A THESIS

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Biological characteristics and rearing conditions of the stick insect, *Ramulus irregulariterdentatus* (Phasmida: Phasmatidae)

대벌레의 생물학적 특성과 사육조건에 관한 연구

BY

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ENTOMOLOGY PROGRAM

DEPARTMENT OF AGRICULTURAL BIOTECHNOLOGY

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UNDER THE DIRECTION OF ADVISER JOON-HO LEE
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Biological characteristics and rearing conditions of the stick insect, *Ramulus irregulariterdentatus* (Phasmida: Phasmatidae)

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ABSTRACT

Public interest in *Ramulus irregulariterdentatus* as a pet insect in Korea is increasing although it is also considered as a potential forest pest. This study was carried out to investigate the biological characteristics and mass rearing technique of *R. irregulariterdentatus*.

In 2016, seasonal occurrence of *R. irregulariterdentatus* was investigated at Guksabong Peak in Goyang, Gyeonggi-do. The first instar nymphs appeared from April 20. The population of the species was peak on May 19 and then declined in late July. No more individual were observed after September. The 62.5 % of individuals were observed from black locust trees and 21.5 % oak trees such as oriental chestnut oak, mongolian oak and chestnut. The amount of daily leaf consumption by one adult was about 4.2 cm^2 (0.09 g). *R. irregulariterdentatus* propagates mainly by parthenogenesis, thus male occurs very rarely in nature, but a male was discovered

which was mating in Goyang, 2013. The color of 1st and 2nd instar nymphs was always light brown and light green, respectively, while 3rd-6th instar nymphs and adults showed green or brown according to their living conditions.

Several unique survival strategies of *R. irregulariterdentatus* were investigated. The duration of death-feigning of nymphs and adults was 336 sec. and 515 sec., respectively. *R. irregulariterdentatus* showed color change from the 3rd instar nymph. After autotomy of one leg, the leg was regenerated in the next or 2nd next molting. At the first phase, the leg was malformed with the symptoms of twisted, thin and short shape. At the next phase, the leg was unfolded normal shape but very short, compared to normal one. The short leg became longer as molting was repeated, and was recovered by being over 90 % of length of the normal leg after 3 times of molting after regeneration of the twisted leg.

Development rates were fitted with a nonlinear Briére model which estimated optimal temperatures to be 24.5 and 26.2 °C with upper development thresholds of 29.3 and 31.4 °C for egg and nymph, respectively. In a linear model, lower development thresholds were estimated as 7.6 and 5.2 °C for egg and nymph, respectively**.** Survivorship was the highest at 21.0 and 22.2 °C for egg and nymph, respectively. Mean fecundity per female life ranged from 14.4 eggs at 17.5 °C to 32.0 eggs at 23.5 °C. It was fitted to an extreme value function. Adult survival and cumulative oviposition rate of *R. irregulariterdentatus* were fitted to a sigmoid function and a two-parameter Weibull function, respectively. These models can be

used to forecast phenology and population dynamics of *R. irregulariterdentatus* in the fields and optimize environmental conditions for rearing *R. irregulariterdentatus.*

The egg hatch rate of *R. irregulariterdentatus* was more than 80 % at 20-25 °C, but 0 % at 30 °C. In nature, *R. irregulariterdentatus* overwinters as an egg, but it appears that cold temperatures are not required for termination of egg diapause. When several treatments were taken to improve the egg hatch rate, in the treatment using floral foam, fermented sawdust and leaves, the rate was 98.2 %. Clover (*Trifolium repens*) was an excellent diet as it was similar to the host plant and could be used as an alternative diet. The rate from 1st instar nymph to adult of *R. irregulariterdentatus* was 88.3 % by using an artificial diet of 25 % black locust leaf powder and 1 % agar. In the density experiments for mass rearing, the survival rate from 1st instar to adult for 60 days was 82.0 % at 50 insects/pot (59 × 18 × 13 cm, planted clover) and the number of oviposition was 710 at 20 adults/cage (49 × 34 × 27 cm).

Key words: *Ramulus irregulariterdentatus*, educational pet insect, survival strategy, development model, oviposition model, rearing condition.

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I. Occurrence, morphological characteristics and survival strategies of *Ramulus irregulariterdentatus*

General introduction

Stick insects are phytophagous. Over 3,000 species of stick insects have been found in the world and most of them are present mainly in tropical regions (Tilgner, 2008; Brock and Hasenpusch, 2009). In Korea, there are 5 species belonging to 2 families, Phasmatidae (*Ramulus irregulariterdentatus* (Brunner von Wattenwyl, 1907), *R. koreanum* (Kwon, Ha et Lee, 1992), *Phraortes illepidus* (Brunner von Wattenwyl, 1907)) and Diapheromeridae (*Micadina phluctaenoides* (Rehn, 1904) and *M. yasumatsui* (Shiraki, 1935)) (Paek et al., 2010). Stick insects are characterized by long body length with body color change ability. For example, Chan's Megastick, a Malaysian species of stick insects, is known to reach 55.9 cm (Gray, 2013). Stick insects can change their body color from green to brown by detecting darkness from the surroundings when they were located on dark background objects (Bückmann, 1977). Some species of stick insects can purposely lose some of their legs to aid their escapes from predators. They can regenerate these lost limbs during successive molts (Tilgner, 2008). In addition to these characteristics, stick insects are sluggish and nonaggressive, and thus are popularly used for educational purpose worldwide (Tilgner, 2008).

Ramulus irregulariterdentatus (Phasmida: Phasmatidae) also has basic characteristics of stick insects. *R. irregulariterdentatus* is wingless and propagates mainly by parthenogenesis. Because *R. irregulariterdentatus* is distributed in Korea and Japan (Kwon et al., 1992), this species is expected to be relatively cold resistant compared to tropical species such as *Didymuria violescens*, *Medauroidea extradentata*, *Phobaeticus kirbri*, and *Phobaeticus serratipes*. Its wingless characteristic is an important property as a pet insect because escape is less likely. Parthenogenesis makes mass rearing much easier. Moreover, because of homomorphism, *R. irregulariterdentatus* can maintain the most popular stick shape (i.e., first instar to adult stages) to be used as a pet insect during a long period (average of 120 days) at room temperature (approximately 24 °C) (Lee et al., 2013).

In Korea, *R. irregulariterdentatus* can be threatening forest pest if outbreak occur, although *R. irregulariterdentatus* has high potential as a pet and educational insect. In 2001, this species caused 21,000 ha of damage at the broad-leaved forest of four provinces in Korea (Korea Forest Research Institute, 2001). However, there is no more report on stick insect damages. Up to date, the ecology of *R. irregulariterdentatus* has been rarely studied. Park et al. (2003) have reported that *R. irregulariterdentatus* occurs one generation per year under natural conditions and overwinters as eggs in Korea. Its overwintering period is very long. Overwintering eggs of *R. irregulariterdentatus* were found from mid-July to mid-April of the next year (Park et al., 2003). The duration of its overwintering eggs was approximately 150 days at 15 °C (Yamaguchi and Nakamura, 2015). Its host range is broad including oak, berry, persimmon, apricot, pear, cherry blossom, chestnut, cherry and so on (Kwon et al., 1992). Its lower developmental threshold and thermal requirement from eggs to adults were estimated to be 6.6 °C and 909.1 degree days, respectively (Park et al., 2003). However, egg development and survival models, non-linear developmental models of each stage, or reproduction models of *R. irregulariterdentatus* have not been reported yet. These models are needed to decide optimal temperature for its mass-rearing, and for a population model of *R. irregulariterdentatus*, which can be useful for forecasting its seasonal occurrence and population dynamics.

Many studies have been carried out on feeding preference tests, body color change depending on the environmental conditions, oviposition behavior, maturation divisions of parthenogenesis, preying by spiders, and leg movements in various stick insects (Pijnacker, 1966; Detlef, 1977; Cassidy 1978; Carlberg, 1984; Eva and Ulrich, 1985; Wolfgang, 1990). But no research was done about its mass rearing method for commercial use.

For mass rearing, researches are needed such as hatching conditions, diets and proper rearing colony size. In relation to hatching, many studies were conducted on various insects such as *Cadra cautella, Nannophya pygmaea* and *Antheraea yamamai* (Kim et al., 2003; Kim et al., 2006; Yoon et al., 2007). They focused mainly the temperature and period for hatching. For rearing *R. irregulariterdentatus,* alternative or artificial diets are very important because their natural diets from broadleaf trees such as black locust trees, oak trees and chestnut trees, are hard to maintain in sufficient quantity all year, particularly in winter. The alternative or artificial diets can be used for sustainable rearing during seasons when it is difficult to obtain the host plant or other food. In addition, optimal rearing colony size should be determined.

Therefore, this study was conducted to investigate the occurrence, morphological characteristics and survival strategies of *R. irregulariterdentatus*, to develop development and oviposition models of *R. irregulariterdentatus*, to determine the best hatching conditions, artificial diets and the optimal mass rearing method of *R. irregulariterdentatus.*

Chapter I.

Occurrence, morphological characteristics and survival strategies of *Ramulus irregulariterdentatus*

1-1. Abstract

In 2016, seasonal occurrence of *R. irregulariterdentatus* was investigated at Guksabong Peak in Goyang, Gyeonggi-do. The first instar nymphs appeared from April 20. The population of the species was peak on May 19 and then declined in late July. No more individual were observed after September. The 62.5 % of individuals were observed from black locust trees and 21.5 % oak trees such as oriental chestnut oak, mongolian oak and chestnut. The amount of daily leaf consumption by one adult was about 4.2 cm^2 (0.09 g).

The periods of first instar nymph to adult of male were 75 days at 19.8 °C and 52 days at 27.0 °C. The color of 1st instar and 2nd instar nymphs was always light brown and light green, respectively, while 3rd - 6th instar nymphs and adults showed green or brown according to their living conditions. Early operculum and early gonapophyses began to develop on 8th and 9th abdominal segments of the 2nd instar nymph. Gonapophyses completely developed in the adult stage and was covered with operculum. The 10th tergum of the male adult was divided into two branches and phallus was projected on the 9th abdominal segment.

Several unique survival strategies of *R. irregulariterdentatus* were investigated. The duration of death-feigning of nymphs and adults was 336 sec. and 515 sec., respectively. When they were in herbaceous place, they were hard to be found. *R.*

irregulariterdentatus showed color change from the 3rd instar. After autotomy of one leg, the leg was regenerated in the next or 2nd next molting. For 1st and 2nd instar nymphs, regeneration occurred mainly at the 2nd molting, with the rate of 71.9 % and 79.5 %, respectively, while for the 3rd instar nymph, 87.9 % of regeneration occurred mainly at the next molting with. At the first phase of regeneration, a twisted, short and deformed leg was occurred. After next molt, the leg was unfolded normal shape but very short. The short leg became longer as molt was repeated, and was recovered over 90 % of length of opposite normal leg after 3 times molt after regeneration of twisted leg.

Key words: *Ramulus irregulariterdentatus*, morphological, survival strategy, death-feigning, color change, regeneration

1-2. Introduction

Stick insects are not dangerous to children touch or catch them and enough slow move possible for observation, so these species are popular as pets and as displays at zoological gardens (Capinera, 2008). They are useful for behavioural, aesthetic and developmental observations for education in the classroom (Lang, 2014). Stick insects routinely shed legs to escape a predator or tangled moult, and these legs are subsequently re-grown (Maginnis, 2006). Their body color can change depending on the environmental conditions; in *Carausius morosus*, the body pigmentation can be affected by visual stimulation of its compound eyes (Detlef, 1977). Many studies have been carried out on feeding preference, oviposition behavior, maturation divisions of parthenogenesis, preying by spiders, and leg movements in various stick insects (Pijnacker, 1966; Cassidy 1978; Carlberg, 1984; Eva and Ulrich, 1985; Wolfgang, 1990). There were very few studies on *R. irregulariterdentatus* which distributed in Asia such as Korea and Japan (Kwon et al., 1992). In Korea, the research about stick insect was focused on pest control because stick insect was regarded forest harmful insect (Kwon T.S., 2000; Kwon T.S., 2002; Park et al., 2003).

Thus, this study was carried out to investigate the occurrence, morphological characteristics and survival strategies such as death-feigning, color change, and leg regenerating of *R. irregulariterdentatus*.

1-3. Materials and methods

1-3-1. Experimental insects

Adults of *R. irregulariterdentatus* were collected from Mt. Mubong in Hwaseong, Gyeonggi-do, Korea. These insects were reared with fresh leaves of black locust (*Robinia pseudoacacia*), oriental chestnut oak (*Quercus aliena*), chestnut (*Castanea crenata var. dulcis*), bush clover (*Lespedeza bicolor*), and clover (*Trifolium repens*) more than four successive generations before they were used in this study. Colonies were maintained in the laboratory at 24-26 °C, 60-70 % RH, and a photoperiod of 16:8 (L:D) h.

1-3-2. Occurrence and leaf consumption

The seasonal occurrence survey of *R. irregulariterdentatus* was carried out at Guksabong Peak in Goyang, Gyeonggi-do. This area was the large outbreak site of *R. irregulariterdentatus* in 2014. In 2016, *R. irregulariterdentatus* was collected for one hour every 2 weeks from April 12 to September 6. In 2014 and 2015, *R. irregulariterdentatus* was collected 2 times (mid-July and early August). The body length of collected *R. irregulariterdentatus* was measured using a ruler in the laboratory.

Thirty adults were starved for 24 hours and were placed individually in each rearing cage (20 × 15 × 15 cm) on the bottom of which wetted paper kitchen towel was placed. The fresh leaves of oriental chestnut oak were supplied as food and leaf area and weight were measured every 24 hours for 3 consecutive days. Fresh leaves were replaced every day. Weight and area of each leaf were measured using the electronic balance (ATX-224, Shimadzu, Japan) and leaf area meter (LI 3100, LI-COR, USA), respectively. Measurement was conducted before leaf supply and after leaf consumption. The base of the leaf was wrapped with wet kitchen paper towel and then paper towel was wrapped with foil.

1-3-3. Morphological characteristics

The specimens of nymphs of each stage and adults (male and female) were made and examined for the external morphology and terminal region of abdomen. Terminal region of abdomen of each stage nymphs and adults were pictured 3 directional views (dorsal, lateral, and ventral). Eggs were observed and pictured under a stereomicroscope (Stereo Discovery V12, Carl Zeiss).

The body length of each stage was measured using a ruler in the laboratory and the insects were same as those of nymphal developmental experiment at 23.5 °C in Chapter 2. The method of the developmental period of male was same as that of nymphal developmental experiment in Chapter 2.

1-3-4. Survival strategies

1-3-4-1. Death-feigning

The stick insects on trees or walls fall down to the ground by lightly touching and played death- feigning. The length of times of death- feigning was measured with 30 nymphs in May and 39 adults in July, 2015 at Guksabong Peak in Goyang, Gyeonggi-do. We checked if we could find them in 10seconds when stick insects fall down in the middle of the herbaceous place not on the ground. Sixty nymphs and 60 adults were surveyed in May and in July, 2015, respectively, at Guksabong Peak.

1-3-4-2. Color change

During the seasonal occurrence survey for *R. irregulariterdentatus*, color of each individual was checked for green or brown.

1-3-4-3. Regeneration

During the seasonal occurrence survey for *R. irregulariterdentatus*, it was examined which leg of collected insects was missing or regenerated. The 1st, 2nd, and 3rd instar nymphs which lost one leg among 6 legs were brought to the laboratory and they were individually reared in each rearing cage (20 × 15 × 15 cm) on the bottom of which wetted paper kitchen towel. The fresh leaves of clover were supplied every day for food. The cages were placed in the rearing rooms at 24-26 °C, 60-70 % RH, and a photoperiod of 16:8 (L:D) h. For each instar, 30 individuals were examined.

