# Dauer Larvae of *Caenorhabditis briggsae* in Axenic Culture[1]

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*Abstract:* The free-living hermaphroditic nematode, *Caenorhabditis briggsae*, enters a dauer stage under certain conditions in axenic culture. Dauer larvae differ from directly-developing third-stage larvae in internal structure, size at time of second molt, morphology of second and third cuticles, separation zone of cuticular caps, and survival at 4 C and 37 C, temperatures fatal to other stages. Males, which occur rarely in liquid medium, may mature under conditions which cause most of the hermaphrodites to go into the dauer stage, resulting in a culture with increased male-to-hermaphrodite ratio.

*Caenorhabditis briggsae* (Dougherty and Nigon) Dougherty, a free-living, protandrous, hermaphroditic nematode, has been maintained in continuous axenic culture since 1954 (2). Its response to nutrition has been studied extensively by observing development of newly hatched larvae during their growth to maturity (4, 8). We now report the occurrence of dauer larvae in axenic culture, compare their molting with the molting of directly developing third-stage larvae and discuss the high ratio of males to hermaphrodites in certain cultures containing large numbers of dauer larvae.

## MATERIAL AND METHODS

Nematodes for this study were obtained from our axenic stock cultures.

MEDIA: A. Chemically defined medium (1) (*C. briggsae* Maintenance Medium, Grand Island Biological Company). B. Medium A supplemented with a proteinaceous growth factor (12). C. A mixture of equal parts of heated liver extract (13) and a liver protein fraction (2). D. Heated liver extract with soy peptone and yeast extract (5). E. Nutrient agar slants supplemented with small pieces of sterile rabbit kidney (3). F. Nutrient agar moistened with medium D.

Selected molts or a sequence of molts were observed in hanging drop cultures. Eight drops of medium of approximately one lambda each were placed on a sterile cover glass and a larva transferred into each drop. The cover glass was edged with vaseline and inverted upon a depression slide. Manipulations were carried out under a dissecting microscope within a sterile enclosure. Larvae and cast cuticles were observed in drops under the high dry objective, but for oil immersion and phase contrast microscopy they were transferred from the drops to flat slides.

Stock cultures are routinely maintained at 20 C. To test survival at higher temperatures, 27 dauer larvae selected from kidney slant culture were transferred to each of two 10 mm tubes, each containing 0.25 ml of medium B and incubated 18 hr at 37 C, then returned to 20 C. Two similar tubes each containing 29 worms including an assortment of sizes from first-stage larvae to adults and excluding dauer larvae were exposed to the same treatment. To test survival of the larvae on agar at various temperatures, loop transfers containing worms of all sizes were made from the kidney slant culture to nutrient agar slants moistened with medium D and incubated at 20 and 37 C. After 18 hrs the 37 C culture was removed to 20 C.

Dauer larvae were held at 20 C and 2 C in medium A and in 0.067 M potassium

Received for publication 24 November 1968.
1 This investigation was supported by PHS Research Grant AI-07359 from National Institute of Allergy and Infectious Disease.
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phosphate buffer, pH 7, for 2 months and in 2% glucose solution for one month at 2 C.

## OBSERVATIONS

OCCURRENCE OF DAUER LARVAE: Dauer larvae were first observed in medium C in hanging drop cultures, each initiated with a newly hatched larva. After 3 weeks the population averaged 70 per drop, of which 40% were dauer larvae.

We then searched for dauer larvae previously unnoticed in our axenic cultures. Terminal populations in tube cultures of medium B contained 25% dauer larvae; none were found in medium A; low numbers were found in media C, D, and F, and large numbers in drops of medium D submerged under oil. A culture on medium E contained many dauer larvae. This culture has been serially transferred for 2 years and provides an abundant supply of dauer larvae.

An attempt was made to induce formation of dauer larvae by inoculating groups of newly-hatched larvae into hanging drops of medium C. With an inoculum of 15, no dauer larvae developed, but some of the progeny became dauer larvae. However, when 70 were used, 12 became dauer larvae. Medium C obtained from a drop containing many dauer larvae did not induce formation of the dauer stage of three new first stage larvae. They matured and reproduced; none of the progeny became dauer larvae, although they grew to the fourth stage.

STORAGE AND SURVIVAL OF DAUER LARVAE: After storage for two months at 20 C in medium A, dauer larvae transferred to media B, C, and D exsheathed, matured and reproduced. A few survived storage in phosphate buffer for 2 months, but did not develop when placed in nutrient media. Larvae stored at 2 C in medium A for 2 months, and in phosphate buffer or 2% glucose for one month, were killed by these treatments.

