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SHORT NOTE

Development of experimental mesocosms for cicada nymphs *Graptopsaltria nigrofuscata*: methodology and research recommendations

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Abstract

The experimental mesocosm, an artificial enclosure system for soil organisms, is a major research tool in soil ecology. Development of a rearing method for target organisms within mesocosms is essential for conducting mesocosm experiments. Cicadas (Hemiptera: Cicadoidea) are assumed to play important roles as root feeders and ecosystem engineers in soil ecosystems. Nonetheless, their ecological roles have not been investigated to the same extent as other root -feeding species, possibly because it is difficult to rear cicadas in mesocosm experiments. Here, we report a rearing method for cicada nymphs (*Graptopsaltria nigrofuscata*) in an experimental mesocosm over a period of 3 months. We prepared eight polyvinyl chloride cages filled with mineral soil in a forest. One final instar (0.33 0.10 g fresh weight [MeanSD]), and one early instar cicada nymph (2.230.16 g fresh weight [MeanSD]) were placed into each mesocosm cage with a seedling of Japanese larch (*Larix kaempferi*) as a food source. After 3 months, 75 % (6 to 8) of final instar cicada nymphs survived and increased in fresh weight by approximately 6 % since the beginning of the experiment, whereas all early instar nymphs had died. This is the first study to provide a detailed rearing method for cicada nymphs in East Asia, a region that harbors a high species diversity of cicadas. Our mesocosm system could be an effective tool for understanding the ecology and ecological roles of final instar cicada nymphs in belowground ecosystems.

Keywords above- and belowground linkage | field experiment | rearing method | root feeders

1. Introduction

In soil ecosystems, there are a large number of species that intricately interact with others, including fungi, microbes, plants (roots), and animals (Bardgett & van der Putten 2014, Tsunoda & van Dam 2017, Thakur et al. 2019). This complexity makes it difficult to understand belowground species interactions, and their roles in ecosystem processes. Experimental mesocosms, artificial enclosure systems of soil organisms, are one of the major research tool in soil ecology (Odum 1984, Kampichler et al. 2001). Mesocosms contribute to a better understanding of belowground species interactions and their roles in soil ecosystem processes by controlling environmental conditions and the number of interacting species (e.g. De Deyn et al. 2007, Makoto et al. 2019). Even though the development of rearing methods for target soil organisms within mesocosms is essential for conducting mesocosm experiments, rearing methods have only been established for a part of soil animals. This bias leads to a skewed understandings of belowground species interactions.

Root-feeding insects are an important group of soil animals that link above- and belowground subsystems, and drive terrestrial ecosystem processes via root

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consumption and changes in physicochemical soil properties (Blossey & Hunt-Joshi 2003, Wardle et al. 2004, Gan & Wickings 2020). Cicadas (Hemiptera: Cicadoidea), one of the largest root feeding insects, are assumed to play an important role as prey and detrital resources derived from their cadavers in terrestrial ecosystems (Hunter 2001, Yang 2004, Tomita 2021); however, their role as root feeders during nymphal stages is mainly investigated by field observation. Thus, there is not a clear causal understanding of their importance as root feeders in terrestrial ecosystems compared to other root feeders such as scarab larvae (Coleoptera: Scarabaeidae) and root aphids (Hemiptera: Aphidoidea) (Karban 1980, Hunter 2001). This knowledge gap is partly due to the difficulty in rearing cicada nymphs for mesocosm experiments (Kato 1956) compared to other root feeders. Most studies test the effects of root- feeding insects on plants and other arthropods (e.g. foliar herbivores) by conducting micro- or mesocosm experiments (e.g. Tsunoda et al. 2018, Soler et al. 2005). Progress in rearing cicada nymphs for mesocosm experiments is hindered by the assumption that they can only grow under mature trees (Dybas & Lloyd 1974). Although it is not realistic to use mature trees for mesocosm experiments, the use of tree seedlings would allow us to easily prepare experiments with a large number of replications (e.g., Logan et al. 2014). Thus, the application of seedlings to mesocosm experiments using cicada nymphs may facilitate progress in experimental approaches to cicada biology.

To the best of our knowledge, there are only two published studies that have reared cicada nymphs: *Amphipsalta zelandica* nymphs in New Zealand at high survival rates (Logan et al. 2014), and *Magicicada* spp. nymphs in North America at very low survival rates (Karban et al. 2000). Additionally, Boulard & Mondon (1995) showed the nymphal length of Cicada orni nymphs by rearing them. This is despite there being an enormous number of cicada species worldwide (Marshall et al. 2018). For example, East Asia harbors a high species diversity of cicadas (Hayashi & Saisho 2012), but rearing methods for cicada nymphs in the region have yet to be established. Therefore, further development of rearing methods for nymphs of other cicada species is important to deepen the understanding of their ecology. So this research focuses on the methodology of rearing conditions of cicadas in mesocosms; thus it is rather a methodological than a pure scientific publication. As the methods constitute the prerequisites for further comprehensive research planned we decided to present our approach and initial results in this paper and report a novel rearing method for nymphs of *Graptopsaltria nigrofuscata* in experimental cages planted with tree seedlings (Fig. 1). Further, we suggest some additional research questions that our mesocosms would be able to examine.

