Survivorship of *Caenorhabditis japonica* dauer larvae naturally associated with the shield bug, *Parastrachia japonensis*

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Caenorhabditis japonica forms an intimate association with the shield bug *Parastrachia japonensis*. Quiescent dauer larvae (DL) are always found on female shield bugs aggregating on leaves in reproductive diapause throughout the year until the next reproductive period in June, which suggests long-term survival of DL on the shield bugs without propagation. To understand the morphological significance of DL on the shield bug, cryo-scanning electron microscopy (Cryo-SEM) was performed. Cryo-SEM observation revealed that the DL appeared to be partly desiccated, but their lateral alae were not shrunk as seen in the anhydrobiotic nematode *Aphelenchoides besseyi*. To test DL survivorship on the shield bug, we collected bugs from the field and kept them under several experimental conditions for 3 months. When bugs were kept at 100% relative humidity (RH), very few nematodes were detected. When bugs were exposed to 97% RH using a K₂SO₄-saturated solution, a small number of nematodes (19 DL/bug) was detected and DL survival was low (33%). At 90% RH in a wooden box, more nematodes (67 DL/bug) were detected and survival was high (54%). These data suggest that DL are able to survive several months on shield bugs by entering a partially desiccated quiescent form. Nematol. Res. 40 (2), 47-52 (2010)

Key words: Cryo-SEM, desiccation, entomophilic, phoresis, survival

INTRODUCTION

Phoresy is a common phenomenon in nematodes. Because of their small size and inability to migrate long distances, many nematodes have formed phoretic relationships with larger, more motile organisms that share their habitat or food source (Timper and Davies, 2004). *Caenorhabditis japonica* Kiontke, Hironaka, and Sudhaus, a diocious bacterial-feeding nematode found on the shield bug *Parastrachia japonensis* Scott (Kiontke *et al.*, 2002), is one of these phoretic nematodes. The life history of *C. japonica* is currently under investigation, but that of the carrier insect with its interesting behaviors such as egg-guarding and provisioning has been well studied (*e.g.*, Gyotoku and Tachikawa, 1980; Tachikawa and Schaefer, 1985; Tsukamoto and Tojo, 1992; Filippi-Tsukamoto *et al.*, 1995). *Parastrachia japonensis* is a univoltine and monophagous insect that spends most of the year aggregating on green leaves without feeding in reproductive diapause, enters reproduction the next spring (around May), and forms new adults in summer (late July). Since the only food of shield bug nymphs is the drupes of the deciduous tree *Schoepfia jasminodora* Sieb. et Zucc., which are available only in early summer (late June to July), the shield bug

seems to have synchronized its reproduction period to that of its host tree. To establish the intimate phoretic relationship, *C. japonica* also seems to have adapted its life cycle to its carrier bug.

A key preadaptation for phoresy in the phylum Nematoda is the formation of dauer larvae (DL) (Sudhaus, 1976; cited in Kiontke, 1996). DL is the stage for dispersal and survival under unfavorable conditions (Riddle, 1988). They do not feed but can survive by using stored nutrients and by reducing their metabolic activity (Burnell *et al.*, 2005). In *C. elegans* Maupas, a closely related species of *C. japonica*, DL survived for 3 months in an old culture (Klass and Hirsh, 1976). DL of *C. japonica* are mainly found on the body surface of adult female bugs throughout the year except during the reproductive period. Because quiescent DL are always found on female bugs, it is suspected that they survive on the bugs without propagation and wait for the bug's next reproductive period for nearly a year, but little is known about the survivorship of *C. japonica* DL on the shield bugs and there has been no morphological and physiological information on phoretic DL on insects, so far. Nematodes in anhydrobiosis, a quiescent form caused by the desiccation, are reported to show changes of surface structures such as shrinkages and deeply indented annulations (Wharton and Marshall, 2002; Otshubo *et al.*, 2006). For such morphological observation, scanning electron microscopy (SEM) is often used, but chemical fixation during the sample processing for SEM may affect the body

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structures. To observe intact body structures of DL, SEM equipped with a cryotransfer system (Cryo-SEM) that does not need any chemical fixation is a powerful tool. In this paper, we observed the body surface structure of DL using Cryo-SEM, and we tested its survival in several experimental conditions to understand the physiological conditions and survivorship of DL on the bug.

