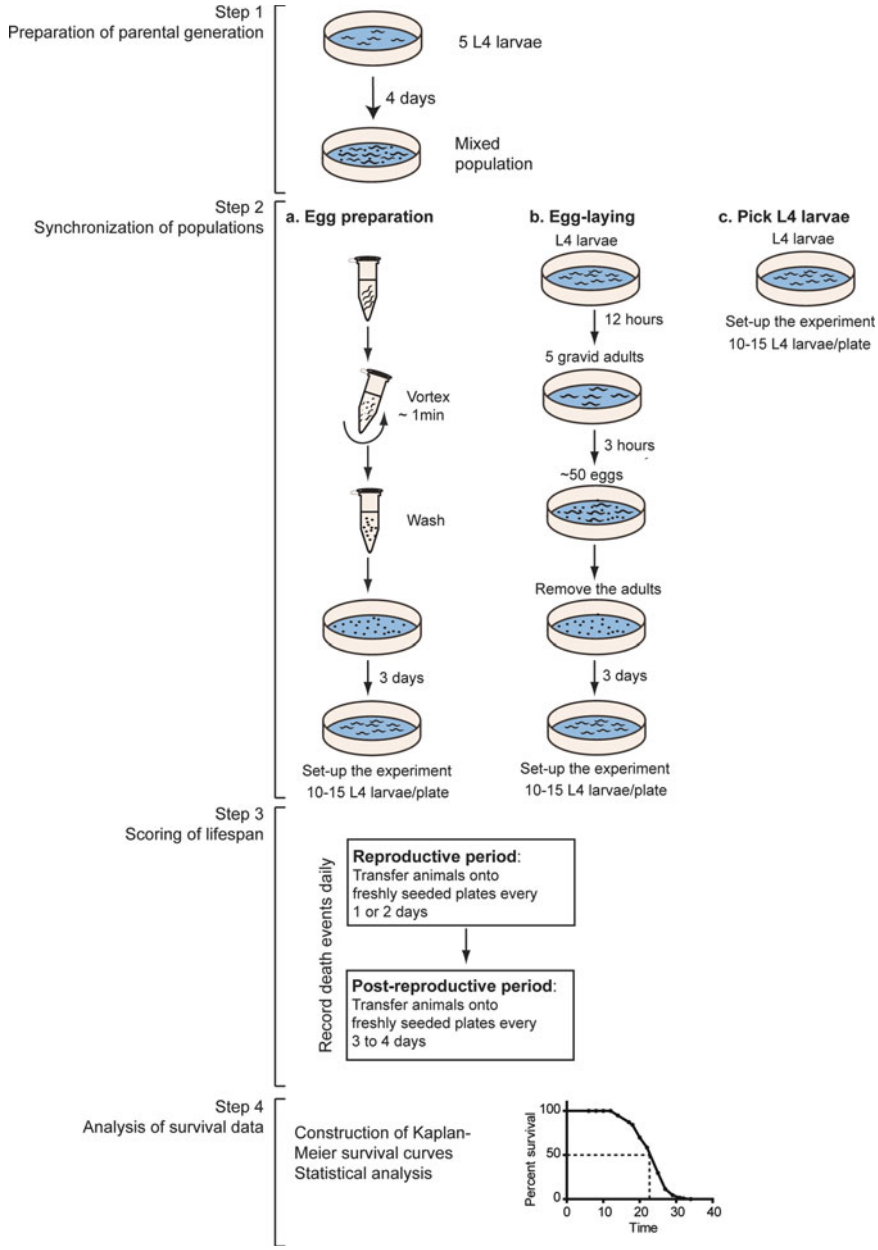


Fig. 1. Lifespan assay on solid media. The schematic shown here summarizes the protocol, including preparation of the parental generation grown under ad libitum conditions, and the subsequent synchronization of experimental populations. Three different procedures are depicted: (**a**) Egg preparation by lysing gravid adult worms, (**b**) egg-laying for a few hours on freshly seeded plates, and (**c**) manual picking of L4 larvae. Worms are transferred to freshly seeded plates every other day during their reproductive period, and every 3–4 days during the post-reproductive period. Death events are recorded daily. The acquired survival data are analyzed using GraphPad Prism software and a Kaplan–Meier survival plot is constructed.