Only dauer larvae survived exposure to 37 C. After exposure for 18 hr in medium B or F they appeared lifeless, but after removal to 20 C or transfer to medium D at 20 C they matured and reproduced.

MORPHOLOGY AND DEVELOPMENT OF DAUER LARVAE: Dauer larvae are initially enclosed in an intact sheath. Occasionally a space was apparent at each end, but usually the sheath was visible only as small thickened areas marking the closed buccal and anal openings, and at the barely perceptible point of the tail. The larvae are straight or slightly curved, narrower and more tapering than non-ensheathed larvae. The digestive tract is slender, with lumen not visible; the pharyngeal bulb is indistinct and non-motile and the mouth is closed. The sheath (molt 2) may be lost. The length ranged 420 $\mu$—600 $\mu$, averaging 510 $\mu$; length of the gonad averaged 62 $\mu$.

Occasionally dauer larvae molted and matured in the original cultures; maturation of 95% of the larvae occurred after transfer to fresh medium. The rate of development for individuals in hanging drops depended upon both the kind of medium of the drop and the culture conditions from which the dauer larva had been transferred. Development was most rapid in medium D. Dauer larvae from media C and E exsheathed within 24 hr and the third molt followed the next day. Dauer larvae from other media developed more slowly; some molted only after 22 days. Exsheathment and third molt were separate in 13 individuals; in 16 others exsheathment and the third molt occurred together, and in 20 larvae the sheaths had already been lost. One larva shed the posterior portions of both cuticles but failed to free itself from the caps. Two remained alive in the drops and after 6 weeks had not

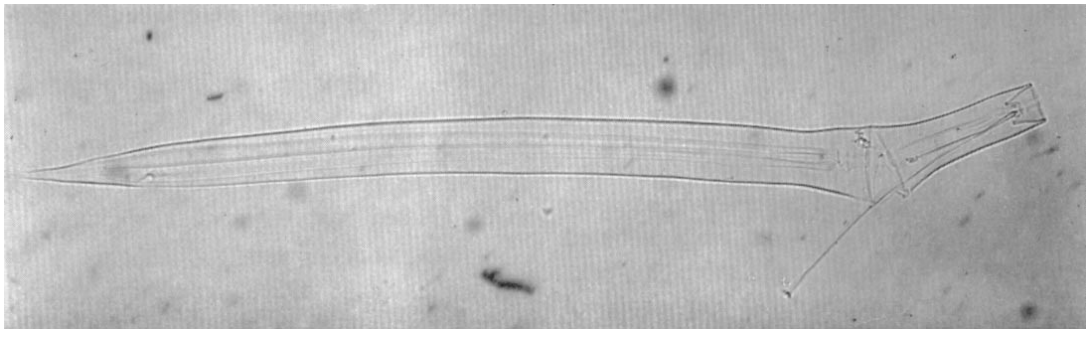

FIG. 1. Photograph of molted cuticle of *Caenorhabditis briggsae* dauer larva (third molt). Length 500 $\mu$. This cuticle shows the partially everted buccal area, the intact oesophageal lining with valvular plates, and the anal lining.

shed either cuticle. Two shed the sheath but did not enter the third molt.

The molted sheath (second molt) was a very thin, usually crumpled, cuticle consisting of a cap with compacted oesophageal lining forming a distinct dot at the anterior end, and a posterior portion with sharply pointed tail. With phase contrast and oil immersion, cuticular annulations, and linings of phasmids, amphids, and excretory duct were discernible. When the sheath was shed with the third molt cuticle, it was seen as a fine, somewhat wrinkled, covering membrane; and particularly visible when, rarely, the break of the sheath did not coincide with that of the third cuticle.

The molted cuticle of the exsheathed dauer larva (third molt) was broken by a partial or complete circular split (Fig. 1). The cap was often partially invaginated during disengagement by the pull of the oesophageal lining, or completely everted with the entire oesophageal lining, including the cuticle of the valvular plates of the terminal bulb, remaining attached. Usually, however, the oesophageal lining was separated in the vicinity of the break in the external cuticle. Whether the portion remaining inside the oesophagus was swallowed, or lost after being shed was not verified. The longitudinal folds of the lateral fields were visible in both portions of the cuticle, except in the zone of separation of the cap. The cap was completely separated from the posterior portion, or both parts somewhat widened and telescoped near the break, making the total length of the cast cuticle less than that of the pre-molted larva. Measurements made in hanging drops are shown in Table 1. The intact buccal-oesophageal lining was found twice, measuring 120 $\mu$ in each case; the buccal portion was 13 $\mu$, and the width of the lining of the valvular plates was 7 $\mu$ (Fig. 1).