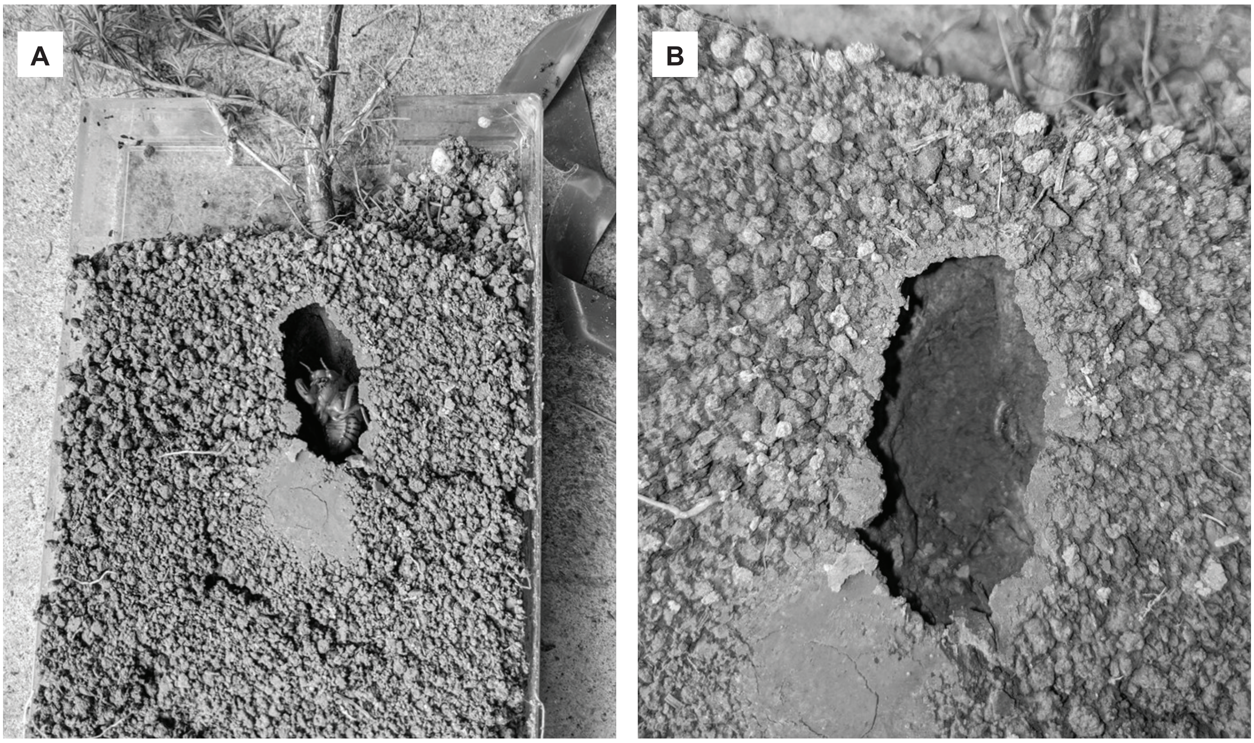

Figure 1. Photographs of the mesocosm experiment. (**A**) a final instar nymph of *Graptopsaltria nigrofuscata* cicada in a mesocosm cage. (**B**) An empty burrow made by a cicada nymph. The nymph might feed on larch root at the interior of burrow. Photographs were taken at the end of mesocosm experiment (7 July).

2. Materials and methods

For our experiment we used *G. nigrofuscata* which is one of the most common cicadas in Japan. The adults of this species oviposit their eggs on dead branches or the bark of tree trunks in summer, and these eggs hatch during the following summer (Moriyama and Numata 2008). The newly hatched nymphs must immediately burrow into the soil to avoid desiccation and predation; moreover, this developmental stage has the highest mortality rate of their life cycle (Moriyama and Numata 2006). Unlike periodical cicadas, *G. nigrofuscata* nymphs are assumed to spend a relatively short period (2–5 years) underground (Hayashi and Saisho 2012).

