Improving rearing methods for Meteorus pulchricornis (Hymenoptera: Braconidae) using Ephestia kuehniella (Lepidoptera: Pyralidae) larvae as substitute hosts

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Improving rearing methods for *Meteorus pulchricornis* (Hymenoptera: Braconidae) using *Ephestia kuehniella* (Lepidoptera: Pyralidae) larvae as substitute hosts

スジコナマダラメイガ幼虫を代用宿主として用いることによる

ギンケハラボソコマユバチの飼育法の改善

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CONTENTS

CHAPTER I

General introduction

1. Biological control

Biological control is an environmentally safe and effective method of pest control that uses other living organisms. Biological control can be conducted by utilizing herbivory, predation, parasitism or other natural mechanisms (Eilenberg et al., 2001).

The first record of the use of an insect species as a biocontrol agent is in the Jin dynasty (304 A.D., China); predatory ants *Oecophylla smaragdina* Fabricius (Hymenoptera: Formicidae) were used to manage hemipteran pests by Chinese citrus farmers (Huang and Yang, 1987).

Likely, the most well-known example of biological control took place in the 1880s, when the invasion of the cottony cushion scale *Icerya purchasi* Maskell (Hemiptera: Monophlebidae) almost devastated California's citrus industry. By introducing natural enemies, i.e., the parasitic fly *Cryptochaetum iceryae* Williston (Diptera: Cryptochaetidae) and the vedalia beetle *Rodolia cardinalis* Mulsant (Coleoptera: Coccinellidae) from Australia in 1888, the damage of the cottony cushion scale was successfully controlled (Metcalf et al., 1973).

Biological control has become a main-stream practice over recent decades for many reasons. For example, first, owing to the long history of using chemical pesticides, some arthropod pests have developed resistance to pesticides forcing farmers to use alternative pest control methods (Gerhardson, 2002). Second, ecological changes caused by continuous use of chemical pesticides may permit the resurgence of pests to become more severe and common (DeBach et al., 1971; Flint and Dreistadt, 1998). Third, consumers have become more aware of health and environmental issues caused by chemical pesticides (WHO, 1990). Therefore, demand has increased for biological control, especially in the adoption of integrated pest management (IPM) programs, as well as the demand for organic cropping. The two main biological control strategies are

classical biological control and augmentation.

1.1. Classical biological control

Classical biological control is the method of introducing exotic control agents in order to control pest organisms by achieving permanent or long-term establishment of natural enemy populations, after a pest has lost its natural enemies in moving to new habitats or by any environmental changes. The aim of classical biological control is to restore and then maintain the balance of pest populations with their natural enemies. This is accomplished by importing parasitoids, predators, or pathogens usually from the original home range of the pests (Eilenberg et al., 2001). Although classical biological control cannot eliminate pest populations entirely, when combined with IPM programs, it can reduce the pest populations and the damage caused, to levels below the economic injury level, thereby providing long-term and sustainable control at minimal recurring cost. Nevertheless, classical biological control measure is usually only effective against invasive pests on perennial crops. For biological control of insect pests on annual crops like vegetables, the following method, augmentation, should be considered.

1.2. Augmentation

Mass release of natural enemies into the fields is called augmentation. There are two types of augmentation methods. One is inoculative release, in which the released natural enemies reproduce themselves for some generations to provide more long-term effects of pest control (Van Driesche and Bellows 1996). The other method is inundative release, also called mass-release. In an inundative release, natural enemies are released in large quantities in order to achieve a rapid effect or immediately control the pest

populations (Eilenberg et al., 2001; Yano, 2018).

The efficacy against target pests is critical in augmentation, but it cannot be successful if the efficient mass-rearing system of natural enemies is not yet established. For that purpose, inexpensive and easily manageable artificial diets, substitute prey, or substitute host insects need to be provided.

1.3. Parasitoids

Parasitoidism is a type of parasitism found in the living organisms whose larvae can develop by feeding on the body of their hosts, eventually kill them. As common natural enemies of various insects, parasitoids have been known to play an important role in insect ecological dynamics and biological control of pest insects (Mills, 2009).

Parasitoids are classified into several types by the oviposition behavior of adults or feeding behavior and development of larvae (Shaw and Huddleston, 1991; Quicke, 2015). Solitary parasitoids feed and develop alone on a host. Whereas, gregarious parasitoids feed and develop together with multiple individuals on a single host. Endoparasitoids develop within the body of their hosts, while ectoparasitoids develop externally on the body of their hosts. Some parasitoids permanently paralyzing their hosts by venom injection at oviposition and providing larvae with an immobilized and static resource, usually concealed in plant tissues, are called idiobionts. Meanwhile, those allowing their hosts to continue to grow and develop until emergence in order to feed on active hosts in open space are known as koinobionts. Parasitic Hymenoptera is one of the most important groups that play valuable roles in pest control programs because about 77% of the known parasitoid insects belong to the order Hymenoptera (Quicke, 1997; Mills, 2009). Due to high host specificity, mass-rearing of parasitoids as biocontrol agents is often forced to use their natural hosts, which often difficult to be

treated in mass-rearing. However, some polyphagous parasitoids, for example, *Trichogramma* spp. (Hymenoptera: Trichogrammatidae), are commercially grown on grain-feeding moths, which do not need daily care and can be reared on dry foods (Laing and Eden, 1990). Grain insects may used as substitute hosts when available and are more cost-effective than using the target pest insects (Hoddle and van Driesche, 2009).

2. *Meteorus pulchricornis*

Meteorus pulchricornis (Wesmael) (Hymenoptera: Braconidae) (Fig. 1-1) is a highly polyphagous, koinobiont endoparasitoid of exophytic caterpillars from 15 families within 10 lepidopteran super-families (Maeto, 2018). It is a common natural enemy of *Helicoverpa, Mamestra, Mythimna* and *Spodoptera* (Lepidoptera: Noctuidae), *Lymantria* (Erebidae), *Lasiocampa* (Lasiocampidae) and other major herbivore pests in East Asia and Oceania (e.g., Marsh, 1979; Huddleston, 1980; Maeto, 1989, 2018; Fuester et al., 1993; Takashino et al., 1998; Suzuki and Tanaka, 2006, 2007; Liu and Li, 2006, 2008; Walker et al., 2016). The natural hosts of *M. pulchricornis* are plant-feeders that move freely on leaves, and thus its host finding and oviposition behavior is triggered by the movement of host insects or even abiotic materials (Yamamoto et al., 2009; Maeto, 2018). This attribute may be one of the reasons why *M. pulchricornis* has such a wide host range. Besides natural hosts in the fields, *Galleria mellonella* (L.) (Lepidoptera: Pyralidae) has been successfully used as a substitute host for rearing *M. pulchricornis* (Askari et al., 1977). The host larvae parasitized *by M. pulchricornis*, as a koinobiont, remain active and continue feeding and growing in the initial phase of parasitism (Fuester et al., 1993; Harvey et al., 2010). After emergence from the host's body, a mature *M. pulchricornis* larva descends as it spins a silk thread attached to the

leaf or twig. It then turns downward, hooks itself to the thread, and forms a spindle-shaped cocoon (Fig.1-2) (Askari et al. 1977).

Fig.1-1. An adult of *Meteorus pulchricornis* (Wesmael) (Hymenoptera: Braconidae)

Fig.1-2. A cocoon of *M. pulchricornis*

Fig.1-3. Adult (left) and larva (right) of the Mediterranean flour moth *Ephestia kuehniella* (Zeller) (Lepidoptera: Pyralidae)

2.1. Parthenogenesis in *Meteorus pulchricornis*

Meteorus pulchricornis has both thelytokous (asexual) and arrhenotokous (sexual) strains (Fuester et al. 1993). Females of arrhenotokous strains can produce both haploid males and diploid females after mating or only haploid males without mating. On the other hand, thelytokous strains produce only diploid females without mating. Both thelytokous and arrhenotokous strains have been identified in Japan by Tsutsui et al. (2014) and Fujie et al. (2019). Thelytoky of *M. pulchricornis* has been revealed to be apomixis, which lacks meiosis and thus maintains heterozygosity in subsequent generations owing to the absence of genetic recombination. Thelytokous strains of *M. pulchricornis* are truly asexual, producing apomictic clones, without any association with symbiotic bacteria (Tsutsui et al., 2014). Thelytokous strains of *M. pulchricornis* can be considered to be promising biological agents, because they are expected to be more effective as biological control agents compare to arrhenotokous (sexual) strains, having the two-fold rate of reproduction (Aeschlimann, 1990; Stouthamer, 1993).

2.2. *Meteorus pulchricornis* as a biological control agent

The first attempt of using *M. pulchricornis* as a biological agent was conducted in the 1970s, by introducing arrhenotokous strains from Europe into North America to control the gypsy moth *Lymantria dispar* (L.) (Erebidae) (Fusco 1981). Although this attempt failed for unknown reasons, it was noted that it may be troublesome to import a polyphagous parasitoid for biological control because it could impact local ecosystems by attacking non-target insects (Howarth, 1991). However, the conservation of existing populations and augmentation use in enclosure conditions are still worthwhile. Recently, *M. pulchricornis* has been examined as a potential biocontrol agent against vegetable and bean crop pests such as *Helicoverpa* and *Spodoptera*

species in East Asia and Oceania (Chen and Hwang, 2015; Liu and Li, 2006; Takashino et al., 1998, 2001; Walker et al., 2016). For augmentation use of *M. pulchricornis*, easily manageable substitute hosts should be used for mass-rearing, because the natural hosts of *M. pulchricornis* are all plant feeders and they are hardly reared in factories.

3. *Ephestia kuehniella*

The Mediterranean flour moth *Ephestia kuehniella* (Zeller) (Lepidoptera: Pyralidae) (Fig. 1-3) is a cosmopolitan storage pest, having been spread throughout the world by international trade (CABI, 2018). Adult *E. kuehniella* lays eggs in flour, grains, semolina, corn, and other stored goods. Inside the stored goods, populations of *E. kuehniella* grow quickly, leading a great deal of waste due to the contamination and infestation of the stored products cause by their larvae, webbing, or cocoons (Jacob and Cox, 1977; Hansen and Jensen, 2002).

3.1. *Ephestia kuehniella* as a substitute host

As a grain pest, owing to its habitat and food source, *E. kuehniella* can be reared in dry conditions and it will grow exponentially without much of care and time. In fact, *E. kuehniella* have been widely used as diets or hosts for mass-rearing of predators and parasitoids in biological control. For example, *Orius* spp. (Heteroptera: Anthocoridae) and other predatory natural enemies of thrips, white flies or mites (Cocuzza et al. 1997; Nagai et al.1998), and the egg parasitoids *Trichogramma* spp. (Hymenoptera: Trichogrammatidae) or some larval ectoparasitoids *Habrobracon* spp. (Hymenoptera: Braconidae) of pest moth are reared on eggs or larvae of *E. kuehniella* (Borzoui et al. 2016; Smith 1996). It is usually considered to be difficult to rear koinobiont parasitoids on substitute hosts owing to its high host-specificity, which is the disadvantage of the

use of koinobionts in augmentation use, although this may not always be true because *M. pulchricornis* is a polyphagous koinobiont (Maeto, 2018).

