Studies on the species-specific and female-biased phoretic behavior of the nematode *Caenorhabditis japonica* to its host insect *Parastrachia japonensis*

(*Caenorhabditis japonica* における、ベニツチカメムシへの種特異的および偏雌的便乗行動に関する研究)

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Contents

Summary

Bacteriophagous *Caenorhabditis japonica* forms a phoretic association with the burrower bug *Parastrachia japonensis*. *C. japonica* dauer larvae (DL), the phoretic stage of the nematode, are mainly found on adult females of *P. japonensis* but not on other arthropods. The aim of the present study is to elucidate the mechanisms of species-specific and female host-biased ectophoresy and of disembarkation from its host insect in *C. japonica*.

Behaviors of *C. japonica* DL to form phoretic association with *P. japonensis* were studied. *C. japonica* DL showed negative gravitaxis that probably enhances upward movement to encounter their host. In experiments using an olfactometer, *C. japonica* DL significantly migrated toward the direction of odors from *P. japonensis* but not from other insects tested. Analyses of odors using gas chromatography-mass spectrometry suggested the presence of *P. japonensis*-specific odors. In loading experiments, *C. japonica* DL embarked onto *P. japonensis* but not other invertebrates tested. There was no difference in association between the sexes of *P. japonensis*. In chemoattraction experiments, *C. japonica* DL were significantly attracted to the hexane-extracts from *P. japonensis* but not to those from other invertebrates. However, there was no significant difference in chemoattraction between the sexes of *P. japonensis*. For disembarkation from *P. japonensis*, high humidity resumed the mobility of *C. japonica* DL on *P. japonensis* but it was not an enough cue for disembarkation and many DL stayed on *P. japonensis*. Among the conditions tested, disembarkation was significantly increased in the presence of *P. japonensis* nymphs. These results suggest the sequence of following behavior in *C. japonica* to associate and dissociate with its host insect: Newly emerged *C. japonica* DL migrate to the soil surface by using

negative gravitaxis, which increases the opportunity to encounter their host insect wandering on the soil surface; *C. japonica* DL recognize species-specific odors and body surface components from *P. japonensis*, move to and embark onto their host, and enter quiescence under low humidity; Quiescent DL recovered their mobility under high humidity in the nest of *P. japonensis* in the litter, and then the presence of nymphs after hatching stimulates the disembarkation. Moreover, these results clearly indicate that *C. japonica* has complex and sophisticated behavior to form a species-specific and female host biased phoretic association with *P. japonensis*.

General Introduction

Nematodes have many types of relationships with other animals. Phoresy (Lesne, 1896) is one of the relationships. Nematodes use larger invertebrates such as insects as vectors for transferring themselves to different sites for reproduction. It is a form of commensalism in which the growth, fecundity, and/or survival of the nematode is enhanced, while the host remains unaffected. Previous studies have revealed the phoretic relationships among the orders in Rhabditida, Diplogasterida, Aphelenchida, Tylenchida (Maggenti 1981; Nickle, 1984; Giblin-Davis et al., 2003; Kiontke and Sadhaus, 2006), and Strongylida (Robinson, 1962).

Some nematode species wait host death on the host, and develop by eating bacteria and nutrition (Rhabditida and Diplogastidae) (Schulte, 1989; Baird et al., 1994; Manegold and Kiontke, 2001), which is called necromeny (Sudhaus and Schulte, 1989). This relationship is different from phoresy with the aspect of constructive use of host nutrition after host death. Necromenic nematodes do not need to leave their host to obtain their food when the host is alive. Phoresy and necromeny are close relationships because both of them use host as a vehicle for dispersal. Phoretic stage in Rhabditida is dauer larvae (DL) (Sudhaus, 1976; Kiontke, 1996; Maggenti, 1981). This is the stage specialized for dispersal and survival under unsuitable environments for nematode development. Unfavorable conditions such as high population density and low food availability induce DL (Riddle, 1988). DL is the developmental diapause stage and does not feed. Natural openings of DL are closed and DL show higher tolerance against surrounding stress (Wharton, 2002). They can survive longer than the nematodes in other developmental stages, by using inner storage energy (Riddle, 1988; Riddle and Albert, 1997; Riddle et al., 1997).

Caenorhabditis japonica, which is a relative species of a model organism *C. elegans*, is a bacteriophagous and dioecism nematode. The species is easily cultivated with *Escherichia coli* OP50 as a food in the laboratory. *C. japonica* was isolated from the burrower bug *Parastrach japonensis* Scott (Heteroptera: Parastrachiidae) (Kiontke et al., 2002). *C. japonica* has been exclusively found on *P. japonensis* (Yoshiga et al., 2013). Moreover, *C. japonica* is predominantly found on body surface of *P. japonensis* females throughout the year but rare on males. *C. japonica* DL survive on *P. japonensis* and propagate during the reproductive period of *P. japonensis* (Okumura et al., 2013).

The host insect, *P. japonensis*, is a subsocial burrower bug with a unique life cycle. After oviposition, female insects straddle, hold, and guard their eggs in the nest under the litters until nymphal hatch (Tachikawa and Schaefer, 1985). Mothers bring drupe seeds of host tree, *Schoepfia jasminodora* Sieb.et Zucc. (Santalales: Olacaceae) to their nest, and feed their nymphs. When nymphs of *P. japonensis* become adult, they climb trees and make aggregation, and enter reproductive diapause for nearly 10 months until the next reproductive season in May. During this period, *C. japonica* DL survive on the insect, and when *P. japonensis* females go under the litter to make nests to lay eggs, they leave host for propagation.

The species-specific and female host-biased phoresy of *C. japonica* implies the specialized association of *C. japonica* with *P. japonensis*. However, there is little information on the behaviors of *C. japonica* DL and their relationship with *P. japonensis*. The aim of the present study is to elucidate the mechanisms of species-specific and female host-biased ectophoresy of *C. japonica* as well as the mechanisms of its disembarkation from host body.

Chapter 1

Negative gravitactic behavior of *Caenorhabditis japonica* dauer larvae

1. Introduction

Gravity is a constant stimulus for life on Earth. Most organisms including animals are able to sense gravitational force and their behaviors are influenced by gravity. Although nematodes are able to perceive and respond to different kinds of stimuli, such as chemical, mechanical, and thermal, as well as light, magnetic fields, and electric currents (Riga, 2004), information on graviperception and response to gravity in nematodes has been controversial (Croll, 1970). Based on early studies on nematode behaviors, a phenomenon of upward movement of nematodes had been observed in plant and animal parasitic nematodes and was considered as a negative gravitactic response (Lees, 1953; Croll, 1970). However, after additional experiments, it was concluded that those responses instead consistent with unbiased random migration (Crofton, 1954; Barraclough and French, 1965). The only known example of negative gravitaxis in nematodes is the vinegar eelworm, *Turbatrix aceti* (Panagrolaimidae). *T. aceti* swims upward in vinegar culture medium due to the dragging effect of its heavy tail (Peters, 1952; Croll, 1970): the gravitaxis is passive in origin. To date, no study has experimentally demonstrated that nematodes actively respond to gravity and show negative gravitaxis

Another typical behavior that is often observed in parasitic and phoretic nematodes is nictation. Nictation, also known as waving, is a behavior in which the nematodes lift their anterior part or more of their body up in the air and wave their body (Croll and Matthews, 1977). This upward movement apparently increases the

opportunity to infect or attach to hosts that move on the soil but information on the mechanisms of induction and regulation of nictation is limited (Lee, 2002). Furthermore, no informations are available on the physiological condition of nictating nematodes.

During *in vitro* culture of *C. japonica* on artificial medium, newly produced DL show active upward migration and nictation (Tanaka *et al*., 2010a), and these behaviors seem to be useful for the nematodes to increase their opportunities of finding and embarking onto host bugs wandering on leaf litter. Because nictating DL migrate up against the direction of gravity, onto their host insects and accumulate readily on pipette tips, I hypothesized that nictating DL could show negative gravitaxis. In this chapter, I demonstrate the negative gravitaxis of *C. japonica* DL, and the influence of physiological condition on this phenomenon.

2. Materials and Methods

Nematodes

C. japonica strain H1 was isolated from an adult female of *P. japonensis* collected from Hinokuma Mountain Prefectural Park, Kanzaki City, Saga Prefecture, Japan. *C. elegans* N2 were obtained from the Caenorhabditis Genetics Center (University of Minnesota, Minneapolis/St. Paul, MN, USA). Nematodes were maintained on nematode growth medium (NGM) plates (1.7% agar) seeded with *Escherichia coli* strain OP50 (Stiernagle, 1999) at 25°C. DL of *C. elegans* and *C. japonica* were collected from NGM plates 20 and 10 days, respectively, after starting nematode culture, depending on the appearance of the DL. Nematodes were treated with 2% sodium dodecyl sulfate (SDS) for 15 min and then washed with distilled water 5–6 times before use to eliminate non-dauer-stage nematodes. Dog food agar (DFA) medium (Hara et al., 1981) seeded with *E. coli* OP50 was used as a nutrient-rich medium to obtain a large number of DL. Nictating *C. japonica* DL were exclusively collected as described by Tanaka et al. (2010a). Briefly, a sterile yellow 200-μl pipet tip (Watson, Fukaekasei Co., Ltd., Tokyo, Japan) was vertically placed such that the tapered side was up, at the center of a 100-ml culture bottle of DFA medium, and *E. coli* OP50 and the nematodes were cultured at 25°C. Approximately 5 days after inoculation, DL started to move upward to the yellow tip. Masses of nictating DL were picked up from the top of the tip using a fine needle, washed with distilled water three times, and used for subsequent experiments. DL from DFA medium, on days 6 and 7 after nematode inoculation, were used as otherwise mentioned. Because *C. elegans* DL did not move upward to the pipet tip, I was unable to use *C. elegans* DL for further assays.

To examine the effect of inoculation medium on negative gravitaxis, nictating

DL were soaked in M9 buffer (Stiernagle, 1999) up to 120 h. Because nematode mortality increased dramatically thereafter, inoculation was terminated at 120 h. Nematodes were collected from M9 buffer at a 24-h interval and used for gravitaxis assay. *T. aceti* obtained from a pet shop was cultured in apple vinegar solution (apple vinegar: water = 2:1) with small pieces of apple at 25°C in the dark.

Negative gravitaxis assay

Nearly 3 μl of nematode suspension containing approximately 20 DL were inoculated onto the center of a 9-cm NGM plate (1.7% agar). Subsequently, water around the inoculated nematodes was absorbed by NGM and the DL started moving on the plate; hence I placed the plate vertically. As a control, we prepared the plates that were placed horizontally. The assay was repeated for 20, 5, 37, and 10 times for non-nictating *C. elegans* DL, non-nictating *C. japonica*, nictating *C. japonica* DL, and nictating *C. japonica* DL (horizontal control), respectively. All assays were performed in a closed room to avoid the influence of air current under the same condition at 25°C. One hour later, I counted the number of nematodes that had moved upward and downward from the inoculation point. Nematodes between the two horizontal lines, 1 cm above and below a horizontal central line (see Fig. 2A), were considered immobile and were not counted. The negative gravitaxis index (NGI) was calculated as follows:
[(number of nematodes migrating upward) − (number of nematodes migrating downward)]/(total number of nematodes inoculated).

To confirm negative gravitaxis, the DL that migrated upward beyond the line 1 cm above the horizontal center line were washed with distilled water and harvested by centrifugation at 3000 × g for 1 min at room temperature. The collected DL were used

to perform a new negative gravitaxis assay as described above.

Negative gravitaxis of an isolated individual was also analyzed in the same manner using a single larva instead of using 20 DL to eliminate any collective effects on the nematode migration. The assay was repeated for 10, 13, and 29 times for non-nictating *C. elegans* DL, non-nictating *C. japonica* DL, and nictating *C. japonica* DL, respectively using different indivisuals. The distribution of nematodes on the assay plate during the negative gravitaxis assay was recorded 1 h after nematode inoculation. Control plates that were placed horizontally were similarly analyzed.

Effect of SDS treatment on negative gravitaxis

To understand the effect of SDS treatment, which is used for killing non-DL and preparing DL for behavior experiments, nictating DL were treated with 2% SDS solution for 15 min and then washed with distilled water 5–6 times. The NGI of SDS-treated or untreated DL were compared as described above. Nictating DL from day 7–9 cultures were used.

Center of gravity of the nematode body

The DL center of gravity was estimated qualitatively as follows. Individual nematodes were heat-killed at 60°C for 1 min. This treatment does not influence the morphology of nematodes and it is commonly used before fixation of the sample for morphological identification of nematodes. Heat-killed nematodes were placed just below the water surface in a 6-cm Petri dish, and they were allowed to sink following Peters (1952). These nematodes were categorized into three groups (head, body, and tail), according to the nematode body part that had first touched the bottom of the dish

after the free sinking.

Statistical analysis

An analysis of variance (ANOVA) with Bonferroni/Dunn test was used for the statistical analysis of negative gravitaxis (StatView Ver. 4.54; Abacus Concepts, Inc., Piscataway, NJ, USA). $P < 0.05$ was considered as the criteria for statistical significance. Statistical analysis of single nematode migration was performed using the chi-square test.

Results

Nictating DL showed negative gravitaxis

When non-nictating DL collected from NGM plates were used, no differences between the number of upward and downward migrations of either *C. japonica* or *C. elegans* were observed, which resulted in low NGI values (Fig. 1A). In contrast, significantly larger numbers of nictating *C. japonica* DL migrated upward than downward ($P<0.0001$), and the NGI value was significantly higher than that of non-nictating DL (Fig. 1A). When I used a single DL for the assay, approximately 70% of the nictating DL migrated upward, which was significantly larger than those that moved downward (Fig. 1B). A comparison of the distribution of nictating and non-nictating DL on the plate showed that the nictating DL moved up a higher than non-nictating DL (Fig. 2), which resulted in a difference in the NGI value between nictating (0.4) and non-nictating DL (0). To confirm the upward movement, the DL that migrated upward on the assay plates were collected and used for a second negative gravity assay. Larger numbers of DL moved upward, and the NGI value increased to approximately 0.6.