When the insect died at the 1st instar nymph, new 1st instar nymph was replenished. The day of molting of regenerating for each stage was investigated every day. The length of regenerated legs and adults was measured using a ruler and the regenerated legs were pictured under a stereomicroscope (Stereo Discovery V12, Carl Zeiss).

1-4. Results

1-4-1. Occurrence

In 2016, *R. irregulariterdentatus* appeared as a 1st instar nymph on April 20, and highest peak was observed on May 19. Its population density maintained for a while, decreased significantly in late July, and further decreased slowly during August and was not observed from September (Fig. 1).

Based on the length of the stick insect, the 1st instar nymphs occurred mainly until early May. The 2nd instar nymphs appeared in mid-May and the 3rd instar nymphs appeared in late May with 5th instar in late June and adults from July.

The first instar nymphs occurred commonly on oriental chestnut oak trees in April and early May. And then, second instar nymphs and adults occurred mainly on black locust trees in mid-May to late August (Table 1). Among 323 stick insects in 2016, 62.5 % of insects were observed on black locust trees and 21.5% of insects on oak trees such as oriental chestnut oak, mongolian oak and chestnut.

The daily amount of leaf consumption of *R. irregulariterdentatus* was shown in Table 2. The 24-hour starving insect had 4.9 ± 2.42 cm^2 (0.11 ± 0.053 g) of leaf on the first day, and on the second and third days, slightly reduced, they ate 4.0 ± 2.32 cm^2 (0.09 ± 0.051 g) and 3.8 ± 2.08 cm^2 (0.08 ± 0.045 g), respectively, and the average was 4.2 ± 2.27 cm^2 (0.09 ± 0.050 g).

The male of *R. irregulariterdentatus* was very rare and has not been reported to be found outdoors (Kwon et al., 1992). However, in 2013, a mating male was first discovered at the military facility in Goyang, Gyeonggi-do (Fig. 2).

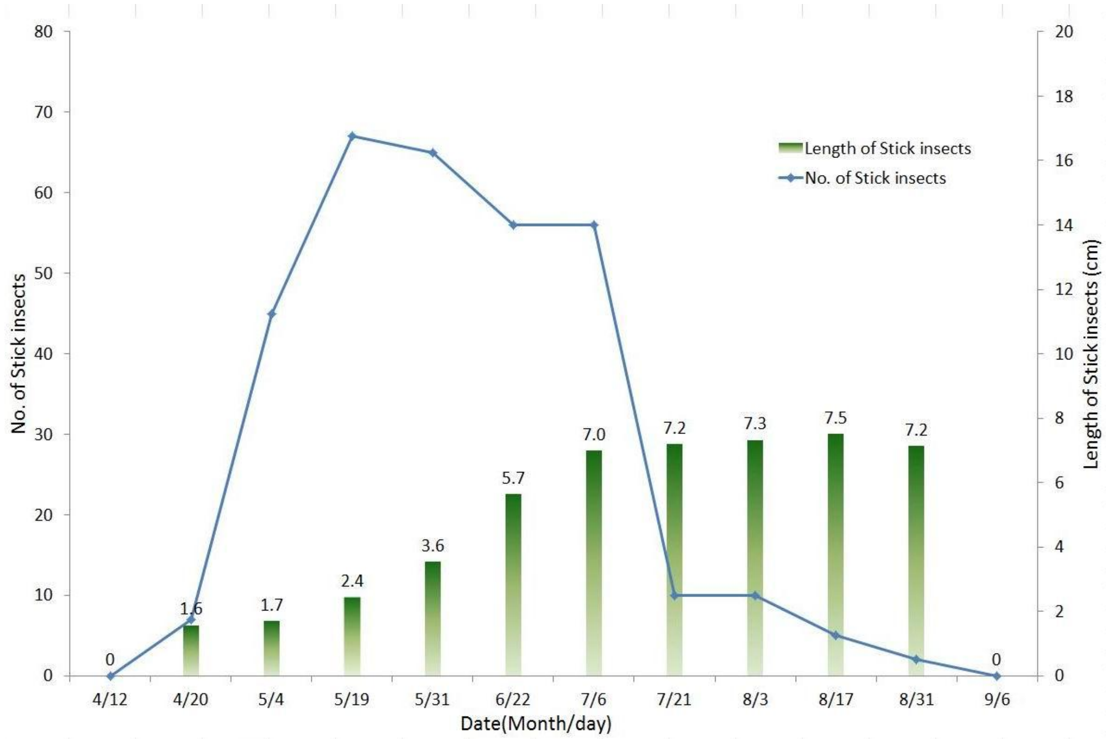

Figure 1. Seasonal occurrence and body length of *R. irregulariterdentatus* at Guksabong Peak in Goyang, Gyeonggi-do, 2016.

※ 2014. July 16 : over 500 insects, August 4 : 215 insects

2015. July 15 : 92 insects, August 6 : 36 insects

Table 1. Relationship between inhabitant plants and occurrence of *R. irregulariterdentatus* at Guksabong Peak in Goyang, Gyeonggi-do.

Inhabitant plants			Date (2016)										Total
Korean name	Common name	Scientific name	4/20	5/4	5/19	5/31	6/22	7/6	7/21	8/3	8/17	8/31	
갈참나무	Oriental chestnut oak	*Quercus aliena*	4	40	12	-	-	2	-	-	-	-	58
신갈나무	Mongolian oak	*Quercus mongolica*	1	1	6	-	-	-	-	-	-	-	8
밤나무	Chestnut	*Castanea crenata*	-	-	3	-	-	-	-	-	-	-	3
아까시나무	Black locust	*Robinia pseudoacacia*	-	4	25	52	50	48	8	9	5	2	202
왕벚나무	Korean flowering cherry	*Prunus yedoensis*	-	-	4	-	-	-	-	-	-	-	4
뽕나무	White mulberry	*Morus alba*	-	-	2	1	1	1	-	-	-	-	5
개암나무	Asian hazel	*Corylus heterophylla*	-	-	3	3	1	-	-	-	-	-	7
산딸기나무	Wild raspberry	*Rubus crataegifolius*	2	-	3	2	3	-	-	-	-	-	10
환삼덩굴	Wild hop	*Humulus japonicus*	-	-	2	2	-	-	-	-	-	-	4
Others*			-	-	7	6	1	5	2	1	-	-	22
Total			7	45	67	65	56	56	10	10	5	2	323

* Herbaceous plants, wall of building, ground, etc.

Table 2. Daily consumption (area : cm^2, mean ± SD and weight : g, mean ± SD) of oriental chestnut oak(*Quercus aliena*) leaves by adult of *R. irregulariterdentatus.*

Leaf consumed by insect	1st day	2nd day	3rd day	Average
Leaf area (cm^2, mean ± SD)	4.9 ± 2.42	4.0 ± 2.32	3.8 ± 2.08	4.2 ± 2.27
Leaf weight (g, mean ± SD)	0.11 ± 0.053	0.09 ± 0.051	0.08 ± 0.045	0.09 ± 0.050

※ Wrap the oak leaf stalk with wet tissue and foil and place in insect starved for 24 hours within rearing cage (20 × 15 × 15 cm).

Figure 2. First report of mating male of *R. irregulariterdentatus* in Goyang, Gyeonggi-do in 2013.

1-4-2. Morphological characteristics

The characteristics of developmental stages are shown in Fig 3. The color of eggs was dark brown. They had an irregular tube shape and micropylar plate in the middle part and operculum at the tip which was gateway at hatching. *R. irregulariterdentatus* was incomplete metamorphosis, so both of their shape of adult and nymph were like slender stick and similar to each other. The legs had dark and light strip and there was no wing. The thoraxes consisted of 3 parts and the abdominal segments were composed of 10 parts. The color of 1st instar nymph was light brown and that of 2nd instar nymph was light green, while that of 3rd-6th instar nymphs and adult was green or brown according to its living circumstances. *R. irregulariterdentatus* reproduced offspring by parthenogenesis, resulting in its male occurred very rarely in nature.

The male was much slender than female, and the color of male was light brown with two white lines from prothorax to 10 abdominal segment. The length of male was shorter than female but the antenna of male was longer than that of female. To classify the wingless 3 species of stick insects in Korea, *Phraortes illepidus* has very long antenna can be distinguished other 2 species and irregular tube shaped egg of *R. irregulariterdentatus* can be differentiate from egg of *R. koreanum* (Kwon, 2000).

The characteristics of terminal region on the stages of nymph and adult are shown in Fig 4. One pair of cercus was projected on 10th abdominal segment. Early operculum and early gonapophyses began to develop on 8th and 9th abdominal segments at 2nd nymph. Gonapophyses completely developed at adult and was covered with operculum. The 10th tergum of male was divided two branches and phallus was projected on 9th abdominal segment.

Bragg (1997) classified the ovipositor of Phasmida to three distinct forms: scoop-shaped operculum, oviscapt ovipositor, and appendicular ovipositor. The female genitalia of *R. irregulariterdentatus* are similar to third forms which have elongated operculum and gonapophyses. The male genitalia of *R. irregulariterdentatus* resemble much with those recently reported as stick insect species: *Micrarchus hystriculeus* (New Zealand) and *Hermarchus leytensis* (Philippines) (Buckley, 2009; Marco and Davide, 2014).

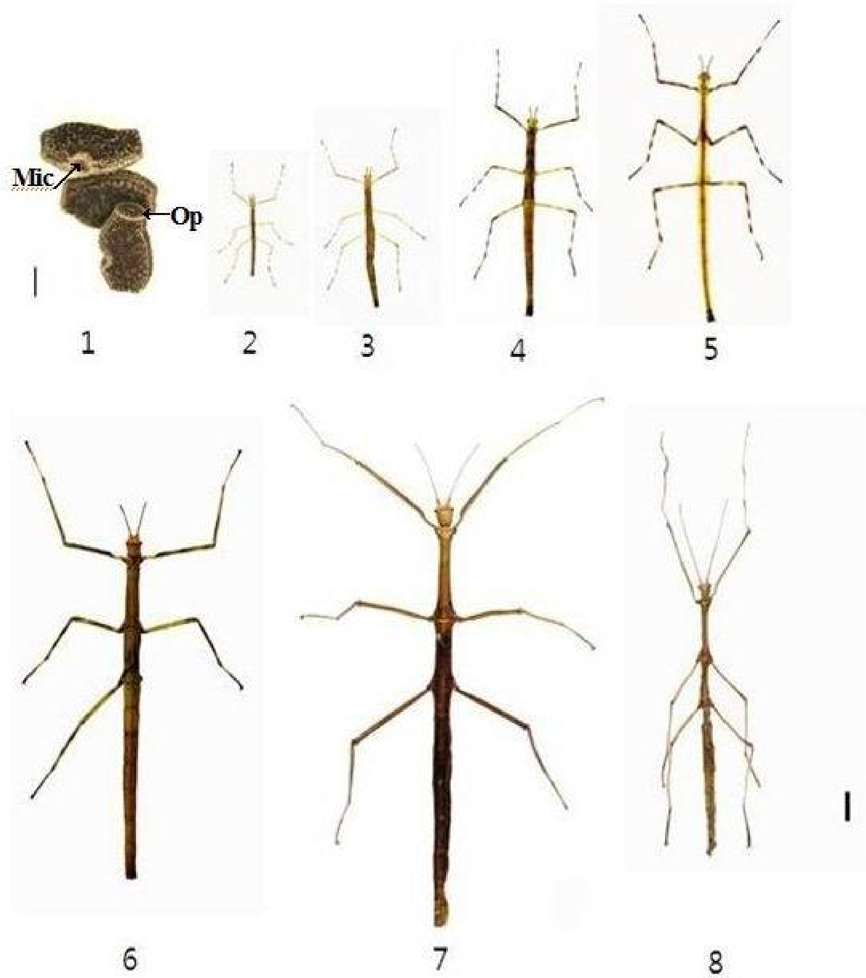

Figure 3. External shape characteristics by developmental stages of *R. irregulariterdentatus*.

(1) Eggs (length 0.33mm); (2-7) Females; (2) 1st instar; (3) 2nd instar; (4) 3rd instar; (5) 4th instar; (6) 5th instar (right hind leg missed); (7) Adult; (8) Male adult; (Op) Operculum; (Mic) Micropylar plate; (Scale bar 1: 1mm, 2-8: 5mm).

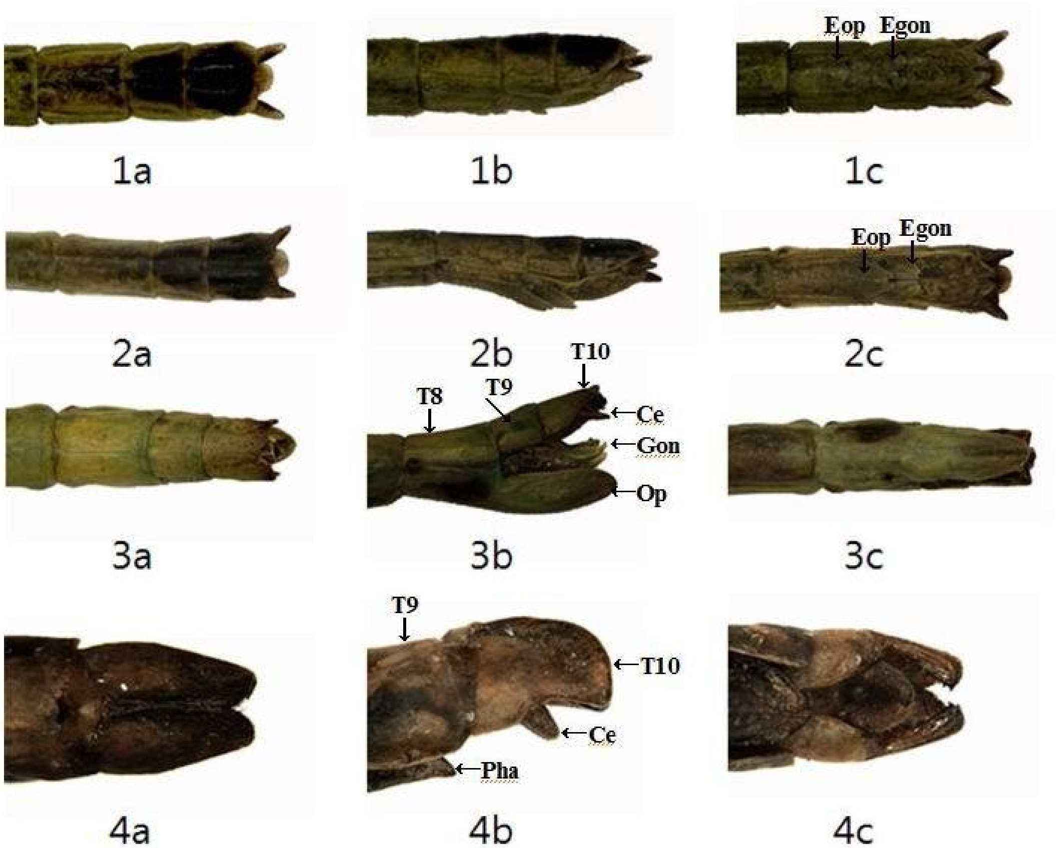

Figure 4. Terminal region on nymph stages and adult of *R. irregulariterdentatus*.

(1) 2nd instar nymph of female; (2) 4th instar nymph of female; (3) female adult; (4) male adult; (a) dorsal view; (b) lateral view; (c) ventral view; (T8-T10) Abdominal tergum 8-10; (Ce) Cercus; (Gon) Gonapophyses; (Op) Operculum; (Pha) Phallus; (Eop) Early operculum; (Egon) Early gonapophyses.