4. Objectives of the study

The goal of this study is to improve the methods of mass-rearing *M. pulchricornis* for augmentation. First, the suitability of *E. kuehniella* as a substitute host for rearing *M. pulchricornis* has been evaluated, and the effects of host species and host body size on the emergence, body size, longevity, and lifetime fecundity of adult *M. pulchricornis* has been examined (Chapter II). Second, the autonomous oviposition of *M. pulchricoris* on *E. kuehniella* larvae has been aimed to be enhanced. The moving of *E. kuehniella* larvae was successfully accelerated with alternate LED lighting, by utilizing the negative phototaxis of the larvae, in order to increase the oviposition activity of *M. pulchricornis* into them. Also, a feasible length of time for oviposition was previously estimated to avoid entanglement in the silk threads of. *E. kuehniella* (Chapter III). Third, in order to further understand the reproductive modes and genetic diversity of *M. pulchricornis* for the use as biocontrol agents for various lepidopteran pests, arrhenotokous (sexual) strains as well as thelytokous (asexual) strains of *M. pulchricornis* have been successfully established in the laboratory (Chapter IV).

CHAPTER II

Ephestia kuehniella larvae as substitute hosts for *Meteorous pulchricornis*

Introduction

Efficient and reliable mass rearing of natural enemies is important for successful biological control, especially through augmentation. Parasitoids are generally reared on natural hosts because of the host specificity, but some polyphagous parasitoids, such as *Trichogramma* spp. (Hymenoptera: Trichogrammatidae), are grown on grain-feeding moths, which can be more cost effective than using the target pest insects (Hoddle and van Driesche, 2009; Wang et al., 2013).

Meteorus pulchricornis (Wesmael) (Hymenoptera: Braconidae) is a solitary and koinobiont endoparasitoid of a wide range of free-living lepidopteran caterpillars (Berry and Walker, 2004; Fuester et al., 1993; Huddleston, 1980; Nishimura et al., 2015; Suzuki and Tanaka, 2006, 2007), and is a common natural enemy of *Helicoverpa*, *Mamestra, Mythimna* and *Spodoptera* (Lepidoptera: Noctuidae), *Lymantria* (Lymantriidae), *Lasiocampa* (Lasiocampidae), *Lemyra* (Arctiidae), and other pest herbivores in East Asia and Oceania (e.g., Liu and Li, 2006, 2008; Maeto, 1989; Marsh, 1979; Takashino et al., 1998; Walker et al., 2016). This polyphagous parasitoid has been reared on natural hosts and also successfully on a non-natural host, *Galleria mellonella* (L.) (Lepidoptera: Pyralidae), for experimental purposes (Askari et al., 1977). Most of the natural hosts of *M. pulchricornis* are plant feeders that eat perishable food; therefore, the rearing of these species is laborious and tends to be unsanitary even with the use of artificial diets. The wax moth *G. mellonella* seems to be a suitable substitute host for biological experiments but not for mass rearing *M. pulchricornis* due to the high cost of the rearing food.

The Mediterranean flour moth *Ephestia kuehniella* (Zeller) (Lepidoptera: Pyralidae) has been widely used for mass rearing of *Orius* spp. (Heteroptera: Anthocoridae) and other predatory natural enemies (Cocuzza et al., 1997; Nagai et al., 1998), as well as

egg parasitoids (e.g., *Trichogramma* spp.) and larval ectoparasitoids [e.g., *Habrobracon* spp. (Braconidae)] (Borzoui et al., 2016; Smith, 1996). This easily reared insect may be a host candidate for mass rearing of *M. pulchricornis*, a highly polyphagous, koinobiont endoparasitoid of lepidopteran larvae.

The host larvae parasitized by *M. pulchricornis* as a koinobiont endoparasitoid remain active and continue feeding and growing in the initial phase of parasitism; finally, the grown host larvae are consumed (Fuester et al., 1993; Harvey et al., 2010). The growth rate of *E. kuehniella* larvae, however, is slow, and the matured larvae are small (Jacob and Cox, 1977) when compared with those of *Spodoptera, Lymantria* and other natural hosts of *M. pulchricornis*. According to the review of Jervis et al. (2007), the effect of initial host size on the development and progeny size of koinobiont parasitoids varies among cases.

In this study, the host suitability of *E. kuehniella* for *M. pulchricornis* was evaluated, and the effects of host body size on the emergence and adult body size of *M. pulchricornis* was examined. Also the body size, longevity, and lifetime fecundity of adult wasps reared on *E. kuehniella* and on a natural host *Spodoptera litura* (Fabricius) (Noctuidae) were compared.

Materials and methods

Insects

The thelytokous strain of *M. pulchricornis* used in the experiments originated from *S. litura* larvae that were collected in Kagawa Prefecture, Japan (Nguyen et al., 2005; KAGAWA_01_U of Abe et al., 2013 and Tsutsui et al., 2014). It had been maintained on *S. litura* larvae that were reared on artificial diets (Insecta LFS, Nosan Corporation, Yokohama). Adult wasps were given with an absorbent cotton ball soaked in 50%

honey solution, and kept at 15–20°C under a 16L:8D photoperiod (16 h light and 8 h dark).

A strain of *E. kuehniella* from the Western Region Agricultural Research Center NARO (WARC/NARO) was examined as an alternative host. Larvae were reared on pressed corn under a 16L:8D photoperiod at 25°C.

A strain of *S. litura* from the Sumika Technoservice Corporation, Takarazuka, was reared on Insecta LFS and used as the natural host for comparison.

Experiment 1: To evaluate the size effects of *E. kuehniella* on parasitism of *M. pulchricornis*

Young to nearly fully-grown larvae (fresh weight: 2.0–30.0 mg) of *E. kuehniella* were placed (and made to move using a pair of tweezers) in front of a female adult of *M. pulchricornis* to encourage oviposition (Fig.2-1) (Yamamoto et al., 2009). The oviposited host larvae were reared individually on pressed corn in transparent plastic cases (40 mm diameter × 25 mm height), at 25°C, under a 16L:8D photoperiod for 28 days or until the emergence of mature *M. pulchricornis* larvae. The fresh weight of the host larvae, the parasitoid cocoons, and the emerged adult wasps were measured using a CPA 64 electronic balance (Sartorius AG, Göttingen). The adult wasps were active and they were each placed in a plastic container for body weight measurement.

Binominal logistic regression analyses were used to compare the emergence success of mature parasitoid larvae and adult wasps against the fresh weight of *E. kuehniella* larvae at the time of oviposition. In addition, the fresh weight of *E. kuehniella* larvae immediately before the emergence of *M. pulchricornis* larvae, the developmental period of *M. pulchricornis* from oviposition to larval emergence, and the fresh weight of cocoons and adults of *M. pulchricornis* were analyzed using simple linear regressions

against the fresh weight of *E. kuehniella* larvae at oviposition.

Experiment 2: To compare the longevity and lifetime fecundity of *M. pulchricornis* reared on *E. kuehniella* with those on *S. litura*

Nearly fully-grown larvae of *E. kuehniella* (fresh weight: > 20.0 mg) were oviposited by *M. pulchricornis* and reared in the same way as in Experiment 1. Adult wasps were weighed immediately after emergence and individually kept in transparent plastic cases (40 mm diameter × 25 mm height) with an absorbent cotton soaked with 50% honey solution at 25°C, under a 16L:8D photoperiod. The honey solution was continuously provided to the adult wasps as a source of carbohydrates and to prolong the lifespan of the specimens (Wu et al., 2008).

The adult wasps were each provided with 10 individuals of the second or third instar larvae of *S. litura* for oviposition. Every two days, the 10 host larvae were removed and replaced with new larvae until the wasps died. The collected host larvae were dissected in 70% ethanol under a stereoscopic microscope to count the number of eggs that had been laid. Because *M. pulchricornis* is a synovigenic endoparasitoid (Fuester et al., 1993), the fecundity was estimated by counting the number of eggs laid in each wasp's lifetime.

Second to third instar larvae (fresh weight: 1.0–6.0 mg) of *S. litura* were oviposited by *M. pulchricornis* and reared on Insecta LFS in transparent plastic cases at 25°C, under a 16L:8D photoperiod. Adult wasps that emerged from the host larvae were treated in the same manner as outlined above.

The fresh weight of adult wasps at emergence, the longevity, and the lifetime fecundity were compared between those reared on *E. kuehniella* and *S. litura* using the two-tailed median test, referring to Fisher's exact probability value. Simple linear

regression analyses were used to assess the longevity and fecundity of adult wasps against the fresh weight at emergence for each host species. Under the assumption of constant linearity throughout the whole range of the fresh weight of adult wasps, an analysis of covariance (ANCOVA) was conducted to test the effects of the host species (*E. kuehniella* vs. *S. litura*), the fresh weight of adult wasps, and their interaction on the fecundity of wasps.

All of the statistical analyses above were performed using IBM SPSS Statistics version 22 for Windows.

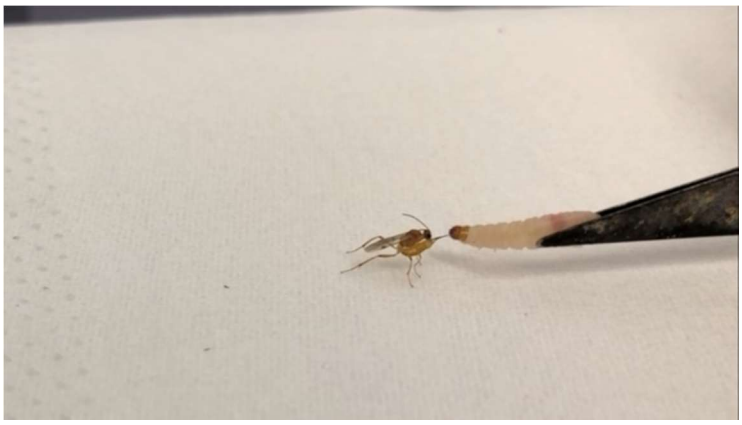


Fig. 2-1. Induced oviposition. *E. kuehniella* were placed (and made to move using a pair of tweezers) in front of a female adult of *M. pulchricornis* to encourage oviposition

Results

Experiment 1

Out of 100 *E. kuehniella* larvae that were oviposited, 4 host larvae pupated normally, 28 host larvae died, 27 host larvae remained alive without parasitoid emergence on the 28th day after oviposition, and 41 host larvae had a single mature larva of *M. pulchricornis* emerge within 28 days after oviposition. The probability of parasitoid

larval emergence increased with the weight of *E. kuehniella* larvae at the time of oviposition (Fig. 2-2A). Out of the 41 emerged parasitoid larvae, 32 larvae span a cocoon, and 29 adult wasps successfully emerged. The probability of adult emergence increased with the weight of *E. kuehniella* larvae at oviposition (Fig. 2-2B), indicating that the adult emergence probability of *M. pulchricornis* should be 80% or higher when the fresh weight of the host larvae at oviposition is greater than 20.0 mg.