When plates were placed horizontally, nictating *C. japonica* DL equally dispersed to all direction on the plate (Fig. 2B) and NGI value for 20 DL was -0.06 (see Fig. 1A)

Effects of nematode condition on negative gravitaxis

Negative gravitaxis of nictating DL collected from cultures inoculated for different periods was compared (Fig. 3). The NGI of nictating DL from younger cultures (days 6 and 7) was significantly higher than that from an older culture (day 9).

After day 9, the number of nictating DL often decreased and collection of DL was difficult.

The effect of SDS treatment, which was used to kill non-DL for collecting DL, was tested. No significant differences were observed in the NGI values between SDS-treated and untreated *C. japonica* DL from day 7 and day 9 cultures (data not shown).

In a separate test to study the influence of inoculation media, nictating DL were collected from the bottom of a flat Petri dish and inoculated in M9 buffer for up to 120 h and then tested for negative gravitaxis, to examine the duration of the negative gravitaxis. An inoculation period of up to 24 h did not affect negative gravitaxis but further inoculation resulted in decreased negative gravitaxis (Fig. 4).

Gravity center of body

To examine the possibilities that gravitaxis is caused by a passive heavy tail dragging mechanism, as for *T. aceti*, I investigated the approximate center of gravity location of *C. japonica* using a sinking experiment. For this, I compared the center of gravity of body of the nematodes. *T. aceti*, which has a heavy tail and used as a control, and sank in the water from the from the posterior part, whereas *C. japonica* DL sank as if the center of gravity was located in the middle of the body, and no difference was observed between nictating and non-nictating conditions (Fig. 5).

3. Discussion

Positive and negative gravitactic behaviors had been reported in many nematodes. However, based on further experiments, they were concluded to be the result of random movement or dragging a heavy tail (Croll, 1970). Since then, no clear evidence showing positive or negative gravitaxis in nematodes has been reported. In the present study, I demonstrated negative gravitaxis in nictating *C. japonica* DL. My results indicate that the negative gravitaxis in *C. japonica* DL continues for several days once started, affected by the age of DL, and does not appear to be a simple passive behavior. This is the first experimental demonstration and characterization of negative gravitaxis in nematodes.

Nictation is a short time movement that continues for seconds to several minutes or longer. It is unlikely that nictation per see is a response to gravity. Rather it is a behavior that allows nematodes to sense and respond gravity. Upward migration and nictation apparently increase the opportunity for DL to attach to hosts in the field. Thus, it is plausible that nictating *C. japonica* DL show negative gravitactic behaviors. Although I still did not clarify the mechanism of inducing negative gravitactic behaviors, I assumed that negative gravitactic behavior of nictating DL might be caused by a change in the center of gravity from a central part of body to a heavy tail through the movement of body fluid and/or hemocytes to the posterior part of the body during upward movement. To test this possibility, I performed a sinking assay. *C. japonica* DL seems to have a central balance and no difference was observed between nematode conditions, indicating that there was no physical change in the center of balance in *C. japonica* DL. In addition, nictating DL showed negative gravitactic behavior, whereas non-nictating DL collected from the same DFA medium did not. These results suggest

that negative gravitactic behaviors were induced and influenced by the age of DL rather than by genetic and nutritional differences. It seems that nictating DL lift their body up on end and wave around. When DL stop lifting themselves, gravity causes the lifted portion of the body to fall, perhaps allowing them to sense the way up and migrate in that direction (on average). Further studies on inducible conditions of nictation and negative gravitaxis will help understand host-finding behaviors and the relationships and consequences of these behaviors.

I demonstrated the change in negative gravitaxis based on the condition of the nematodes. NGI values of waving DL collected from older plates were lower than those collected from younger plates. Furthermore, NGI values of nictating DL that were inoculated for more than 48 h and 120 h in solution were lower than those of nictating DL inoculated for 24 h. Although there were no significant differences between in NGI values 24, 72, and 96 h inoculation times, the values tended to become lower in longer inoculation times. I am uncertain why the NGI values tended to be lower after inoculation for 48 h. Among the 11 plates used for experiments at 48 h, NGI values from 2 plates were negative with unknown reasons, which may have caused lower NGI values. However, NGI values were significantly lower after inoculation for 120 h, and all plates showed similar tendencies. Longer inoculation times caused nematode death, and nematode mortality was higher for inoculation times greater than 120 h (data not shown; see Tanaka et al., 2012). Swimming in solution as well as nictation appears to consume a large amount of energy because of the vigorous movement involved. The movement of *C. japonica* DL was much faster than that of other DL such as *C. elegans*. Although dauer is the survival stage under unfavorable conditions, *C. japonica* DL have a shorter survival period than *C. elegans* (Tanaka et al., 2012). The decrease in negative

gravitaxis may be related to physiological changes including depletion of the energy reservoir, and fatigue after vigorous host-finding behavior. In a previous study, my colleagues and I found that the amount of triacyl glycerol did not significantly decrease, but protein carbonyl concentration, an indicator of oxidative damage and aging, significantly increased during the 6-day incubation of *C. japonica* DL (Tanaka et al., 2012). This suggests that oxidative stress could lower the survival rate of *C. japonica* DL and may be related to the decrease of negative gravitactic behavior. Very little informations are available on negative gravitaxis in invertabrates. In *Drosophila*, negative gravitaxis is influenced by aging because of age-related decline in locomotor speed (Gargano et al., 2005; Simon et al., 2006; Rhodenizer et al., 2008). My results together with these data indicate that aging may have a great influence on locomotion and negative gravitactic behavior in invertebrates including nematodes.

C. japonica DL have to locate *P. japonensis* wandering on the ground, and they must embark onto the the insect after encountering them to form a phoretic association. Without an association with the carrier insect, the survival of *C. japonica* dramatically decreases (Tanaka et al., 2012). In addition, I found that nictating DL are able to recognize *P. japonensis* by its chemicals and embark specifically onto the carrier (Okumura et al., 2013a). This results in their transfer to another place for propagation (Okumura et al., in press). Thus, both nictation and upward migration appear to be important for *C. japonica* DL to form a phoretic association with the host insect, and *C. japonica* seems to have acquired and developed these behaviors.

Because nictation is a typical host-finding behavior often observed in parasitic and phoretic nematode species (Croll and Matthews, 1977), it is possible that other nematodes also perceive and respond to gravity. The ability to detect and respond to

gravity occurs through the action of mechanoreceptors in many animals (Barbercheck and Duncan, 2004). In *Drosophila*, negative gravitaxis requires Johnston's organ, a mechanosensory structure located in the antenna, which also detects near-field sound (Sun et al., 2009; Kamikouchi et al., 2009). Although the existence of graviperception mechanism in *C. elegans* has been reported (Beckingham et al., 2005), there is no information about where and how gravity is perceived in nematodes. Lee et al. (2012) found that nictation is regulated by IL2 neurons in *C. elegans*. Thus, IL2 neurons may be related to graviperception in nematodes. In the present study, I tried collecting nictating *C. elegans* DL. However, to obtain a sufficient number of *C. elegans* DL for analysis was difficult because *C. elegans* DL are not as active as *C. japonica* DL. Therefore, I need to establish a method to collect a sufficient number of nictating *C. elegans* DL for further analysis. Further studies on gravitactic behaviors in other nematodes including *C. elegans* are necessary to understand the mechanisms of graviperception and response to gravity in nematodes.

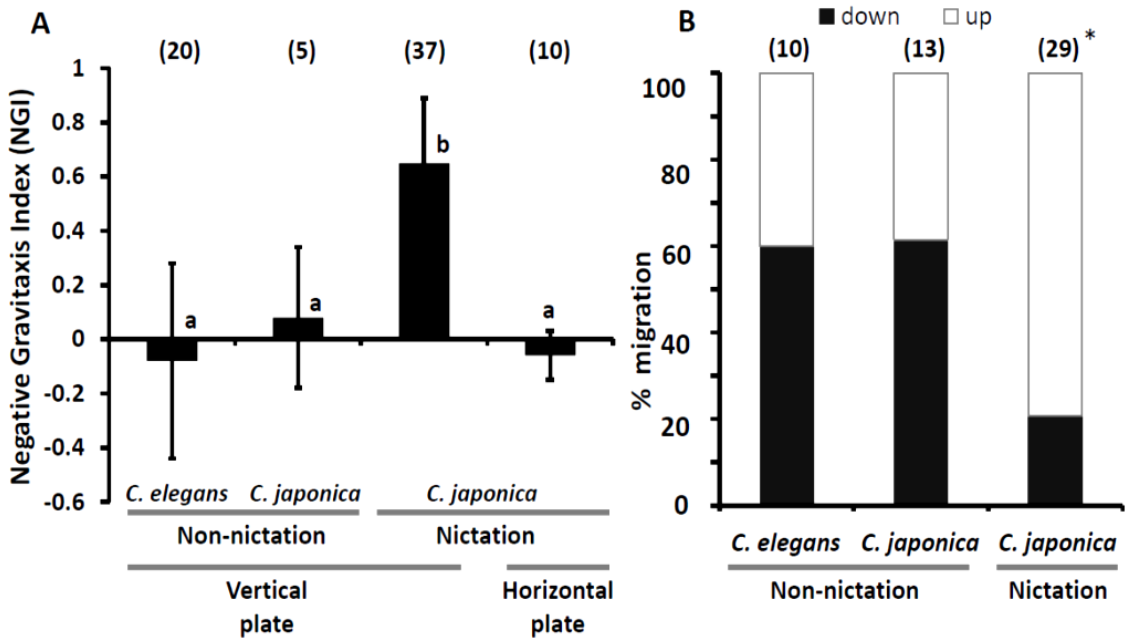

Fig. 1. Comparison of negative gravitaxis between *Caenorhabditis japonica* and *C. elegans* dauer larvae (DL). The distribution of nematodes on the assay plate during the negative gravitaxis assay was recorded 1 h after nematode inoculation. A, approximately 20 DL were used for each assay. Bars indicate standard deviation. Different letters above the columns indicate statistically significant differences detected by ANOVA with Bonferroni/Dunn test (P<0.0001). B, a single DL was used for assay. Asterisk indicates significant difference by the chi-square test (P<0.0001). Values in the parentheses indicate the number of replicates.

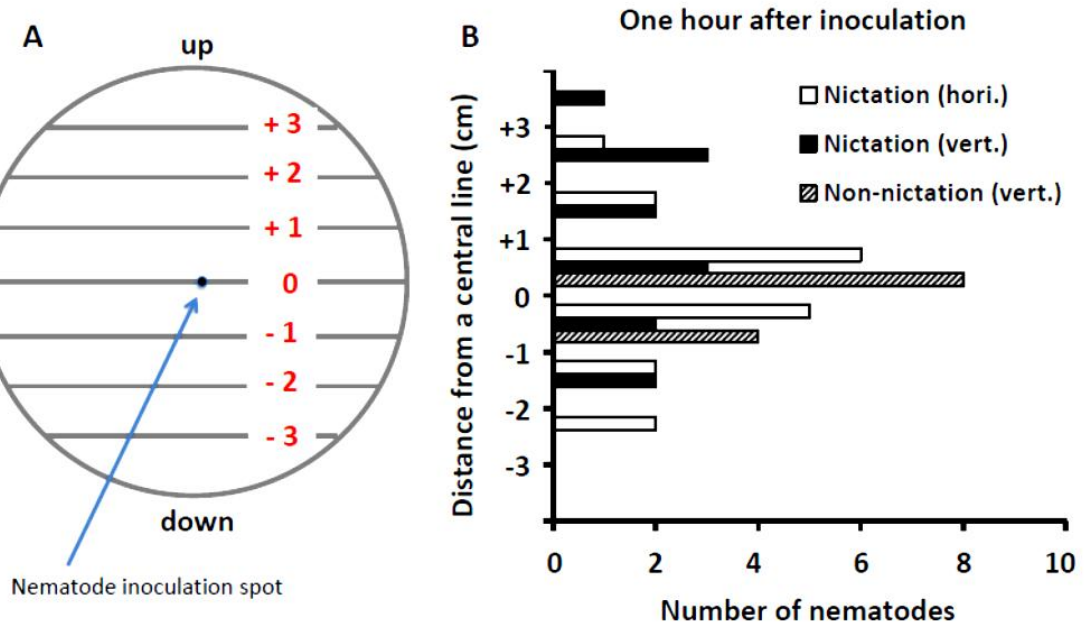

Fig. 2. Comparison of distribution between nictating and non-nictating *C. japonica* DL on a nematode growth medium (NGM) plate. A, diagram of the NGM plate used for the distribution assay. −3, −2, −1, +1, +2, and +3, indicate the distance in cm from the horizontal center line. B, distribution of DL on the assay plate 1 h after starting the experiment. The results of three different experiments were combined and presented. Larger numbers of nictating DL moved upward while non-nictating DL did not on the plates were set vertically (vert.). When plates were set horizontally (hori.) as a control, nematode distribution was equal and was not biased.

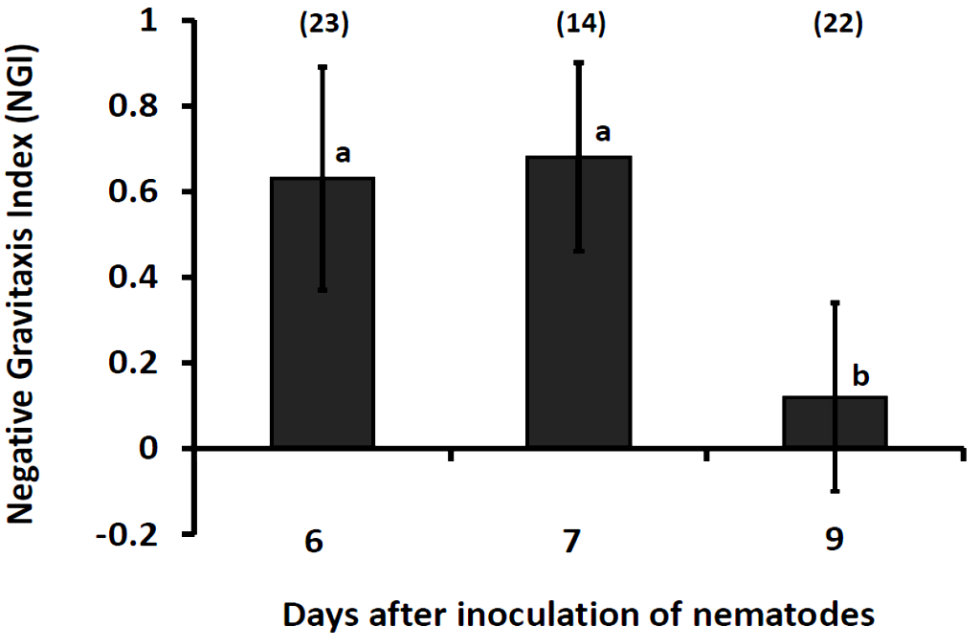

Fig. 3. Comparison of negative gravitaxis index of nictating *C. japonica* DL collected on different days. The NGI of nictating DL from a younger culture (day 6 and 7) was significantly higher than that from an older culture (day 9). After day 9, the number of nictating DL often decreased and collecting DL was difficult. Bars indicate standard deviation. Different letters above the columns indicate a statistically significant difference as detected by ANOVA with Bonferroni/Dunn test. The values in the parentheses indicate the number of replicates.