The nymph lengths (1-6 instars) were 1.6 ± 0.2, 2.4 ± 0.1, 3.4 ± 0.1, 4.5 ± 0.1, 5.8 ± 0.4 and 6.6 ± 0.3 cm, respectively (Table 3). The body lengths were clearly separable according to the instar and the deviation in length was very low. The body length was most suitable to distinguish nymphal stages because head width and body weight could be overlapped (Park et al., 2003). Two males occurred accidentally during the temperature development test, and the length was 4.7 cm. The periods of first instar nymph to adult of male were 75 days at 19.8 °C and 52 days at 27.0 °C (Table 4).

Table 3. Body length (cm, mean ± SD) of each stage of *R. irregulariterdentatus.*

Egg	Sex	Nymph stages (instar)						Adult
		1st	2nd	3rd	4th	5th*	6th**	
0.33 ± 0.0	Female	1.6 ± 0.2	2.4 ± 0.1	3.4 ± 0.1	4.5 ± 0.1	5.8 ± 0.4	6.6 ± 0.3	7.5 ± 0.4
	Male***	-	-	-	-	-	-	4.7

※ Measurement of the length immediately after molt.

* 5th instar: 62.9%

* 6th instar: 37.1%

*** Coincidental occurrence of two males during breeding

Table 4. Developmental period (days) on nymph stages of male of *R. irregulariterdentatus* at two temperatures.

Temperature. (°C)	Nymph stages (instar)					1st instar to adult
	1st	2nd	3rd	4th	5th	
19.8	19	11	14	13	18	75
27.0	9	7	8	18	10	52

1-4-3. Survival Strategies

1-4-3-1. Death-feigning

When *R. irregulariterdentatus* was touched, it fall down to the ground and pretended to be dead. Duration of death-feigning of second and third instar nymphs was the highest with 56.7 % at 1-5 minutes, the average time was 336 sec and the maximum time was 1,347 sec (Table 5). The time of adults was the highest with 33.3 % at 5-10 minutes, the average time was 515 sec and the maximum time was 2,169 sec.

In order to validate whether this death-feigning is effective to hide, we examined the probability of discovery when the stick insect behaved death-feigning after falling in the middle of the herbaceous place. In second and third instar nymphs, the probability of discovery was 31.7 % which means 68.3 % of the stick insects can survive thank to death-feigning (Table 6). In adult, the probability of discovery was high with 73.3 % because of its bigger size than nymph.

Table 5. Duration of death-feigning of *R. irregulariterdentatus* in the field condition at Guksabong Peak in Goyang, Gyeonggi-do.

Developmental stage	Time of death-feigning (%)					Average
	1 min. under	1-5 min.	5 -10 min.	10-20 min.	20 min. over	
Nymph (2nd-3rd instar)	6.6	56.7	20.0	13.3	3.3	336 sec. (Max. 1,347 sec.)
Adult	10.3	30.8	33.3	15.4	10.3	515 sec. (Max. 2,169 sec.)

Table 6. Probability of discovery within 10 seconds of *R. irregulariterdentatus* behaved death-feigning after falling in the middle of the herbaceous place at Guksabong Peak in Goyang, Gyeonggi-do.

Developmental stage	Probability of discovery (%)
Nymph (2nd-3rd instar)	31.7
Adult	73.3

1-4-3-2. Color change

R. irregulariterdentatus showed color change from the third instar nymph and 23.2 % at the fifth instar nymph (Table 7).

Table 7. Seasonal dynamics of color change by *R. irregulariterdentatus* at Guksabong Peak in Goyang, Gyeonggi-do, 2016.

Contents	Date									
	4/20	5/4	5/19	5/31	6/22	7/6	7/21	8/3	8/17	8/31
Rate of color changed insects (%)	0	0	0	13.8 (9)*	23.2 (13)	10.7 (6)	20.0 (2)	10.0 (1)	60.0 (3)	100 (2)
Developmental stage	1st instar	1st instar	2nd instar	3rd instar	5th instar	Adult	Adult	Adult	Adult	Adult

* Numbers in parentheses indicate the numbers of color-changed insects investigated.

1-4-3-3. Regeneration

The rate of missing leg was the highest in July at 53.0 %, with the average of 39.7 % (Table 8). The regeneration rate of missing leg was 12.3 %. The missing leg occurred mainly at foreleg with 54.1 % at foreleg, 21.6 % at midleg and 24.3 % at hind leg (Table 9).

Table 8. The rates of missing and regeneration leg of *R. irregulariterdentatus* at Guksabong Peak in Goyang, Gyeonggi-do, 2016.

Contents	Season				
	May	June	July	August	Total
Rate of missing leg (%)	38.5 (25)*	26.8 (15)	53.0 (35)	35.3 (6)	39.7 (81)
Rate of regeneration leg (%)	16.0 (4)	20.0 (3)	8.6 (3)	0 (0)	12.3 (10)

* Numbers in parentheses indicate the numbers of color-changed insects investigated.

Table 9. The rate of missing leg relative to the leg position of *R. irregulariterdentatus* at Guksabong Peak in Goyang, Gyeonggi-do, 2016.

Leg location	Foreleg	Midleg	Hind leg
Rate of missing leg (%)	54.1	21.6	24.3

After autotomy of one leg, the leg was regenerated through next or after next molt. When first instar nymph and second instar nymph missed legs, regeneration occurred mainly at second molt, showing the rate of 71.9 % and 79.5 %, respectively (Table 10). When 3rd instar nymph missed leg, regeneration rate was 87.9 %, occurred mainly at first molt. At the first phase, the leg was malformed with the symptoms of twisted, thin and short shape (Fig 5). At the next phase, the leg was unfolded normal shape but very short comparing with normal contralateral leg. The short leg became longer as molt was repeated, and was recovered over 90 % of length of contralateral leg after 3 times molt after regeneration of twisted leg (Table 11). Body lengths of adult developed from missing leg nymph were 7.5-7.6 cm, no difference in body length of normal adult (Table 12).

Table 10. The occurrence rate (%) of regeneration stage after missing leg of *R. irregulariterdentatus*.

Missing leg stage	Regeneration stage			
	2nd instar	3rd instar	4th instar	5th instar
1st instar	21.9	71.9	6.3	-
2nd instar	-	20.5	79.5	-
3rd instar	-	-	87.9	12.1

1 2a 2b 3

Figure 5. Process of regeneration after missing leg (left 3rd) of *R. irregulariterdentatus*.

(1) Missing leg (2nd instar); (2a, 2b) Regenerated but malformed twisted leg (3rd instar); (3) Straight but short leg (4th instar: regenerated left 3rd leg 1.6cm, normal right 3rd leg 2.7cm).

Table 11. The recovery rate (%, mean ± SD) of regenerated leg compare with original leg of *R. irregulariterdentatus*.

Missing leg stage	Molt times after regeneration of twisted leg			
	1 time	2 times	3 times	4 times
1st instar	64.3 ± 8.64	81.6 ± 6.55	91.1 ± 6.15	93.1 ± 4.77
2nd instar	67.8 ± 12.73	85.7 ± 12.30	94.7 ± 4.53	-
3rd instar	68.3 ± 9.24	89.0 ± 6.92	95.5 ± 5.45	-

Table 12. Body length (cm, mean ± SD) of adult developed from missing leg nymph of *R. irregulariterdentatus*.

Missing leg stage	Length of adult
1st instar	7.5 ± 0.22
2nd instar	7.6 ± 0.26
3rd instar	7.6 ± 0.24

1-5. Discussion

In general, the stick insect was known as an insect pest in the forest and mainly at oak trees (Bae, 2002). In July 2014, when the stick insect heavily occurred at Guksabong Peak in Gyeonggi-do, it was broadcasted under the title 「Subtropical forest insect pest "Stick insect", the first outbreak in the metropolitan area」 (KBS, 1994). The number of collected *R. irregulariterdentatus* for one hour at Guksabong Peak reached more than 500 individuals in 2014, but the number decreased to 92 in 2015 and 10 in 2016 (Fig. 1). Since an adult ate about 4 cm^2 (0.09 g) of leaves of oriental chestnut oak a day (Table 2) and it tended to prefer black locust to oak trees (Table 1), the damage to oak trees by stick insects, the main trees in Korea, can be said to be not severe. Kwon (2002) reported that it was the best control strategy of the stick insect to use the density regulation of ecosystem not chemical control except for damage to crops or people.

Although the stick insects can be a potential pest in the forest, they are highly likely to be industrialized as educational pet insects thanks to their behavioural or morphological characters. For industrialization of educational and pet purpose, it is very important that the unique characteristics of stick insect have to be emphasized. Stick insects are thin, weak, not poisonous and not biting, but have extraordinary survival strategies.

Stick insects take action of death-feigning. They pretend to be dead in crisis, can survive from the natural enemies who like living food and, especially, if they are in a forest or herbaceous place, it is hard to find them (Table 6).

Stick insects can change body color depending on circumstance where they live (Table 7). It will be a good defensive system to avoid the predators that their body shape resembles the twig and their body color change with background.

Stick insects can purposely lose some of their legs to aid their escape from a predator's grasp and can regenerate the lost limbs during molts (Table 10). But leg regeneration requires a lot of energy and it takes three or four molts to recover more than 90 % of the original leg (Table 11). Maginnis (2006) reported that leg regeneration stunted wing growth and hindered flight performance in a stick insect (*Sipyloidea sipylus*). It was inferred that as regeneration progressed, the adult would be shorter than normal adult, but the adult size was unaffected by regeneration occur or not in this study (Table 12). Wrinn and Uetz(2007) reported that leg regeneration of field-caught spiders exhibited reduced mass and molt interval but laboratory-reared spiders exhibited increased molt intervals but no difference in mass after regeneration.

Stick insects mainly reproduce through parthenogenesis without mating with male (Pijnacker, 1966). Parthenogenesis has the advantage of doubling productive female offspring to thrive. If male has to concern mating always, female has to have the chance for meeting to male and the 50 % of offspring will be male. *R.*

irregulariterdentatus mainly reproduce through parthenogenesis, but male exist and mate (Fig. 2). Although bisexual reproduction has the advantage of increasing genetic diversity, the role of male of *R. irregulariterdentatus* is a task to be studied.

Stick insects are great camouflager. Their body resembles the twig and sometimes moves back and forth in the wind like real twig (Fig. 6). Their eggs resemble the seed, so carnivore predators have little interest in the eggs. Their ability to remain motionless, resembling stick, as a primary mechanism of defense can make them difficult to find (Brock and Hasenpusch, 2009).

Figure 6. The egg and adult of *R. irregulariterdentatus* resembling the seed and twig, respectively.

Consequentially, these morphological characteristics and survival strategies of *R. irregulariterdentatus* will be a good educational content to teach students about the mysteries of life. Stick insects now are being used as pet or exhibition insects at the museum or insect zoo in the world (Capinera, 2008; Lang, 2014), and will be excellent educational pet insects in Korea.

Chapter II.

Temperature-dependent development and oviposition models of *Ramulus irregulariterdentatus*

2-1. Abstract

Public interest in *Ramulus irregulariterdentatus* as a pet insect in Korea is increasing, although it is also considered as a potential forest insect pest. The objective of this study was to construct development and oviposition models of *R. irregulariterdentatus*. Development rates were fitted with a nonlinear Briére model which estimated optimal temperatures to be 24.5 and 26.2 °C with upper development thresholds of 29.3 and 31.4 °C for egg and nymph, respectively. In a linear model, lower development thresholds were estimated as 7.6 and 5.2 °C for egg and nymph, respectively**.** Survivorship was the highest at 21.0 and 22.2 °C for egg and nymph, respectively. Mean fecundity per female life ranged from 14.4 eggs at 17.5 °C to 32.0 eggs at 23.5 °C. It was fitted to an extreme value function. Adult survival and cumulative oviposition rate of *R. irregulariterdentatus* were fitted to a sigmoid function and a two-parameter Weibull function, respectively. These models can be used to forecast phenology and population dynamics of *R. irregulariterdentatus* in the fields and optimize environmental conditions for rearing *R. irregulariterdentatus.*

Key words: *Ramulus irregulariterdentatus,* pet insect, development model, oviposition model

2-2. Introduction

Ramulus irregulariterdentatus (Phasmida: Phasmatidae), one of Asian species, also has basic characteristics of stick insects. *R. irregulariterdentatus* is wingless. It propagates mainly by parthenogenesis. It is also expected to be relatively cold resistant compared to tropical species such as *Didymuria violescens*, *Medauroidea extradentata*, *Phobaeticus kirbri*, and *Phobaeticus serratipes* based on its worldwide distribution. Its wingless characteristic is an important property as a pet insect because escape is less likely. Parthenogenesis makes mass rearing much easier. Moreover, *R. irregulariterdentatus* can maintain the most popular stick shape (i.e., first instar to adult stages) during a long period (average of 120 days) at room temperature (approximately 24 °C) (Lee et al., 2013).

In Korea, *R. irregulariterdentatus* is considered as a potential forest pest, although *R. irregulariterdentatus* has high potential as a pet and educational insect. *R. irregulariterdentatus* caused 21,000 ha damage of forests in 2001 (Korea Forest Research Institute, 2001). Although there have been no reports regarding damage by this stick insect since then, there is still a possibility of its outbreak under conditions of warm overwintering temperature. Up to date, the ecology of *R. irregulariterdentatus* has been rarely studied. Park et al. (2003) have reported that *R. irregulariterdentatus* occurs one generation per year under natural conditions and overwinters as eggs in Korea. Its overwintering period is very long. Overwintering eggs of *R. irregulariterdentatus* were found from mid-July to mid-April of the next year (Park et al., 2003). The duration of its overwintering eggs was approximately 150 days at 15 °C (Yamaguchi and Nakamura, 2015). Its host range is broad including oak, berry, persimmon, apricot, pear, cherry blossom, chestnut, cherry and so on (Kwon et al., 1992). The lower developmental threshold and thermal requirement from eggs to adults were estimated to be 6.6 °C and 909.1 degree days, respectively (Park et al., 2003). However, egg development and survival models, non-linear developmental models of each stage, and oviposition models of *R. irregulariterdentatus* have not been reported yet. These models are needed to decide optimal temperature for its mass-rearing, and for a population model of *R. irregulariterdentatus*, which can be useful for forecasting its seasonal occurrence and population dynamics.

Therefore, the objective of this study was to develop development and oviposition models of *R. irregulariterdentatus*. Stage-specific survival models of its immature stages are also studied.

2-3. Materials and methods

2-3-1. Experimental insects

Adults of *R. irregulariterdentatus* were collected from Mt. Mubong in Hwaseong, Gyeonggi-do, Korea. These insects were reared with fresh leaves of black locust (*Robinia pseudoacacia*), oriental chestnut oak (*Quercus aliena*), chestnut (*Castanea crenata var. dulcis*), bush clover (*Lespedeza bicolor*), and clover (*Trifolium repens*) more than four successive generations before they were used in this study. Colonies were maintained in the laboratory at 24-26 °C, 60-70 % RH, and a photoperiod of 16:8 (L:D) h.

2-3-2. Experimental procedures

Development of *R. irregulariterdentatus* eggs was determined at five temperatures (15.4, 20.3, 22.9, 24.9, and 29.3 °C) and that of nymphs was determined at six temperatures (17.5, 19.8, 22.9, 23.5, 27.0, and 31.4 °C). Oviposition of *R. irregulariterdentatus* was tested at five temperatures (17.5, 19.8, 22.9, 23.5, and 27.0 °C). Ovipositional experiment was conducted with survived individuals from nymphal developmental experiment. However, nymphal developmental experiment was conducted independently from egg developmental experiment because eggs needed very long time to complete their development (up to 209 days). All experiments were conducted under a photoperiod of 16:8 (L:D) h with RH of 50-80 % using GC-300 environmental chambers (Jeio Tech., Daejeon-si, Korea) for eggs and Gaooze environmental chambers (Korea Scientific Technique Industry Co., Suwon-si, Korea) for nymphs and adults. Temperature and relative humidity inside chambers were monitored using HOBO data loggers (U12-012, OnSet Computer Corp., Pocasset, MA, USA). Average temperatures were used for data analyses.