1. One day before egg-laying, select L4 larvae (starting from a mixed population, see Subheading 3.2) and transfer them to freshly seeded NGM plates.
2. Let the larvae mature overnight at 20°C, until they start laying eggs.
3. Transfer five adults per plate and let them lay eggs for 3 h.
4. Remove the adults from the plate and let the eggs hatch, to begin a synchronous population. When working with the standard wild-type strain (N2), this procedure will give approximately 50 eggs/plate (see Note 7). Day 1 of the assay can be set as either the day of hatching, or the first day of adulthood.

3.3.3. Picking L4 Larvae

This type of synchronization is chosen when the duration of the post-embryonic development period is not included in the assay. L4 animals are selected from mixed populations (prepared in Subheading 3.2). Pick with a platinum wire and transfer to separate plates to start the experiment. Day 1 of this experiment will be the first day of adulthood (Fig. 1, step 2c).

3.4. Setting up the Experiment

An adequately sized population for a lifespan assay typically includes about 100 animals (see Note 8). Usually, experiments are started with populations of more than 100 worms, because it is expected that about 20% of animals will be removed from the assay later on as "censored" (see Subheading 3.5.3).

1. Prepare ten freshly seeded plates per worm population/strain (see Note 9).
2. Transfer 10–15 worms per plate.
3. Annotate plates with numbers from 1 to 10, in order to follow worms on each plate separately (see Note 10).
4. Prepare a chart where the number of alive, dead, and censored worms will be noted each day.

3.5. Scoring Lifespan

In summary, this part of the experiment includes the following steps:

1. Counting of worms daily and transferring when necessary (see below) onto fresh pre-annotated plates.
2. Removing dead and censored worms form the plates.
3. By the end of the experiment, when the worm population shrinks, pool noncontaminated worms from different plates on the same plate.

3.5.1. Reproductive Period: Progeny Separation

1. During the egg-laying period (approximately the first week of adulthood), worms should be transferred to fresh plates daily or every other day, to separate them from their progeny. The egg-laying period may differ between different strains, so the daily transfer routine can be extended or shortened accordingly.

During the reproductive period, it is important that the worms are monitored and recorded every day.

2. To maintain population synchrony without having to transfer parental worms every day, strains with temperature-sensitive fertility defects have been used (for example *fer-15(b26)* or *glp-4(bn2)* mutants). Both these mutants are fertile when grown at 15°C but become sterile when grown at 25°C, starting at the L1 stage. The *fer-15(b26)* allele blocks sperm production at high temperature, while the *glp-4(bn2)* mutation prevents efficient germ line proliferation (7, 8). These fertility mutants have been also used in cases where the genetic trait to be monitored causes deleterious phenotypes (i.e., hatching of eggs inside the uterus) (9).
3. Alternatively, 5-fluoro-2-2deoxyuridine (FUdR), an inhibitor of DNA synthesis that blocks egg-hatching may be used. In this case, L4 worms are transferred to FUdR-containing plates (concentration 120 μM) (see Note 11). The drug can cause complete sterility by inhibiting egg-hatching, within 5 h of its application (10). Lower concentrations of FUdR (25 μM) have also been used successfully (11). However, recent data have shown that FUdR in some cases can affect the overall lifespan of the animals, so it must be used with caution (12, 13).

3.5.2. Post-reproductive Period: Scoring of Lifespan

After, animals stop laying eggs, they can be transferred every 3–4 days or even once a week, depending on the availability and the quality of the food source (see Note 12). However, worms should be counted daily, for deaths to be scored on time and for the removal of censored worms from the plate.

3.5.3. Censoring Individual Death Events

Animals are excluded from analysis (censored) when they display obvious defects that interfere with normal aging, or have been otherwise compromised by experimental mishandling or accidents. Examples of such censoring instances can be:

1. Internal hatching of eggs (bag-of-worms phenotype or bagging): Hermaphrodites carrying internally hatched eggs usually do not move. Upon inspection, newly hatched worms can be spotted moving inside the animal. The mother is literally eaten by its progeny, up until larvae escape by penetrating the cuticle. It is important to remove these animals early on, because worms that escape from bagging parents may already be old enough to be mixed with their parental generation on the plate, altering the results of the experiment.
2. Vulva protrusion: The vulva protrusion phenotype should be dealt with caution as it can also be a trait accompanying some mutant strains. In general, it is advisable to remove animals with vulva protrusion that also show additional signs of sickens such as impaired movement or paralysis.