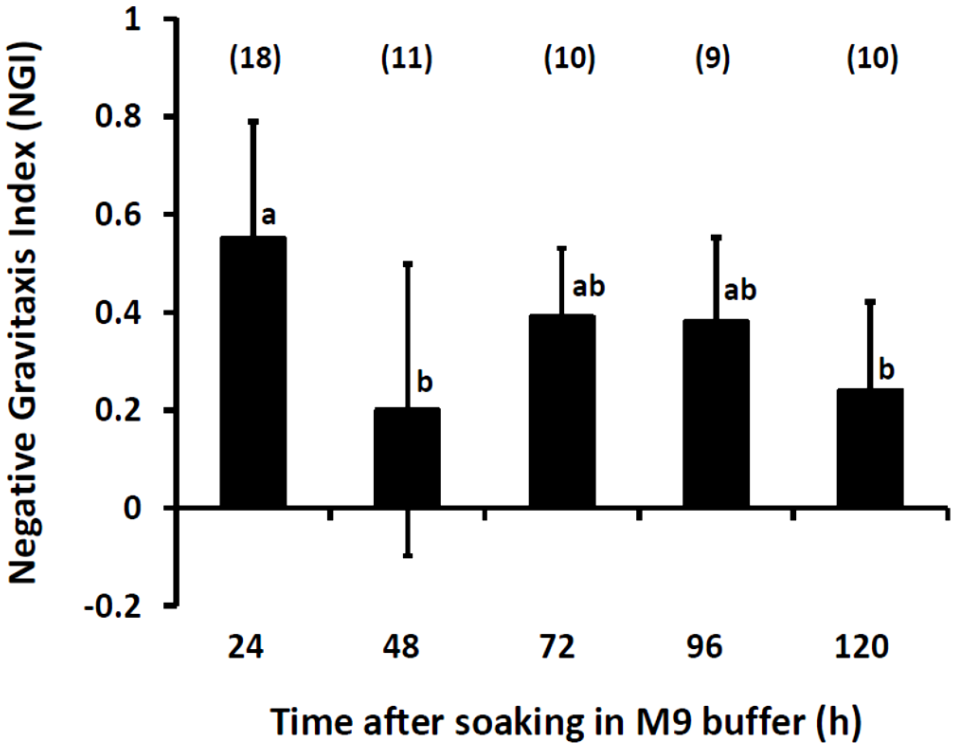

Fig. 4. Effect of the period of inoculation in buffer on negative gravitaxis of *C. japonica* DL. Negative gravitaxis was kept until 120 h inoculation in M9 buffer. Inoculation of nematodes was stopped at 120 h because of the high mortality thereafter (see Tanaka et al., 2012). Bars indicate standard deviation. Different letters above the columns indicate a statistically significant difference as detected by ANOVA with Bonferroni/Dunn test. The values in the parentheses indicate the number of replicates.

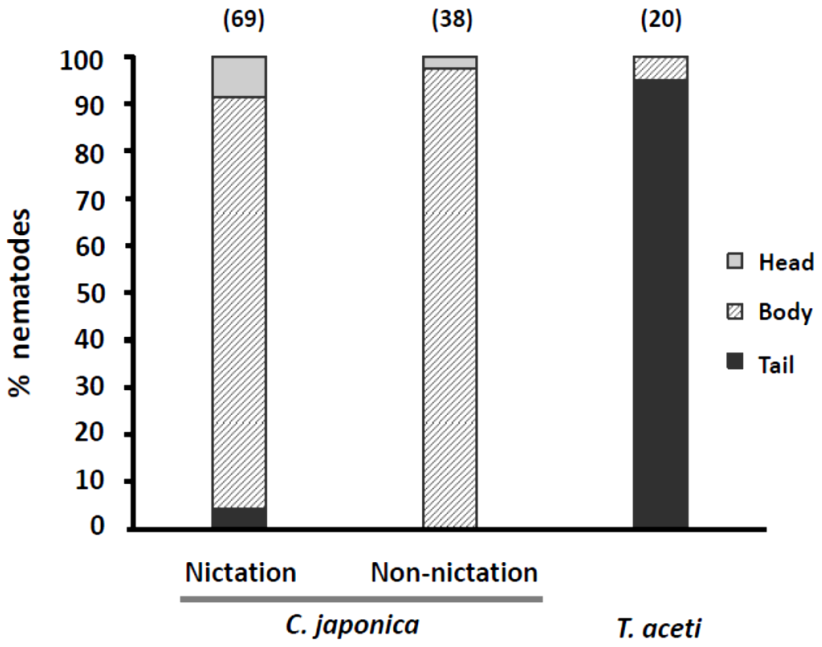

Fig. 5. Qualitative comparison of the center of gravity off-set. Nematodes were categorized into three groups (head, body, and tail) according to the body part that first touched the bottom of the plates after free sinking. *C. japonica* DL sank from the middle of the body, and no difference was observed between nictating and non-nictating condition while *T. aceti* sank from the posterior part.

Chapter 2

Species-specific recognition of the carrier insect by dauer larvae of *Caenorhabditis japonica*

1. Introduction

Although some host preferences exist, certain nematode species have been often isolated from different types of invertebrates (Barrière and Félix, 2005; Kiontke and Sudhaus, 2006; Herrmann et al., 2006). This indicates the nematode-invertebrate specificity is low in many cases. In addition, informations on species-specific phoretic associations are limited (Kiontke, 1997; Herrmann et al., 2006), and there are very few reports in which the specificity of association has been demonstrated experimentally (Baird, 1999).

DL are likely to use cues from their host insects, *e.g.*, chemicals (volatile and water soluble) and physical contact (Grewal et al., 1993). However, there are little information on whether DL are able to recognize their host insects, and if so, the mechanism involved in this recognition. Chemoattraction has been studied in many nematodes, and the chemotaxis of *C. elegans* has been clarified in depth both at the molecular and cellular levels (Bargmann, 2006). In addition, host-specific chemoattraction has been reported in *Pristionchus* nematodes (Hong and Sommer, 2006). However, these studies in *C. elegans* and *Pristionchus* spp. used the adult stage, and thus, no information is available on chemoattraction of DL to host insects, which is the actual phoretic stage, probably because DL are unresponsive to attractants in chemotaxis assays (Riddle, 1988). Recently, chemotaxis has been studied in DL of entomopathogenic nematodes (Rasmann et al., 2005; Hiltpold et al., 2010; Ali et al.,

2010; 2011). However, these studies focused on herbivore-induced volatiles and used indirect host cues. CO_2 acts as a direct host cue for nematodes, but very little information is available on the chemicals involved in direct host recognition (Hallem et al., 2011). Moreover, no information is available on species-specific kairomones that are released from host insects and directly attract DL.

The biology and life history of *Caenorhabditis japonica* are currently under investigation. According to my observations, this nematode appears to have a species-specific phoretic association, because *C. japonica* has never been detected from other invertebrates thus far. To establish such an intimate association, *C. japonica* may have developed mechanisms to recognize and associate with its carrier burrower bug *P. japonensis*. The aim of the present chapter is to investigate whether *C. japonica* DL are able to recognize and embark onto their carrier insect in a species-specific manner. I used loading experiments in which *C. japonica* DL and a invertebrate were inoculated together is a small Petri-dish to demonstrate that *C. japonica* DL specifically associate with their carrier insect. I also demonstrated the presence of kairomones, which are host-specific attractants, on the host insect for *C. japonica* DL.

2. Materials and Methods

Nematodes

Phoretically active *C. japonica* DL were collected from DFA using a worm picker, washed with distilled water three times, and used for the experiments.

Insects

Adults of *P. japonensis* were collected from Hinokuma Mountain Prefectural Park during 2007–2009. Females and males in reproductive diapause were collected from aggregations of burrower bugs between autumn and spring. As a control insect on an olfactometer experiment, I collected nymphs of *Acanthocoris sordidus* Thunberg (Hemiptera: Coreidae), green and brown colored unsexed adults of *Acrida cinerea* Thunberg (Orthoptera: Acrididae) and unsexed adults of *Oxya yezoensis* Shiraki (Orthoptera: Acrididae) from the campus of Saga University, Saga City, Saga Prefecture, Japan, in summer 2010. For chemical analysis, I also collected *Macroscytus japonensis* Scott (Heteroptera: Cydnidae), which has the same habitat as *P. japonensis*.

For loading experiments, two other species of burrower bugs, *M. japonensis* and *Erthesina fullo* Thunberg (Hemiptera: Pentatomidae), were used for comparison. *M. japonensis* was chosen because it is a burrower bug that is present in the same habitat as *P. japonensis* in Hinokuma Mountain Park and it was relatively easier to collect. A phoretic nematode species (not *C. japonica*) was occasionally present on adult *M. japonensis*. *C. japonica* has never been found on *M. japonensis* (Yoshiga et al., 2013). *E. fullo* was selected because it was a similar size to *P. japonensis* and it was readily available. No nematodes have been detected in this insect species (unpublished data). A species of terrestrial isopods, *Armadillidium vulgare* Latreille (Isopoda:

Armadillidiidae), was also used in the loading experiment because isopods were often present in the area where *P. japonensis* was found, and phoretic nematodes (not *C. japonica*) were often found in terrestrial isopods. Adults of *M. japonensis* were collected in the autumn from below the litter near an aggregation of *P. japonensis*, whereas *E. fullo* was collected on the campus of Saga University, in autumn 2007. *Armadillidium vulgare* was collected on the campus of Saga University in July 2012.

Olfactometer

To investigate whether volatiles from insects provoke nematode attraction to the insects, I made an olfactometer modified from the study of Japanese horntail *Urocerus japonicus* (Hymenoptera: Siricidae) (Matsumoto and Sato, 2007). The olfactometer consists of two Y-shaped glass tubes (shaped glan air pump, two flow meters, and two chambers as illustrated in Fig. 6. The air pump was set to obtain air flow at a rate of 0.8-1.0 L/min. Air was passed through distilled water to remove odors in the air and to keep a high humidity, and then divided into two ways by the first Y-tube. Each flow meter was adjusted to 0.4 L/min by valves. Two air lines were passed through the test and control glass bottles. The test bottle contains a test insect or CO_2, and the control bottle nothing. The air passed through the test and control bottles was connected to an experimental Y-tube, whose half volume was filled with 1.5% water agar. Thirty–100 DL picked up from the tip of a yellow tip on the DFA were inoculated at the center of the experimental Y-tube, and then the Y-tube was set vertically. Air flow was kept for 10 min at a rate of 0.8–1.0 L/min. Ten min after starting the experiment, I disconnected the experimental Y tube and the nematode numbers in each area (test, control, inoculation area, and downward; see Fig. 6) were counted.

Collection of odors

To collect the odors vapored from the females, males, and nymphs of *P. japonensis*, adults of *M. japonensis* and *S. jasminodora*, I made a collecting system as illustrated in Fig. 7. An air pump was connected to a series of glass bottles containing silica gels, molecular sieves, or granular activated charcoals for removing air trashes. The air was passed through a Tenax TA glass tube for air cleaning, and then the sampling bottle, and odors were caught with a Tenax TA glass tube. Silicon tubes were used for connections. Air was flown at a rate of 0.8-1.0 L/min for 10 min during the odor collection. Different samples were collected from each species (N = *P. japonensis* female: 3, male: 4, nymph: 4, and *M. japonensis* unsexed adult: 4). The seeds of *S. jasminodora* were collected from Hinokuma mountain in summer 2010, and three seeds were used as 1 seed sample, I prepared 4 samples. As a control, the air passed through the sampling bottle without any organisms was also collected.

Analysis of odors by GC-MS

Collected odor samples were analyzed by Gas Chromatography Mass Spectroscopy (GC-MS) [GC: Agilent 6890 with an HP-5MS capillary column of 30 m long, 0.25 mm inner diameter, and 0.25 μm film thickness; MS: Agilent 5973 mass selective detector, 70 eV, equipped with a thermal desorption cold trap injector (TCT) (CP4010; Chrompack, Bergen op Zoom, The Netherlands)] at Center for Ecological Research, Kyoto University, Japan.

Loading experiments

About 1000 DL were inoculated on a filter paper in a 3-cm plastic Petri dish, and then a *C. japonica*-free insect (male or female *P. japonensis*, unsexed adults of *M. japonensis*, *E. fullo*, or *A. vulgare*) or a pair of *P. japonensis* (male and female) was released in the dish. Twenty-four hours after the inoculation at 25°C, the insects were dissected and their body parts were placed in water for 24 h to release DL then the nematodes were counted under the stereomicroscope. Nematode numbers were compared among insect species and between male and female *P. japonensis*.

Because *C. japonica* DL are usually associated with *P. japonensis*, preparation of *C. japonica*-free *P. japonensis* was necessary before carrying out the loading experiments. To prepare nematode-free *P. japonensis* samples, adults of *P. japonensis* were partly soaked in tap water for 3 days and rehydrated nematodes on the bugs were washed off (Tanaka et al., 2010a). All DL were removed using this method. The removal of nematodes was also confirmed by observations using a stereoscopic microscope. Other burrower bugs and the isopod were used after rinsing with distilled water.