Eggs (< 1 day old) were collected by placing one layer of moistened kitchen towels on the bottom of a rearing cage (48 × 33.5 × 26 cm) (Wooju Chemical, Busan-si, Korea) in which 20 adults were reared with 20-30 clover plants (*T. repens*). A total of ten rearing cages were used for egg collection. Twenty to 22 eggs were placed on a dental pad (4 × 4 cm) (Deahan Medical, Chungju-si, Korea) with bottom covered by a disposable weighing dish (4.8 × 4.8 × 1 cm) (Korea Ace Scientific, Seoul-si, Korea). This weighing dish was placed on a moistened filter paper (9 cm diameter) (Tokyo Roshi Kaisha Ltd., Tokyo, Japan) in petri-dishes (9 cm diameter × 1.5 cm height) (SPL Sciences, Pocheon-si, Korea). Five petri-dishes were allocated at 22.9 °C. At other temperature conditions, three petri-dishes were allocated for each temperature. These petri-dishes were checked daily for egg hatching and for replenishing water.

To examine nymphal development, ten petri-dishes (approximately 200 unknown aged eggs) were placed at each temperature by the same method as described above. Newly hatched nymphs (< 1 day old) were then transferred individually onto a moistened filter paper (9 cm in diameter) in petri-dishes (10 cm in diameter × 4 cm in height) (SPL Sciences, Pocheon-si, Korea). Experiment was started with 60 nymphs at each temperature. New nymphs (< 1 day old) were added in case of nymphal death at relatively early developmental stage (up to fourth instar) to ensure enough number of adults for oviposition experiment. For preparation of new nymphs, one petri-dish with 20 eggs was added at each temperature every week. At 31.4 °C, all second instars did not survive. To obtain proper data set for generating stage-specific developmental models of nymph of *R. irregulariterdentatus*, ten individuals (< 1 day old) of third, fourth, fifth, and sixth instar nymphal stage reared at 27.0 °C were tested at 31.4 °C to check the development of respective stage. One clover plant wrapped with moistened kitchen towels was supplied as food source for each nymph. From the third instar, each individual was moved to a larger cage (30 × 10 × 10cm, custom-made with acryl) to minimize space stress. Other conditions were same except for using larger clover plants to provide enough food and multiple filter papers to cover the bottom of cage. These cages were checked daily for nymphal development and death and for replenishing food or water.

The oviposition experiment was started with individuals that completed their development to adults in the nymphal development experiment. Thus, the oviposition experimental conditions were also maintained the same as those of larger instar nymphal development experiment. Female adult survival (longevity) and the number of eggs laid were examined daily.

2-3-3. Model development and data analysis

The effect of temperature on the development of eggs and nymphs was tested with PROC GLM in SAS (SAS Institute, 2011). Means were separated by Tukey student range test ($P < 0.05$; SAS Institute, 2011). The effect of temperature on occurrence of the 6th instar nymphal stage was tested with Chi-square test (SAS Institute, 2011). These means were also separated by Chi-square test for all pairwise combinations ($P < 0.05$; SAS Institute, 2011).

Development rates of eggs and nymphs were expressed as the reciprocal of developmental times (in days) of each stage by applying a linear model (Davidson, 1994) and a non-linear model (Briére et al., 1999). A linear model was applied to fit the linear portion of developmental rate for each stage using PROC REG in SAS (SAS Institute, 2011). The linear model was:

$$y = ax + b \qquad (1)$$

where y was the developmental rate at temperature of x (°C), a was the slope, and b was y-intercept. Lower developmental threshold was calculated as $-a/b$ (Arnold, 1959). Thermal constant in degree-day (DD) required to complete development was calculated as $1/a$ (Campbell et al., 1974).

A non-linear model was used to fit the development rate data over the entire temperature range. The non-linear model (Briére et al., 1999) used in this study was:

$$R(T) = n \times T(T - T_{\mathrm{b}})(T_{\mathrm{L}} - T)^{1/2} \quad (2)$$

where $R(T)$ was the rate of development at temperature T (°C), n was an empirical constant, T_b was the lower developmental threshold (°C), and T_L was the upper developmental threshold. The optimum temperature (T_{opt}) at which the maximal rate of development occurs was estimated using the equation suggested by Briére et al. (1999). The operative thermal range (B_{80}) indicating ≥80 % performance of maximal rate was determined using the protocol of Lutterschmidt and Hutchison (1997). The operative thermal range (B_{80}) may be more applicable rather than the optimal temperatures (i.e., T_{opt}, T_m, and T_f) for selecting temperature conditions for insect rearing, natural enemy release, optimal sampling timing, and so on (Baek et al., 2017; Lutterschmidt and Hutchison, 1997).

Variation in developmental time for each stage was described by normalized cumulative distribution of the frequency of developmental time. Development times were standardized by physiological age (px) of immatures using Eq. (3):

$$p_x = \sum_{i=1}^{n} r(T_i) \quad (3)$$

where $r(T_i)$ was the developmental rate at temperature T (°C) at day i. Two-parameter Weibull function was then applied:

$$p(px) = 1 - exp^{-(px/\alpha)^{\beta}} \tag{4}$$

where $p(px)$ was the cumulative proportion of individuals that completed development at physiological time (px), and α and β were fitted constants.

Stage-specific survival rate (percentage) was calculated by dividing the number of individuals survived to the next stage by the initial number of individuals at each temperature. The following model (Eq. 5) suggested by Sanchez-Ramos et al. (2015) was used:

$$S(T) = 100 - exp^{a+bT+cT^2} \tag{5}$$

where $S(T)$ was the survival rate (%) at temperature T (°C), and a, b, and c were fitted parameters. Operative thermal range (B_{80}) with ≥ 80 % performance of maximal rate was determined using protocols recommended by Lutterschmidt and Hutchison (1997). The operative thermal range (B_{80}) might be more applicable to the temperature changing conditions (i.e., insect rearing, pesticide application, natural enemy release, optimal sampling timing, and so on) and the conflicting issues (i.e., selection rearing temperature based on different optimal developmental, ovipositional, and survivorship temperatures) compared to the optimum temperature (T_{opt}) (Baek et al., 2017). However, the survivorship model of 6th instars could not be developed because all or no individual developed to adults over temperature regimes tested.

The effect of temperature on longevity, fecundity, and pre-oviposition period was tested with PROC GLM (SAS Institute, 2011). Means were separated by Tukey student range test ($P < 0.05$; SAS Institute, 2011).

For the purpose of modelling, adult longevity was regarded as adult development. Adult development rate, the reciprocal of longevity in days as a function of temperature (°C), was fitted to a function from the library of TableCurve (Jandal Scientific, 1996). The following equation (Eq. 6) was used:

$$R(T) = a + bT + c \cdot exp^{(T)} \quad (6)$$

where R(T) was the developmental rate at temperature T (°C), and a, b, and c were fitted constants.

Total number of eggs per female (fecundity) was obtained by summing eggs daily laid by an adult female during its whole life span. The mean fecundity was calculated based on females examined in this study. The relationship between mean total fecundity and temperature (°C) was fitted to the following extreme value function (Eq.

7) of Kim and Lee (2003):

$$f(T) = a \cdot exp^{[1+(b-T)/k-exp^{(b-T)/k}]} \quad (7)$$

where *f*(*T*) was the mean number of total eggs produced by a female at temperature *T* (°C), a was the maximum reproductive capacity, *b* was the temperature (°C) at which the maximum reproduction occurred, and *k* was a fitted constant.

The cumulative oviposition rate according to physiological time was calculated at each temperature examined. Each cumulative oviposition rate was scaled to the maximum. Physiological time (*px*) of females was then calculated using Eq. (3). Two-parameter Weibull function (Eq. 4) was used to model scaled cumulative oviposition rates. This model indicates age-specific cumulative oviposition rate defined as the cumulative proportion of eggs laid by a female adult until physiological time (*px*).

Age-specific survival (proportion) of females at physiological time (*px*) was fitted to the following function (Eq. 8):

$$s(px) = exp^{(\gamma-px)/\delta} \quad (8)$$

where *s*(*px*) was the percentage of live females at physiological time (*px*), γ was the physiological time at 50% survival, and δ was a fitted constant. These parameters of the adult developmental rate model were estimated by TableCurve (Jandal Scientific, 1996). Other non-linear models used in this study were estimated using PROC NLIN in SAS (SAS Institute, 2011).

2-4. Results

2-4-1. Development of immature stages

Eggs of *R. irregulariterdentatus* could hatch at 15.4-24.9 °C, but not at 29.3 °C. Nymphs could complete their development at 17.5-27.0 °C. At 31.4 °C, few individuals of first instar could develop up to third instar without further development (Table 1).

Linear portions of development rate data were well fit to the linear model with the following results: egg (F = 198.0; df = 1, 3; P = 0.005 ; r^2 = 0.99), first instar (F = 77.5; df = 1, 4; P = 0.003; r^2 = 0.96), second instar (F = 55.3; df = 1, 4; P = 0.005; r^2 = 0.95), third instar (F = 25.2; df = 1, 4; P = 0.015; r^2 = 0.89), fourth instar (F = 91.9; df = 1, 4; P = 0.002; r^2 = 0.97), fifth instar (F = 130.8; df = 1, 4; P = 0.001; r^2 = 0.98), sixth instar nymphs (F = 46.4; df = 1, 4; P = 0.007; r^2 = 0.94), and total nymph stage (first instar to adult) (F = 123.5; df = 1, 4; P = 0.002; r^2 = 0.98) (Fig. 1; Table 2). Lower developmental thresholds for egg, first, second, third, fourth, fifth, sixth instar nymphs and total nymph stage were 7.6, 8.5, 8.1, 8.6, 9.5, 8.5, 6.4, and 5.2 °C, respectively. Thermal requirements (DD) to complete development for egg, first, second, third, fourth, fifth, sixth instar nymphs, and total nymph stage were 1,593.3, 186.9, 139.1, 139.3, 151.3, 193.8, 203.9 and 1,097.4 DD, respectively.

The non-linear Briére model provided a good fit for developmental rate data over the entire temperature: egg (F = 531.5; df = 2, 4; $P = 0.002$; $r^2 = 0.99$), first instar (F = 653.5; df = 2, 6; $P < 0.001$; $r^2 = 0.99$), second instar (F = 476.6; df = 2, 6; $P < 0.001$; $r^2 = 0.99$), third instar (F = 110.3; df = 2, 6; $P = 0.001$; $r^2 = 0.99$), fourth instar (F = 103.9; df = 2, 6; $P = 0.002$; $r^2 = 0.99$), fifth instar (F = 240.4; df = 2, 6; $P < 0.001$; $r^2 = 0.99$), sixth instar nymphs (F = 269.1; df = 2, 6; $P < 0.001$; $r^2 = 0.99$), and total nymph stage (F = 405.2; df = 2, 6; $P < 0.001$; $r^2 = 0.99$) (Fig. 1). Lower thresholds (T_b) for egg, first, second, third, fourth, fifth, sixth instar, and nymph stages were 8.9, 7.5, 8.2, 10.9, 11.5, 10.8, 9.4, and 8.7 °C, respectively. Upper thresholds (T_L) for egg, first, second, third, fourth, fifth, sixth instar, and nymph stages were 29.3, 35.4, 34.0, 31.4, 31.4, 31.4, 31.4, and 31.4 °C, respectively (Table 3). Thus, operative thermal ranges (T_b - T_L) of nymphs (8.7-31.4 °C) were slightly wider than those of eggs (8.9-29.3 °C). Optimal temperatures (T_{opt}) indicated by the maximal rate for egg, first, second, third, fourth, fifth, sixth instar, and nymph stages were 24.5, 29.2, 28.1, 26.5, 26.6, 26.5, 26.3, and 26.2 °C, respectively. Thermal ranges (B_{80}) with ≥ 80 % of the maximum value for egg, first, second, third, fourth, fifth, sixth instar, and nymph stages were 19.9-27.6, 23.2-33.2, 22.5-31.9, 21.8-29.6, 22.0-29.7, 21.8-29.6, 21.3-29.5, and 21.0-29.5 °C, respectively.

Table 1. Developmental time (day, mean ± SD) of *R. irregulariterdentatus* at five or six constant temperatures.

Temperature (°C)	Egg	Temperature (°C)	1st instar	2nd instar	3rd instar	4th instar	5th instar	6th instar	1st to adult
15.4	204.5±4.74a* (14/65)**	17.5	21.2±2.62a (60/91)	15.2±2.64a (60/70)	15.5±3.10a (59/64)	18.0±3.73a (54/59)	22.0±3.00a (51/54)	21.5±0.71a (2/2)	92.0±8.60a (51/105)
20.3	115.8±6.46b (23/60)	19.8	16.1±2.79b (59/82)	11.6±2.30b (59/63)	12.5±2.67b (59/61)	14.5±2.73b (57/59)	16.5±2.21b (54/57)	16.8±1.72b (9/9)	73.9±8.49b (54/86)
22.9	100.9±4.2c (36/103)	22.9	13.7±3.34c (60/79)	10.0±2.99c (60/63)	11.0±3.99c (60/60)	12.3±3.45c (59/62)	14.1±3.09c (56/59)	14.8±1.47b (11/11)	63.9±7.93c (56/84)
24.9	90.5±6.90d (20/63)	23.5	11.6±2.06d (59/81)	8.3±1.16d (59/65)	8.3±1.16d (59/60)	10.7±3.83c (59/64)	12.4±3.80d (57/59)	12.4±1.96c (25/25)	57.4±6.27d (57/94)
29.3	-***	27.0	10.2±2.70d (59/107)	7.5±1.79d (59/62)	7.7±2.22d (59/62)	8.4±1.74d (58/62)	10.5±2.55e (52/58)	11.5±1.21c (31/31)	51.3±5.88e (52/116)
		31.4	10.5±1.00cd (4/177)	8.8±2.22bcd (4/64)	-	-	-	-	-

* Means within a column followed by the same letter are not significantly different ($P > 0.05$; Tukey's HSD test at 95% confidence intervals)

** Numbers in parentheses indicate the numbers of individuals that survived / total number of individuals tested

*** No individuals survived

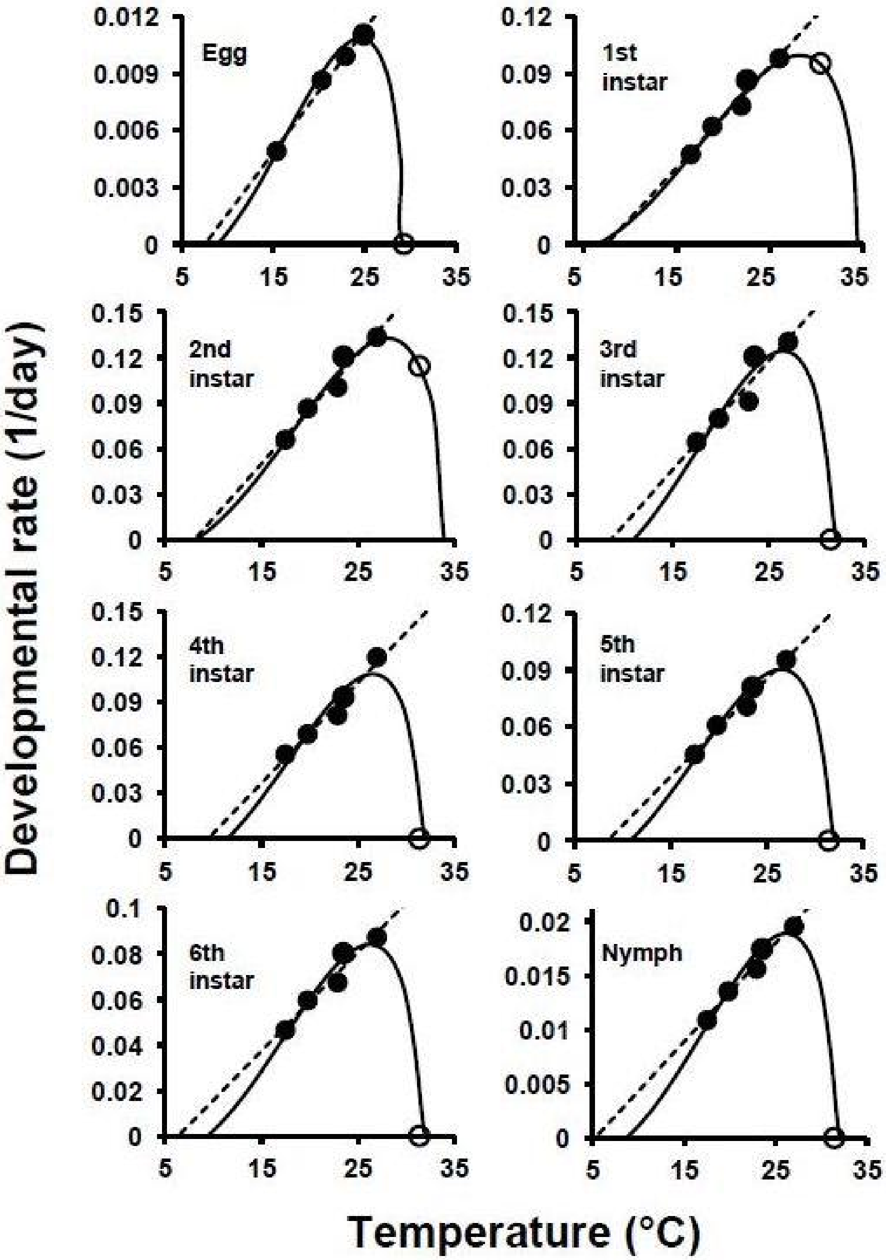

Figure 1. Linear and nonlinear development model for temperature-dependent rates of *R.irregulariterdentatus* (●, Observed; ○, Excluded in the linear model; ----, linear model; —, Nonlinear model).