3. Vulva rupture: These worms appear to drag their intestine that has spilled out of their body cavity through the opening of the vulva.
4. Paralysis or uncoordinated movement: These phenotypes, when not attributed to mutant alleles carried by the strain itself, indicate injury or experimental mishandling, which can affect animal lifespan and skew the outcome of the assay.
5. Bacterial or fungal contamination: Contamination of NGM plates with bacteria not indented for feeding or with fungi may have a detrimental impact on animal survival (see Note 13).

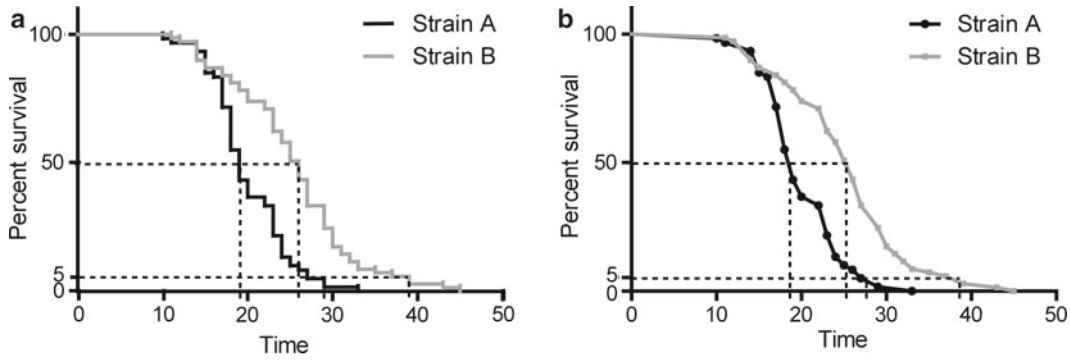
3.5.4. Scoring Death Events

As they grow older worms gradually stop moving and only forage for food by just moving their head. Ultimately they also cease foraging, but they may remain alive for several more days. Worms are scored as dead if they fail to respond to repeated light prodding on the head and tail.

3.6. Data Analysis

The Kaplan–Meier analysis is the most common method used for processing and plotting survival data. The graph generated presents the percentage of animals surviving at each observation point plotted against time. The advantage of the Kaplan–Meier survival analysis is that it takes into account censoring events. The curve usually comprises horizontal steps with progressively lower magnitude (beginning at 100%; Fig. 2). In this case, survival in between different sample observation points is considered steady. Two Kaplan–Meier survival curves corresponding to different populations can be compared by means of several statistical tools like the log-rank test (or Mantel-Cox test), the Gehan-Breslow-Wilcoxon test, and the Cox hazard regression. The key parameters, derived from survival analysis, which are used to interpret and compare survival data are the mean and maximum lifespan values. Mean lifespan is the time interval by which half of the population (50% of the total number of animals, nor including censored individuals) is dead. Maximum lifespan is defined by the time of death of the longest-lived survivor within the population. Maximum lifespan is also alternatively calculated as the mean lifespan of the longest-lived 10% of the population (Fig. 2).

There are several software applications that perform survival data analysis based on the Kaplan–Meier method. Microsoft Excel (Microsoft Corporation, Redmond, WA, USA) is an extensively used application for a wide range of statistical analyses. Specific Excel add-ins can be obtained and installed for easier analysis of survival data (14). Origin is another software package (OriginLab Corporation, Northampton, USA) for scientific graphs and data analysis that is capable of generating Kalpan–Meier plots. GraphPad Prism (GraphPad Software Inc., San Diego, USA) is an additional, useful software application for scientific graphing, including survival curve construction and analysis.



	A	B
Median lifespan:	19	26
Maximum lifespan (10%):	28	39
Absolute Maximum lifespan:	33	45

Fig. 2. Examples of survival curves. The GraphPad Prism software was used for the construction of Kaplan–Meier survival curves for each of the two hypothetical strains A and B. The same set of data is used for the construction of the two different types of curves: the staircase type shown in panel (**a**), where points are connected with steps, and the point-to-point fit in panel (**b**), where the points are connected with *straight lines*. Two *dashed lines* starting from 50 and 5% survival (*Y* axis) intersect the two curves at different points. For each curve, the projections of these points on the *X* axis, indicate the median lifespan of the total population, and the median lifespan of the upper 10% of the longest-lived survivors in the population (maximum lifespan), respectively. The median and maximum lifespan of each strain are shown in panel (**c**). Both the Log-rank test and the Gehan-Breslow-Wilcoxon test estimate the difference between the two survival curves to be statistically significant, with p values < 0.001.