Chemoattraction experiments

I modified the chemotaxis assay method developed for *C. elegans* to investigate the nematodes' response to the hexane extracts containing body surface components of burrower bugs (Matsuura et al., 2005). Test and control spots (1-cm circle) were set in a 6-cm nematode growth medium (NGM) plate, and 3 μl of hexane extracts containing body surface components of insects and only hexane were spotted at the center of test and control spots, respectively. Next, approximately 20–30 DL in 2–3

μl of water were placed at the center of the plate. Nematodes in the test and control spots were counted at intervals of 10 min for 60 min. The chemoattraction index (CI) value [(number of nematodes in the test spot−number of nematodes in the control spot)/total number of nematodes] was calculated as described in Bargmann et al. (1993). Sodium azide, used in the original method to keep nematodes on the spots, was omitted in these experiments because *C. japonica* DL actively moved on the plate and was sometimes trapped by sodium azide during random movement. Hexane extracts containing body surface components of insects were prepared by soaking a bug in hexane in a glass tube with a lid for 10 min. Insects were weighed in advance, and only those with an average weight of 0.05±0.02 g for *M. japonensis*, 0.13±0.03 g for male *P. japonensis*, 0.17±0.03 g for female *P. japonensis*, and 0.3±0.05 g for *E. fullo* were used in the assays. Based on the average insect sizes, 90, 300, 350, or 530 μl of hexane was used for *M. japonensis*, male *P. japonensis*, female *P. japonensis*, or *E. fullo*, respectively. For 5th instar nymphs of *P. japonensis*, 350 μl of hexane was used for extraction.

Test for the arrest of DL dispersal

I investigated the effects of hexane extracts containing body surface components of *P. japonensis* on arresting DL dispersal. One circle (1-cm diameter) was made at the center of an NGM plate, and 3 μl of hexane or hexane extracts was spotted at the center of the circle. As soon as the hexane evaporated and/or was absorbed on the plate, DL were inoculated directly onto the center of the circle. The number of nematodes left in the circle was counted every 10 min for 60 min.

Statistical analysis

Generalized linear model was used for the statistical analysis for olfactmeter experiments. ANOVA with Bonferroni/Dunn tests was used for statistical analysis of chemoattraction (StatView Ver. 4.54; Abacus Concepts, Inc.).

3. Results

Olfactometer

DL moved to the direction of *P. japonensis* odors and 43% of inoculated DL were found in the test area (Fig. 8). The percentage of *C. japonica* DL moved toward the air from *P. japonensis* was significantly higher than toward control air (Fig. 8, GLM, $P<0.001$). On the other hand, more than half of inoculated DL stayed in the inoculation area when *A. sordidus*, *A. cinerea*, *O. yezoensis*, or CO_2 was used. There was no significant difference in the percentages of DL that were found in the test and control areas.

Analyses of Collected Odors

By comparing the odor components from *P. japonensis*, *M. japonensis*, and *S. jasminodora* collected by a Tenax TA glass tube, I found 7 peaks that are specific for *P. japonensis*. Among them, dodecanoic acid, pentadecanoic acid, and octadecanoic acid were identified by their mass spectrum (Fig. 9).

Loading experiments

During the loading experiments with *P. japonensis*, I observed DL crawling up the legs and abdominal parts of both male and female of *P. japonensis*. After dissection of the insects, aggregates of DL were found between the body segments and the wings where naturally associating DL are usually found. At maximum, 333 DL were found from *P. japonensis*. In contrast, nematodes were scarcely found on the body surfaces of *M. japonensis*, *E. fullo*, and *A. vulgare* (maximum =3, minimum =0; maximum =21, minimum =0; maximum =16; and minimum =0, respectively), and there

were significant differences between the nematode numbers on the insects (Fig. 10A).

The number of DL associated with male and female *P. japonensis* was not significantly different when a single male or female burrower bug was placed in a 3-cm dish (Fig. 10B) and when a pair of male and female burrower bugs was placed in a 3-cm dish (Fig. 10C).

Attraction of bugs to hexane extracts

The *P. japonensis*-specific embarkment in the loading experiments may imply the presence of some specific attractant cues. To test whether there were any differences in attraction toward the chemicals from the borrower bugs, chemoattraction of *C. japonica* DL to the hexane extracts containing body surface components of bugs was compared. Hexane extracts containing body surface components of *P. japonensis* moderately attracted *C. japonica* DL, and the CI values reached a plateau (about 0.3) within 60 min after the start of experiments (Fig. 11). In contrast, hexane extracts containing body surface components of *M. japonensis* and *E. fullo* did not affect nematode behavior and CI values were less than 0.03 (Fig. 11). The mean response of *P. japonensis* was significantly higher than that of the others at every time point (ANOVA, F=33.297, P<0.0001). When attraction was compared among the different stages, sexes, and physiological conditions of *P. japonensis*, the CI values for nymphs were relatively high and were statistically higher than those for males (ANOVA, F=3.024, P=0.0429). However, no significant differences were observed in CIs among males, reproductive diapause females (where *C. japonica* DL are usually associated), and provisioning females (where *C. japonica* DL are not found) (Fig. 12).

Arrest of dispersal by hexane extracts

I released DL directly on the site where hexane extracts were spotted to evaluate the arresting effects of hexane extracts. When only hexane or hexane extracts containing body surface components of *M. japonensis* were used, DL rapidly dispersed on the plate (Fig. 13). In contrast, when hexane extracts containing the body surface components of *P. japonensis* were used, >50% of DL remained in the inoculation area even 60 min after the nematode inoculation and the mean response of *P. japonensis* was significantly higher than that of others at any time point (ANOVA, F=78.083, P<0.0001). Hexane extracts containing the body surface components of *E. fullo* paralyzed DL on the spot after nematode inoculation possibly because of a toxin on its body surface, which resulted in high percentages (82% and 62% at 10 and 20 min after the nematode inoculation, respectively). Thus, these results were omitted from the analyses. When arrest of dispersal effect was compared among different *P. japonensis* stages, sexes, and physiological conditions, no differences among the developmental stages or sexes of *P. japonensis*, except between male and nymph were found (ANOVA, F=5.858, P=0.0024) (Fig. 14).

4. Discussion

In the present study, I demonstrated that *C. japonica* DL move toward the odors from their carrier insect *P. japonensis* but not toward those from other insects or CO_2. Moreover, they specifically associate with their carrier burrower bug *P. japonensis*, but not with *M. japonensis*, *E. fullo*, and *A. vulgare*. Hexane extracts containing body surface components of *P. japonensis* significantly attracted *C. japonica* DL and arrested their dispersal after contact, whereas DL were not attracted to hexane extracts containing body surface components of other bugs. These results indicate that *C. japonica* DL recognize their carrier through some specific chemicals from the carrier insect, thereby enabling them to associate with the carrier. To the best of my knowledge, this is the first report demonstrating the species-specific orientation and embarkment of DL onto their host and the direct chemical recognition of the host by DL. These findings strongly suggest the presence of species-specific kairomones in nematodes.

Allelochemicals elicit a physiological or behavioral response between members various nematode species (Huettel, 1986; Riga, 2004). Kairomone is an allelochemical that elicits a positive response from the receiving organism. Although many nematodes form phoretic or parasitic associations with insects, very little information is available on kairomones other than CO_2 with regard to direct host recognition cues (Hallem et al., 2011). In the present study, I found that *C. japonica* DL were attracted to hexane extracts containing only body surface components of their host *P. japonensis*, indicating that the chemical components from their host appear to contain specific kairomones for the association of *C. japonica* DL with their carrier insect. Although the ecology of *C. japonica* is currently under investigation, the nematode has been isolated so far only from *P. japonensis*. *P. japonensis* feeds only on the drupes of *S. jasminodora* and is one

of only two species comprising the genus *Parastrachia* (Schaefer et al., 1991; Sweet and Schaefer, 2002). The specialized trophic ecology and evolutionary independence of *P. japonensis* may help to develop the specific kairomone for *C. japonica*. However, further studies on the characterization and identification of the kairomones are essential to understand the evolution of this species-specific phoresy and the mechanisms of host recognition.

Although phoresy is commonly found in nematodes, there are very few reports on sex-specific or sex-biased associations; female-biased association has been reported in *Fergusobia* nematode/*Fergusonia* fly mutualism (Currie, 1937; Davies et al., 2001) and *Sphaerularia* nematode/*Bombus* bee or *Vespa* hornet parasitism (Bedding, 1984; Sayama et al., 2007). However, no information is available on the mechanisms of female-biased association. In the field, *C. japonica* DL are mostly found on female *P. japonensis* but seldom on males. I expected that there would be some differences in attraction to male and female *P. japonensis*. However, no significant difference was observed in both loading and chemoattraction experiments. These results suggest that attraction of nematodes to both male and female insects does not differ. The differences in nematode association in the field could be due to factors other than chemoattraction. One possible reason is the difference in DL survival on male and female insects. Male bugs are smaller than female insects and DL on male insects may face more severe desiccation than those on female insects, resulting in the death of DL; these dead DL would then fall off the insect. Another possibility is behavioral differences between the sexes. We observed grooming behavior in *P. japonensis* individuals in which they would open their wings and rub their body, antennae, and legs with their hind legs. Thus, DL

on male insects could be removed during grooming. The frequency of the grooming behavior and/or the pattern of grooming may differ between sexes. Additional *C. japonica* and *P. japonensis* studies in the field are necessary to understand the female-specific association of DL, including studies on ecological and behavioral differences as well as on survivorship.

Table 1. List of arthropods used in olfactometer and GC-MS analyses

Arthropods	Experiments used	
Insecta/Hemiptera		
Acanthocaris sordidus (nymph adult)	olfactometer	
Macroscytus japonensis (unsexed adult)	olfactometer	GC-MS
Parastrachia japonensis Female (reproductive diapause)	olfactometer	GC-MS
Insect/Orthoptera		
Acrida cinerea (green and brown colored adult)	olfactometer	
Oxya yezonsis (unsexed adult)	olfactometer	

Table 2. List of arthropods used in loading, chamoattraction, and arrest of dispersal experiments.

Arthropods	Experiments used		
Insecta/Hemiptera			
Erthesina fullo (unsexed adult)	Loading	Chemoattraction	Arrest of dispersal
Macroscytus japonensis (unsexed adult)	Loading	Chemoattraction	Arrest of dispersal
Parastrachia japonensis			
Male (reproductive diapause)	Loading	Chemoattraction	Arrest of dispersal
Female (reproductive diapause)	Loading	Chemoattraction	Arrest of dispersal
Female (provisioning)		Chemoattraction	Arrest of dispersal
Nymph (5th instar)		Chemoattraction	Arrest of dispersal
Malacostraca/Isopoda			
Armadillidium vulgare (unsexed)	Loading		

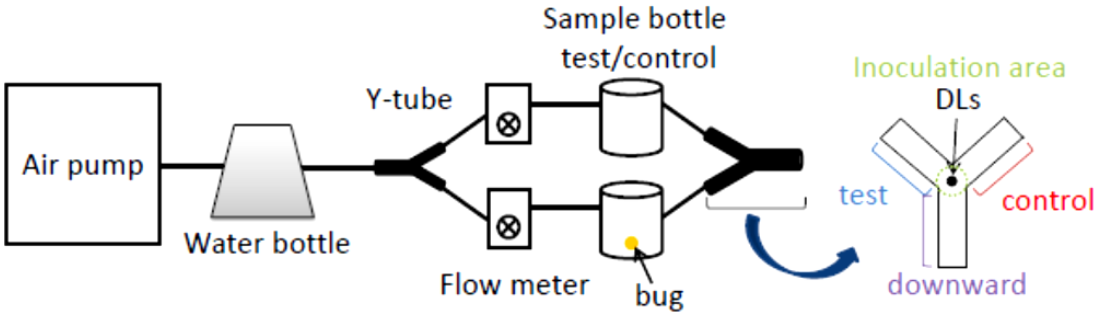

Fig. 6. Olfactometer. The olfactometer consists of two Y-shaped glass tubes (φ12 mm), an air pump, two flow meters, and two chambers. Air pump was set to obtain airflow at a rate of 0.8-1.0 L/min. Each flow meter was adjusted to 0.4 L/min by valves. Thirty–100 DL picked up from the tip of the yellow tip on the DFA were inoculated at the center of the experimental Y-tube, and was set vertically. Ten min after starting experiment, nematode number was counted. Areas were divided into test, control, inoculation area, and downward.

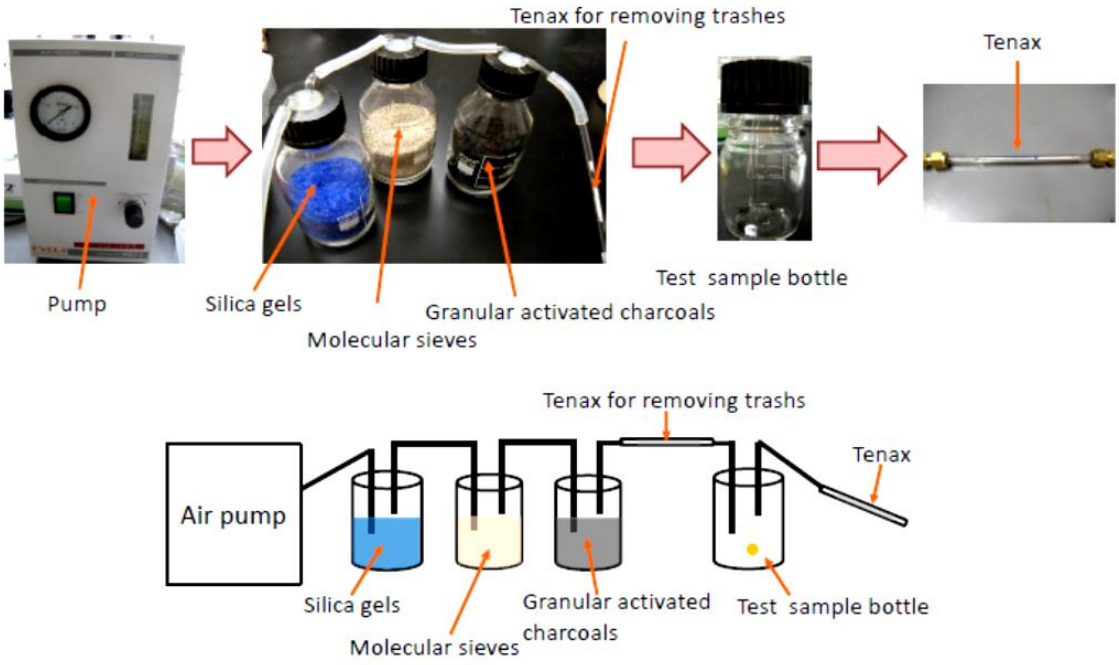

Fig. 7. A system for collecting odors. Air was passed through silica gels, molecular sieves, and granular activated charcoal to remove trashes in the air. Air was flown at a rate of 0.8-1.0 L/min for 10 min. Insects and plant odors were collected by a Tenax TA glass tube.