Table 2. Parameter estimates (± SEM) of the linear development model, lower temperature threshold, and thermal requirement for each stage of *R. irregulariterdentatus.*

Stage	Parameters		r^2	Lower temperature threshold (°C)	Thermal requirement (Degree-days)
	a	*b*			
Egg	$6.4821 \times 10^{-4} \pm 6.8840 \times 10^{-5}$	$-4.9100 \times 10^{-3} \pm 1.4200 \times 10^{-3}$	0.99	7.6	1,542.7
1st instar	$5.3500 \times 10^{-3} \pm 6.0814 \times 10^{-4}$	$-4.5250 \times 10^{-2} \pm 1.3610 \times 10^{-2}$	0.96	8.5	186.9
2nd instar	$7.1900 \times 10^{-3} \pm 9.6728 \times 10^{-4}$	$-5.7950 \times 10^{-2} \pm 2.1650 \times 10^{-2}$	0.95	8.1	139.1
3rd instar	$7.1800 \times 10^{-3} \pm 1.4300 \times 10^{-3}$	$-6.1870 \times 10^{-2} \pm 3.2040 \times 10^{-2}$	0.89	8.6	139.3
4th instar	$6.6100 \times 10^{-3} \pm 6.8951 \times 10^{-4}$	$-6.2580 \times 10^{-2} \pm 1.5430 \times 10^{-2}$	0.97	9.5	151.3
5th instar	$5.1600 \times 10^{-3} \pm 4.5129 \times 10^{-4}$	$-4.3670 \times 10^{-2} \pm 1.0100 \times 10^{-2}$	0.98	8.5	193.8
6th instar	$4.3300 \times 10^{-3} \pm 6.3549 \times 10^{-4}$	$-2.7610 \times 10^{-2} \pm 1.4220 \times 10^{-2}$	0.94	6.4	230.9
1st to adult	$9.1124 \times 10^{-4} \pm 8.2010 \times 10^{-5}$	$-4.7800 \times 10^{-3} \pm 1.8400 \times 10^{-3}$	0.98	5.2	1,097.4

Table 3. Parameter estimates (± SEM) of the nonlinear development model for each stage of *R. irregulariterdentatus.*

Stage	Parameters			r^2
	n	T_b	T_L	
Egg	$1.30 \times 10^{-5} \pm 1.13 \times 10^{-6}$	8.9413 ± 1.0834	$29.3000 \pm 7.0150 \times 10^{-9}$	0.99
1st instar	$6.30 \times 10^{-5} \pm 1.30 \times 10^{-5}$	7.5261 ± 2.3790	35.4239 ± 1.0513	0.99
2nd instar	$9.80 \times 10^{-5} \pm 1.80 \times 10^{-5}$	8.2093 ± 2.2623	33.9570 ± 0.6602	0.99
3rd instar	$1.36 \times 10^{-4} \pm 2.70 \times 10^{-5}$	10.9361 ± 2.2867	$31.4000 \pm 1.6740 \times 10^{-8}$	0.99
4th instar	$1.23 \times 10^{-4} \pm 2.40 \times 10^{-5}$	11.4548 ± 2.1543	$31.4000 \pm 1.6920 \times 10^{-8}$	0.99
5th instar	$9.80 \times 10^{-5} \pm 1.30 \times 10^{-5}$	10.8010 ± 1.5846	$31.4000 \pm 1.1400 \times 10^{-8}$	0.99
6th instar	$8.40 \times 10^{-5} \pm 1.20 \times 10^{-5}$	9.4179 ± 1.8660	$31.4000 \pm 1.1270 \times 10^{-8}$	0.99
1st to adult	$1.80 \times 10^{-5} \pm 2.24 \times 10^{-6}$	8.6554 ± 1.7013	$31.4000 \pm 9.3860 \times 10^{-9}$	0.99

Developmental variations (i.e., distribution of development time) of all stages were well described by the two-parameter Weibull function: egg (F = 870.0; df = 1, 47; $P < 0.001$; $r^2 = 0.97$), first instar (F = 3,162.0; df = 1, 62; $P < 0.001$; $r^2 = 0.99$), second instar (F = 3,444.8; df = 1, 56; $P < 0.001$; $r^2 = 0.99$), third instar (F = 4,083.6; df = 1, 59; $P < 0.001$; $r^2 = 0.99$), fourth instar (F = 5,102.4; df = 1, 66; $P < 0.001$; $r^2 = 0.99$), fifth instar (F = 5,579.3; df = 1, 65; $P < 0.001$; $r^2 = 0.99$), sixth instar nymphs (F = 522.9; df = 1, 29; $P < 0.001$; $r^2 = 0.97$), and total nymph stage (F = 6,975.0; df = 1, 122; $P < 0.001$; $r^2 = 0.99$) (Fig. 2, Table 4).

The relationship between survival and temperature was also well described by this model: egg (F = 743.6; df = 2, 4; $P = 0.001$; $r^2 = 0.99$, first instar (F = 641.0; df = 2, 6; $P < 0.001$; $r^2 = 0.99$), second instar (F = 1,079.2; df = 2, 6; $P < 0.001$; $r^2 = 0.99$), third instar (F = 14,374.9; df = 2, 6; $P < 0.001$; $r^2 = 0.99$), fourth instar (F = 1,812.9; df = 2, 6; $P < 0.001$; $r^2 = 0.99$), fifth instar (F = 15,644.1; df = 2, 6; $P < 0.001$; $r^2 = 0.99$), sixth instar nymphs (F = 15,644.1; df = 2, 6; $P < 0.001$; $r^2 = 0.99$), and total nymph stage (F = 222.3; df = 2, 6; $P < 0.001$; $r^2 = 0.99$) (Fig. 3, Table 5). Temperatures (T_m) with the maximal survival (%) for egg, first, second, third, fourth, and fifth instar nymphs, and total nymph stage were estimated to be 21.0, 21.4, 23.0, 22.8, 22.2, 21.3, and 22.2 °C, respectively. Thermal ranges (B_{80}) with ≥ 80 % of the maximum value for egg, first, second, third, fourth, fifth instar, and nymph stages were 17.0-25.0, 15.6-27.3, 16.7-29.3, 16.1-29.5, 15.5-29.0, 13.8-28.7, and 17.2-27.2 °C, respectively.

Temperature had a significant effect on the occurrence of sixth instar nymph (X^2 =

36.9; df = 4; $P < 0.001$). With increasing temperature, the proportion of sixth instar occurrence was also increased from 3.9% at 17.5 °C to 59.6% at 27.0 °C (Table 6). All individuals that became sixth instar successfully completed development to adults.

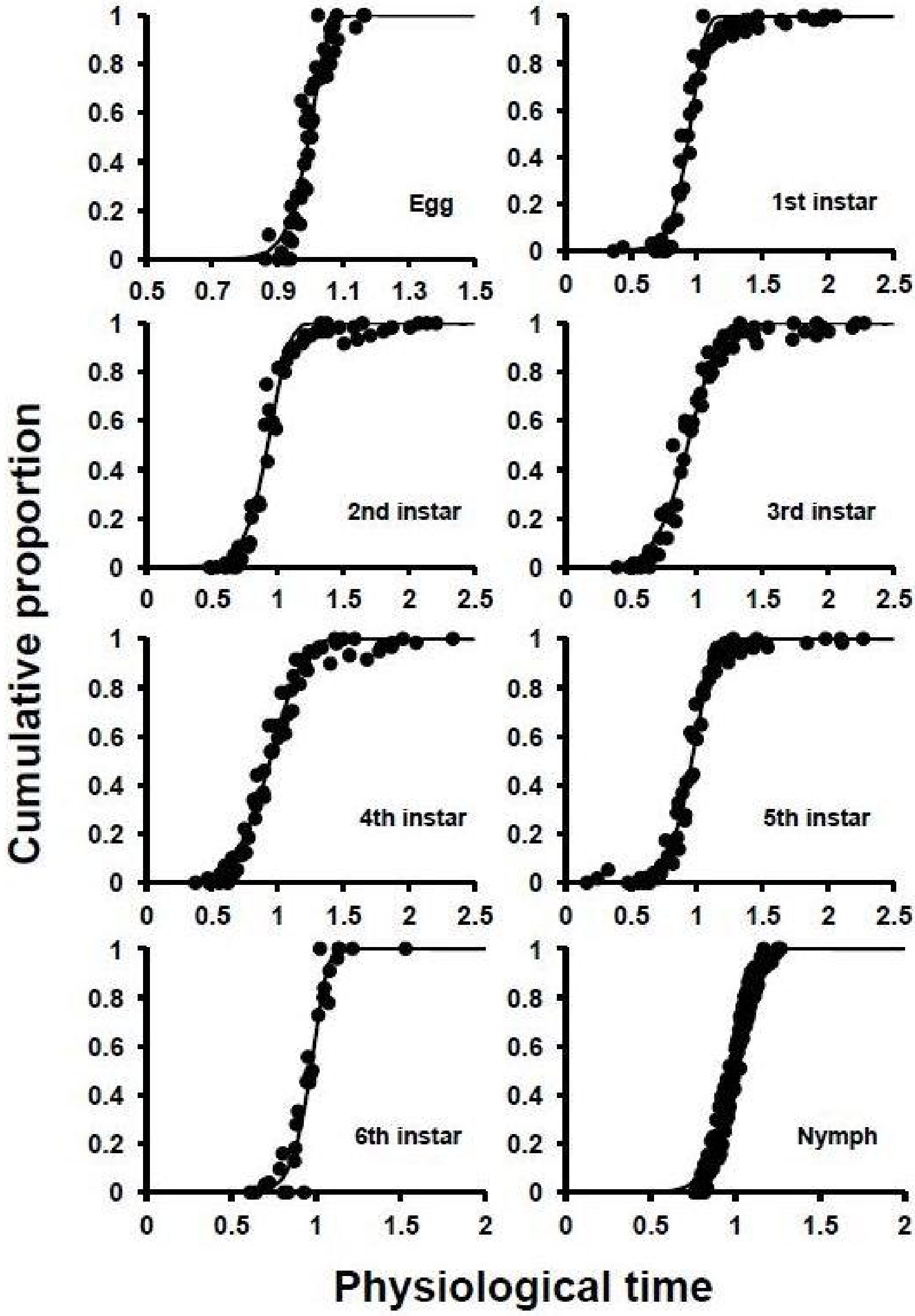

Figure 2. Cumulative proportions of development in each stage of *R.irregulariterdentatus* versus physiological time (●, Observed; —, Expected).

Table 4. Parameter estimates (± SEM) of the distribution model of developmental time for each stage of *R. irregulariterdentatus*.

Stage	Parameters		r^2
	α	β	
Egg	1.0100 ± 0.0029	24.1621 ± 2.2259	0.97
1st instar	0.9752 ± 0.0053	10.1323 ± 0.6822	0.99
2nd instar	0.9689 ± 0.0065	7.5901 ± 0.4932	0.99
3rd instar	0.9928 ± 0.0072	5.5999 ± 0.3005	0.99
4th instar	1.0095 ± 0.0063	5.2487 ± 0.2295	0.99
5th instar	1.0059 ± 0.0043	8.1325 ± 0.3676	0.99
6th instar	0.9953 ± 0.0073	12.9852 ± 1.5839	0.97
1st to adult	1.0307 ± 0.0023	9.9989 ± 0.2905	0.99

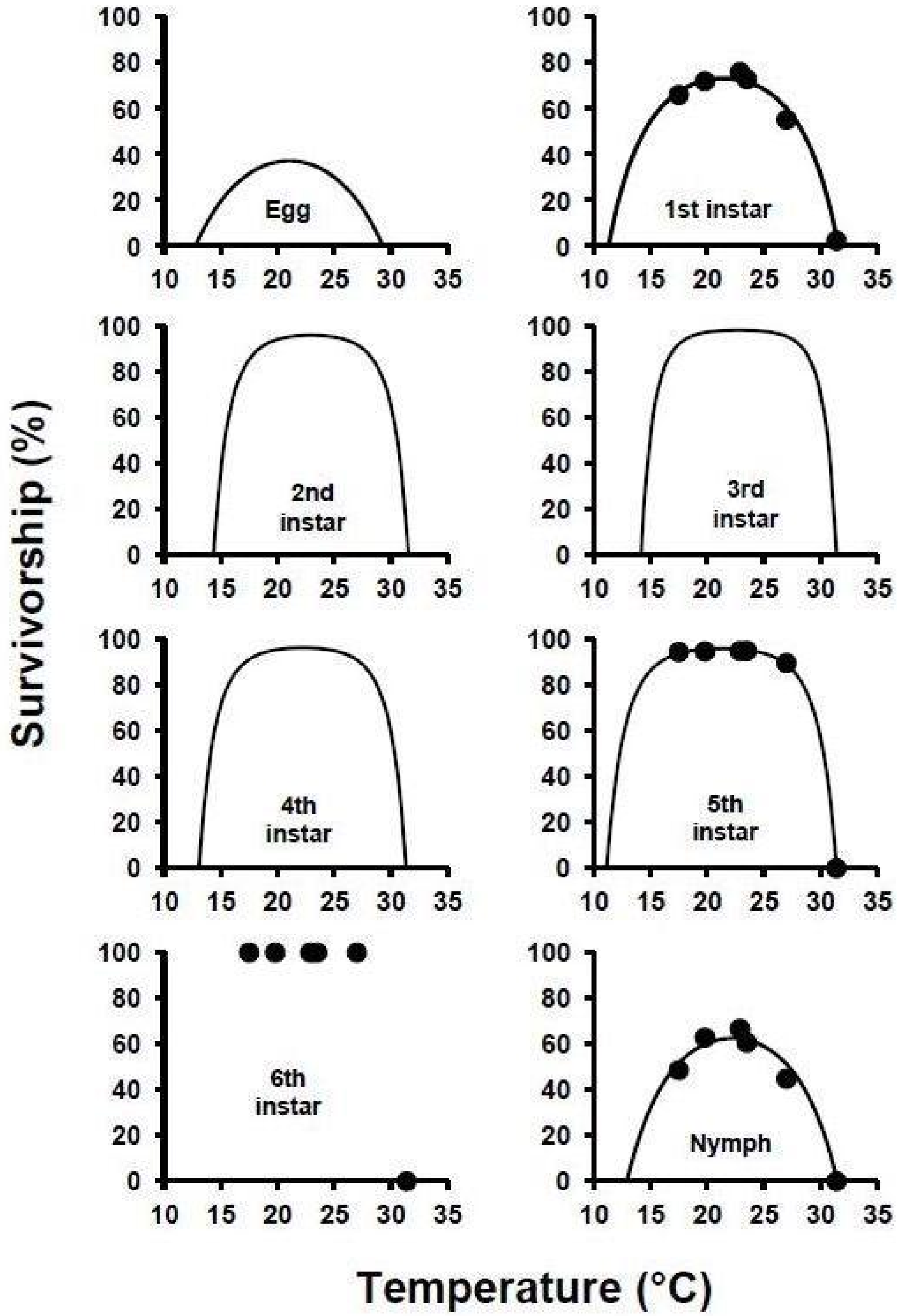

Figure 3. Temperature-dependent model for survival (%) of *R. irregulariterdentatus* (●, Observed; —, Expected).