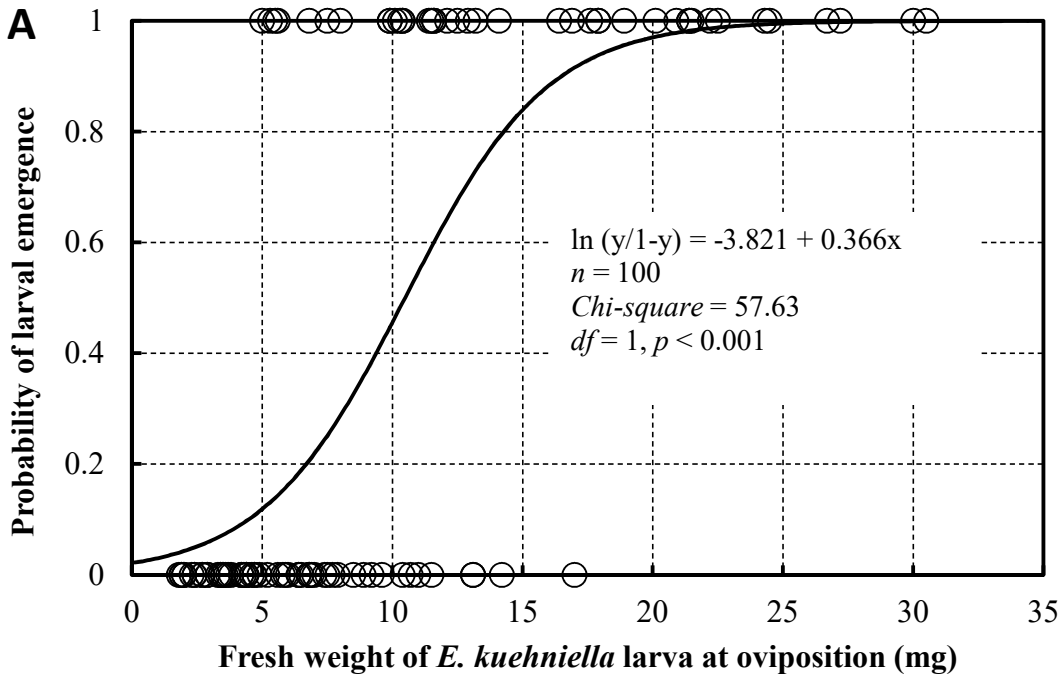
The fresh weight of *E. kuehniella* larvae immediately before the emergence of the parasitoid larvae was usually less than that at oviposition (Fig. 2-3A). The developmental period to parasitoid larval emergence decreased with an increase in the fresh weight of *E. kuehniella* larvae at oviposition (Fig. 2-3B), and reached a constant duration of approximately 10–12 days when the fresh weight of host larvae was over 20.0 mg. The fresh weight of parasitoid cocoons and adult wasps at emergence increased with an increase in the fresh weight of host larvae at oviposition (Fig. 2-3C).

Experiment 2

The fresh weight of adult wasps reared on *E. kuehniella* was approximately 60% of that of wasps reared on *S. litura*, and this difference was found to be significant (Table 1). The median longevity of wasps reared on *E. kuehniella* appeared to be shorter than that on *S. litura*, while this difference was not significant (Table 1). The lifetime fecundity of wasps reared on *E. kuehniella* was approximately half of that of wasps reared on *S. litura*, and this difference was significant (Table 1).

The linear regression analyses did not indicate a relationship between the longevity of wasps and the fresh weight of those reared on *E. kuehniella* or *S. litura* (Fig. 2-4A). Positive regressions were found for the lifetime fecundity of wasps and the fresh weight, and were both marginally significant for wasps reared on *E. kuehniella* and *S. litura*

(Fig. 2-4B). The ANCOVA revealed that the effect on lifetime fecundity was not significant for the type of host species (*E. kuehniella* vs. *S. litura*) ($F = 0.143$, $df = 1, 12$, $p = 0.712$), was significant solely for the fresh weight ($F = 8.183$, $df = 1, 12$, $p = 0.014$), and was not significant for their interaction ($F = 0.024$, $df = 1, 12$, $p = 0.880$). The results suggest that the fecundity of wasps simply depends on the body weight regardless of host species; however, this is a hypothetical assessment because the range of fresh weight (covariate) did not overlap between that of wasps reared on *E. kuehniella* and *S. litura*, as was critically mentioned by Gotelli and Ellison (2004).



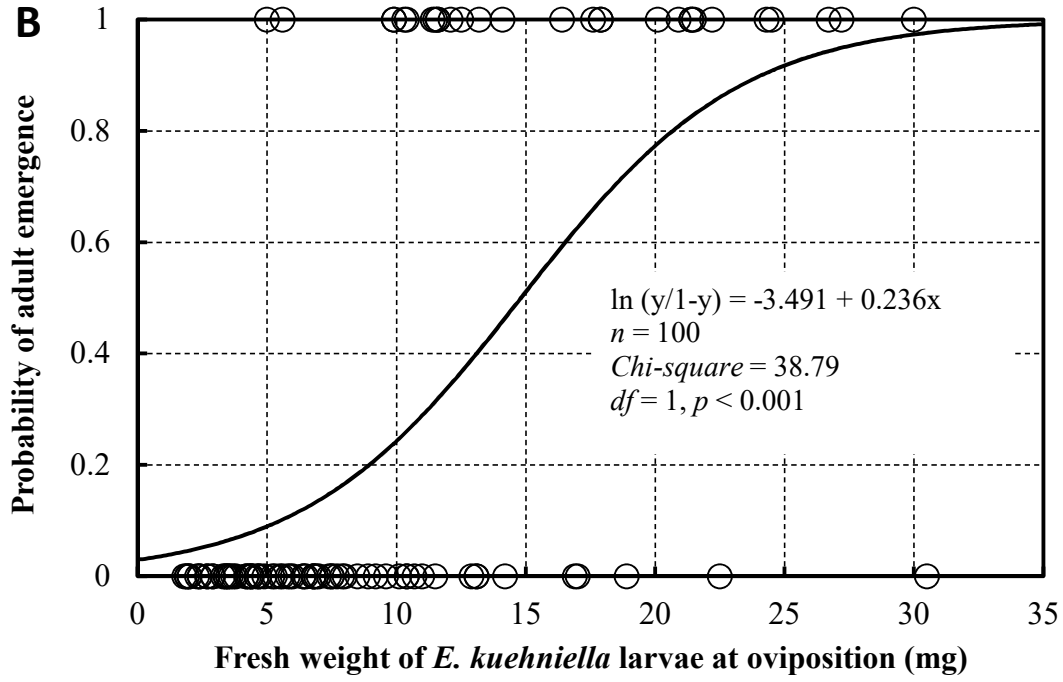
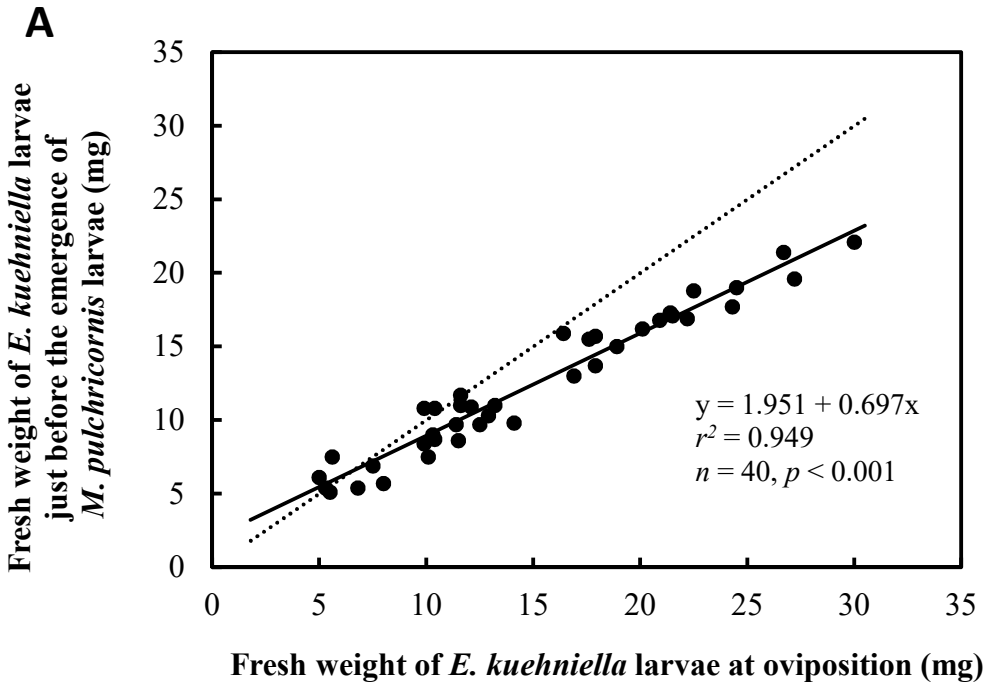
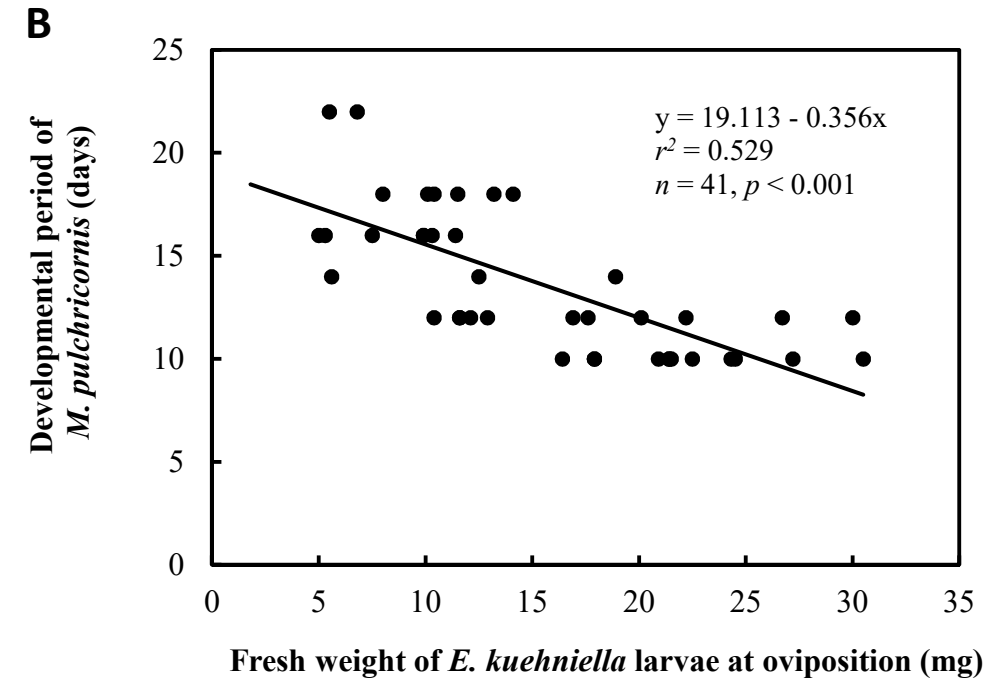


Fig. 2-2 Logistic regressions of mature larval emergence (A) and adult emergence (B) of *Meteorus pulchricornis* against the fresh weight of *Ephestia kuehniella* larvae at oviposition. Each point represents an individual of *M. pulchricornis* that either successfully emerged (1) or did not (0)


A
Fresh weight of E. kuehniella larvae just before the emergence of M. pulchricornis larvae (mg)
y = 1.951 + 0.697x
r2 = 0.949
n = 40, p < 0.001
Fresh weight of E. kuehniella larvae at oviposition (mg)


B
Developmental period of M. pulchricornis (days)
y = 19.113 - 0.356x
r2 = 0.529
n = 41, p < 0.001
Fresh weight of E. kuehniella larvae at oviposition (mg)