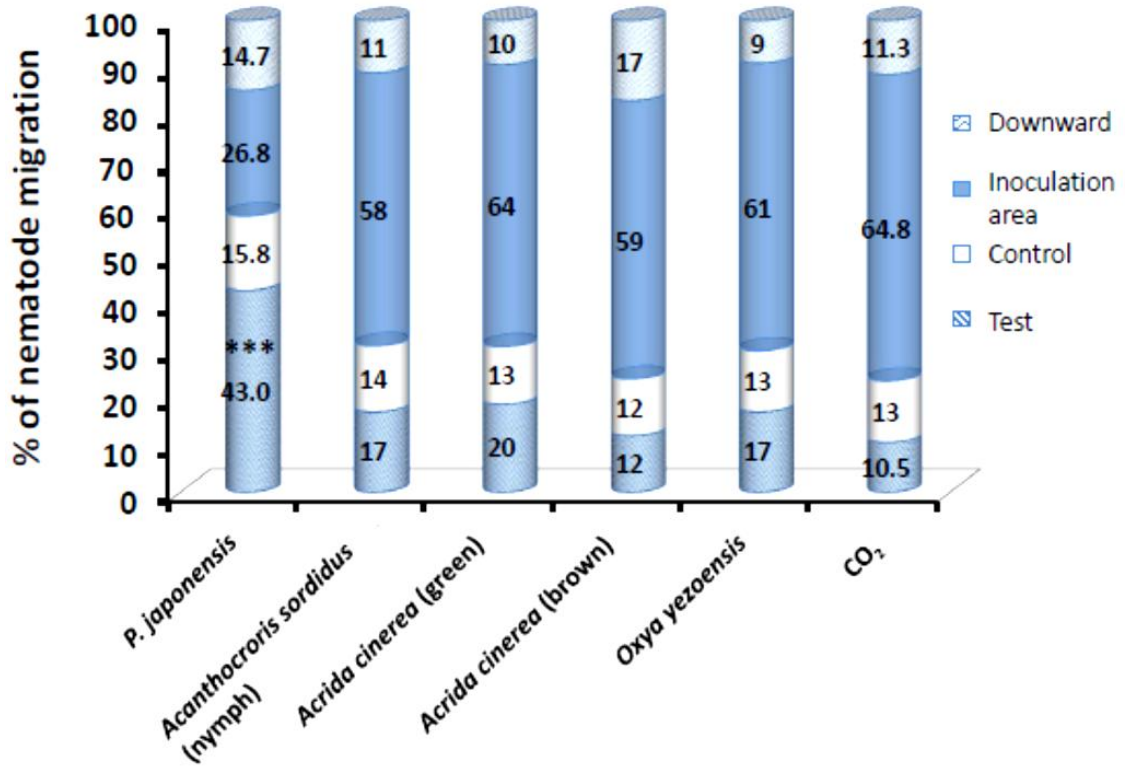

Fig. 8. Olfactory response to 4 insect species and CO_2. Significantly larger number of DL moved toward the odors from *P. japonensis*. On the other hand, more than half of inoculated DL stayed in the inoculation area when other insects or CO_2 was used and there was no attraction to other insects or CO_2. Data were analyzed by GLM (Generalized linear model). *** $P<0.001$.

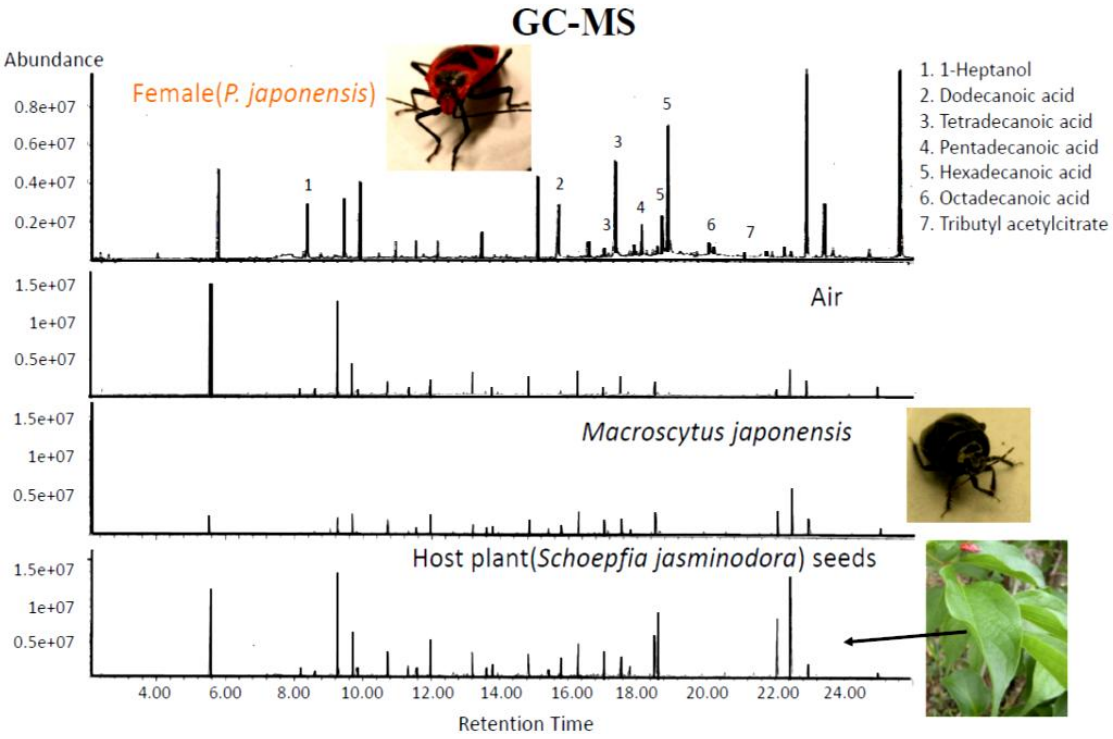

Fig. 9. Analyses of collected odors. Odors from female of *P. japonensis*, *Macroscytus japonensis*, and *Schoepfia jasminodora* fruits with analyses of GC-MS. At least 7 components that were specific for *P. japonensis* odors were found.

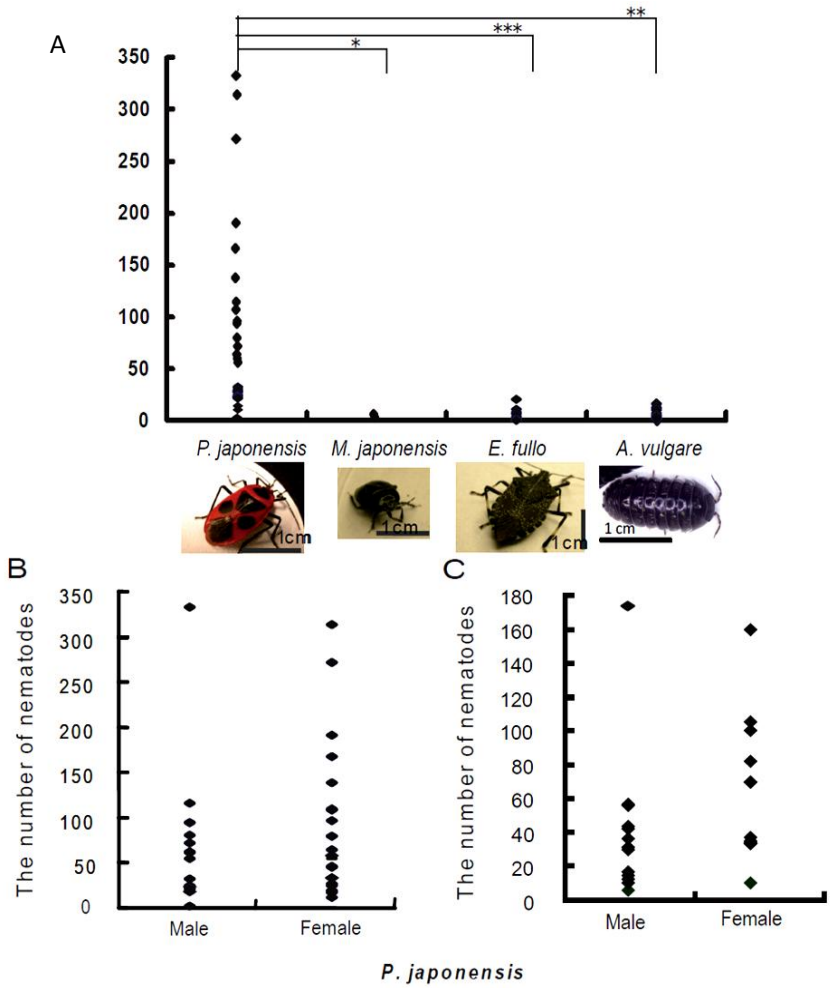

Fig. 10. Loading experiments of *Caenorhabditis japonica* DL. A, Comparisons among the numbers of nematodes loading onto the three burrower bugs and the pill bug. Significantly higher numbers of nematodes embarked on *P. japonensis* compared with *M. japonensis*, *E. fullo*, and *A. vulgare*. B, One female or male *P. japonensis* was placed individually in a dish. No significant difference was observed between the number of nematodes that embarked. C, A pair (female and male) of *P. japonensis* were placed in a dish. No significant difference was observed between the numbers on male and female *P. japonensis*. (ANOVA, $*P > 0.01$, $***P > 0.0001$). N = 17, 21, 5, 21, and 10 for *P. japonensis* males, *P. japonensis* females, *M. japonensis*, *E. fullo*, and *A. vulgare*, respectively.

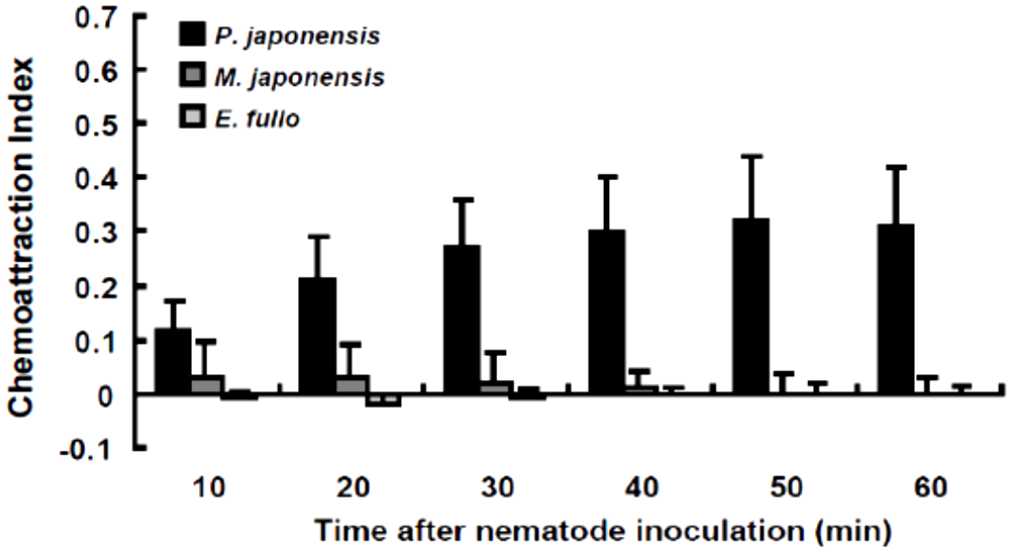

Fig. 11. Comparison of the chemoattraction response to the hexane extracts from three bugs. *C. japonica* DL were attracted to the extract containing body surface components of *P. japonensis*, but not to the extracts from *M. japonensis* and *E. fullo*. The mean response of *P. japonensis* was significantly higher than that of the others at every time point. N=30 (*P. japonensis*), 10 (*M. japonensis*), and 2 (*E. fullo*) (ANOVA, F=33.297, P<0.0001). Error bars indicate s.d.

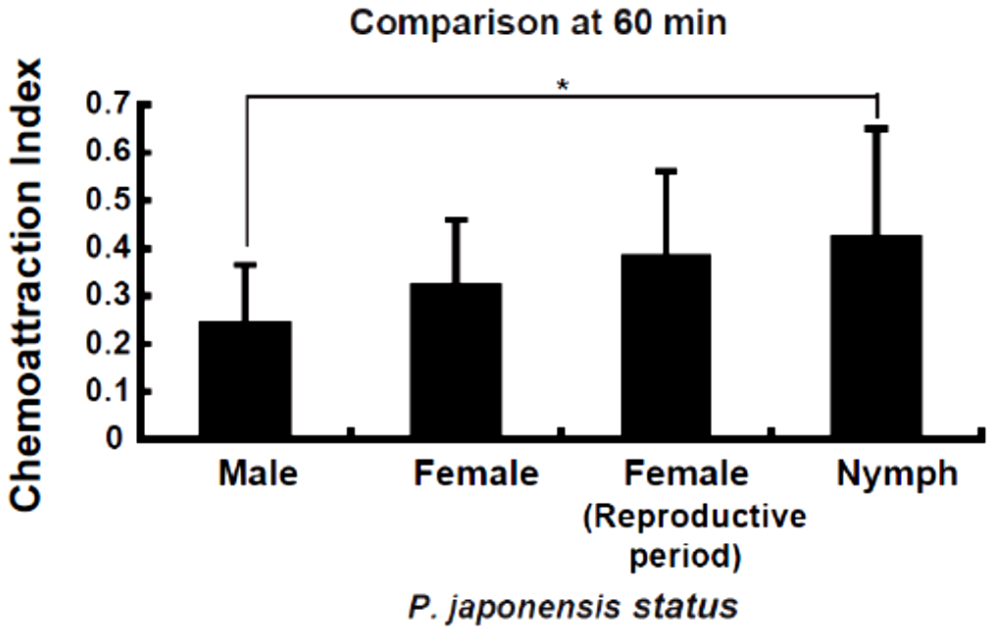

Fig. 12. Chemoattraction response to four types of hexane extract containing body surface components of *P. japonensis* at 60 min. *C. japonica* DL were attracted to four types of *P. japonensis* extracts. Reproductive diapauses males and females, reproductive females, and nymphs were tested (N=10, 10, 10, and 8, respectively). The mean for nymphs was significantly higher than that for males (ANOVA, F=3.024, P=0.0429. *<0.01).