Table 5. Parameter estimates (± SEM) of the survival rate model for each stage of *R. irregulariterdentatus.*

Stage	Parameters			r^2
	a	*b*	*c*	
Egg	7.1506 ± 0.1762	-0.2861 ± 0.0162	$6.80 \times 10^{-3} \pm 3.57 \times 10^{-4}$	0.99
1st instar	9.1662 ± 1.1370	-0.5486 ± 0.0926	$1.28 \times 10^{-2} \pm 1.81 \times 10^{-3}$	0.99
2nd instar	24.6076 ± 4.8015	-2.0249 ± 0.4144	$4.41 \times 10^{-2} \pm 8.35 \times 10^{-3}$	0.99
3rd instar	29.4010 ± 2.7608	-2.5351 ± 0.2396	$5.56 \times 10^{-2} \pm 4.84 \times 10^{-3}$	0.99
4th instar	21.0249 ± 4.3035	-1.7774 ± 0.3605	$4.00 \times 10^{-2} \pm 7.15 \times 10^{-3}$	0.99
5th instar	15.6418 ± 1.4520	-1.3410 ± 0.1159	$3.15 \times 10^{-2} \pm 2.23 \times 10^{-3}$	0.99
6th instar	-*	-	-	-
1st to adult	9.2493 ± 1.1877	-0.5064 ± 0.0980	$1.14 \times 10^{-2} \pm 1.94 \times 10^{-3}$	0.99

* No fitted

Table 6. Proportion (%) of sixth instar occurrence at six constant temperatures.

Temperature (°C)	17.5	19.8	22.9	23.5	27.0	31.4
Proportion (%)	3.9c*	16.7b	19.6b	43.9a	59.6a	-**

※ There was a linear relationship between temperature (°C) and proportion (%) of sixth instar occurrence ($y = 5.7889x - 99.4250$; y was proportion (%) of sixth instars developed from fifth instars, x was temperature (°C); $r^2 = 0.88$).

* Means within a row followed by the same letter are not significantly different ($P > 0.05$; chi-square test at 95% confidence intervals)

** No individuals survived

2-4-2. Adult longevity and fecundity

Temperature had a significant effect on the longevity of female adults of *R. irregulariterdentatus* ($F = 4.1$; df = 4, 265; $P = 0.003$). The longevity of female adults decreased with increasing temperature (Table 7). Adult developmental rate over tested thermal ranges were well described (Fig. 4a, $F = 34.9$; df = 2, 2; $P = 0.028$; $r^2 = 0.98$, Table 8). Adult survival rate expected over adult physiological time was well described by sigmoid function (Fig. 4d, $F = 11,278.1$; df = 1, 186; $P < 0.001$; $r^2 = 0.99$, Table 8).

The pre-oviposition period of adult females was the highest at 17.5 °C among temperature regimes in this study ($F = 19.9$; df = 4, 163; $P < 0.001$, Table 8). The relationship between fecundity and temperature was well described by the extreme value function (Fig. 4b, $F = 56.5$; df = 2, 8; $P < 0.001$; $r^2 = 0.97$, Table 5). Fecundity was the highest at temperature of 23.5 °C. It was the lowest at temperature of 17.5 °C ($F = 2.7$; df = 4, 265; $P = 0.032$, Table 7). Temperature (T_f) with the maximal fecundity was estimated to be at 22.6 °C. Thermal range (B_{80}) with ≥ 80 % of the maximum value was determined to be 19.8 to 26.1 °C. Age-specific cumulative oviposition rate over physiological time of female adults was well fitted by the two parameter Weibull function (Fig. 4c, $F = 73,335.6$; df = 1, 447; $P < 0.001$; $r^2 = 0.99$, Table 8).

Table 7. Longevity, fecundity, and pre-oviposition period (mean ± SE) of female *R. irregulariterdentatus* at 6 constant temperatures.

Temperature (°C)	*n*	Longevity (day) (min – max)	Fecundity (min – max)	Pre-oviposition period (day)
17.5	51	42.4 ± 4.20a* (2 – 135)	14.4 ± 2.41b (0 – 61)	24.3 ± 0.91a
19.8	54	38.8 ± 3.57a (1 – 118)	18.6 ± 3.58ab (0 – 93)	18.6 ± 0.63b
22.9	56	42.1 ± 3.84a (1 – 109)	27.3 ± 5.32ab (0 – 129)	18.5 ± 0.71b
23.5	57	37.2 ± 3.62ab (4 – 122)	32.0 ± 5.94a (0 – 172)	15.1 ± 0.73b
27.0	52	24.5 ± 2.22b (2 – 69)	17.7 ± 3.90ab (0 – 106)	15.9 ± 1.19bc

* Means within a column followed by the same letter are not significantly different ($P > 0.05$; Tukey's HSD test at 95% confidence intervals)

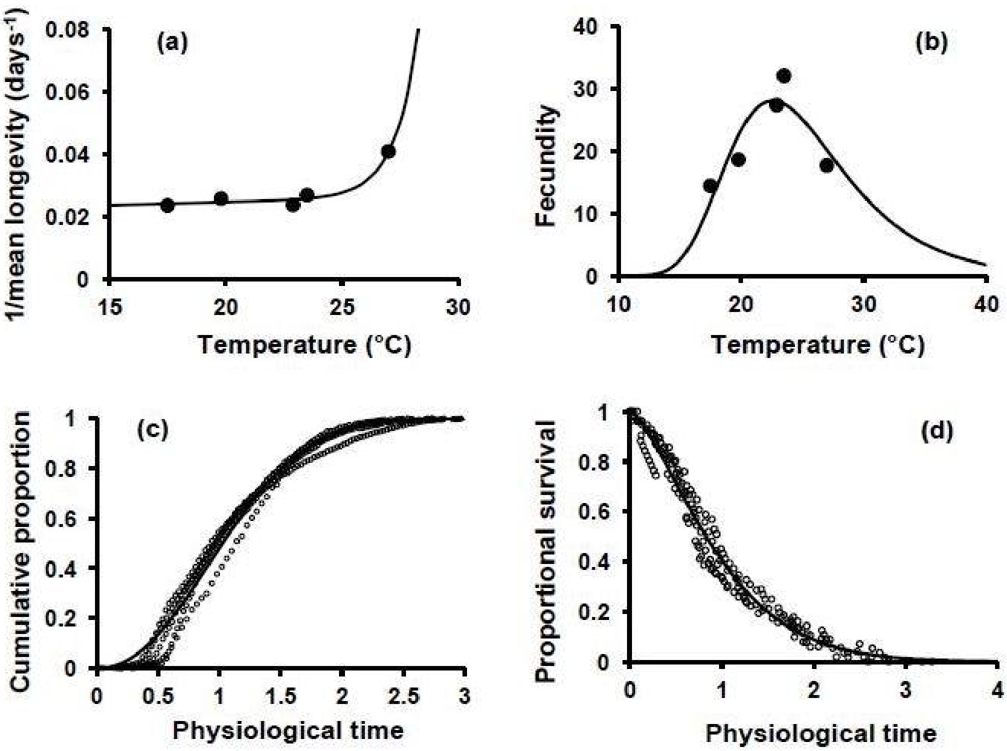

Figure 4. Temperature dependent oviposition models of *R. irregulariterdentatus*. (a) Adult development rate curve (1/mean longevity) (●, Observed; —, Estimated), (b) Temperature-dependent fecundity curve (●, Observed; —, Estimated), (c) Age-specific cumulative oviposition rate curve (○, Observed; —, Estimated), and (d) Age-specific adult survival rate curve (○, Observed; —, Estimated).

Table 8. Parameter estimates (± SEM) of the oviposition models of *R. irregulariterdentatus.*

Models	Parameters	Estimated values	r^2
Developmental rate model	a	$2.0563 \times 10^{-2} \pm 7.5882 \times 10^{-3}$	0.97
	b	$2.0300 \times 10^{-4} \pm 3.6200 \times 10^{-4}$	
	c	$2.7800 \times 10^{-14} \pm 5.6100 \times 10^{-15}$	
Fecundity model	a	27.9807 ± 3.3979	0.98
	b	22.5954 ± 1.0138	
	k	4.7020 ± 1.2924	
Cumulative oviposition rate model	α	1.2166 ± 0.0045	0.99
	β	2.1828 ± 0.0255	
Survival rate model	γ	1.0731 ± 0.0107	0.99
	δ	1.4339 ± 0.0310	

2-5. Discussion

In this study, we presented the temperature-dependent development, survival, and oviposition models of *R. irregulariterdentatus* by applying linear or non-linear models. Different lower development thresholds (T_b) for *R. irregulariterdentatus* were estimated from linear and nonlinear models. Overall, estimates of T_b of *R. irregulariterdentatus* from the nonlinear model were higher than those form the linear models, and estimates from the linear model appear to be more proper by considering results from Park et al. (2003). The thermal requirement (DDs) of *R. irregulariterdentatus* eggs were estimated as 1,542.7 DD with a T_b of 7.6 °C. However, for predicting its occurrence of fist instar nymphs, this thermal requirement for eggs may not be used directly beginning from January 1st in the current year because its overwintering eggs should have proceeded embryonic development before entering diapause since eggs of *R. irregulariterdentatus* are laid from July (Park et al., 2003). Embryonic diapause of many Orthopteran species such as *Melanoplus sanguinipes* halts morphological development (Fielding, 2006). For prediction of its nymphal occurrence time in spring, further physioecological study of embryonic diapause of *R. irregulariterdentatus* is needed.

Previously, nymphal development of *R. irregulariterdentatus* from the first instar to adult has been conducted by Park et al. (2003). They reported that T_b of the nymphal stage was 6.6 °C. This value was slightly higher than our result (5.2 °C). Such discrepancy might be mainly caused by different diets and experimental populations: clover plants and laboratory colony in our study vs. leaves of acacia and field population in the study of Park et al. (2003). The effect of different diets on development of Phasmatidae has been shown in *Medauroidae extradentata* by Boucher and Verady-Szabo (2005). The different colony effects on fecundity was proven by Van Lenteren and Noldus (1990) with whiteflies which were reared at different hosts during three generations. Other contributing factors may include difference in experimental conditions (e.g., photoperiod, relative humidity, moisture source, and precision of temperature control, and so on), origin of the test insect, and data range selected for linear regression analysis (Baek et al., 2014). It was noteworthy that the linear model herein was developed with more number of data sets compared to that used in Park et al. (2003). Therefore, findings in our study might have more accurate prediction of *R. irregulariterdentatus* development.

In our study, various thermal parameters (i.e., the upper threshold, optimal thermal range for survivor, development, and fecundity) were newly provided for *R. irregulariterdentatus*. The upper threshold (T_L) of insect development has been commonly used to more accurately calculate degree days by applying horizontal cutoff techniques (Roltsch et al., 1999). Overestimated upper development threshold can lead to erroneous prediction of stage emergence (earlier than actual emergence in the field), particularly when seasonal temperatures are hovering near the upper threshold temperature (Baek et al., 2014). Thermal ranges (B_{80}) with ≥ 80 % of the maximum value of development rates for eggs and nymphs, B_{80} of the survival rates of eggs and nymphs, and B_{80} of female adult fecundity could be applicable to rearing system for *R. irregulariterdentatus.* Based on thermal ranges, rearing temperatures for *R. irregulariterdentatus* are highly recommended to be 21.0 - 26.1 °C (Figure 5). In this temperature range, *R. irregulariterdentatus* might show high reproduction, rapid development, and high survival rates of eggs and nymphs.

The proper storage stage for *R. irregulariterdentatus* can be determined based on the lower developmental threshold as one potential industrial insect such as pet insect and educational material. Eggs would be better for storage than other developmental stages not only because eggs do not need food, but also because they have more tolerance to low temperature. Moreover, overwintering eggs without chilling periods can be stored much longer at constant temperature such as 25 °C (Yamaguchi and Nakamura, 2015). In this study, the egg survivorship was lower than the nymph's one. However, the egg survivorship could be increased up to 98.2 % by providing mist, floral foam, fermented sawdust, and fallen leaves for eggs (Lee et al., 2013). Egg stage is also recommended as a shipping stage. Eggs have slightly narrow thermal range (B_{80}) than nymphs. However, they are more tolerant to low temperature, not needing food, and more resistant to impact during shipping. Specially, adults delivered during the period of decreasing daytime can oviposit overwintering eggs. These eggs may not hatch at room temperature.