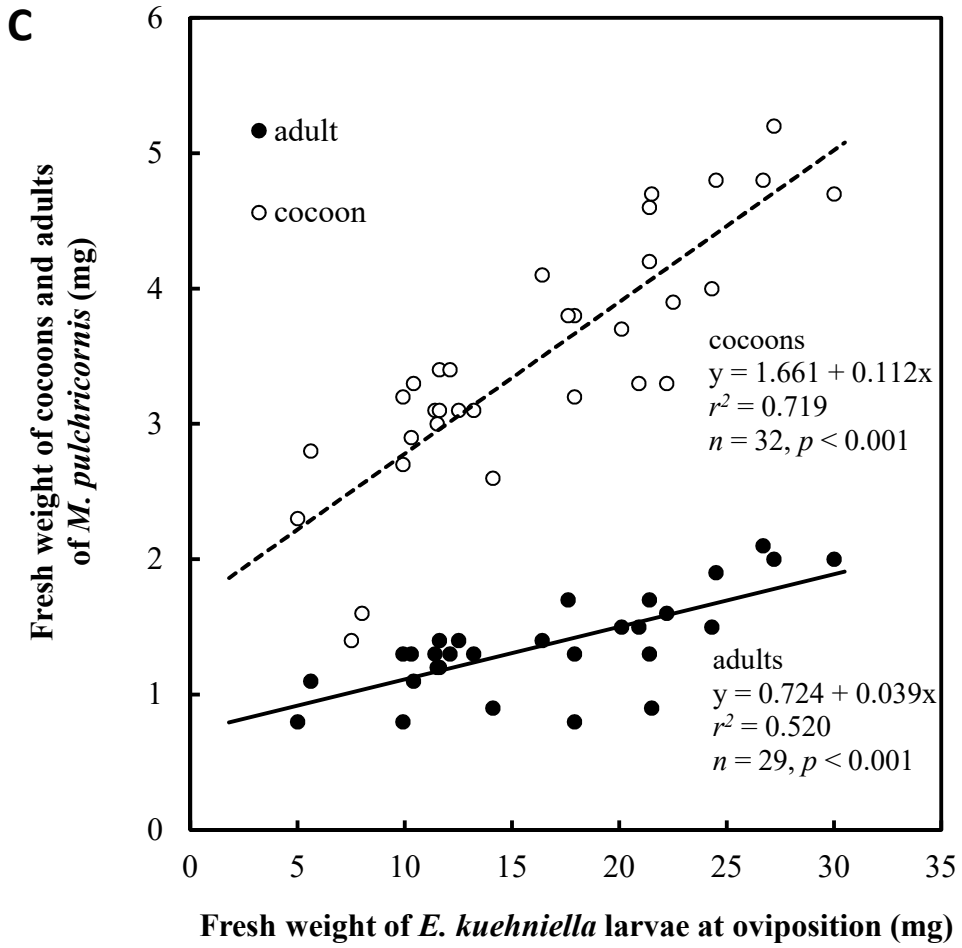


Fig. 2-3 Linear regressions of the fresh weight of *Ephestia kuehniella* larvae immediately before the emergence of *Meteorus pulchricornis* larvae (A), the developmental time of *M. pulchricornis* from oviposition to larval emergence (B), and the fresh weight of cocoons or adults of *M. pulchricornis* (C), all against the fresh weight of *E. kuehniella* larvae at oviposition. Dots in (A) indicate the line of equality

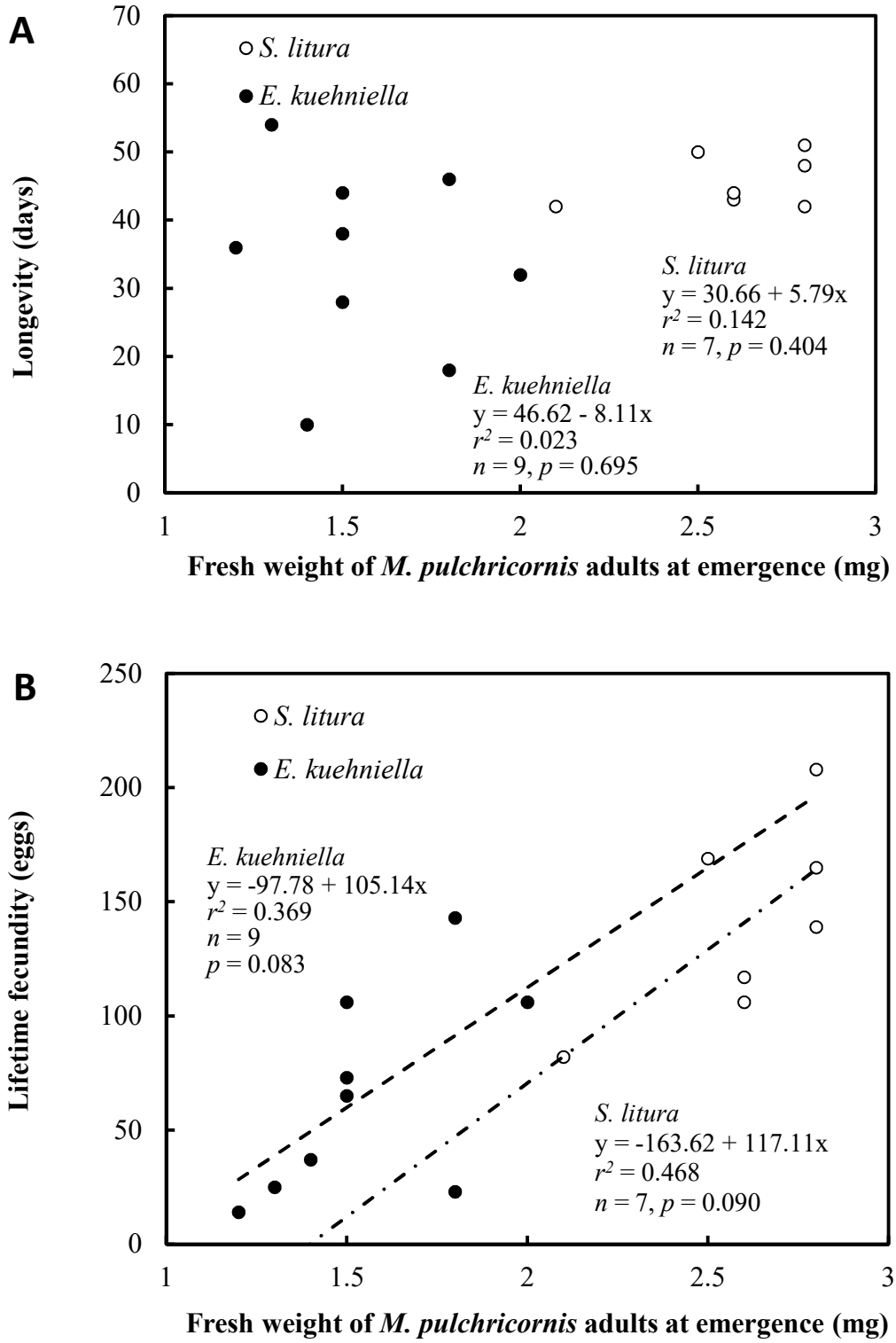


Fig. 2-4 Relationships of the longevity (A) and the lifetime fecundity (B) of *Meteorus pulchricornis* adults reared on *Ephestia kuehniella* or *Spodoptera litura* to the fresh weight of adults at the time of emergence

Discussion

This study shows that the nearly fully-grown larvae of the Mediterranean flour moth *E. kuehniella* are potential alternative hosts of *M. pulchricornis* for mass rearing practices. By providing larvae of *E. kuehniella* with a fresh weight of over 20.0 mg for oviposition, the possibility of adult emergence is expected to be 80% or higher. However, the period from oviposition to mature larval emergence at 25°C (10–12 days) was somewhat longer than that for wasps reared on *G. mellonella* (ca. 9 days) or *Mythimna separate* (Walker) (Noctuidae) (8–9 days) (Askari et al., 1977; Suzuki and Tanaka, 2007). Importantly, the body weight of emerged adult wasps was approximately 60% of that reared on the natural host *S. litura*, and probably due to the small body size, the lifetime fecundity of the wasps (median: 65 eggs) was approximately half of that reared on *S. litura* (median: 139 eggs). These fecundity values are also small when compared to the fecundity of wasps reared on *Lymantria dispar* (L.) (Lymantriidae), which was reported to be 268 eggs on average (Fuester et al., 1993). It is well known that the egg load and lifetime fecundity of parasitoid wasps usually depend on the female body size (Jervis et al., 2007). Small body size may also diminish the dispersal and searching ability of parasitoid wasps (Ellers et al., 1998). One aspect of using *E. kuehniella* in the mass rearing of *M. pulchricornis* that requires attention is the small body size of the emerged wasps. As indicated by the result of ANCOVA, probably there is a positive regression of the lifetime fecundity of *M. pulchricornis* on wasp body weight irrespective of host species.

In Experiment 2, the body weights of the *E. kuehniella* larvae that were provided for oviposition (> 20.0 mg) were much larger than those of the *S. litura* larvae (< 6.0 mg). However, the body weights of adult wasps reared on *E. kuehniella* (median: 1.5 mg) were reversely only approximately 60% of those of wasps reared on *S. litura* (median:

2.6 mg). Adults of *M. pulchricornis* usually attack young instars of "fast-growing" hosts, such as *S. litura* larvae, and the mature larvae of wasps emerge from enlarged host larvae. As shown by Harvey et al. (2010) for *M. separate* and *Plutella xylostella* (L.) (Lepidoptera: Plutellidae), the body weight of parasitized host larvae greatly increased until the wasp larvae emerged. The body weight of parasitized *S. litura* larvae was seen to increase up to 30 fold until the emergence of the wasp larvae (S. Nakano unpublished data). In contrast, *E. kuehniella* is a "slow-growing" species, and the body weight of the host larvae was decreased at the time of the emergence of the wasp larvae (Fig. 2-3A). This study revealed that the body size of the parasitoid wasp *M. pulchricornis* is limited by the body size of *E. kuehniella* larvae at oviposition. To obtain *M. pulchricornis* wasps that are more effective biocontrol agents, I suggest that enlargement of *E. kuehniella* larvae for parasitoid oviposition by improving rearing conditions or selective breeding will be necessary.

Another aspect requiring a solution is how to make wasps oviposit on *E. kuehniella* larvae, which are usually hidden among grains. As a parasitoid of free-living caterpillars exposed on plant leaves, adult *M. pulchricornis* attack moving hosts using visual cues (Yamamoto et al., 2009). In the experiments of the present study, oviposition was induced by moving a host larva with tweezers in front of the wasps; however, this methodology is not practical for the mass rearing of wasps without an ingenious contrivance to make the host larvae move autonomously to encourage the wasps to attack. In addition, the defensive behaviors of *E. kuehniella* larvae against wasp oviposition are not known, but may need to be considered (Zhou et al., 2017).

In conclusion, the Mediterranean flour moth may serve as a positive host candidate for the mass rearing of *M. pulchricornis*; however, further studies are needed to provide wasps of sufficient quality for practical use of this species as a biocontrol agent.

CHAPTER III

Increasing oviposition on *E. kuehniella* larvae by *M. pulchricornis* with alternate LED lighting

Introduction

Efficient mass-rearing systems are essential for the successful augmentation of biological control agents. Various inexpensive and easily manageable substitute prey or host insects are applied to the rearing systems of predators or parasitoids. For example, the Mediterranean flour moth *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) (CABI, 2018) has been widely used for the propagation of idiobiont parasitoids of lepidopteran eggs and larvae (Borzoui et al., 2016; Smith, 1996) as well as for rearing predators of thrips, white flies, or mites (Cocuzza et al., 1997; Nagai et al., 1998). It is usually difficult to rear koinobiont parasitoids using substitute host insects because of their high host-specificity, which is one of the disadvantages of using koinobionts in augmentation, but this is not always the case.