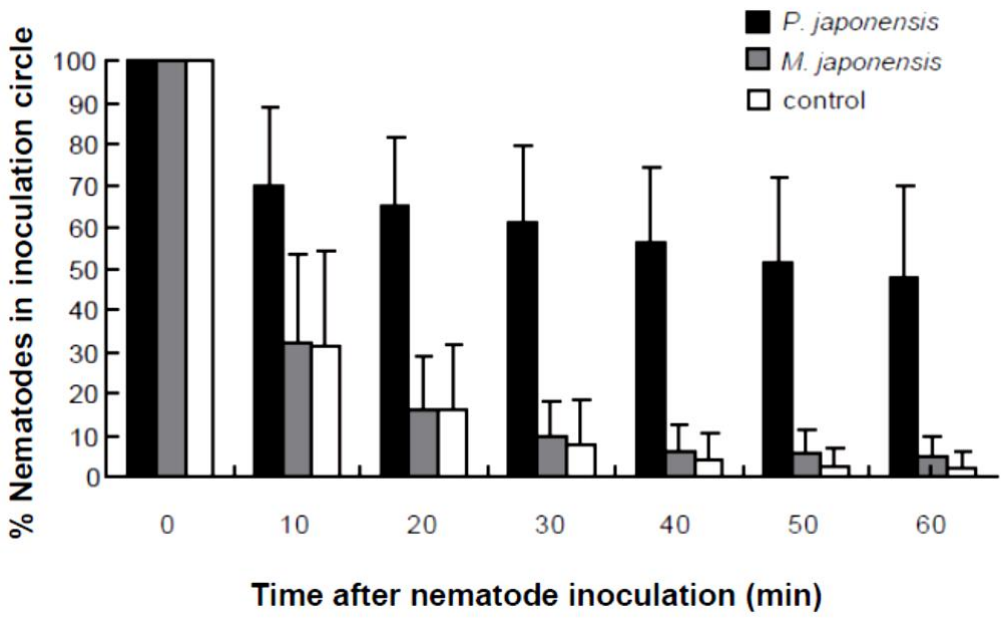

Fig. 13. Arrest of dispersal experiments. Comparisons between the two insects. *C. japonica* DL dispersal was arrested or they crawled on the extract containing body surface components of *P. japonensis* but not on the other extracts. The mean response of *P. japonensis* was significantly higher than that of the others at any time point. N=31 (*P. japonensis*), 10 (*M. japonensis*), and 40 (control) (ANOVA, F=78.083, P<0.0001). Error bars indicate s.d.

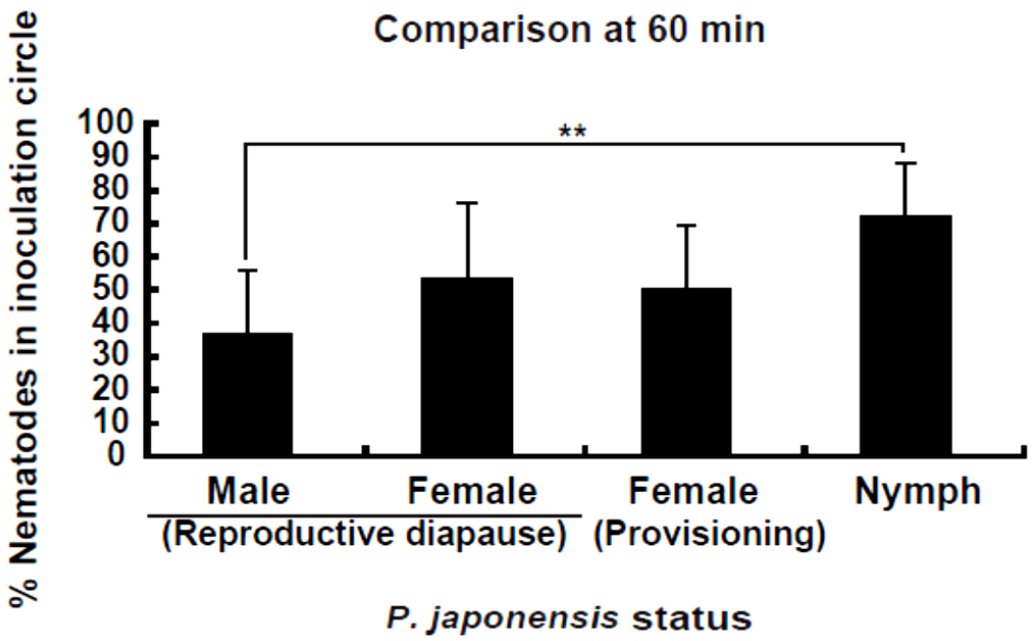

Fig. 14. Arrest of dispersal experiment. Comparisons among the four types of extract containing body surface components of *P. japonensis* at 60 min. Reproductive diapauses males and females, reproductive females, and nymphs were tested (N=10, 11, 10, and 8, respectively). The proportion of nematodes that remained in the inoculation circle was significantly higher when nymph extracts (ANOVA, F=5.858, P=0.0024). Error bars indicate s.d. ** P<0.001.

Chapter 3

Condition for disembarkation of *Caenorhabditis japonica* from its carrier insect *Parastrachia japonensis*

1. Introduction

Necromeny is a special type of phoresy in which nematodes on the insect carrier wait for the death of the carrier and develop by feeding on bacteria present on the carrier's decomposing corpse (Sudhaus and Schulte, 1989). Although many different groups of nematodes have phoretic associations with different insect species (Poinar, 1975; Kiontke, 2006), researches of the mechanisms underlying the mechanisms of embarkation and disembarkation of phoretic nematodes are limited (Kiontke, 1996; Baird, 1999).

DL is a non-feeding and developmental diapause stage, which is specialized for dispersal and survival in unfavorable conditions. According to my observation, *C. japonica* use *P. japonensis* as a vehicle for transferring to places for reproduction, the nest of carrier insect. *C. japonica* propagate on the egg carcass and nymphal cadavers, which is suggesting phoretic and necromenic association with the insect (Okumura et al., 2013a). The carrier insect *P. japonensis* is a monophagous and gregarious hemipteran insect with a unique biology (Tsukamoto and Tojo, 1992). *P. japonensis* spends most of the year aggregating and forming clusters on the green leaves during their reproductive diapause. They mate in March and during July, the gravid females *P. japonensis* lay eggs as an egg mass in her nest within the leaf litter. The mother insects carry an egg mass that protect the eggs from predators and microbes, and they provisions the nest with drupes from the deciduous tree *Schoeofia jasminodora* Sieb et Zucc. after

hatching.

In a previous study, I demonstrated that *C. japonica* on female *P. japonensis* resume their mobility in high humid conditions, when they disembark and propagate in the nests of *P. japonensis* (Okumura et al., 2013a). In the present study, I tested for possible disembarkation cues in *C. japonica* DL. I also compared the behavioral changes of *C. japonica* DL during their association with *P. japonensis*. Upward movement of *C. japonica* DL was observed during nematode culture (Tanaka et al., 2010a), which suggested negative gravitaxis in *C. japonica*, although DL on the carrier insect have to move down from their carrier. I hypothesized that the gravitactic behavior of *C. japonica* changed during the association and then compared the gravitactic behavior of DL and its possible role in disembarkation.

2. Materials and Methods

Nematodes and insects

C. japonica DL were maintained on dog food medium (Hara et al., 1981) seeded with *E. coli* strain OP50 at 25˚C. Phoretically active DL were collected using a worm picker, washed with distilled water three times before use.

Reproductive diapause females of *P. japonensis* were collected from Hinokuma (October-December 2008 and March 2009) and were used for experiments.

C. japonica DL were artificially loaded on *P. japonensis* as described by Tanaka et al. (2010b). In short, nematode-free reproductive diapauses female *P. japonensis* was prepared by partly soaking in tap water for 3 days and rehydrated nematodes on the insects were washed off. After 24 h inoculation of a reproductive diapauses female of *P. japonensis* with approximately 1,000 *C. japonica* DL in a 6-cm plastic Petri dish, the *P. japonica* loaded with *C. japonica* DL were placed in a desiccator with 97% relative humidity (RH) for 3 days and then in a desiccator with 85% RH for 1 day at 25˚C to obtain quiescent DL with a condition similar to field conditions. K_2SO_4- and KCl-saturated solutions were used to produce 97% and 85% RH conditions at 25˚C, respectively, according to Winston and Bates (1960). After the desiccation treatment, I used *P. japonensis* for disembarkation experiments.

Disembarkation of *C. japonica* from their carrier

I placed a single *P. japonensis* female into a plastic cup (diameter = 7 cm at the bottom and 10.3 cm at the top; deep = 4.3 cm) and the insect was covered with a short stainless steel pipe (diameter = 7.2 cm, height = 3.4 cm), the top end of which was covered with wire netting (pore size: 1 mm^2). A piece of wet Kimwipe S-200

(Kimberly-Clark Co., supplied by Kulesia, Tokyo, Japan) was placed in the cup (Fig. 15A). The plastic cup was covered with a lid and placed in a large plastic box (23×16 ×8.5 cm) with a wet Kimtowel (Kimberly-Clark Co., supplied by Kulesia, Tokyo, Japan) to maintain high humidity in the box. Nematodes that disembarked from insects (N = 10) were collected by washing Kimwipe and small plastic cup at 24-h intervals for 13 days for naturally associated condition because *P. japonensis* hatching occurs about 12 days after oviposition in the field. Collection of nematodes from artificially loaded condition was stopped after 10 days because nematode disembarkation was not observed 8 days after starting experiment. The piece of Kimwipe was replaced every day. After 13 days, the insects were dissected and placed in tap water in a 6-cm Syracuse watch glass for 24 h to separate the nematodes from the insects. The nematodes that remained on the insects were counted using a stereomicroscope. The percentage of disembarkation was calculated based on the total number of nematodes that disembarked from an insect and remained on an insect.

To test possible bacterial disembarkation cues, a 3-cm plate containing NGM (Liuzzi et al., 2012) with a fresh *E. coli* OP50 lawn was also placed in the stainless steel pipe in the plastic cup (Fig. 15A). The total numbers of disembarked nematodes were calculated 13 and 10 days after starting the experiments for naturally associated and artificially loaded *P. japonensis* (N = 10 and 10), respectively.

To examine the disembarkation in soil, I filled a plastic cup (diameter = 3 cm at the bottom and 4.2 cm at the top; deep = 2.4 cm) with soil collected from *P. japonensis* habitat. A few drops of water were added to the cup to maintain high humidity, and a single female *P. japonensis* naturally associated with *C. japonica* was placed in the cup (N = 6). After covering with a lid, the cup containing the single female insect was

placed in a high humidity plastic box (23×16×8.5 cm) with the lid side of the cup facing down. The plastic box was inoculated at 25˚C. Ten days later, I counted the number of *C. japonica* in the soil and on the *P. japonensis*. The nematodes in the soil using the Baermann funnel technique, the number of *C. japonica* was counted using the stereomicroscope. I then calculated the proportion of *C. japonica* that disembarked from their carrier. In a preliminary experiment, I confirmed the absence of *C. japonica* in the soil.

I used the system shown in Fig. 15B to evaluate the effect of *P. japonensis* hatching on disembarkation of *C. japonica*. Reproductive diapause females of *P. japonensis* (N = 9) that are naturally associated with *C. japonica* were incubated individually in 100% RH for 10–14 days in a plastic cup (diameter = 7 cm at the bottom and 10.3 cm at the top; deep = 4.3 cm) before starting the experiment. The nematodes that disembarked during this incubation period were collected and counted using a stereomicroscope each day. A single female of *P. japonensis* was placed in a plastic tube (diameter = 1.5 cm, length = 3 cm) with one side was sealed with a nylon mesh (pore size = 5 μm) and the other side was capped. A small piece of wet Kimwipe was placed inside the tube. Four or five tubes were placed in a plastic cup (diameter = 6 cm at the bottom and 8 cm at the top; deep = 4 cm) with wet cotton wool at the center. A mature egg mass with a surface partly covered by symbiotic bacteria provided for the *P. japonensis* by the mother insect was hung in the center. Synchronized hatching was stimulated intermittently by motor vibration for 20 min, which mimicked the behavior of the mother *P. japonensis*. Synchronized hatching was observed from 10 min after starting the vibration. Nematodes that disembarked from insects during hatching were collected by washing the tubes and Kimwipes about 30 to 90 min after stopping the

vibration, depending on the hatching conditions.

After the hatching experiment, individual *P. japonensis* were placed in plastic cups with high humidity for another 12 days. Nematodes that disembarked during the 12-days inoculation were collected and counted. Next, I placed a tube containing *P. japonensis* and two 3rd-instar nymphs in a plastic cup in high humidity for 24 h, *P. japonensis* was dissected and placed in 10 ml of tap water on a 6 cm Syracuse watch glass for 24 h to release the nematodes from the insects. The nematodes were counted using a stereomicroscope. I calculated the number of nematodes that disembarked during 10-14 days in 100% humidity before starting the experiment, and the total number of nematodes during this period.