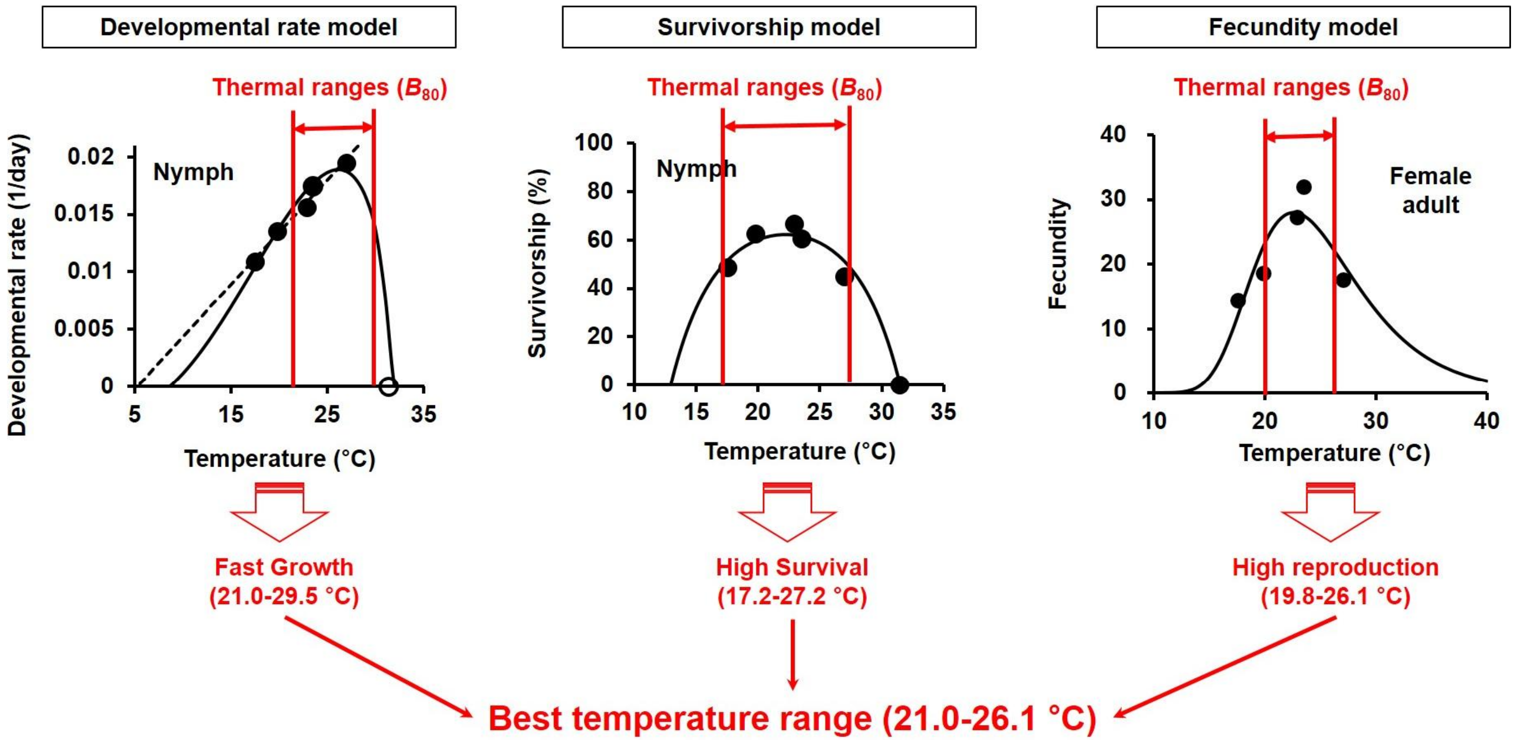

Figure 5. Best temperature range for rearing system for *R. irregulariterdentatus* based on thermal ranges (B_{80}) with ≥ 80 % of the maximum value of development rates for eggs and nymphs, B_{80} of the survival rates of eggs and nymphs, and B_{80} of female adult fecundity.

In summary, our study quantitatively demonstrated the effect of temperature on development and oviposition of *R. irregulariterdentatus*. Such information is fundamental for optimizing rearing methods and predicting seasonal phenology of *R. irregulariterdentatus* in the field. In addition, when combined with environmental data, models developed in this study might be useful for simulating its distribution, abundance, and occurrence pattern in the future, similar to those used in previous studies (Hance et al., 2007; Dixon et al., 2009).

Chapter III.

Determining conditions for mass rearing of *Ramulus irregulariterdentatus*: Egg hatching, diets and rearing densities

3-1. Abstract

This study was carried out to determine the optimum egg hatching conditions, the alternative or artificial diet and the optimal mass rearing method of *R. irregulariterdentatus*. Egg hatching rate of *R. irregulariterdentatus* was over 80 % at 20-25 °C, but about 18.3 % at 27.5 °C and 0 % at 30 °C. In nature, *R. irregulariterdentatus* overwinters as an egg, but it appears that cold temperatures were not required for termination of egg diapause. When several treatments were taken to improve the egg hatch rate, in the treatment using floral foam, fermented sawdust and leaves, the rate was 98.2 %. Clover (*Trifolium repens*) was an excellent diet as it was similar to the host plant and could be used as an alternative diet. The rate from 1st instar nymph to adult of *R. irregulariterdentatus* was 89.8 % and survival rate of adult over 30 days was 94.3 % by using an artificial diet of 25 % black locust leaf powder and 1% agar. Lifespan of adult feeding artificial diet and natural diet were 100.4 ± 36.8 days and 49.7 ± 16.0 days, respectively. The number of eggs of *R. irregulariterdentatus* feeding artificial diet and natural diet were 145.0 ± 63.0 and 109.5 ± 70.5, respectively. In the density experiments for mass rearing, the survival rate from 1st instar to adult for 60 days was 82.0 % at 50 insects/pot (59 × 18 × 13 cm, planted clover) and the number of oviposition was 710 at 20 adults/cage (49 × 34 × 27cm).

Key words: *Ramulus irregulariterdentatus*, egg hatching characteristics, alternative diet, artificial diet, mass rearing

3-2. Introduction

Many studies on stick insects have been mainly carried out on feeding preference, body color change depending on the environmental conditions, oviposition behavior, maturation divisions of parthenogenesis, preying by spiders, leg movements in various stick insects and regeneration of limb (Pijnacker, 1966; Detlef, 1977; Cassidy 1978; Carlberg, 1984; Eva and Ulrich, 1985; Wolfgang, 1990; Maginnis, 2006). But no research was done about a mass rearing method required for commercial use of stick insects.

For mass rearing, information are needed on proper egg hatching conditions, diet and rearing densities. In relation to hatching, many studies were conducted about various insects such as *Cadra cautella, Nannophya pygmaea* and *Antheraea yamamai* (Kim et al., 2003; Kim et al., 2006; Yoon et al., 2007). They focused mainly the temperature and period for hatching.

In *R. irregulariterdentatus,* the alternative or artificial diets were very important because their natural diets, broadleaf trees such as black locust, oak trees and chestnut, are hard to manage all year, particularly in winter. The alternative or artificial diets can be used for sustainable rearing during seasons when it is difficult to obtain the host plant or other food. To economically produce as many insects as possible area, optimal rearing density should be determined. In *R. irregulariterdentatus*, the egg is small and brown color so it is difficult to find the egg in the soil. The nymph density for general rearing and the adult density for oviposition are different.

Thus, this study was carried out to determine the best egg hatching condition, the alternative or artificial diet and the optimal mass rearing method of *R. irregulariterdentatus.*

3-3. Materials and methods

3-3-1. Experimental insects

Adults of *R. irregulariterdentatus* were collected from Mt. Mubong in Hwaseong, Gyeonggi-do, Korea. These insects were reared with fresh leaves of black locust (*Robinia pseudoacacia*), oriental chestnut oak (*Quercus aliena*), chestnut (*Castanea crenata var. dulcis*), bush clover (*Lespedeza bicolor*), and clover (*Trifolium repens*) more than four successive generations before they were used in this study. Colonies were maintained in the laboratory at 24-26 °C, 60-70 % RH, and a photoperiod of 16:8 (L:D) h.

3-3-2. Egg hatching characteristics

To investigate the hatch rate of *R. irregulariterdentatus* after a period of exposure to cold temperatures, 16 treatments were conducted. The rearing cage (30 × 20 × 20 cm) was half-filled with soil and covered with fallen leaves, and the 30 eggs were scattered on the leaves and re-covered with fallen leaves (Fig. 1). The hatching of eggs of *R. irregulariterdentatus* was very difficult; hence, in this study, we considered the environmental conditions observed in the natural habitat of *R. irregulariterdentatus*. Five cages were placed in each of 3 growth chambers set to a temperature of 0, 4, or 8 °C and after 30, 60, 90, 120 or 150 days 1 cage from each chamber was placed in the rearing room at 24-26 °C. One cage was retained in the rearing room at 24-26 °C without cold treatment. The eggs were checked every day to see if they had hatched.

Figure 1. The setting cages for hatching of *R. irregulariterdentatus.*
: half-filled with soil, covered with fallen leaves, scattered eggs and re-covered with fallen leaves.

To investigate the hatch rate of *R. irregulariterdentatus* under different hatching conditions, 4 treatments were conducted. In the 1st treatment, rearing cage (30 × 20 × 20 cm) were half-filled using fermented sawdust, which is generally used to rear *Allomyrina dichotoma*, and the eggs were spread on the sawdust. The 2nd treatment was similar to 1st treatment, except that the eggs were covered using fallen leaves. The 3rd treatment was similar to the 1st except that the floral foam, which is commonly used for flower arrangement, was embedded in the sawdust. The 4th treatment was similar to the 3rd except that the eggs covered using fallen leaves. The cages were placed in the laboratory at 24-26 °C, relative humidity (RH) of 60-70 %, and a photoperiod of 16:8 (L:D) h. The eggs were checked every day to see if they had hatched.

To investigate the hatch rate of *R. irregulariterdentatus* under different temperatures, the eggs treated using the methods from the 3rd treatment of previous experiment were put on a weighing dish (3 × 3 cm). One cage each was placed in rearing rooms set at 20, 22.5, 25, 27.5, or 30 °C and relative humidity (RH) of 60-70 %, and a photoperiod of 16:8 (L:D) h. The eggs were checked every day to see if they had hatched.

3-3-3. Alternative and artificial diets

To select an alternative diet, leaves of several plants were tested such as black locust, oriental chestnut oak, chestnut, bush clover, clover, grape, pepper, gyeol-myeong-ja, rice, corn, adlay, and bean. A nymph was reared on a diet of each test plant in the rearing room for 3 days. If the nymph ate the plant, then the test was continued. This was to determine whether the insects that consumed the test plant leaves developed to the next stage or not.

To develop an artificial diet, black locust leaf powder was used as the main host plant source and agar was used as the coagulating agent. Fresh black locust leaves were dried using a drier at 50 °C for 48 h and were ground into powder by using electric grinder. After boiling in 100 mL distilled water, 1, 2 or 3 g of agar corresponding to 1, 2, or 3 % treatments, respectively, were added and boiled for further 10 min. Leaf powder in the amount of 20 and 25 g corresponding to 20 and 25 % treatments, respectively, were added and mixed along with several additives (Table 1). The rate of leaf powder was decided on the preliminary examination. This composition of artificial diet was modified from the composition of artificial diet of *Spodoptera litura* (Im et al., 1988).

Table 1. Composition of artificial diet of *R. irregulariterdentatus.*

Ingredient	Water	Yeast	Malt	Vitamin	Sorbic acid	Salt mixture	Ascorbic acid	MPH
Quantity	100 ml	7.5 g	14 g	0.9 g	0.3 g	1.0 g	1.9 g	0.6 g

3-3-4. Optimal breeding density for mass rearing system

In the preceding studies for characteristics and diets, stick insect was reared only one insect per one cage to observe efficiently. For mass rearing, the pots (59 × 18 × 13 cm) were planted with clover, set with 4 sticks (about 30 cm) at 4 corners of the pot, and covered with net (Fig. 2). 50, 100, 150 and 200 first instar nymphs of *R. irregulariterdentatus* were let in each pot and placed in the laboratory at 24-26 °C, relative humidity (RH) of 60-70 %, and a photoperiod of 16:8 (L:D) h. If the insects consumed all the clover, they were moved to new pot set by clover and net. The survival rates of the insect were studied after 30 and 60 days.

For oviposition, the bottom of cages (49 × 34 × 27 cm) was covered with wet tissue to maintain humidity and set the center with twig to utilize the space for climbing (Fig. 3). 20, 30 and 40 adults of *R. irregulariterdentatus* were let in each cage and placed in the laboratory at 24-26 °C, relative humidity (RH) of 60-70 %, and a photoperiod of 16:8 (L:D) h. The clovers planted at 10-15 cm pots were supplied with diet. The oviposition was checked every week.


Figure 2. Clover (*Trifolium repens*) as an alternative host plant for mass rearing of *R. irregulariterdentatus.*


Figure 3. The cages for oviposition of *R. irregulariterdentatus.*

3-4. Results

3-4-1. Egg hatching characteristics

The hatch rates of *R. irregulariterdentatus* were 70.0 % after 30 days, 66.7 % after 60 days, 10.0 % after 90 days, 6.7 % after 120 days, and 0 % after 150 days at 8 °C (Table 2). And the rate was 73.3 % at 25 °C without any cold temperature.

The hatch rates of *R. irregulariterdentatus* were more than 80 % at 20-25°C, but 18.3 % at 27.5 °C and 0 % at 30 °C (Table 3).

The hatch rate of *R. irregulariterdentatus* was 98.2 % in the treatment comprising floral foam, fermented sawdust, and leaves and 92.0 % in the treatment comprising the fermented sawdust and leaves (Table 4).

Table 2. Hatch rate (%) of *R. irregulariterdentatus* eggs when maintained at rearing room conditioned at 24-26 °C and 60-70 % RH after cold exposure of 3 low temperatures and 6 different periods combinations.

Cold temperature (°C)	Control	0					4					8				
Period (days)		30	60	90	120	150	30	60	90	120	150	30	60	90	120	150
Hatch rate (%)	73.3	63.3	60.0	30.0	26.7	0	50.0	46.7	53.3	13.3	10.0	70.0	66.7	10.3	6.7	0

※ For each treatment, the cages (30 × 20 × 20 cm) were set half-filled with soil, covered with fallen leaves, scattered eggs and re-covered with fallen leaves.

※ Control : No cold temperature treatment.

Table 3. Hatch rate (%) of *R. irregulariterdentatus* under different temperatures.

Temperature (°C)	*n*	20	22.5	25	27.5	30
Hatch rate (%)	60	85.0	83.3	80.0	18.3	0

※ For each treatment, 30 × 20 × 20 cm cages with floral foam, fermented sawdust, egg dish, and eggs were placed in a rearing room maintained at relative humidity (RH) of 60-70 %, and a photoperiod of 16:8 (L:D) h.

Table 4. Hatch rate of *R. irregulariterdentatus* under four different hatching conditions using fermented sawdust, floral foam and leaves.

Treatments	*n*	RH in cage (%)	Hatch rate (%)
Fermented sawdust and eggs	50	85.8	70.0
Fermented sawdust, eggs and leaves	50	91.6	92.0
Floral foam, fermented sawdust and eggs	50	92.7	86.0
Floral foam, fermented sawdust, eggs and leaves	55	95.8	98.2

※ For each treatment, 30 × 20 × 20 cm cages were placed in a rearing room maintained at 24-26 °C, relative humidity (RH) of 60-70 %, and a photoperiod of 16:8 (L:D) h.

3-4-2. Alternative and artificial diets

R. irregulariterdentatus did not feed on rice, corn, adlay, or bean leaves. While the insects ate grape, pepper, and gyeol-myeong-ja leaves, they did not develop to the next stage (Table 5). Clover was an excellent diet, as was black locust, oriental chestnut oak, chestnut and bush clover.

Table 5. Potential of alternative diet plants for *R. irregulariterdentatus.*

Potential	Plants (Leaves)
5*	Black locust, oriental chestnut oak, chestnut, bush clover, clover
3	Grape, pepper, gyeol-myeong-ja
1	Rice, corn, adlay, bean

* 5: Very high (as host plant); 3: Consumed but no normal growth; 1: Not consumed

When *R. irregulariterdentatus* ate the artificial diets with different rate of media and leaf powder, the length of body was 7.7 ± 0.3 - 8.0 ± 0.3 and had no difference between treatments. *R. irregulariterdentatus* had the chewing mouthpart suitable to eat the artificial diet and liked that such as natural diet (Fig. 4). The rate of reaching adulthood for *R. irregulariterdentatus* was 88.3 % and survival rate of adult over 30 days was 94.2 % by using an artificial diet of 25 % black locust leaf powder and 1 % agar (Table 6). The developmental periods of nymph when fed artificial diets with different rate of media and leaf powder were 50.7 ± 2.2 - 55.5 ± 4.2 and the developmental period of nymph when fed natural diet was 55.3 ± 4.6 (Table 7). Lifespan of adult feeding artificial diet and natural diet were 100.4 ± 36.8 days and 49.7 ± 16.0 days, respectively. The number of eggs of *R. irregulariterdentatus* feeding artificial diet and natural diet were 145.0 ± 63.0 and 109.5 ± 70.5, respectively (Table 8).

Figure 4. Adult of *R. irregulariterdentatus* eating the artificial diet.

Table 6. Characteristics of adult of *R. irregulariterdentatus*, when fed different artificial diets composed of different rate of media and leaf powder.