Meteorus pulchricornis (Wesmael) (Hymenoptera: Braconidae) is a solitary koinobiont endoparasitoid of exposed-living lepidopteran larvae (Maeto, 1989, 2018), and is a common natural enemy of *Helicoverpa*, *Mamestra, Spodoptera* (Lepidoptera: Noctuidae), *Lymantria*, *Orgyia* (Erebidae), and other pest herbivores (e.g., Liu and Li, 2006, 2008; Marsh, 1979; Takashino et al., 1998; Walker et al., 2016). Due to its wide range of hosts, *M. pulchricornis* could be a potential biocontrol agent for various lepidopteran pests. Nakano et al. (2018) has demonstrated that this parasitoid can be successfully reared on the final-instar larvae of *E. kuehniella* as a substitute host in the laboratory. In their experiments, however, egg oviposition of *M. pulchricornis* into *E. kuehniella* larvae was artificially induced by shaking a larva in front of a wasp. Autonomous oviposition of *M. pulchricornis* on the larvae of *E. kuehniella* has not been confirmed.

The natural hosts of *M. pulchricornis* are caterpillars moving freely on plants, and its oviposition behavior is triggered by the movement of host larvae or even abiotic

materials (Maeto, 2018; Yamamoto et al., 2009). However, the larvae of *E. kuehniella* are concealed among dry plant materials sewed with silk threads, due to negative phototaxis (Brandt, 1934; Staddon, 2016). Thus, inactive larvae removed from food materials are less attractive for oviposition; however, the oviposition behavior can be enhanced if the larvae are stimulated to become more active. Another critical problem is that *M. pulchricornis* adults often get entangled in the silk threads spun by the larvae of *E. kuehniella* before oviposition.

The aim of this study is to enhance the autonomous oviposition of *M. pulchricoris* on *E. kuehniella* larvae. The moving of *E. kuehniella* larvae was accelerated with alternate LED lighting, by utilizing the negative phototaxis of the larvae (Brandt, 1934; Shimoda and Honda, 2013), in order to increase the oviposition activity of *M. pulchricornis* into them. Also, a feasible length of time for oviposition was previously estimated to avoid entanglement in the silk threads of *E. kuehniella*.

Materials and methods

Insects

A strain of *E. kuehniella* donated by the Western Region Agricultural Research Center (WARC/NARO, Japan) was reared on pressed corn at 25°C under a 16L:8D photoperiod (16 h light and 8 h dark). For all the experiments, final-instar larvae (fresh weight: 15.0 - 25.0 mg) were provided.

A thelytokous strain of *M. pulchricornis* originating from *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae) in Japan (Nguyen et al. 2005; KAGAWA_01_U of Abe et al., 2013 and Tsutsui et al., 2014; mtCOI haplotype 21 of Fujie et al., 2019) was used in experiments 2 and 3. It was maintained on *S. litura* larvae reared on artificial diets (Insecta LFS, Nosan Corporation, Yokohama, Japan). Adult wasps were

given an absorbent cotton ball soaked in a 50% honey solution and kept at 15°C under a 16L:8D photoperiod. Adult wasps of less than three-weeks-old were used in the experiments.

Experimental device

Two LED lights (CLEAR LED POWER III 600, GEX Corporation, Osaka, Japan) were set up along the upper lateral edges of the outer container (Fig. 3-1). Four transparent plastic boxes (156 × 24 × 24 mm, Clear pen case, Lindexs, Shanghai, China) for enclosing insects for examination were set in parallel on the bottom of the container. A shading film was placed in the middle of the container to reduce light spread. Video recordings were obtained with a web camera (1280 × 720 pixels, ELP-USBHD06H-FV, Ailipu Technology Corporation, Shenzhen, China) set on top of the outer container. This experimental device was placed in a dark room at 25°C.

The LED lights had white (400 – 700 nm) and red (600 – 700 nm) modes. The white or red mode was applied for constant lighting, in which the photon flux density (average ± SD, n = 15) at the both ends and the center of the plastic cases was 35.56 ±1.28μmol m^{-2} s^{-1} (white) / 1.40 ± 0.13 μmol m^{-2} s^{-1} (red) and 37.52 ± 1.53 μmol m^{-2} s^{-1} / 1.58 ± 0.08 μmol m^{-2} s^{-1}, respectively. The white mode was set to alternate lighting at 5 min intervals, in which the illumination at the light end, the center, and the dark end of the plastic cases was 35.45 ±1.32 μmol m^{-2} s^{-1}, 19.00 ± 1.28 μmol m^{-2} s^{-1}, and 0.08 ± 0.00 μmol m^{-2} s^{-1}, respectively. The photon flux density was measured by LI-190SA Quantum Sensor and LI-250A Light Meter (LI-COR Biosciences, Lincoln, Nebraska).

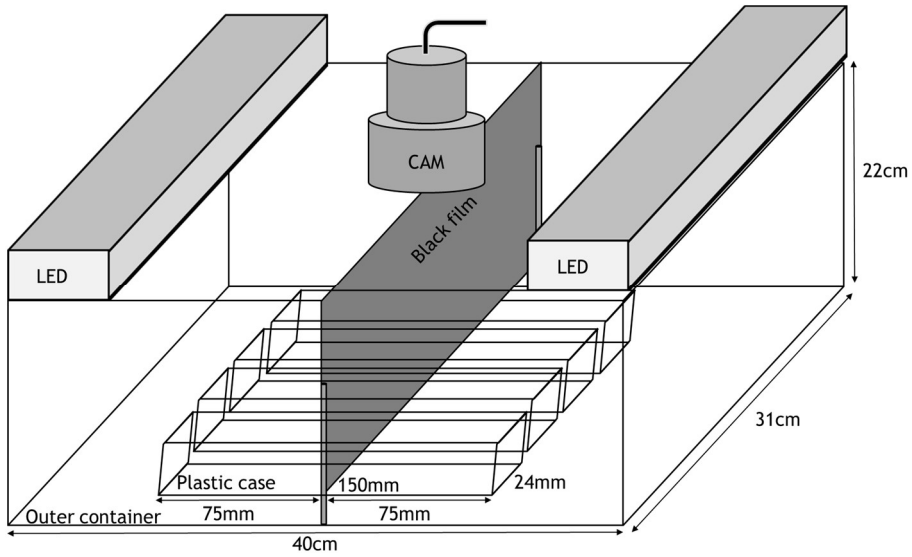


Fig. 3-1a. Set-up of the experimental device.

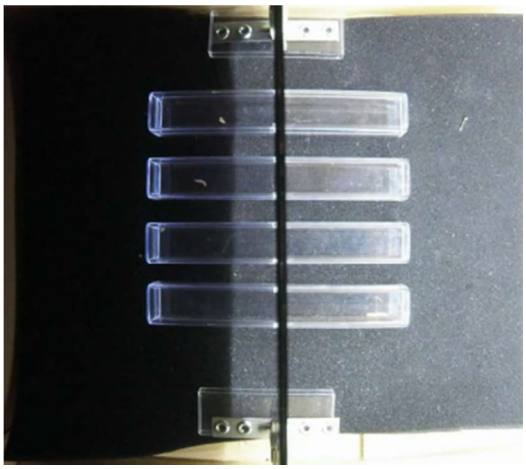


Fig. 3-1b. Example of video data

Experiment 1: to increase the mobility of *E. kuehniella* larvae by alternate lighting

One *E. kuehniella* larva was put in the transparent box for 24 h. Three lighting conditions, i.e., constant lighting of white mode (CW) or red mode (CR) and alternate lighting of white mode at 5 min intervals (AW_5), were provided to compare the mobility of larvae. The total length of larval movement was calculated by the visual observation of video recordings. It was compared among the three lighting conditions by the generalized linear mixed model (GLMM) with the gamma distribution and log-link function. A total of 31 larvae were examined only once. The time of examination, when at most four individuals were simultaneously observed in the device, was treated as a random factor. Multiple comparisons between CW, CR and AW_5 were conducted with the sequential Bonferroni correction.

Experiment 2: to estimate the timespan free from silk-thread entanglement

One wasp and one *E. kuehniella* larva were put together in the transparent box for 24 h. Two lighting conditions, constant lighting of white mode (CW) and alternate lighting of white mode at 5 min intervals (AW_5), were provided for the observation of the wasp's movement. The time (in hours) when a wasp became crippled, mainly due to silk-thread entanglement, was determined by the visual observation of video recordings. The upper and lower 95% confidence intervals of the proportion of intact (non-crippled) wasps every hour were estimated by the Kaplan-Meier method. Overall proportion of intact wasps was tested between CW and AW_5 using the log-rank test.

Experiment 3: to increase oviposition on *E. kuehniella* larvae by alternate lighting

One wasp and one *E. kuehniella* larva were put together in the transparent box for 6 h since the number of intact wasps had decreased markedly after that as shown in

experiment 2 (Fig. 3-4). Two lighting conditions, constant lighting (CW) and alternate lighting at 5 min intervals (AW_5), were provided for the observation of wasp oviposition. After 6 h the wasp and larva were removed from the box, and the larva was reared with pressed corn in a transparent plastic case (40 mm diameter × 25 mm height). After three days, the larva was dissected in 70% ethanol under a stereoscopic microscope in order to count the number of eggs or hatched larvae of wasps.

A total of 20 wasps were used in this experiment after being trained for oviposition by giving them ten *S. litura* larvae together for three days beforehand. They were repeatedly used for examinations after two or more days of rest. The number of eggs or hatched larvae was compared between CW and AW_5 by GLMM with the Poisson distribution and log-link function. Individual wasps and the time of examination were treated as random factors. All the statistical analyses were performed using IBM SPSS Statistics version 22 for Windows.

Results

Experiment 1

Under alternate white lighting (AW_5), *E. kuehniella* larvae were often observed moving from the light side to the dark side, indicating negative phototaxis (Supplementary Video 1, 64 times speed). The estimated marginal mean of the total length of larval movement in 24 h was 662.3 cm, 577.3 cm, and 1841.5 cm under CW, CR, and AW_5, respectively (Fig. 3-2) (GLMM, $F = 8.847$, $df = 2, 28$, $p = 0.001$). The mobility of larvae under alternate white lighting (AW_5) was significantly different from that under constant lighting (CW, CR), while it was not significantly different under constant lighting between white mode (CW) and red mode (CR) (Fig. 3-2).

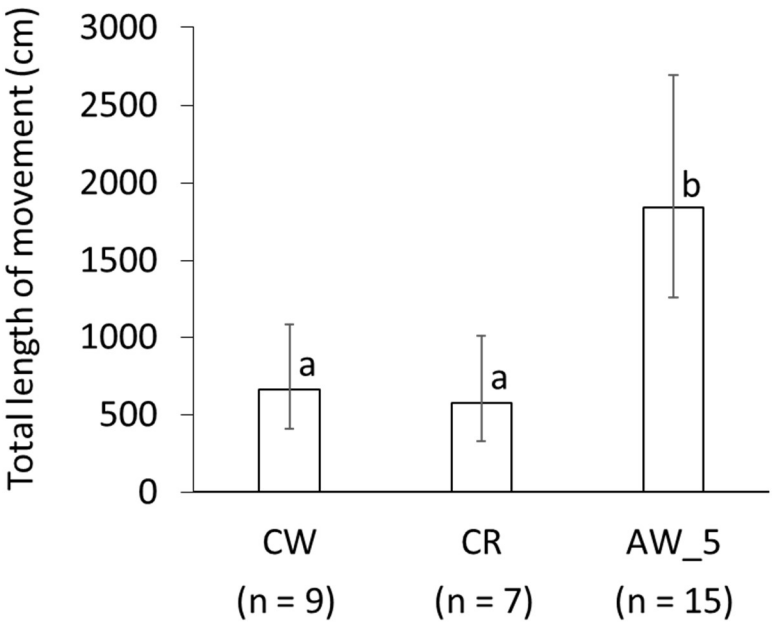


Fig. 3-2 Estimated marginal means of the total length of *E. kuehniella* larval movement in 24 h under constant white mode (CW) and red mode (CR) lighting and under alternate lighting of white mode at 5 min intervals (AW_5). The vertical lines indicate 95% confidence intervals. Different letters indicate a significant difference between treatments after sequential Bonferroni correction ($p < 0.05$).