The lining of the distal portion of the excretory duct was exactly at the separation zone, sometimes on the cap and sometimes on the posterior section. It appeared as a small, crooked tubule connected to the excretory pore. Linings of the amphids,

TABLE 1. Molted cuticles of *C. briggsae* measured in hanging drop cultures.

| | Posterior Cuticles | | Caps | | |
|---|---|---|---|---|---|
| Molt No. | Number | Av. Length $\mu$ | Number | Av. Length $\mu$ | Av. Width at Break $\mu$ |
| Direct | | | | | |
| 1 | 37 | 264 | 23 | 13 | 11 |
| 2 | 11 | 343 | 4 | 14 | 17 |
| 3 | 11 | 450 | 4 | 20 | 23 |
| 4 | 27 | 620 | 3 | 6 | 18 |
| Dauer | | | | | |
| 3 | 18 | 380 | 20 | 70 | 20 |

phasmids and rectum were visible. Annulations were distinct, gradually increasing in width posteriorly from 1.0 $\mu$ to 1.5 $\mu$. A pair of structures, each consisting of a 2 $\mu$-diameter circular plaque in the focal plane of the annulations, was noted dorsal of and adjacent to the lateral fields, approximately 225 $\mu$ posterior to the cuticular break. From the plaque a flask-shaped structure extended inward, ending in what appeared to be a short duct and pore.

The fourth-stage larva, emerged from the dauer stage, was indistinguishable in appearance and later development from the fourth stage in direct development.

MOLTING IN DIRECT DEVELOPMENT: Study of the molting sequence during direct (non-dauer) development provided details not previously reported. In the first three molts the anterior portion of the cuticle separated by a circular split as reported (8). This occurred at a level near the posterior end of the buccal cavity and the resulting cap was shed along with all or part of the buccal and oesophageal lining.

The fourth molt was more prolonged. The larva stopped swimming and made a succession of attempts to shed the loosened cuticle by alternately sucking in and pushing out the oesophageal lining. Finally the circular split occurred, very close to the anterior end, and the cap and oesophageal lining were either swallowed or ejected. One worm was observed to suck in the cuticle, then forcefully eject it from the mouth. It remained as a crumpled mass adhering to the anterior edge of the posterior cuticle. Several such cuticles were found, but the usual condition was a smooth anterior edge with no cap present in the drop. It is assumed the cap is frequently swallowed with the oesophageal lining as described in *Diploscapter coronata* (6). Since the circular break is so far forward, the open end of the posterior cuticle is narrow and often appeared as a constriction passing posteriorly as the cuticle slipped off. The posterior cuticle sometimes split longitudinally and was more easily discarded.

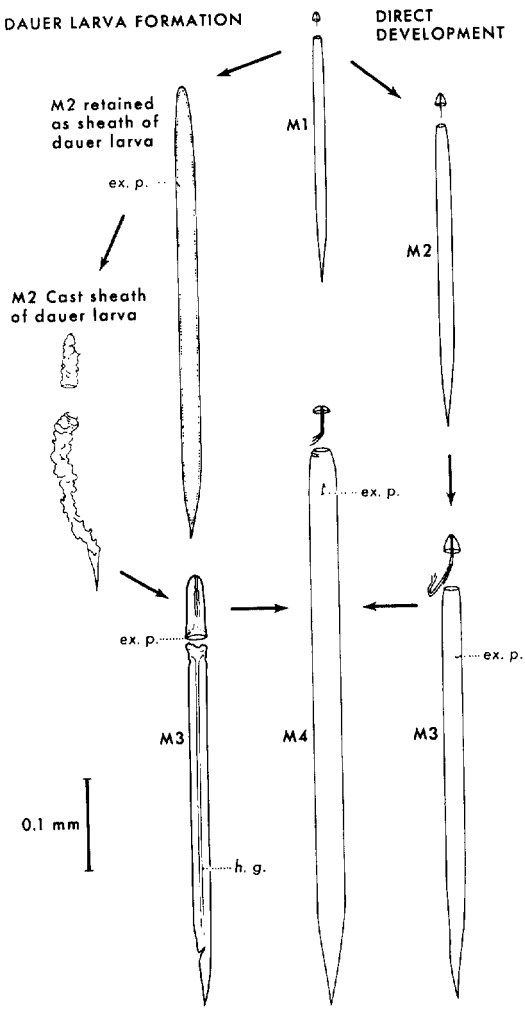


FIG. 2. Diagram of succession of cuticles cast in the molts of *C. briggsae*, developing either directly or through the dauer stage. During the first molt (M 1), L 1 becomes L 2; the cuticle of M 2 becomes the sheath of the dauer larva, or in direct development is cast by the emerging L 3. Cuticles are distinctive at M 3 in the alternative developments but appear similar when 4th stage larvae become adults at M 4.