MATERIALS AND METHODS

Insects:

The shield bug *P. japonensis* was collected from Hinokuma Mountain Prefectural Park, a natural secondary forest in Saga, Japan in October 2007 and 2008. The reproductive diapause of these bugs, which emerged in August, becomes steady and constant numbers of DL are found on them by October. To avoid population decline, we tried to minimize the number of bugs used in the experiments.

Scanning electron microscopy:

For the investigation of the body surface structures of *C. japonica* DL, a scanning electron microscope equipped with a cryotransfer system (Cryo-SEM) was used to evade the effect of chemical fixation. DL on the backs of the bugs or nictating DL on the point of a pipette tip on dog food agar medium (Tanaka *et al.*, 2010) were picked up using a worm picker (a 1-cm piece of platinum wire mounted on the tip of a Pasteur pipette). Nematodes were mounted on aluminum stubs using a piece of carbon double-sided tape (Nisshin EM Co., Ltd., Tokyo, Japan), plunged into liquid nitrogen, and set into an Alto 1000 cryotransfer system (Gatan, UK). After the surface water evaporated, the nematodes were coated with gold using a sputter coater in the cryotransfer system for 45 sec at 20 mV. Nematodes were observed at 15 kV using a Hitachi S4300 scanning electron microscope (Hitachi Ltd, Tokyo, Japan). For comparison, mixed stages of *Aphelenchoides besseyi* Christie harvested from nematode culture on *Rizoctonia solani* Kühn AG-4 on a 1/2 Perlcore potato dextrose agar (Eiken, Japan) plate were directly desiccated on a piece of No.1 filter paper (Advantec, Japan) and directly used for conventional SEM observation. Because we were able to observe the surface of the dried *A. besseyi* even without any fixation and sputtering, conventional SEM observation was performed.

Survival of DL on bug (Experiment 1):

Sixty adult bugs (30 females and 30 males) were put in a cage composed of a pair of wire baskets (10 cm dia. at the bottom, 14 cm dia. at the top, 5 cm height) and the cage was placed in a glass vessel (10 l) containing approximately 500 ml of water or K₂SO₄-saturated solution at the bottom to

keep a constant 100% or 97% RH at 25°C, respectively (Fig. 1A). The cage was set above the solution to avoid direct contact with it. The glass vessel with water or K₂SO₄-saturated water was kept for 7 days at 25°C before the start of the experiment to reach proper humidity (Winston and Bates, 1960). Since the bugs were in reproductive diapause and did not feed, only water was supplied in a small glass bottle (5 mm dia., 3 cm height) containing wet cotton with the bottle opening covered with a sheet of Parafilm in the wire cage to minimize humidity disturbances. The bottle was changed every 7 days. Dead bugs were removed as soon as possible to avoid propagation of nematodes on the cadavers and embarking of nematodes during the experiments. Three months later, the bugs were dissected and each individual was floated on 5 ml water in a Syracuse watch-glass for 12 hr. Nematodes from the bugs were harvested and the numbers of dead and live nematodes were counted.

Survival of DL on bug (Experiment 2):

A plastic cage (22 × 15 × 15 cm) containing 30 adult females and 30 adult males was kept in a box (43 × 67 × 47 cm) made of wooden boards at 20°C with a 12-hr light (300 lux)/dark photoperiod. A plastic box (16.5 × 23 × 9 cm) containing 300 ml of tap water was put in the wooden box to keep the humidity high (Figs. 1B, 1C). The humidity in the box was checked by a thermo-hygrometer (TRH-CA SHINYEI Co., Ltd., Japan).

Statistical analysis:

The relationship between the numbers of nematodes on the bugs and nematode survival was analyzed by coefficient of correlation (Kaleida Graph 4.0J, Synergy Software, USA).

RESULTS

SEM observation of DL:

Active DL collected from the dog food agar medium were fully hydrated (Figs. 2B, 2E). On the other hand, the body surfaces of the quiescent DL freshly picked up from the back of the bugs were partially depressed in some areas but the surfaces looked relatively smooth (Figs. 2A, 2D). The lateral alae of DL on the bug appeared not to be shrunken, looked similar to that of hydrated DL, but differed from that of *A. besseyi*, whose lateral alae were shrunken by desiccation (Figs. 2C, 2F).