Experiment 2

The proportion of intact wasps of *M. pulchricornis* decreased mainly due to them being crippled by silk-thread entanglement (Fig. 3-3). Wasps, once crippled, hardly ever recovered. The decrease was marked after about 10 h especially under constant lighting (CW). Overall proportion of intact wasps was significantly different between CW and AW_5 (Fig. 3-4).

Experiment 3

The estimated marginal mean of the number of *M. pulchricornis* eggs (including hatched larvae) laid per wasp in 6 h was 0.67 under constant lighting (CW) and 1.11 under alternate lighting (AW_5), and was significantly different between them (GLMM, $F = 4.184$, $df = 1, 111$, $p = 0.043$) (Fig. 3-5).

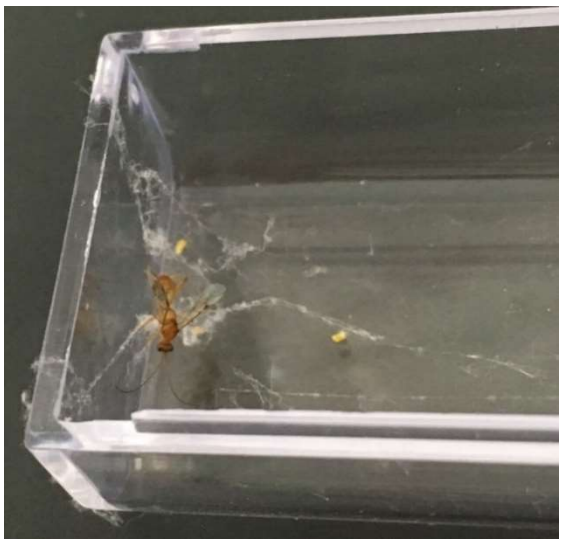


Fig. 3-3 The wasp crippled and killed by silk-thread entanglement

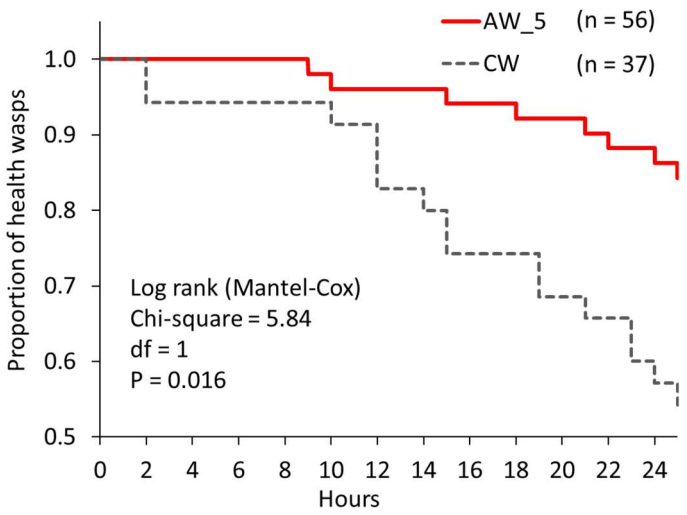


Fig. 3-4 The proportion of intact wasps estimated by the Kaplan-Meier method. Overall proportion of intact wasps was tested between constant (CW) and alternate lighting (AW_5) using the log-rank test.

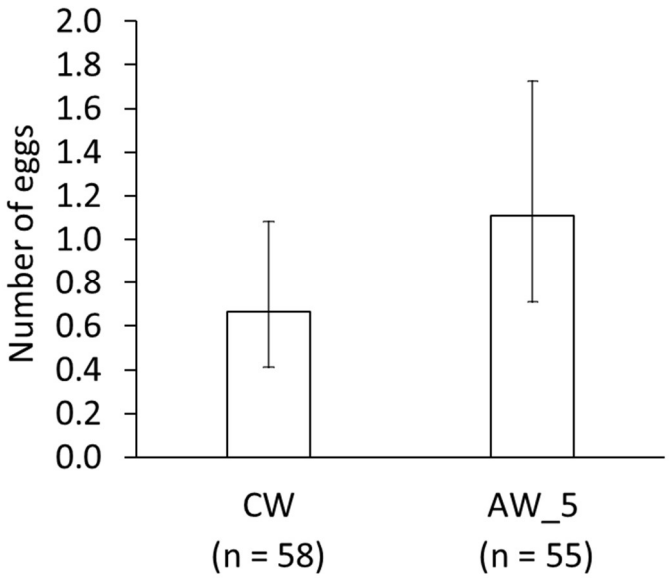


Fig. 3-5 Estimated marginal means of the number of eggs laid per *M. pulchricornis* adult in 6 h under constant (CW) and alternate lighting (AW_5). The vertical lines indicate 95% confidence intervals.

Discussion

Increasing the mobility of *E. kuehniella* larvae

This study shows that the mobility of *E. kuehniella* larvae can be enhanced by using alternate lighting. In experiment 1, the total length of larval movement under alternate lighting (AW_5) was increased about three-fold when compared to the larvae under constant lighting (CW) (Fig. 3-2). This is most probably caused by the negative phototaxis of *E. kuehniella* larvae (Brandt, 1934).

Besides phototaxis, I am also interested in photokinesis, which controls the activity of insects without any particular direction when stimulated by light (Fraenkel and Gunn, 1961). The comparison of adult movement under continuous white light (CW) and red light (CR) in experiment 1 was conducted to identify the negative or positive photokinesis because insects are generally not sensitive to red light. Consequently, observing the same level of activity under both light conditions (Fig. 3-2) would not indicate any obvious photokinesis of *E. kuehniella* larvae.

Avoiding silk-thread entanglement

As observed in experiment 2, *M. pulchricornis* wasps were often crippled by the silk-threads spun by *E. kuehniella* larvae for constructing nest webs. The wasps could hardly recover from the silk-thread entanglement and thus they were unlikely to be successful in oviposition. However, the decrease of intact wasps owing to the silk-thread entanglement was comparatively slow under alternate lighting (Fig. 3-4), probably because added time of moving could decrease time for spinning threads. Even under alternate lighting, intact wasps began to decrease after around ten hours. Therefore, to avoid silk-thread entanglement, oviposition should be conducted within less than that time under alternate lighting.

Increasing autonomous oviposition

According to Yamamoto et al. (2009), it is expected that increased host movement accelerates the oviposition behavior of *M. pulchricornis*. In fact, I have confirmed that its autonomous oviposition on *E. kuehniella* larvae can be enhanced by alternate lighting, which approximately doubled the number of eggs laid per one wasp in six hours up to about 1.1. However, this is one-third of the potential fecundity per 1/4 day of this species (Fuester et al., 1993). There is much room for improvement in the oviposition method.

In experiment 3, only one host larva was provided to each wasp for oviposition, whereas *M. pulchricornis* females could induce a short-term host paralysis for the avoidance of multiple oviposition (self-superparasitism) (Chau and Maeto, 2009). This may cause *E. kuehniella* larvae to interrupt their movement after oviposition and decrease their chance of being oviposited again. Also, according to Sheng et al. (2014), a high host density has a positive effect on *M. pulchricornis* host searching behavior and oviposition. Providing multiple hosts to each wasp should be investigated for practical oviposition.

On the other hand, the positive phototaxis of *M. pulchricornis* may also increase the chance of wasps coming into contact with host larvae under alternate lighting. Further investigation will be necessary to understand such effects on wasp oviposition.

Further consideration

Alternate lighting is proven to increase the movement of *E. kuehniella* larvae, which are substitute hosts for mass rearing of the herbivore parasitoid *M. pulchcircornis*, and therefore to relieve the silk-thread entanglement and enhance the oviposition behavior of the parasitoid wasp. Further investigations are yet necessary to determine the best

timing of light alternation, the light wavelength and intensity, the number of host larvae provided at the same time, and other details for practical oviposition.

Although I have examined the effects of light radiation of 400 - 700 nm, the reaction of host larvae and parasitoid wasps to ultraviolet radiation should be also examined. This is because ultraviolet radiation may be more stimulative for increasing host activity. Also, the wasps are relatively nocturnal (Nishimura et al., 2015) and thus may be highly sensitive to weak ultraviolet illumination during ovipositional behavior.

In these experiments, host larvae were previously taken out from nest tunnels by hand, but this process needs be automated also. Intense light, heat and/or vibration will be examined to drive them out for subsequent oviposition.

CHAPTER IV

Establishment of arrhenotokous *M. pulchricornis* strains

Introduction

Meteorus pulchricornis (Wesmael) (Hymenoptera: Braconidae) is a solitary and koinobiont endoparasitoid of a wide range of lepidopteran pest in East Asia and Oceania (e.g., Liu and Li, 2006, 2008; Maeto, 1989; Marsh, 1979; Takashino et al., 1998; Walker et al., 2016), having both thelytokous (asexual) and arrhenotokous (sexual) strains in nature (Tsutsui et al., 2014; Fujie et al., 2019).

Thelytoky is a type of parthenogenesis in which females can produce diploid female offspring without mating, occasionally occurring in Hymenoptera (Aeschlimann, 1990). The cytogenetic research on thelytoky of *M. pulchricornis* was conduct by Tsutsui et al. (2014), first in the family Braconidae. Arrhenotoky is the most common mode of reproduction mode (haplodiploidy) in Hymenoptera (Heimpel and de Boer, 2008), in which unfertilized eggs develop parthenogenetically into haploid males and fertilized eggs develop into diploid females. Tsutsui et al. (2014) identified both thelytokous and arrhenotokous strains in Japan, revealing that the thelytokous strains were truly asexual apomictic clones, not induced by any kind of symbiotic bacteria. Abe et al. (2013) showed marked genetic differentiation in polymorphic melanism among thelytokous strains in Japan. Also, deuterotoky (females producing parthenogenetic males and females) has rarely been observed (Fujie et al., 2019) and nuclear genome differentiation between sympatric sexual and asexual strains is not distinct in Japan (unpublished data), suggesting that gene-flow between sexual and asexual strains may occasionally occur in nature condition.

In order to further understand the reproductive modes and genetic diversity of *M. pulchricornis* for the use as biocontrol agents for various lepidopteran pests, arrhenotokous (sexual) strains as well as thelytokous (asexual) strains of *M. pulchricornis* should be established.

Materials and methods

Insect rearing

Collected *M. pulchricornis* were maintained on *S. litura* larvae that were reared on artificial diets (Insecta LFS, Nosan Corporation, Yokohama). Adult wasps were given an absorbent cotton ball soaked in 50% honey solution and kept at 15–20°C under a 16L:8D photoperiod (16 h light and 8 h dark).

Experiment 1: Collecting and identifying the reproductive mode of wild *M. pulchricornis*

Field surveys were conducted three times during 2017 to 2018 at Takamatsu City in Kagawa Prefecture, Japan (Fig.4-1). Individuals of *M. pulchricornis* were collected by finding their cocoons under the leaf of bean plants (Fig.4-2.) or gathering their host larvae of *S. litura.* The larvae of *S. litura* were reared in 25°C under a 16L:8D photoperiod until wasp emergence of the wasp. One male wasp and multiple female wasps were reared in a petri dish (90 mm diameter × 15 mm height) (Fig.4-3.) for 7 days. Mated female wasps were each provided with 10 individuals of the second or third instar larvae of *S. litura* for oviposition. Every two days, the 10 host larvae were removed and replaced with new larvae at least four times. The sex of adult *M. pulchricornis* was obtained following the method of Fujie et al., (2019) and the reproductive mode of the strain was determined. For example, if male individual was identified, the strain was defined as a sexual strain. Correspondingly, female offspring were divided into two groups, one group paired with males, the other were allowed to perform parthenogenesis. Their reproductive model was confirmed from the sex of third generation of the wasps.