Negative gravitaxis experiments

P. japonensis females collected in October (N = 10) and December (N = 13) 2008, and March (N=10) 2009 were immediately used for experiments. The *P. japonensis* females artificially loaded with *C. japonica* DL (N = 5) were kept for 3 days at 25°C after desiccation treatment and were used for experiments. *C. japonica* DL were picked up from *P. japonensis* using a nematode picker and were soaked in M9 buffer to revive them from their quiescent state. About 20 DL were inoculated onto the center of a 9-cm NGM plate. After the water around the DL had absorbed by a NGM plate and they started moving, I set the plate in a vertical position. After 1 h, I counted the number of nematodes that moved upward or downward from the inoculation point. The negative gravitaxis index was calculated as follows: (number of nematodes in upward direction –number of nematodes in downward direction) / total number of inoculated nematodes (Okumura et al., 2013b). Nematodes ± 1 cm from the central line were excluded from

the dataset.

Statistical analysis

ANOVA was used for analyses of disembarkation and negative gravitaxis experiments. Values of negative gravitaxis index were compared using Bonferroni/Dunn test. Non parametric ANOVA, Mann-Whitney's U-test was used analyses for disembarked DL with *E. coli* experiments, and Kruskal-Wallis test was used analysis for disembarked DL with egg hatching and nymphs. All statistical analyses were performed using StatView Ver. 4. 54 (Abacus Concepts, Inc.).

3. Results

Effect of bacterial cues for disembarkation of *C. japonica* DL from its carrier insect

Reproductive diapause *P. japonensis* females with naturally associated *C. japonica* DL on their body surfaces were placed in 100% RH condition; the DL resumed their mobility, although not all nematodes left their carriers within 13 days (Fig. 16). Over 70% of nematodes diesmbarked from three of 10 insects, whereas <50% of the associated nematodes disembarked from the other seven insects. Less than 60% of the nematodes left the insects during the inoculation period after artificial loading.

To determine possible disembarkation cues other than high humidity, I used a small NGM plate with *E. coli* lawn as a food cue. Many nematodes remained on the carrier insects in the presence of *E. coli*, and the percentages of nematodes that disembarked from the insects the presence or absence of *E. coli* were not significantly different in naturally associated nematodes and in those grown on *E. coli* and artificially loaded onto the insects (Mann-Whitney's U-test, P = 0.9698, and 0.0013, respectively) (Fig. 17). Fresh soil from *C. japonica* habitat also failed to stimulate disembarkation, from the soil and 24% *C. japonica* DL disembarked from their carrier insect within 10 days of starting the experiments (data not shown).

Effect of hatching and nymphs on *C. japonica* DL disembarkation from their carrier insects

Before hatching, a mean of 7.7% (minimum = 1.8%; maximum = 15.4%) of DL left from the *P. japonensis* during the 14-days inoculation period in high humidity conditions. Very few nematodes disembarked in response to the *P. japonensis* hatching stimuli (Fig. 18). After hatching, *P. japonensis* was placed in 100% RH condition for a

further 12 days, but very few nematodes (maximum = 7 DL) disembarked during this period. In contrast, when the *P. japonensis* females that were inoculated for 12 days and exposed to nymphs, significantly high percentages (6.1% -76.3%) of DL disembarked from the carrier insect within 24 h, comparing to the 14-day inoculation and hatching stimuli (Kruskal-Wallis, $P < 0.0001$) (Fig. 18).

Changes in gravitactic behavior of *C. japonica* DL

C. japonica DL showing nictation had a tendency to move upward which is opposite movement from disembarkation. I compared the gravitactic behavior of *C. japonica* DL collected from *P. japonensis* under different conditions (Fig. 19). *C. japonica* DL exhibited upward movement, and their NGI values were positive from 3 days after artificial loading. In contrast, significantly larger numbers of nematodes on insects collected in the field during October, December, and March moved down ward on an NGI plate (ANOVA, $P < 0.001$). The NGI values for October, December, and March were negative and significantly lower than that of 3 days after artificial loading.

4. Discussion

C. japonica DL are phoretically associated with their carrier insect *P. japonensis* females, they have to leave their carrier insect for their propagation. In a previous study, I demonstrated that *C. japonica* DL disembarked and propagated in *P. japonensis* nest (Okumura et al., 2013a). A high humidity is necessary condition for resumption of nematode mobility, but there may be cues for disembarkation. In this chapter, I tested other possible disembarkation cues in the form of soil, food bacteria, *P. japonensis* hatching, and *P. japonensis* nymphs. The presence of nymphs was the most effective cue for disembarkation by *C. japonica* DL. I also found that duration of the association with the insect affected the gravitactic behavior. These results suggest that the presence of nymphs in high humidity as well as a gravitactic behavioral change of DL on their carrier are important factors for disembarkation of *C. japonica*.

Humidity is one of the most important abiotic factors that affects nematode activity. It is also a useful cue for phoretic nematodes on their carrier because it indicates that they have reached their propagation site. A key factor disembarkation cue for *C. remanei* is humidity, and *C. remanei* DL easily disembark from their carriers in high humidity condition (Baird, 1999). However, in my thesis, I found that high humidity was not a sufficient cue for *C. japonica* DL disembarkation.

C. japonica is a bacterial feeding nematode, and thus, I thought that the presence of *E. coli* as a food source or habitat soil containing microbes, leaf litter, and plant roots, may trigger the disembarkation. However, the presence of *E. coli* and soil did not stimulate disembarkation by *C. japonica* DL. When *C. japonica* propagates in the nest of *P. japonensis*, there are fresh egg carcasses and nymphal cadavers of *P. japonensis* soon after hatching and *C. japonica* seems to feed on fresh nutrients from *P.*

japonensis and not bacteria (Okumura et al., 2013a). These previous observations and the present results suggest that the presence of bacteria is not a preferred cue for disembarkation *C. japonica* DL.

In the previous study, very few DL were detected on mother insects after hatching (Okumura et al., 2013a). Immediately before hatching, the mother insect spreads symbiotic bacteria on the surface of the egg mass. The symbiotic bacteria on the egg mass are ingested by the *P. japonensis* nymphs while feeding on trophic eggs provided for the nymphs, which are used for nitrogen recycling in *P. japonensis* (Hosokawa et al., 2010). Thus, I considered that a disembarkation cue may be provided by the symbiotic bacteria. Another possibility was that hatching itself could trigger nematode disembarkation. Unexpectedly, however, almost no disembarkation was observed in the presence of symbiotic bacteria and hatching stimuli.

The most effective DL disembarkation cue was the presence of *P. japonensis* nymphs. Nymphs actively feed on drupes that they metabolize for their growth. Odors from the nymphs could be an important disembarkation cue ensuring the presence of food for *C. japonica* DL. Physical stimuli such as movements and/or sounds by the nymphs may also contribute. In the present study, I was not able to prepare *P. japonensis* females that were treated with neither hatching nor nymphal stimuli for comparison because of the limitation of *P. japonensis* numbers. Thus, I cannot deny the possibility that not only the presence of nymphs but also the combinations of incubation in high humidity, hatching stimuli, and the presence of nymphs, stimulate the disembarkation. There is also a possibility that a longer incubation period also stimulates disembarkation. In my preliminary observation, however, longer incubation in high humidity did not stimulate disembarkation very well. The presence of nymphs

enhanced disembarkation, but not all DL disembarked. Thus, a combination of symbiotic bacteria, hatching, the presence of nymphs, and/or the consequences of these phenomena may constitute the overall disembarkation cue, which indicates favorable conditions for *C. japonica*.

I used reproductive diapause females because of the difficulty of preparing sufficient numbers of gravid females in the present study. In a preliminary study using gravid *P. japonensis* females, the disembarkation profile in high humidity conditions was similar to that with reproductive diapause *P. japonensis* females. However, I could not exclude the possible effect of the physiological condition of the mother insect from the present study.

Nictating *C. japonica* DL show a negative gravitactic behavior (Okumura et al., 2013b). The negative gravitactic behavior of DL appears to increase the opportunity to encounter its carrier insects wandering on the ground and useful for embarkation. In the present study, *C. japonica* DL which artificially loaded onto *P. japonensis* for 3 days still showed the negative gravitactic behavior. On the other hand, DL from *P. japonensis* collected in October, December, and March exhibited positive gravitaxis. New adults of *P. japonensis* emerge in late June to early August. The DL from *P. japonensis* in October, December, and March had been associated with *P. japonensis* for more than 2 months. The length of duration of the association may have altered the behavioral change. I do not know the mechanism that underlies this gravitactic change, but this behavioral change appears to be important for the disembarkation from their carrier. In the present study, I used not only naturally associated DL but also artificially loaded DL for negative gravitaxis experiments because it was difficult to obtain *P. japonensis* with *C. japonica* DL immediately after nematode embarkation in the field. However, because

artificially loaded DL were similarly found at the body parts of *P. japonensis* and were morphologically similar to naturally associated ones, my results are suggestive of the natural phenomenon in the field. Further study is necessary to elucidate the mechanisms of changes of the gravitactic behavior under controlled conditions.

In my previous studies, we found that *C. japonica* forms a species-specific and female-host biased phoretic/necromenic association with *P. japonensis* (Okumura et al., 2012, 2013a; Yoshiga et al., 2013). The survivorship of *C. japonica* DL is maintained high over months on *P. japonensis* while is low without *P. japonensis* (Tanaka et al., 2010b, 2012). Moreover, my present results suggest that the disembarkation of *C. japonica* DL is controlled by complex mechanisms to ensure its association with *P. japonensis*. These data indicate that the life cycles of *C. japonica* and *P. japonensis* are synchronized and *C. japonica* has sophisticated mechanisms to associate with *P. japonensis*.

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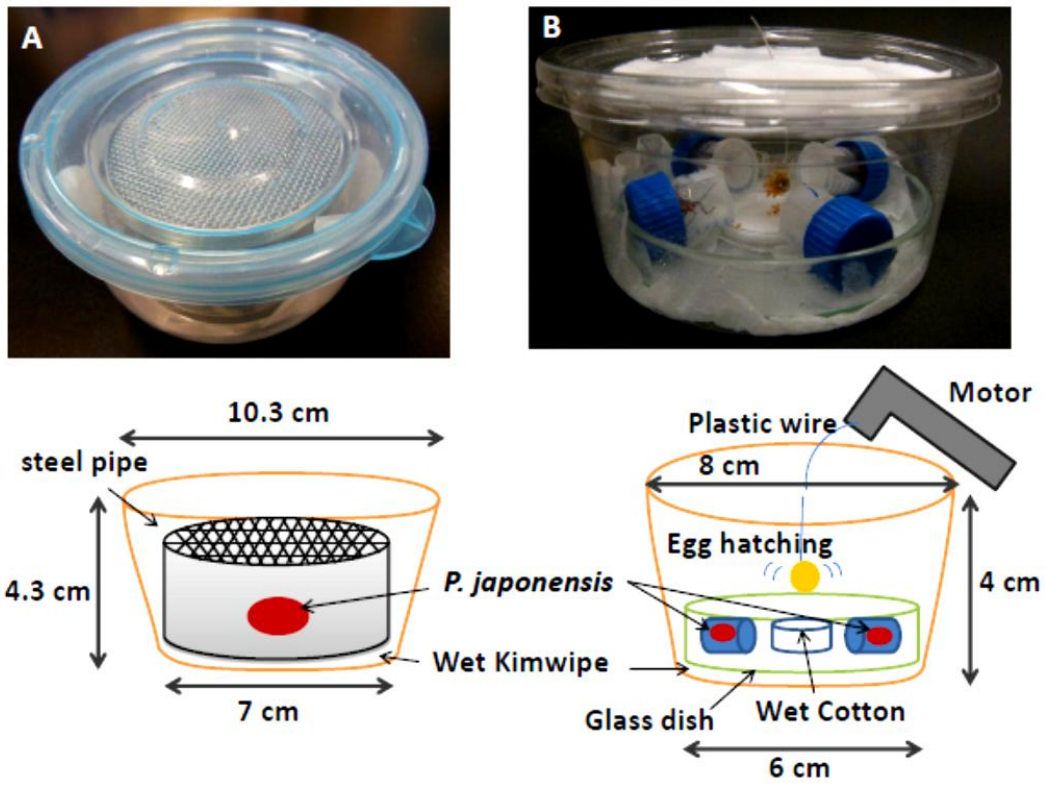

Fig. 15. Systems used for disembarkation. To keep high humidity, wet Kimwipe was put at the bottom of cups. A, a plastic cup for disembarkaion experiments. A *P. japonensis* female was placed in a steel pipe in the plastic cup. The plastic cup was used for most experiments, except the hatching experiment. B, an equipment for hatching experiment. Four or five tubes each containing a female *P. japonensis* were placed in the cup. An egg mass was hung by a nylon fishing line from the center of a lid and synchronized hatching was stimulated intermittently by motor vibration for 20 min.

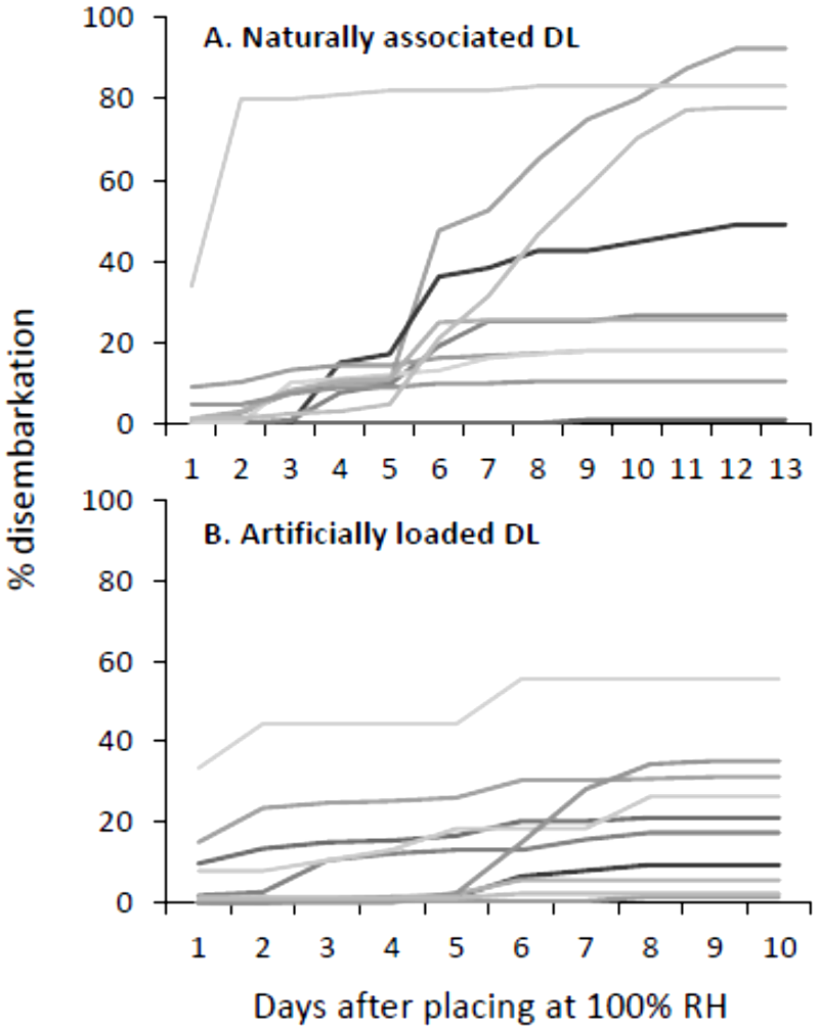

Fig. 16. Disembarkation profiles of *C. japonica* DL from their *P. japonensis* carrier in high humidty conditions. Reproductive diapause females that naturally associated with *C. japonica* DL and artificially loaded females were exposed to 100% humidty (Fig. 15A) and the daily disembarkation rate was recorded. Each line indicates the accumulated percentage of disembarked nematodes from a single *P. japonensis*. Ten insects were used for each condition.