Survival of DL on bug (Experiment 1):

Soon after the bugs were collected from the field, 10 bugs were sacrificed and the numbers of DL on each bug

Fig. 1. Glass vessel and wooden box for controlling humidity. A, vessel containing about 500 ml distilled water or K₂SO₄-saturated water (Experiment 1). B, wooden box used in Experiment 2. C, top view of inside the wooden box.

Fig. 2. Cryo-SEM observation of *Caenorhabditis japonica* dauer larvae (DL) and anhydrobiotic *Aphelenchoides besseyi*. A, clump of quiescent DL picked up from the back of a bug. B, active DL collected from culture plates. C, dried *A. besseyi*. D, E, and F are the enlargement of a part of quiescent *C. japonica* DL (Fig. 2A), active DL, and dried *A. besseyi* (Fig. 2C), respectively. Arrowhead indicates lateral alae.

Fig. 3. Relationship between the numbers of nematodes on the bugs and their survival rates. Bugs with nematodes were kept in a 97% RH condition (Experiment 1) (A) or in a wooden box to maintain 90% RH (Experiment 2) (B).

Table 1. Effect of humidity on survival of bug, number of DL and survival of DL

RH (%)	% Survival of bug	Number of DL/bug Average (min - max)	% Survival of DL Average (min - max)
100 ¹	100	3 (0 - 11)	33 (0 - 100)
97 ²	53	19 (5 - 37)	33 (12 - 52)
90 ³	100	67 (16 - 306)	55 (21 - 86)

¹ Humidity was kept by the glass vessel with water.

² Humidity was kept by the glass vessel with K₂SO₄ solution.

³ Humidity was kept by the wood box with water.

were counted before the start of the experiments. About 218 DL/bug (146–277 DL/bug) were detected, all of which were alive. Using the same bug population, DL survivability on the bugs in the experimental conditions was examined. When bugs were exposed to the 100% RH condition for 3 months, all of the bugs survived but very few DL were detected on them (average of 3 DL/bug; range, 0–11 DL/bug) (Table 1). The survival of bugs exposed to the 97% RH condition in which humidity was controlled by the saturated K₂SO₄ solution for 3 months was 53%. An average of 19 DL/bug (range, 5–37 DL/bug) was detected and the rate of DL survival was 33% (range, 12%–52%). There was no correlation between nematode numbers and their rates of survival (Fig. 3A).

Survival of DL on bug (Experiment 2):

In Experiment 1, both bug and nematode survival were low. To obtain better survival conditions, a wooden box was used to keep the humidity relatively high and the temperature was set at 20°C. The humidity in the box was constant at around 90% RH during the experiment. As a result, all of the bugs used in the experiments survived. From these bugs an average of 67 DL/bug (range, 16–306 DL/bug) was found and DL survival on the bug was about 55% (range, 20.9%–85.3%). There was no correlation between the number of nematodes on the bug and nematode survivability (Fig. 3B).

DISCUSSION

Many nematodes have a phoretic association with insects. However, no information is available on the physiological conditions of DL phoretically associated with insects as well as DL survivorship. In this paper, we tried Cryo-SEM and observed the partially desiccated surface structure of DL on bugs. In addition, we demonstrated that *C. japonica* DL naturally associated with the shield bug were able to survive for at least 3 months. To our knowledge, this is the first paper to demonstrate the long-term survival of DL on shield bugs under experimental conditions.

Nematodes are essentially aquatic organisms that have adapted well to the soil environment, and most stresses associated with their quiescence involve either immobilization or removal of water (Wormersley *et al.*, 1998). Quiescent *C. japonica* DL found on the bugs appeared to be partly desiccated, and we speculated that quiescent DL on the bugs are in an anhydrobiotic state since nematodes under anhydrobiotic conditions survive for long periods of time. In our preliminary experiments, however, *C. japonica* did not have strong desiccation tolerance and DL on the bugs died easily when the bugs were exposed to desiccation conditions (< 40% RH) (data not shown). In addition, the host bug *P. japonensis* lives in a mixed secondary forest, with a variety of deciduous trees, broad-leaf evergreens and some conifers, where humidity is constantly high throughout the year. Thus, we tried several high humidity conditions to mimic their environmental conditions to demonstrate the survivorship of *C. japonica* on the bug. When the bugs were kept in the 100% RH condition, very few nematodes were found on them. Quiescent DL on the bugs seem to resume their activity at 100% RH, disembarking from the bugs and/or dying during the 3 months. In the field, DL on the bugs are usually quiescent, and a slightly desiccated condition seems to be important for keeping the quiescent state and for their long-term survival. When bugs collected in the field were kept in the glass vessel with water saturated with K₂SO₄ to keep the bugs under high humidity, the surface of the bugs looked sticky and crystals of K₂SO₄ were sometimes observed on the bugs. About half of the insects died during the experiment and DL survival was low (31%). Although this method of using K₂SO₄-saturated water has been used for biological experiments, it seems to be harmful for not only the bugs but also the nematodes on the bugs, especially in the long-term experiments. Among the conditions we tested, the wooden box in which the humidity was kept constant at about 90% was best and both bug and DL survival were better.