Experiment 2: Establishment of arrhenotokous *M. pulchricornis* strains

Adult of the strains performing arrhenotokous reproduction were paired for mating. Single female and single or multiple males were reared in the same petri dish (Fig.4-3.) for 7 days. After the mating process, mated female wasps were each provided with 10 individuals of the second or third instar larvae of *S. litura* for oviposition. Every two days, the 10 host larvae were removed and replaced with new larvae at least four times. The offspring of mated wasps were paired with other strains in equal proportions, in order to prevent inbreeding depression.

Results

Experiment 1

From the surveys in Kagawa Prefecture, 124 individuals of *M. pulchricornis* were obtained. After the mating process they were reared for a least three generations, and the reproductive mode of 48 female individuals was confirmed. During these surveys, 21 arrhenotokous individuals and 27 thelytokous individuals were collected, as shown in Table 4-1.

Experiment 2

After the mating process, four arrhenotokous strains have been successfully reared for nine generations (Fig. 4-4).

Table. 4-1 List of female individuals collected and their reproductive modes

Individuals	Reproductive modes	Location	Collected date
T17-22	Arrhenotoky	Kagawa Prefecture	9/13/2017
T17-23	Thelytoky	Kagawa Prefecture	9/13/2017
T17-24	Thelytoky	Kagawa Prefecture	9/13/2017
T17-25	Thelytoky	Kagawa Prefecture	9/13/2017
T17-27	Thelytoky	Kagawa Prefecture	9/13/2017
T17-29	Thelytoky	Kagawa Prefecture	9/13/2017
T17-44	Thelytoky	Kagawa Prefecture	9/13/2017
T17-53	Thelytoky	Kagawa Prefecture	9/13/2017
T17-57	Arrhenotoky	Kagawa Prefecture	9/13/2017
T17-60	Thelytoky	Kagawa Prefecture	9/13/2017
T17-63	Thelytoky	Kagawa Prefecture	9/13/2017
T17-68	Thelytoky	Kagawa Prefecture	9/13/2017
T17-70	Thelytoky	Kagawa Prefecture	9/13/2017
T2F1	Arrhenotoky	Kagawa Prefecture	9/23/2018
T2F2	Arrhenotoky	Kagawa Prefecture	9/23/2018
T2F3	Uthelytoky	Kagawa Prefecture	9/23/2018
T2F4	Arrhenotoky	Kagawa Prefecture	9/23/2018
T2F6	Thelytoky	Kagawa Prefecture	9/23/2018
T2F8	Arrhenotoky	Kagawa Prefecture	9/23/2018
T2F10	Arrhenotoky	Kagawa Prefecture	9/23/2018
T2F11	Thelytoky	Kagawa Prefecture	9/23/2018
T2F12	Thelytoky	Kagawa Prefecture	9/23/2018
T2F13	Arrhenotoky	Kagawa Prefecture	9/23/2018
T2F15	Arrhenotoky	Kagawa Prefecture	9/23/2018
T2F16	Thelytoky	Kagawa Prefecture	9/23/2018
T2F17	Arrhenotoky	Kagawa Prefecture	9/23/2018
T2F18	Arrhenotoky	Kagawa Prefecture	9/23/2018
T2F19	Thelytoky	Kagawa Prefecture	9/23/2018
T2F21	Arrhenotoky	Kagawa Prefecture	9/23/2018
T2F22	Arrhenotoky	Kagawa Prefecture	9/23/2018
T2F23	Thelytoky	Kagawa Prefecture	9/23/2018
T2F24	Arrhenotoky	Kagawa Prefecture	9/23/2018
T2F26	Thelytoky	Kagawa Prefecture	9/23/2018
T2F28	Thelytoky	Kagawa Prefecture	9/23/2018
T2F33	Thelytoky	Kagawa Prefecture	9/23/2018
T2F35	Arrhenotoky	Kagawa Prefecture	9/23/2018
T2F36	Thelytoky	Kagawa Prefecture	9/23/2018
T2F38	Arrhenotoky	Kagawa Prefecture	9/23/2018
T2F39	Thelytoky	Kagawa Prefecture	9/23/2018
T2F40	Arrhenotoky	Kagawa Prefecture	9/23/2018
T2F42	Thelytoky	Kagawa Prefecture	9/23/2018
T2F43	Arrhenotoky	Kagawa Prefecture	9/23/2018
T2F46	Arrhenotoky	Kagawa Prefecture	9/23/2018
T2F48	Arrhenotoky	Kagawa Prefecture	9/23/2018
T2F49	Thelytoky	Kagawa Prefecture	9/23/2018
T2F50	Arrhenotoky	Kagawa Prefecture	9/23/2018
T2F51	Thelytoky	Kagawa Prefecture	9/23/2018
T2F52	Thelytoky	Kagawa Prefecture	9/23/2018

Fig. 4-1. A bean farm in Takamatsu City in Kagawa Prefecture

Fig. 4-2. A spindle-shaped cocoon of *M. pulchricornis* in a soybeanfield

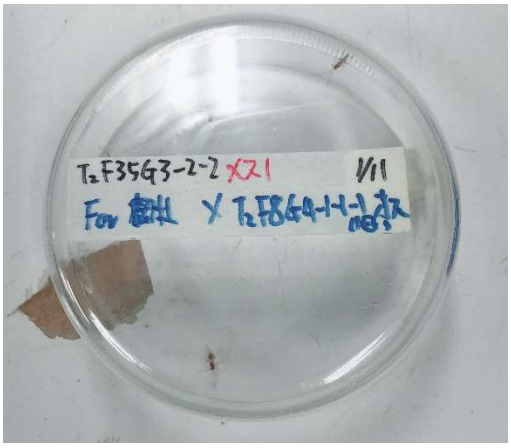


Fig. 4-3 Rearing of paired *M. pulchricornis* in a petri dish

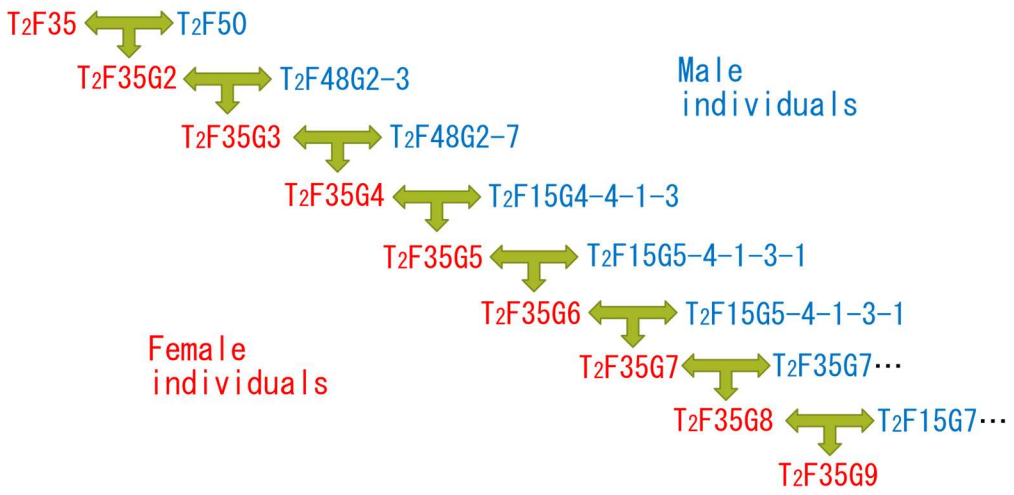


Fig. 4-4. Crossing between arrhenotokous strains of *M. pulchricornis*

Discussion

There are three hypotheses for the evolution of asexuality: 1) the recessive gene hypothesis, 2) the hybridization hypothesis, and 3) the cytoplasmic element hypothesis, They are considered for *M. pulchricornis*.

First, the recessive gene hypothesis explains that asexuality is induced by the homozygous state of nuclear recessive genes within sexual populations. For example, automictic thelytoky in *Apis mellifera* capensis Eschscholtz (Lattorff et al., 2005), *Lysiphlebus fabarum* (Marshall) (Sandrock and Vorburger, 2011), and probably also in *Venturia canescens* (Gravenhorst) (Beukeboom and Pijnacker, 2000; Schneider et al., 2003; Mateo Leach et al., 2009).

Second, some reports indicate that an asexual strain without meiosis can be accidentally born by the crossing of distantly related strains, which may be the case for apomictic thelytoky of *Trichogramma cacoeciae* Marchal (Hymenoptera: Trichogrammatidae) (Vavre et al., 2004). This may be also the case for the apomictic thelytoky occurring in *M. pulchricornis* populations (Tsutsui et al., 2014), because the

sexual and asexual strains are neighboring or sharing the habitats (Fujie et al., 2019).

Third, the cytoplasmic element hypothesis explains that asexuality is induced by cytoplasmic factors. For instance, bacterial symbionts such as *Wolbachia* and *Rickettsia* are known to induce automictic or apomictic thelytoky in micro-hymenopterans (e.g., Stouthamer and Kazmer, 1994; Tagami and Miura, 2007; Adachi-Hagimori et al., 2008) and in the braconid *Asobara japonica* Belokobylskij (Kremer et al., 2009). However, this hypothesis is likely not be the case for *M. pulchricornis.* PCR assay with bacterial universal primers was negative and generations of antibacterial treatment did not produce any male offspring indicate that thelytoky in *M. pulchricornis* is not cause by bacterial symbionts (Tsutsui et al., 2014).

In this study, I successfully established the arrhenotokous strains of *M. pulchricornis,* in order to test the above-mentioned recessive gene hypothesis, although there was no thelytokous strain from crossing sexual strains; this may be simply because the strains obtained from the surveys did not contain the required genes, due to lack of individuals from deferent strains or because of the poor successful rate in mating (the sexual strains I maintained only came from 4 pairs of wasps). In hindsight, the poor success rate in mating may be caused by inbreeding depression or owning to appearance of diploid males. Further examination of this topic may bring more insight into the evolution of asexuality. Thus, more scheduled surveys are needed in order to obtain more sexual (arrhenotokous) strains of *M. pulchricornis* for more crossing experiments.

CHAPTER V

General discussion

Use of *E. kuehniella* larvae as substitute hosts for *M. pulchricornis*

There are three ways of rearing natural enemies: 1) on the natural host, 2) on artificial diet and 3) on substitute hosts. In augmentation, inoculative releases as well as inundative releases, the mass-rearing system of natural enemies are needed to be established beforehand. Due to high host specificity, koinobiont parasitoids are often forced to use their natural hosts for rearing, while the natural hosts are usually difficult to be treated in mass-rearing. However, *M. pulchricornis* still has potential as a biological agent. This study confirmed that the nearly fully-grown larvae of the Mediterranean flour moth *E. kuehniella* could be a substitute host for *M. pulchricornis* in mass rearing practices (Chapter II; Nakano et al., 2018). Also, by improving the mobility of *E. kuehniella* larvae through alternative lighting, oviposition behavior of *M. pulchricornis* can be accelerated (Chapter III).