The linings of the excretory duct, rectum, phasmids and amphids were observed on the cuticles of each of the four molts; the vulva is closed until after the last molt [see (8)]. Sizes of molted cuticles are given in Table 1. Comparing molting in the alternative types of development, both the sheath (M2) and the cuticle of the dauer larva were about the same total length as the cast cuticle of the third stage larva in direct development, whereas the directly developing second-stage larva shed a shorter M 2 cuticle (Fig. 2). In the molts of the dauer larva, the sheath usually separated at the same point as the dauer larval cuticle, at the level of the excretory pore. In both the second and third molts of direct development the break occurred at the level of the buccal-oesophageal junction, far anterior to the excretory pore. The cast sheath and cuticle of the dauer larvae differed in thickness from cuticles shed during the direct molting sequence. The sheath cuticle was much thinner and the dauer larval cuticle was thicker.

OCCURRENCE OF MALES: Males occurred in our axenic cultures of *C. briggsae* in liquid media at the rate of 1–2%. In contrast to this, one culture on a slant of medium E containing a large number of dauer larvae showed 75% males among mature individuals. The few adult hermaphrodites usually had a mucous protrusion at the vulva similar to that seen in copulated females of some dioecious rhabditids. This culture had been initiated with 3 young hermaphrodites from medium C. At 4 weeks, dauer larvae were noticed and at 6 weeks there was a high number of males. In a count of 137 worms there were 66% dauer larvae, 18% smaller larvae, 12% adult males and 4% adult hermaphrodites. In several successive subcultures made at monthly intervals more than half of the adults were males. From dauer larvae taken from the original culture on medium E, 30 adults developed of which only four were males. Thus of the larvae undergoing direct development on the slants, a large proportion were males, whereas those that went into the dauer stage were largely hermaphrodites.

Four mature hermaphrodites with fertilization plugs, isolated from slants to hanging drops, produced 61 offspring. These offspring, isolated individually to hanging drops, developed into 22 males, 24 hermaphrodites and two non-reproducing adults of the hermaphrodite form; 13 failed to develop. Thus, in culture where males were numerous, most hermaphrodites were fertilized by males, giving rise to progeny in which the numbers of males to hermaphrodites was approximately equal.

## DISCUSSION

Formation of dauer larvae in free-living rhabditids was attributed by Maupas (9) to scarcity of food, but this does not seem to be the explanation in *C. briggsae*. Dauer larvae do not appear in slowly growing cultures in deficient media but develop when there is rapid growth of a large population in a favorable medium; i.e., dauer larval formation is a response to crowding in a good nutritional environment. Thus it would appear that different kinds of stimuli induce dauer formation: in Maupas' experiments, removal from a thriving culture to water; on our agar slants and in the drop cultures, perhaps accumulation of metabolic products. However, it is possible that in Maupas' experiments the larvae in the thriving culture were already incipient dauer larvae.

The sizes of the cast cuticles of successive larval stages shown in Fig. 2 suggest that dauer larval formation is determined early in larval development. The course

of development proceeds either directly into the second molt, or the larva continues to grow without molting until it reaches the size of the third-stage larva, the molted cuticle being then retained as a sheath around the resulting dauer-stage larva. Exsheathment, or the shedding of this second cuticle, occurs readily. However, shedding of the cuticle of the dauer larva (3rd molt) does not take place without some stimulus such as transfer to fresh medium. Rogers (10) has suggested chemo-receptive structures for detecting suitable conditions for exsheathing. That the dauer larvae of *C. briggsae* do not resume growth until the third molt occurs suggests that the postulated chemo-receptors may be more useful for the third molt than for exsheathment. The pair of structures found dorsal of the lateral fields of the dauer larval cuticle may be evidence of such receptors. They resemble hypodermal glands described in the Aphasmidia (7) (14), and were recently shown in *Xiphinema index* to be nerve-connected receptors containing modified cilia (11).

Observation of the four sequential molts in individuals in direct development showed that the shortest cap is that of the fourth molt rather than that of the first as had been assumed (8).

The numbers of males and hermaphrodites are approximately equal in offspring of fertilized hermaphrodites. The preponderance of males among the adults in the slant culture giving rise to dauer larvae indicates that males are able to mature under conditions which cause most of the potential hermaphrodites to go into the dauer stage. The high proportion of males among the adults on the slants can be explained by the fact that the few maturing hermaphrodites were fertilized by males, thus continuing the high male ratio. In liquid media occurrence of a male does not lead to an increase in the number of males, indicating that copulation does not occur.

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