In the field, the bugs spend time aggregating on leaves from late July until the next June, when mated female bugs lay eggs and the nematodes on the female bugs disappear (Tanaka *et al.*, unpublished data). Nematodes seem to propagate during the reproductive period by infesting the dead eggs and nymphs in the bug nests. The bugs used in the experiments were collected in October, and we demonstrated that many DL survived for 3 months (until January) on the bugs. However, they have to survive on the bug for another 5 months to reach the reproductive period of the bug in June. We were not able to demonstrate longer-term survival because it was difficult to maintain the bugs and nematodes in good condition for a longer time. We tried

several experiments to demonstrate longer-term DL survival on the bugs by keeping the bugs in a big net in the field, but bug survival was very low (Tanaka *et al.*, unpublished data). In addition, it was difficult to maintain optimum conditions because the area was too far away to visit often enough to maintain the population under good conditions. Thus, controlling these conditions appears to be difficult. The temperature used in Experiment 2 was set at a constant 20°C to test survivability for the convenience of laboratory study. Lower temperatures suppress metabolism, which increase DL survival. Because the temperature of the bug's natural habitat fluctuates and becomes lower than 20°C from the autumn to spring, experiments under lower temperatures should increase survival of DL and are needed to demonstrate longer-term survival of DL. Since the number and survival of DL on the bugs were lower than those of DL in the field, there is the possibility of new embarkment on the bugs in the field. However, we were able to demonstrate that DL on the bugs survive at least for 3 months under relatively high temperatures compared to those in the field during the winter, and many of them seemed to be able to survive on the bugs until reproduction occurred in the field.

In anhydrobiotic nematodes, morphological changes in the body surface of desiccated nematodes are reported (Wharton and Marshall, 2002; Otsubo *et al.*, 2006). Thus, we examined body structures of the DL on the bugs using Cryo-SEM so as to observe the intact structure and evade the affects of chemical fixation. The bodies of DL were partially depressed and looked partly desiccated, but their body surfaces were smooth and the lateral alae were not shrunken, which differed from the anhydrobiotic nematode *A. besseyi* with its lateral alae shrunken under desiccation conditions. In *Ditylenchus dipsaci* (Kühn) Filipjev low-temperature field emission scanning electron microscopy (FESEM) showed that desiccation causes morphological changes of J4s: annulations are deeply indented and lateral alae are shrunken (Wharton and Marshall, 2002). In *Aphelenchus avenae* Bastian the body surface of desiccated nematodes exhibited a regular shrinkage (Otsubo *et al.*, 2006). Because *C. japonica* did not survive under high desiccation conditions, the body structure and physiological changes of DL are different from those anhydrobiotic nematodes. Morphological changes of body surface structures found in anhydrobiotic nematodes may be important for obtaining high desiccation tolerance. Further physiological and morphological studies of desiccation tolerances of non-anhydrobiotic nematodes including *C. japonica* are necessary for understanding the relationship between morphological changes of the body structure and desiccation toler-

ance under moderate desiccation conditions. In *Anguina amsinckiae* (Steiner and Scott) Thorne, presence of a thin external lipophilic coating secreted by the nematode was reported on desiccated nematodes but has not been studied in detail (Womersley *et al.*, 1998). In Diplogasteridae, DL are frequently coated with a thin layer of an oily substance that makes them stick together or float on the surface of water (Poinar, 1975). In *C. japonica* DL on the bug, the presence of such a coating is also suggested because of its glistening appearance, which masks the morphology of the annuli under higher magnification in Cryo-SEM observation (data not shown). Further research with detailed ultrastructural observation under high magnification is necessary for understanding the morphological adaptation and for analysis of the presence of the lipophilic coating in *C. japonica* DL.

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Received: September 30, 2010