Considerations for the mass-rearing of *M. pulchricornis*

Natural enemies, such as predators and parasitoids in augmentative control, are mass-reared to ensure that they are affordable and reliably for suppression of the targets or establishment of the natural enemy populations. The efficacy of biological control is closely related to the quality of mass-reared parasitoids (Ridgway and Morrison, 1985). Visser (1994) shows the effect of body size of the parasitoid *Aphaereta minuta* (Nees) (Hymenoptera: Braconidae). The quality (fitness) of parasitoid can be determined by its 1) lifetime fecundity, 2) longevity, and 3) host-finding abilities (Visser, 1994). In the case of *Trichogramma,* their fitness is closely related to body size. For example, large hosts produce large parasitoids and lifetime fecundity and longevity are correlated with the body size of the wasp (Bai, 1986; Bai et al., 1992; Pavlik,1993), thus using host with large body size would be an important part of mass-rearing parasitoids. In this case,

from the results of my study, there are two major problems still need to be addressed in order to utilize *E. kuehniella* larvae as substitute hosts for the mass-rearing of *M. pulchricornis*.

Small body size of *M. pulchricornis*

Chapter II has revealed that the body size of the parasitoid wasp *M. pulchricornis* is limited by the body size of *E. kuehniella* larvae at oviposition (Nakano et al., 2018). The small size of *E. kuehniella* in nature may decrease the general reproductive ability of *M. pulchricornis* as a biocontrol agent.

The body weight of emerged adult wasps which came from *E. kuehniella* larvae was approximately 60% of those reared on the natural host *S. litura*. The small body size may diminish the dispersal or host-finding ability of parasitoid wasps (Ellers et al. 1998). Also, the egg load and lifetime fecundity of parasitoid wasps usually depends on the body size of females (Jervis et al. 2007). The small body size could be the reason why the lifetime fecundity of the wasps (median, 65 eggs) from *E. kuehniella* was approximately half of those reared on the natural host *S. litura* (median, 139 eggs). Moreover, the fecundity was much smaller compared to those reared on *Lymantria dispar* (L.) (Lymantriidae) (average, 268 eggs) (Fuester et al. 1993).

To solve these problems, any approaches to enlarge *E. kuehniella* larvae should be considered. For example, by giving juvenile hormone analog (JHA) to *E. kuehniella* larvae to prolong the larva stage, the body size of the full-grown larvae could be increased (Keisuke Okazawa, unpublish data). Alternatively, an artificial diet could be another way to increase body size of the larvae (Kondo, 2011). For example, in *Spodoptera*, larvae given a high-quality protein diet had higher survival rates and bigger body size (Lee et al., 2008). Further studies are needed to obtain larger *E. kuehniella*

larvae constantly for the mass-rearing of *M. pulchricornis*.

Low oviposition rate of *M. pulchricornis*

Chapter III has revealed that by utilizing the negative phototaxis of *E. kuehniella* larvae (Brandt, 1934), autonomous oviposition of *M. pulchricornis* on *E. kuehniella* larvae can be enhanced by alternate lighting of white LEDs. This practice has approximately doubled the number of eggs laid per wasp. However, there are two issues needed to be addressed.

First, *M. pulchricornis* wasps were often crippled by the silk-threads spun by *E. kuehniella* larvae for constructing nest webs. The wasps could hardly recover from the silk-thread entanglement and died easily after struggle for short period of times, thus they were unlikely to be successful in oviposition. In the alternate lighting conditions, however, the decrease of intact wasps owing to the silk-thread entanglement was comparatively slow. This may probably because added time of moving could decrease time for spinning threads or high density of threads in same places. However, even under alternate lighting, intact wasps began to decrease after around ten hours. Therefore, to avoid silk-thread entanglement, oviposition should be conducted within less than ten hours under alternate lighting.

Second, the autonomous oviposition rate of *M. pulchricornis* to *E. kuehniella* larvae represented only one-third of the potential fecundity of this species (Fuester et al., 1993). There is still much room remines for improvement in the oviposition method. In this study, only one host larva was provided to each wasp for oviposition, whereas *M. pulchricornis* females could induce a short-term host paralysis for the avoidance of multiple oviposition (self-superparasitism) (Chau and Maeto, 2009). This may cause *E. kuehniella* larvae to interrupt their movement after oviposition and decrease the chance

of being oviposited again. Also, according to Sheng et al. (2014), a high host density has a positive effect on *M. pulchricornis* host searching behavior and oviposition. Providing multiple hosts to each wasp for practical oviposition and to determine new oviposition time for higher silk tangle rate are major issues that need to be addressed for designing a practical mass-rearing method. In other hand, parasitoids also locate their host by detecting chemical cues left in the environments. Yamamoto et al., 2009 shows that host finding behavior of *M. pulchricornis* depends on movement of the host. However, it did not exclude the use of chemical cues during host searching by this species. For example, some volatiles released from host plants as defense against herbivores, or the chemicals and excretion from the host themselves are crucial factors for host finding behavior of parasitoids (Lewis and Tumlinson, 1988; Morehead and Feener, 2000). Finally, the positive phototaxis of *M. pulchricornis* may also increase the chance of wasps coming into contact with host larvae under alternate lighting. Further investigation will be necessary to understand such effects on wasp oviposition.

Establishment of sexual strains of *M. pulchricornis*

In Chapter IV, in addition to the presently asexual (thelytokous) strains, some sexual (arrhenotokous) strains have been successfully established in the laboratory. Although no asexual strain originated from crossing sexual strains, demonstrating that the recessive gene hypothesis was not supported, this may be simply that the strains I used did not contain required genes due to a small number of original individuals. In hindsight, the poor mating success rate may also be caused by inbreeding depression or appearance of diploid males (Cook and Crozier, 1995; Heimpel and de Boer, 2008). Further examination may bring more insight of the evolution of asexuality, for which more intensive crossing experiments with much more sexual strains are needed.

Conclusion

This study shows that *E. kuehniella* is a suitable substitute host for rearing *M. pulchricornis*, though it would be a much better host if the host body size were increased. Also, alternate LED lighting was shown to increase the movement of *E. kuehniella* larvae, resulting in an increased oviposition rate of *M. pulchricornis*. Further investigations are still necessary to determine the best timing of light alternation, light wavelength and intensity, the number of host larvae provided at the same time, and other details for practical oviposition.

Summary

Chapter I

I have reviewed the research subjects and problems to be solved related to biological control using parasitoid wasps. Biological control is an environmentally safe and effective way against pests by utilizing herbivory, predation, parasitism, or other natural mechanisms (Eilenberg et al., 2001). Biological control has become a main-stream practice over recent decades (DeBach et al., 1971; Flint and Dreistadt, 1998; Gerhardson, 2002), thus the demand of biological control, especially by the adoption of integrated pest management (IPM) programs, as well as the demand of organic cropping are increasing.

The release of natural enemies to control pests is a type of biocontrol called augmentation. By using commercially available species to prevent the pest population increases, or to suppress a pest population. The efficient mass-rearing system of natural enemies is a critical factor to conducting an efficient augmentation against target pest. For that purpose, mass-rearing system with optimized conditions including inexpensive and easily manageable artificial diets, substitute prey, or substitute host insects need to be provided.

Meteorus pulchricornis (Wesmael) (Hymenoptera, Braconidae) is a solitary endoparasitoid of free-living lepidopteran larvae, with a wide range of host families (Maeto, 2018). *M. pulchricornis* has been considered to be a promising biological agent, due to the existence of the thelytokous strains, which are expected to be more effective as biological control agents than arrhenotokous (sexual) strains with the two-fold reproduction (Aeschlimann, 1990; Stouthamer, 1993). It is usually considered to be difficult to rear koinobiont parasitoids on substitute hosts due to high host-specificity, though this may not always be true because *M. pulchricornis* is a polyphagous koinobiont.

The Mediterranean flour moth *Ephestia kuehniella* (Zeller) (Lepidoptera: Pyralidae) is a cosmopolitan storage pest and can be reared in dry conditions and it grows exponentially without much of care and time. Thus, the eggs and larvae of *E. kuehniella* have been widely used as diets or hosts for mass-rearing of predators and parasitoids in biological control (Borzoui et al., 2016; Smith, 1996).

The goal of this study is to design a better way of mass-rearing *M. pulchricornis* for augmentation with the following objects (1) evaluated the host suitability of *E. kuehniella* for *M. pulchricornis*, (2) establish a better mass-rearing method for *M. pulchricornis* on *E. kuehniella* and (3) establish sexual strains in the laboratory, in order to further understand the reproductive modes and genetic diversity of *M. pulchricornis* for use as biocontrol agents.

Chapter II

In this chapter, the host suitability of *E. kuehniella* on *M. pulchricornis* was examined. The body size, longevity, and lifetime fecundity of adult wasps reared on *E. kuehniella* and on a natural host *Spodoptera litura* (Fabricius) (Noctuidae) were compared. I observed that there is a positive relation between the body size of the host at oviposition and that of *M. pulchricornis*. As the flesh weight of *E. kuehniella* larva increase, the adult emergence rate of *M. pulchricornis* become higher, too. This chapter revealed that the nearly fully-grown larvae of the Mediterranean flour moth *E. kuehniella* could be potential substitute hosts for *M. pulchricornis* in mass rearing practices. However, the lifetime fecundity is lower when using *E. kuehniella* as hosts and the autonomous oviposition of *M. pulchricornis* on *E. kuehniella* is difficult.

Chapter III

This chapter confirmed that autonomous oviposition of *M. pulchricornis* on *E. kuehniella* larvae is enhanced by alternate lighting. According to Yamamoto et al. (2009), it is expected that increased host movement accelerates the oviposition behavior of *M. pulchricornis*. This experiment confirmed that the mobility of *E. kuehniella* larvae was enhanced by using alternate lighting of LEDs. The total length of larval movement under alternate lighting increased about three-fold when compared to the larvae under constant lighting. Consequently, was approximately doubled the number of eggs laid per wasp.

Chapter IV

In this chapter, the sexual (arrhenotokous) strains of *M. pulchricornis* were successfully established. In the field surveys in Kagawa Prefecture during 2017 to 2018, 124 individuals of *M. pulchricornis* were obtained. After mating experiments, the reproductive mode of 48 female individuals was confirmed, showing 21 sexual (arrhenotokous) and 27 asexual (thelytokous) strains. Sexual (arrhenotokous) strains have been reared and crossed for nine generations. There was no asexual strain obtained from crossing sexual strains, thus, the recessive gene hypothesis for the origin of asexuality (thelytoky) was not supported.

Chapter V

Finally, I discuss the results of this study and problems that remain. In conclusion, this study shows that *E. kuehniella* is a suitable substitute host for rearing *M. pulchricornis*.

However, the small size of *E. kuehniella* in nature may decrease the general

reproductive ability of *M. pulchricornis* as a biocontrol agent. Because the body size of the parasitoid wasp *M. pulchricornis* is limited by the body size of *E. kuehniella* larvae at oviposition, the life time fecundity of *M. pulchricornis* from *E. kuehniella* was less than natural hosts. Thus, enlargement of *E. kuehniella larvae* is a potential solution to this problem.

On the other hand, alternate LED lighting increased the movement of *E. kuehniella* larvae, which consequently increases the oviposition rate of *M. pulchricornis* on them. Although the oviposition rate of *M. pulchricornis* on *E. kuehniella* larvae is lower compared to its natural hosts, it could be improved by giving it multiple hosts for oviposition. Further investigations are necessary to determine the best timing of light alternation, the light wavelength and intensity, the number of host larvae provided at the same time, and other details for practical oviposition.

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