4. Notes

1. Caution: do not flame or autoclave!
2. Caution: do not flame or autoclave! Store at 4°C in a dark bottle or wrap the bottle in aluminum foil since it is photosensitive.
3. Do not spread the bacterial lawn close to the edges of the plate. Worms tend to stay within the bacterial lawn and by extending it near the ends, the probability of worms crawling on the sides of the plate and dying by desiccation increases.
4. It is not uncommon that genetic or environmental manipulations have different effects on lifespan at different temperatures. For this reason a comprehensive analysis should include lifespan assays at different temperatures (within the 15–25°C range of rearing temperatures).
5. Several studies with diverse species indicate that parental age or stress experienced by the parental generation affects progeny physiology and lifespan (3, 15–17). For this reason it is important that synchronous worm populations assayed derive from synchronous parents grown under optimal conditions.

6. Egg preparation from gravid adult worms: gather animals from plates containing mixed worm populations, using 1 mL of sterile water per plate and transfer them to 1.5 mL microcentrifuge tubes. Spin down shortly and resuspend worms in sodium hypochlorite solution (bleach) (0.5 N NaOH, 20% bleach). Vortex for about 1 min, until animal bodies are completely dissolved (check the tube under a dissecting microscope to monitor when lysis is complete). Eggs, protected by the egg shell, are resistant to this treatment. When only eggs are visible in solution, spin down and wash extensively the egg preparation (2–3 times) with sterile water. Washing off the bleaching solution is important for the survival of embryos.
7. If more embryos are needed, the number of plates, the number of gravid adults per plate, or the duration of the egg-laying can be increased accordingly.
8. Largest populations provide more statistically reliable survival data.
9. Given that the quality of bacterial food and the composition/condition of the solid medium, which are important components of the microenvironment that can cumulatively affect survival lifespan of a population, it is important to keep these parameters steady throughout the study. Plates should be no more than 2 weeks old and always freshly seeded. Moreover, it is important to use the same batch of seeded NGM plates for all different worm populations within one experiment, to ensure that all populations age within comparable microenvironments and survival data are directly comparable.
10. Annotation of the plates confers better control over the course of an experiment. For example, animals on specific plates can be monitored separately from the rest of the population allowing to animals in contaminated plates to be discarded without adversely affecting the outcome of the experiment.
11. Preparation of FUdR-containing plates: make a stock solution of FUdR at a concentration 40 mM in dd H_2O (by adding 984 mg in 100 mL dd H_2O). Filter-sterilize the solution, aliquot and freeze at –20°C. Add 3 mL of the stock solution in 1 L NGM agar after it cools down to 55°C, just before pouring plates.
12. If drugs are used in the food source or if RNAi by feeding is performed, the stability of the compounds examined or the ability of the HT115 *E. coli* strain to induce double-stranded RNA after several days should be taken into account, to determine the frequency of transferring. Typically, for feeding RNAi experiments, dsRNA levels and consequently target gene knockdown is not considered efficient after 4 days on the same plate. Therefore transferring frequency should be adjusted accordingly.

13. Contamination problems are already mitigated by the use of the *E. coli* strain OP50-1, a uracil auxotroph resistant to streptomycin, and also by the addition of streptomycin and nystatin in NGM agar plates. However, contaminations may still appear during the course of an experiment, originating from the NGM agar plates, the feeding bacterial cultures used or airborne contaminants. Hence, sterilization practices should be meticulously implemented and all necessary safety measures taken when performing animal transfers and population observations. In cases of extensive contamination, affected plates should be removed from the experiment. Transferring worms from contaminated plates would risk spreading the contamination to the rest of the population. If contamination is less extensive, transferring animals more often or passing them through a clean intermediate plate for about an hour prior to moving them to their final destination may rid animals of contaminants. However, if contamination persists for several days, contaminated animals should be removed from the experiment.

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