On 14 April 2021, we collected soil-dwelling *G. nigrofuscata* nymphs from a forest in southern part of Hokkaido, northern Japan (42°12’N, 140°45’E). The forest mainly consists of elm (*Ulmus davidiana*) and cherry trees (*Prunus sargentii*). Prior to collecting samples, we found that a large amount of cicadas had already emerged from this forest based on evidence we observed in August 2020, including exuviae and cicada songs. It is important for effective sampling of cicada nymphs to pay attention to the aboveground evidence because the nymphs do not produce any sound signals and thus their presence cannot be directly confirmed without digging up the soil. We collected cicada nymphs by digging up the soil (~20 cm depth and 2–3 m^2 area at several locations, a total of 10 m^2) using a shovel. As a result, we could collect 20 final instar and 10 early instar nymphs. After collecting the cicada nymphs, we carefully transported them to the experimental site by placing them in a cooler box (45 cm long, 28 cm wide and 36 cm deep). We added the collected soil and some fragmented roots as their food to the cooler box to mitigate large fluctuations in soil temperature and provide a food resource during transportation. The abovementioned collection of cicadas was conducted by one author (K.T.) for a duration of about three hours, indicating that collecting belowground cicada nymphs is relatively easy if a location with high nymphal density is selected.

Our mesocosm experiment was conducted from 16 April to 6 July, 2021 in a Japanese larch stand (*Larix kaempferi*) in the Teshio Experimental Forest (TEF) of Hokkaido University in northern Hokkaido (44°57′N, 142°01′E). The mean soil temperature in mid-April was 5.9°C at the cicada collection site and 5.6°C at the experimental site in the TEF, indicating that the thermal environment for cicada nymphs did not differ considerably between the two sites. The mean daily soil temperature at the experimental site during our mesocosm experiment ranged from 4.0°C to 16.0°C (Fig. S1).

On 16 April 2021, after two days from when sampling cicada nymphs, we prepared eight polyvinyl chloride cages (29 cm long, 19 cm wide and 3 cm deep), filled with the sieved mineral soil. We planted a larch seedling (0.810.12 cm stem diameter and 35.784.39 cm height) and placed two cicada nymphs (one final instar and one early instar nymphs into each mesocosm cage. The samples were placed in a cooler box until the experiment. We made some 2 mm diameter hole on the bottom of the mesocosms for water drainage. Early instar nymphs were assumed to be unable to escape from the mesocosm through these holes because their abdomen width was generally greater than 2 mm (Author *unpublished data*). Each mesocosm contained 1.2 kg of wet soil (gravimetric moisture content was approximately 40 %) after sieving (with a 4.75 mm sieve) to homogenize the soil and remove gravel, fine roots, and coarse woody debris (as conducted in Makoto et al. 2019). In April, the mean fresh weights of cicada nymphs in this study differed substantially between the two nymphal stages (early instar: 0.33 0.10 g, final instar: 2.230.16 g), although we were not able to identify the age of early instar nymphs correctly. Eight ditches were made at 5 m intervals to bury the mesocosms at the experimental site. The mesocosms were buried in the soil so that the base of the mesocosms reached a depth of 25 cm. Burying the mesocosm cages in soil is important as it ensures that the soil temperature range in the cages is similar to that of natural field conditions (Logan et al. 2014). Given that *G. nigrofuscata* usually starts emerging from mid-July (Hayashi & Saisho 2012), the end of the mesocosm experiment was 6 July 2021. After the experiment, we measured fresh weight of the surviving nymphs. To examine whether cicada nymphs grew or not during the experimental period (80 days), we compared the fresh weight of the nymphs between the beginning and the end of the mesocosm experiment using a paired-samples t-test.

3. Results and Discussion

Whereas all early instar cicada nymphs had disappeared by the end of the mesocosm experiment, 75 % (6 to 8) of the final instar cicada nymphs had survived and increased fresh weight by 6 % on average since the beginning of the experiment (16 Apr. : 2.230.16 g, 6 July: 2.370.17 g). A paired t-test indicated a significant increase in the fresh weight of the nymphs from April to July (Fig. 2, t = -2.8334, df = 5, p-value = 0.03653). This implies that the final instar nymphs of cicadas successfully grew within our mesocosms. This is the first report showing that final instar cicada nymphs gain weight even during their emergence year. Previously, it had been assumed that nymphs were preparing for the emergence without an increase in body mass during this developmental stage (Kato 1956, White & Lloyd 1975).

Unfortunately, all early instar nymphs disappeared from the mesocosm cages. Given that we found their cadavers in the cages, and the diameter of the cages' drainage holes were smaller than their abdominal width (Authors *personal observations*), we concluded that early instar nymphs died in the cages during the mesocosm experiment. One reason for the difference in survival rates between instars may be age differences in tolerance to translocation. Cicada nymphs could not feed on roots for at least two days during translocation from the sampling site to the experimental site. Although they survived at the beginning of the mesocosm experiment, translocation might debilitate early nymphs more than final instar nymphs, possibly due to starvation. Another possible cause of high mortality in the early instar nymphs is predation by organisms invading into the cage. The development of a rearing method for early instar nymphs is a problem to be resolved by future studies.