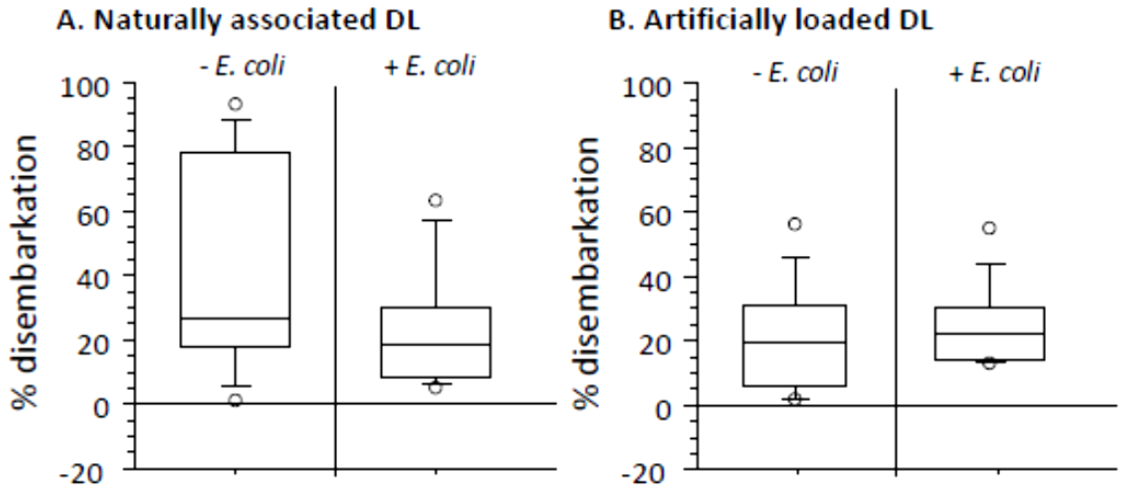

Fig. 17. Effect of bacteria on embarkation of *C. japonica* DL. *Escherichia coli* strain OP50-containing culture was placed in a plastic cup (Fig. 15A) with reproductive diapause *P. japonensis* females. Ten *P. japonensis* females that were naturally associated with or artificially loaded *C. japonica* DL, were used for each experiment. Total numbers of disembarked nematodes were calculated 13 and 10 days after starting experiments for naturally associated and artificially loaded DL, respectively. The line inside the box is the median, the top of box is third quartile, the bottom of the box is first quartile, the upper bar is 90th percentile, the lower bar is 10th percentile, and the white circle is outlier. Error bars indicate s. d. There were no statistical differences (Mann-Whitney's U-test, A, P= 0.9698; B, P= 0.0013).

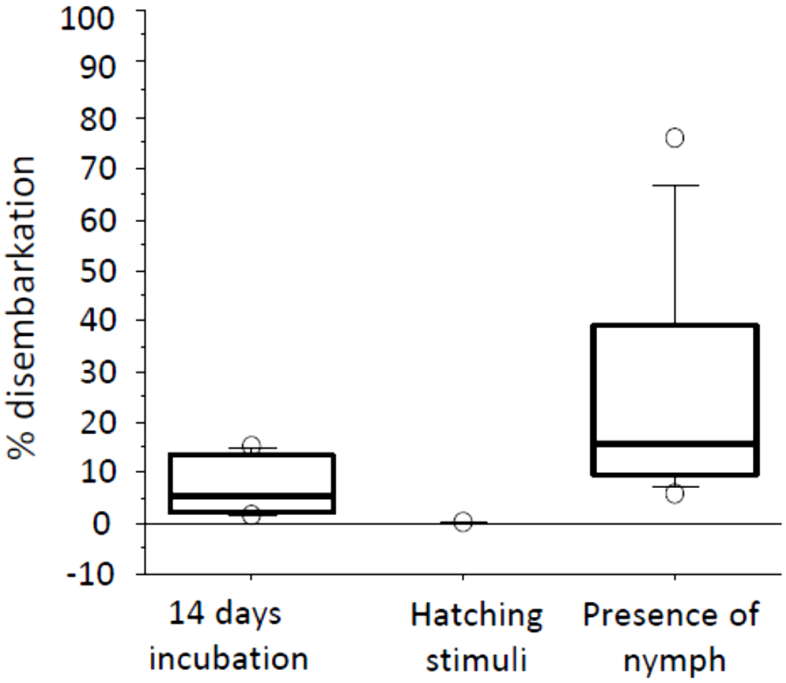

Fig. 18. Comparison of *C. japonica* DL disembarkation from *P. japonensis* in different conditions. The extent of disembarkation during the first 14 days in the presentce of high humidity condition, hatching, and 3rd instar *P. japonensis* nymphs were compared (N = 9). *P. japonensis* females were initially inoculated in high humidity conditions for 14 days to remove DL before being exposed to hatching stimuli. After being exposed to the hatching stimuli, the insects were inoculated for another 12 days in high humidity condition (Fig. 15A) and then exposed to nymphal stimuli (Fig. 15B). No nematodes disembarked (except 1 sample in which a single nematode disembarked) during the 12-days inoculation period. The line inside the box is median, the top of box is third quartile, the bottom of the box is first quartile, the upper bar is 90th percentile, the lower bar is 10th percentile, and the white circle is an outlier. Error bars indicate s. d. (Kruskal-Wallis, $P < 0.0001$).

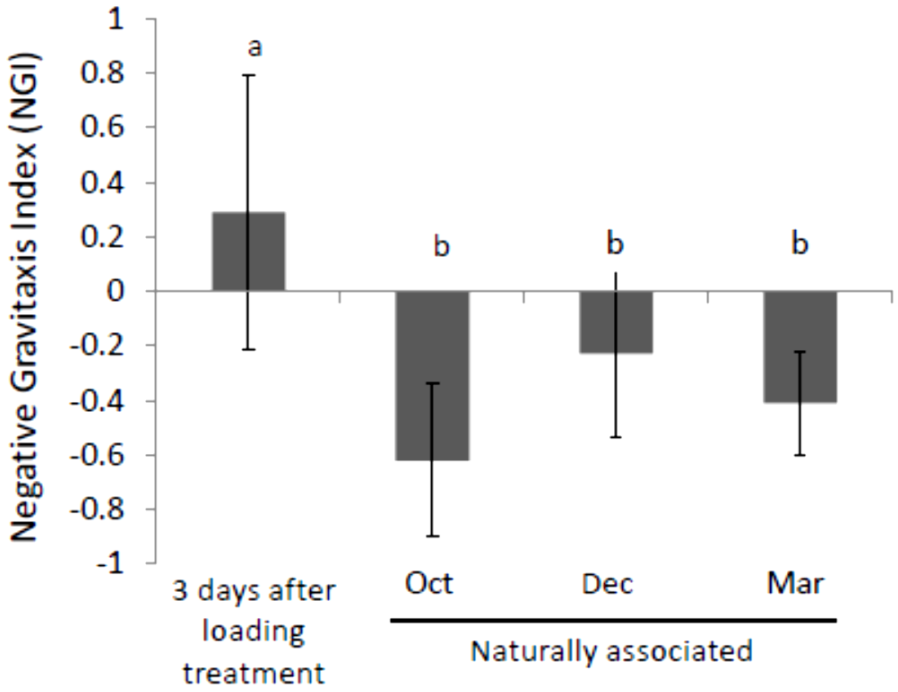

Fig. 19. Comparison of the gravitactic behavior of *C. japonica* DL in different conditions. *C. japonica* DL were collected from *P. japonensis* 3 days after artificial loading with *C. japonica* and desiccation treatment (N = 5) and from *P. japonensis* collected during October, December, and the following March (N = 10, 13, and 10, respectively). Error bars indicate s. d. Different letters above the columns indicate a statistically significant difference as detected by ANOVA with Bonferroni/Dunn test (P<0.0083).

General Discussion

Many nematode species have different kinds of relationships with other animals. Phoresy is one of such relationships. Phoretic nematodes use other animals for transportation to obtain fresh resources and to protect themselves from unsuitable biotic and abiotic environments (reviewed in Timper and Davies, 2004; Barbercheck and Duncan, 2004). To form a phoretic association, nematodes need to find a carrier. However, there is little information on how they reach, find, and recognize their hosts. In this thesis, I studied host-finding behavior of *C. japonica* DL. I demonstrated that *C. japonica* DL show negative gravitactic behavior, attraction to odors and body surface components from *P. japonensis*, and embarkation onto the insect. In addition, I found out the conditions for disembarkation of from *P. japonensis*. The results of my thesis clearly indicate that *C. japonica* has established complex and sophisticated behaviors to form a species-specific and female host biased phoretic association with *P. japonensis*. In this chapter, I discuss the behavioral sequence of nematodes to form the association with their host based on the results from my *C. japonica* study.

The first important step to form phoretic or parasitic association is host searching. Increasing opportunities to encounter with hosts lead to the success of association with their hosts. In my study, I found two important behaviors in *C. japonica* DL: negative gravitaxis and orientation to host odors. The negative gravitaxis will help DL migrate upward to reach the ground. DL on the ground may nictate to find the direction to *P. japonensis* and/or touch to their host. The olfactory experiments indicated that *C. japonica* DL recognize and orient to *P. japonensis* odors. These behaviors apparently increase the opportunities to encounter *P. japonensis* wandering on the ground. Parasitic nematodes respond to CO_2, which is often suggested as a cue from

hosts (Sciacca et al., 2002). However, because CO_2 is released from many different organisms and abiotic factors, it may be not a definite cue for phoretic and parasitic nematodes. Although neither negative gravitaxis nor orientation to host odors by host searching stages have not been reported in other nematode species, these behaviors may be observed in other phoretic and parasitic stages of nematodes.

The second step is the host recognition and embarking. When phoretic or parasitic stages of nematodes encounter with other organisms, they have to distinguish their appropriate hosts from others. In case of *C. japonica*, DL have to recognize and embark onto *P. japonensis*. Chemoattraction and loading experiments demonstrated the species-specific recognition and embarkation in *C. japonica*. I found that surface components of *P. japonensis* attracting and arresting the dispersal of *C. japonica* DL may be used by DL to distinguish *P. japonensis* from other animals. I was not able to identify the chemicals but the recognition of the chemicals must be the key for *C. japonica* to form the specific phoresy. There is no information on such species-specific phoresy or parasitism in nematodes other than *C. japonica*. *C. japonica* is exceptionally specific to a single species of host insect while other nematodes are usually less specific and have wider host ranges in many cases. For examples, *C. elegans* that forms phoretic and necromenic associations with organisms is often found in association with diverse such as snails, isopods, and myriapods (Barrière and Félix, 2005; Caswell-Chen et al., 2005; Chen et al., 2006; Kiontke and Sudhaus, 2006). Species in the genus *Steinernema* and *Heterorhabditis* are known as entomopathogenic nematodes and are able to infect wide ranges of insects (reviewed in Tanada and Kaya, 1993). However, these nematodes also have to recognize their hosts to embark onto or enter them. Further researches of *C. japonica* host recognition may provide an insight to elucidate the host recognition

mechanisms as well as the host range determinations in other phoretic and parasitic nematodes.

The third step is disembarkation. This step is specific for phoretic nematodes. *C. japonica* DL on *P. japonensis* have to disembark for their propagation because of the lack of food on the insects. My question was how they decide to disembark from their hosts and what is the cue for disembarkation. From my results, the presence of at least 3 important factors was indicated: behavioral change to gravity, humidity, and chemical stimuli. Negative gravitaxis of *C. japonica* DL is an important behavior for embarkation, which ensures the association. On the other hand, it may inhibit the disembarkation for nematode propagation. A change of gravitaxis of DL from negative to positive after the association with *P. japonensis* is a remarkable finding to understand the behavioral change in DL. Even after the behavioral change, the association of DL with *P. japonensis* is secured physically by humidity. Moreover, even in high humidity, many DL are still associated with *P. japonensis* until hatching of offspring. Very limited information is available on disembarkation thus far (Baird, 1999). Findings indicate the presence of complex mechanisms to ensure the timing of disembarkation. Further researches clarifying molecular basis of the mechanisms are necessary to understand the evolution of such complex regulatory mechanisms.

I revealed the specialized host-searching behaviors of *C. japonica* DL to form phoretic and necromenic association with *P. japonensis*. *C. japonica* is a close relative of a model organism *C. elegans*. The established methods as well as large amount of useful information from *C. elegans* researches can be applied to further researches of *C. japonica*. There are many nematode species that are parasitic to animals and plants, but studies on the parasitic and phoretic behaviors are not yet studied very well. Further

molecular and cellular studies will elucidate the mechanisms not only of the phoretic behaviors in *C. japonica* but also of other phoretic and parasitic behaviors of nematodes.

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