Contents of leaf powder	Contents of agar	Length (cm, mean ± SD)	Rate of adult development (%)	Survival rate of adult over 30 days (%)
20 %	1 %	7.7 ± 0.4	78.3ab*	66.0ab
	2 %	7.8 ± 0.2	75.0ab	63.7ab
	3 %	7.7 ± 0.3	66.7b	52.5a
25 %	1 %	7.8 ± 0.4	88.3a	94.2a
	2 %	7.9 ± 0.4	83.3a	77.4ab
	3 %	8.0 ± 0.3	85.0a	88.6a

※ The length of body of stick insect fed natural diet (leaf of black locust) was 7.5cm.

* Means within a column followed by the same letter are not significantly different ($P > 0.05$; Tukey's HSD test at 95% confidence intervals)

Table 7. Developmental period of each stage of *R. irregulariterdentatus,* when fed different artificial diets composed of different rate of media and leaf powder.

Content of leaf powder	Content of agar	Developmental period of nymph(days, mean ± SD)						
		1st instar	2nd instar	3rd instar	4th instar	5th instar	6th instar	1st to adult
20%	1%	12.3 ± 1.5	8.4 ± 1.3	9.2 ± 1.1	9.7 ± 1.3	12.1 ± 1.4	14	52.0 ± 3.9
	2%	12.0 ± 1.2	8.7 ± 1.0	8.9 ± 1.3	9.5 ± 1.1	11.6 ± 1.8	-	50.7 ± 2.2
	3%	11.9 ± 1.2	8.4 ± 1.3	8.5 ± 0.8	10.0 ± 1.2	12.1 ± 2.1	-	50.8 ± 2.2
25%	1%	13.2 ± 1.1	9.2 ± 1.3	9.3 ± 1.0	10.2 ± 1.3	12.0 ± 1.5	13	54.4 ± 3.9
	2%	12.7 ± 1.3	9.4 ± 1.3	9.0 ± 1.0	10.0 ± 1.3	13.0 ± 2.0	-	54.1 ± 3.2
	3%	12.1 ± 1.1	9.6 ± 1.6	9.4 ± 1.3	10.3 ± 1.1	13.6 ± 1.1	15.5	55.5 ± 4.2
Natural diet		11.6 ± 2.1	8.3 ± 1.2	8.3 ± 1.2	10.7 ± 3.8	12.4 ± 3.8	12.4 ± 2.0	57.4 ± 6.3

※ Natural diet and leaf power: leaf of black locust

Table 8. Lifespan of adult and fecundity of *R. irregulariterdentatus* relative to the diet types.

Kind of diets	Lifespan of adult (days, mean ± SD)	Number of eggs/female (ea, mean ± SD)
Artificial diet	100.4 ± 36.8	145.0 ± 63.0
Natural diet	49.7 ± 16.0	109.5 ± 70.5

※ Artificial diet: Leaf power of leaf of black locust 25%, Agar 1%

Natural diet: Leaf of black locust

3-4-3. Optimal breeding density for mass rearing

The survival rates from 1st instar to adult for 60 days increased with decreasing density and were 82.0, 43.0, 34.7 and 24.7% at 50, 100, 150 and 200 insects/pot, respectively, when *R. irregulariterdentatus* of four densities were raised in the pot for mass rearing (Fig. 5).

The number of oviposition of *R. irregulariterdentatus* according to three densities at 49×34×27cm cage were 710, 707 and 359 at 20, 30 and 40 adults/cage, respectively (Table 9).

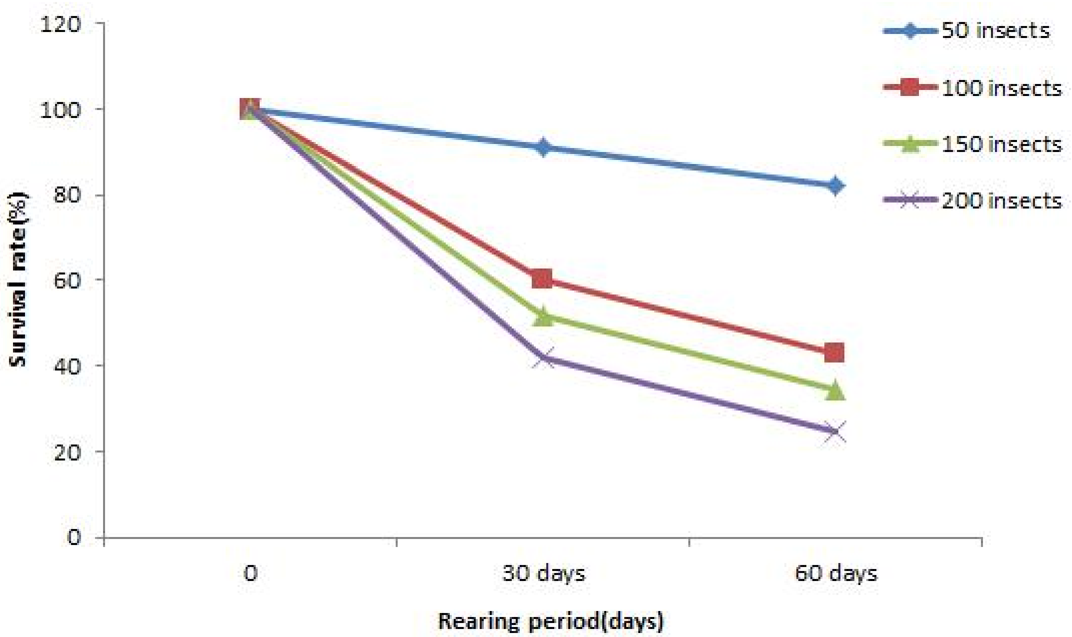

Figure 5. Survival rate of *R. irregulariterdentatus* to adult from 1st instar according to four densities at 59×18×13cm pot planted clover.

Table 9. Fecundity of *R. irregulariterdentatus* under different densities at 49 × 34 × 27cm cage.

Density (adults/cage)	Weeks during oviposition												Total
	1st	2nd	3rd	4th	5th	6th	7th	8th	9th	10th	11th	12th	
20	2.7	46.7	116	133	127	83.7	76.7	65.3	37.3	11.3	7.7	2.3	710a*
30	5.0	64.7	150	103	83.7	86.7	79.3	64.7	40.0	17.0	11.0	1.7	707a
40	0	24.3	44.3	37.3	50.0	53.0	59.7	47.3	31.0	10.7	1.0	0	359b

* Means within a column followed by the same letter are not significantly different (P > 0.05; Tukey's HSD test at 95% confidence intervals)

3-5. Discussion

In nature, *R. irregulariterdentatus* overwinters as an egg and it may be thought that cold temperature treatment is necessary. In result of cold and 20-30 °C temperatures, cold temperatures were not required for hatching (Table 2) and high temperature, such as 30°C, disturbed the hatching (Table 3). The best results for hatching the eggs of *R. irregulariterdentatus* was to use a rearing cage set with floral foam, fermented sawdust and fallen leaves (Table 4). For the hatching of *R. irregulariterdentatus* eggs, humidity is very important, and floral foam can help maintain the moisture. Kim et al. (2011) reported that when hatching the Emma field cricket, *Teleogryllus emma*, floral foam was moistened frequently with water so that the hatching of the eggs would not be affected by moisture loss.

It is known that black locust, oriental chestnut oak, chestnut, and bush clover are the host plants of *R. irregulariterdentatus* (Bae, 2002; Park et al., 2003). The host plants are deciduous woody plants, so it is difficult to harvest them for the diet during the winter in Korea. Clover, however, is an herbaceous plant and easy to manage at a green-house in winter; hence, it could be promising as an alternative diet for mass rearing of *R. irregulariterdentatus* (Table 5). It is important that the alternative diet should be obtained cheaper and easier than the natural host plant such as peanuts and beans as potential alternative diets for rearing *Poecilocoris lewisi* (Kim and Seol, 2003).

Many studies have been carried out to develop an artificial diet for the harmful insect or industrial insect, for example, *Matsumuraeses phaseoli* and *Teleogryllus emma* (Jung et al., 2007; Kim et al., 2007). Artificial diet can be used for sustainable rearing in all seasons when it is difficult to obtain the host plant or other food. In particular, artificial diet is necessary for small-scale breeder who grows a stick insect as a pet. To rear nymphs and adults of *R. irregulariterdentatus*, natural diet such as black locust, oriental chestnut oak, chestnut, or bush clover leaves can be used during from spring to autumn, an alternative diet such as clover can be used in all season including winter, and an artificial diet can be used for breeder of insect as pet.

In the survival rates of *R. irregulariterdentatus* from first instar to adult according to rearing density, the treatment of 50 insects/pot was appropriate for mass rearing (Fig. 5). In the number of eggs by adult density, the treatment of 20 insects/cage was optimal for oviposition (Table 9). Because the adult of *R. irregulariterdentatus* scatter eggs at trees to the ground and it is difficult to collect the eggs in the soil, it is necessary to manage for oviposition.

As a result of the above, we proposed a manual for mass rearing of *R. irregulariterdentatus*. The manual for mass rearing of stick insect was comprised of egg, nymph and adult management. In the egg management, the cages (30 × 20 × 20 cm) were half-filled with fermented sawdust and covered with fallen leaves, and the eggs were scattered on the leaves and re-covered with fallen leaves. Water was sprayed 1-2 times per week. The eggs would be hatching after about 100 days. In the nymph management, the pot (59 × 18 × 13 cm) were planted with clover, set with 4 sticks (about 30 cm) at 4 corners of the pot, and covered with net. About 50 nymphs are able to live a pot. If the insects consumed all the clover, should be moved to new pot. In the adult management, the bottom of cages (49 × 34 × 27 cm) were covered with wet white tissue to maintain humidity and set the center with twig to utilize the space for climbing. About 20 adults are able to live a cage. The clovers planted at 10 - 15 cm pots were supplied with diet. The oviposition should be checked every week.

In conclusion, in this study, the biological characteristics and rearing conditions of *R. irregulariterdentatus* will be essential for use as educational pet insect and it is expected that the industrialization of *R. irregulariterdentatus* will contribute to develop the insect industry in Korea.

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대벌레의 생물학적 특성과 사육조건에 관한 연구

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초록

대나무를 닮았다고 해서 이름이 붙은 대벌레는 산림에서는 해충을 여겨지지만 애완용 곤충으로도 관심이 높아지고 있다. 이 연구는 국내에 서식하는 대벌레인 *Ramulus irregulariterdentatus* 의 생물학적 특성과 사육조건을 연구하여 학습애완용 곤충으로 개발하기 위하여 수행하였다.

2016 년 경기도 고양시 국사봉에서 대벌레 발생을 조사한 결과, 부화한 약충이 4 월 20 일에 최초로 발견되었고, 5 월 19 일 조사에서 최고를 보이고 7 월 하순에 감소하여 9 월에는 발생하지 않았다. 62.5 %가 아까시나무에서 서식하였고, 참나무류에는 21.5 %만 서식하였으며, 성충이 하루에 먹는 갈참나무잎은 약 4.2 cm^2 (0.09 g)이었다. 대벌레는 암컷이 단위생식하여 번식하는 종으로 수컷의 자연발생이 매우 드문 종인데 2013년 수컷이 교미하는 장면을 처음으로 발견하였다. 1 령 약충의 색은 옅은 갈색이었고, 2 령 약충은 밝은 녹색이지만, 3 령 이후 약충과 성충은 밝은 녹색 또는 주위환경에 의해 갈색을 나타내었다. 대벌레는 위기상황에서 가사행동을 하는데 약충의 가사행동 시간은 평균 336 초, 성충은 515 초이었다. 대벌레는 주위 환경에 따라 체색변이를 나타내는데 3 령부터 색이 변하기 시작하였으며 5 령기인 6 월에는 23.2 %가 갈색으로 변하였다. 대벌레는 위기상황에서 다리가 탈락되고 이후 재생되는 특성을 갖는다. 1 령과 2 령에서 탈락되었을

때는 주로 두 번째 탈피 후, 3 령에서 탈락되었을 때는 주로 첫 번째 탈피 후 다리가 재생되었다. 재생되는 다리는 처음에는 짧고 뒤틀린 형태이며, 다음 번 탈피 후에 짧지만 펴진 형태를 갖게 되고, 3번째 탈피 후에 정상의 90 % 이상 길이의 다리로 재생되었다.

발육속도는 비선형 Briére model에 적합하였으며 알과 약충의 고온발육 임계온도는 각각 29.3 °C 와 31.4 °C 이었고, 최적온도는 24.5~26.2 °C 로 추정되었다. 선형모델에서 저온발육 임계온도는 알과 약충에서 각각 7.6 °C 와 5.2 °C 이었다. 생존율은 알과 약충에서 각각 21.0 °C 와 22.2 °C 에서 최고를 나타내었다. 평균 산란수는 17.5 °C 에서 14.4개, 23.5 °C 에서 32.0개의 범위를 나타내었다. 성충 생존율과 누적산란율은 각각 각각 시그모이드 함수와 2파라미터 Weibull 함수에 적합하였다. 이러한 모델은 야외에서 대벌레의 발생(phenology)과 개체군 동태를 예측하고, 대량사육에서 환경조건을 최적화하는데 이용할 수 있다.

대벌레는 알로 월동하지만 부화를 위해 저온처리를 필요로 하지 않았으며, 부화율은 20~25 °C에서 80 % 이상 이었지만, 30 °C 에서는 부화하지 않았다. 부화율은 발효톱밥, 플로랄폼, 낙엽을 이용하였을 때 98.2 %까지 증가하였다. 기주식물인 참나무나 아까시나무를 대신할 수 있는 대체먹이로 클로버를 선발하였으며, 동절기와 애완용 사육을 위한 인공먹이를 개발하였다. 대량사육을 위해서 59 × 18 × 13 cm 사각화분에 클로버를 재식하여 약충을 50마리 밀도로 사육하였을 때 성충까지 가는 비율이 82.0 % 이었고, 산란을 위해 49 × 34 × 27 cm 사육상자에 성충 20마리 밀도로 사육하였을 때 산란수가 710개로 가장 많았다. 이러한 결과를 종합하여 대벌레 대량사육 매뉴얼을 제시하였다.

검색어 : 대벌레, 학습애완용 곤충, 생존전략, 발육모델, 산란모델, 사육조건

감사의 글

이 한편의 논문이 나오기 위해서 오랜 시간이 걸렸습니다. 힘들었던 시간 동안 부족한 제자를 위해 많은 가르침과 격려해 주신 이준호 교수님께 감사 드립니다. 부족한 논문을 가다듬고 심사해 주신 제연호 교수님, 이광범 교수님, 정철의 교수님, 박홍현 박사님께 감사의 말씀 전합니다. 반가운 마음으로 함께 해준 곤충생태학 실험실 남화연, 김민중, 김규순, 박유정, 임재성과 특히, 학문적 소양으로 본 논문에 많은 도움을 준 백성훈에게 고마운 마음 전합니다. 직장생활과 병행하면서 어려운 여건 속에서 힘을 주신 박인태 국장님과 강창성 과장님께 감사 드립니다. 같은 팀원으로 동고동락하고 있는 유기농업팀 임갑준 팀장님, 장재은 연구사, 황지은 연구사와 특히, 곤충을 전공하여 저 때문에 더욱 고생을 많이 한 윤승환 박사에게 감사의 마음 전합니다. 대벌레를 선발하여 연구의 시작을 마련해 준 이영수 연구사, 사육실에서 곤충이 잘 자라도록 세심한 정성을 주신 김예경 님, 박귀자 님, 문미영 님 고맙습니다. 연천에서 곤충 채집 함께하고 무럭무럭 자라나게 사육해 주신 박화용 주무관님, 이운권 주무관님, 서애경 님, 박현옥 님 고맙습니다. 학교에서, 사무실에서 제 연구에 도움을 주신 모든 분들께 감사의 마음 전합니다. 동생 잘 되기만을 바라면서 격려해

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