Traditionally, it has been assumed that the nymphs of forest-dwelling cicadas grow only under mature trees because they generally inhabit mature forests (Dybas & Lloyd 1974). Thus, tree seedlings are assumed to be incapable of nourishing cicada nymphs as they provide insufficient nutrition. This assumption hinders the progress of research on cicada ecology because it is difficult to use mature trees for mesocosm studies since they are too large for experimental treatment. However, we found that seedlings (~ 30 cm in height) were sufficient for the growth and survival of *G. nigrofuscata* nymphs for at least several months. We believe that this finding is a step forward in the study of cicada ecology. We should further explore whether cicada nymphs can survive under tree seedlings for longer periods (over 1 year) to understand the long-term role of cicadas in soil ecosystems and how to rear early instar nymphs in a mesocosm.

Although we only succeeded in rearing final instar nymphs for several months, our mesocosm system has the potential to answer questions about basic cicada biology, and above- and belowground species interactions. First, our mesocosm allowed us to evaluate the differences in individual growth patterns of cicada nymphs among host tree species. Cicadas utilize a wide variety of host species, but their density usually differs among tree species (Dybas & Lloyd 1975, Tomita & Hiura 2021, Lee et al. 2010). One possible mechanism driving difference in nymphal growth rates among host species is variation in xylem sap quality of host trees. We can test this mechanism by preparing mesocosms planted with a wide variety of tree species.

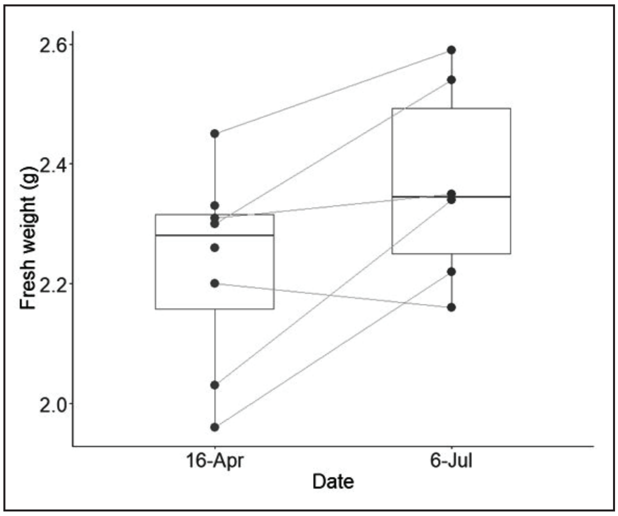

Figure 2. A paired box plot for comparing fresh weight (gram) of final instar *Graptopsaltria nigrofuscata* nymphs between the beginning (16-Apr, Blue) and the end (6 Jul, Red) of the mesocosm experiment. Two points of 16 Apr data with no line connected to 6 Jul are fresh weight of cicada nymphs that died until the end of experiment. Paired t-test indicated a significant increase in fresh weight of the nymphs from April to July (t = -2.8334, df = 5, p-value = 0.037).

Second, our mesocosm can be used to understand ecological roles of cicada burrows. Cicada burrows provide an aerobic habitat for other soil animals (e.g. Maezono & Miyashita 2000). Excretion from cicada nymphs may also affect other soil organisms, such as microbes and detritivores which play important roles in soil ecosystem processes by altering soil water and nutrient conditions around cicada burrows. Alteration of soil water and nutrient conditions due to cicada nymph excretion may feedback to host trees via root foraging. Moreover, an understanding how long the ecological functions of cicada burrows persist after cicada emergence shed light on the legacy or afterlife effects of root- feeding insects (Gan & Wickings 2020). Although it is impossible to extract and observe cicada burrows in a non-destructive manner under natural conditions, our mesocosm can be used to elucidate the functions and post-emergence persistence of cicada burrows.

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Supplementary Figure 1

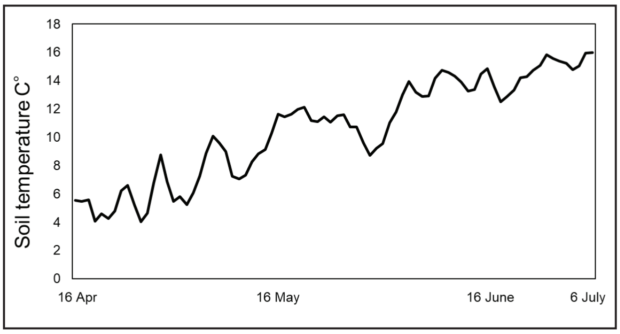

Figure S1. Mean daily soil temperature during the mesocosm experiment (16 April to 6 July 2021).