Hemocyte Types Based on Total and Differential Counts in *Samia cynthia ricini* (Lepidoptera; Saturniidae) Reared on Host Plants Versus an Artificial Diet

Jatuporn Tungjitwitayakul* and Nujira Tatun

School of Science, University of Phayao, Phayao, 56000, Thailand

* Corresponding author. E-mail address: jatuporn.tu@up.ac.th

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Abstract

Hemocytes are an important component of the insect immune system because of their involvement in coagulation, phagocytosis, and encapsulation. The larvae of the eri silkworm, *Samia cynthia ricini*, has been successfully used as insect model to study the innate immune response and antibacterial activity. This insect grows on host plants, such as cassava and castor leaves, and on artificial diets. Several studies have revealed that artificial diets alter the insect's immune responses. Hence, it is important to assess the concentration of hemocytes in *S. cynthia ricini* reared on the host plant (castor leaves) and an artificial diet. The hemocytes of *S. cynthia ricini* are classified into five types: prohemocytes (PRs), plasmatocytes (PLs), granulocytes (GRs), spherulocytes (SPs), and oenocytoids (OEs). The total hemocyte count (THC) was studied in four developmental stages, including 3rd-, 4th-, and 5th-instar larvae, in addition to pupae. The results indicated that the THC in larvae reared on castor leaves decreased gradually in later developmental stages. In contrast, the THC in larvae reared on an artificial diet were at lower levels in all stages. The differential hemocyte count (DHC) indicated that ratio of each hemocyte type was comparable during larval and pupal stages in which PLs were most abundant, followed by GRs, SPs, PRs, and OEs in both groups. Furthermore, the diet had different effects on the percentage of PLs, GRs, and PRs during larval and pupal stages. Since the immune system of eri silkworm was interfered, this artificial diet may not suitable for rearing system in immunological study aspect.

Keywords: eri-silkworm, hemocytes, castor leave, artificial diet, immunity

Introduction

The eri silkworm, *Samia cynthia ricini* (Saturnidae), is a non-mulberry silkworm, and the larvae feed on several host plant species, including castor oil plants, cassava, ailanthus, kesseru, and plumeria. Eri silkworm larvae have been successfully used in bioassays and analyses to evaluate the abilities of plants to defend against herbivorous insects (Hirayama, Konno, Wasano, & Nakamura, 2007). In addition, *S. cynthia ricini* was used as an insect model to study innate immune responses and antibacterial activity (Kishimoto, Fujimoto, Matsumoto, Yamano, & Morishima, 2002; Bao, Yamano, & Morishima, 2005; Bao, Yamano, & Morishima, 2007; Onoe, Matsumoto, Hashimoto, Yamano, & Morishima, 2007; Hashimoto, Yamano, & Morishima, 2008a, 2008b).

An artificial diet for the polyphagous mutant silkworm, Silkmate L4M, has been used to rear several lepidopteran insects for research purposes, including *S. cynthia ricini* (Bao et al., 2005; Onoe et al., 2007; Hashimoto et al., 2008a, 2008b; Suzuki et al., 2015). This diet contains a low percentage of mulberry leaf powder (4%) (Fukuzawa, Tatsuki, & Oshikawa, 2004). However, it has been reported that mulberry leaves are highly toxic to generalist caterpillars that do not feed on mulberry trees as host plants, such as the *S. cynthia ricini* and cabbage moth, *Mamestra brassicae.* This is due to the latex ingredients exuded from damaged leaf veins (Konno et al., 2006). Furthermore, sugar-mimicking alkaloids in mulberry latex are toxic to eri silkworm larvae because they inhibit midgut sucrase and trehalase activity (Hirayama et al., 2007).

Based on the above-mentioned reports, a comparison of the biological and biochemical properties of *S. cynthia ricini* reared on artificial and natural diets has been recently reported (Tungjitwitayakul & Tatun, 2017). The lengths of the larval and pupal periods of *S. cynthia ricini* fed on the artificial diet were longer than those fed on cassava leaves. In addition, protein and lipid concentrations in the hemolymph and α-amylase activity in *S. cynthia ricini* fed on an artificial diet were lower than those in *S. cynthia ricini* fed on cassava leaves (Tungjitwitayakul & Tatun, 2017). Hence, an artificial diet containing mulberry leaves affects many biological and biochemical processes in this insect. Accordingly, we assume that an artificial diet may alter the immune system of *S. cynthia ricini*.

Insect hemocytes perform various biological functions, including phagocytosis, encapsulation, detoxification, storage, and distribution of nutritive materials. In larval stage of Lepidoptera, granulocytes and plasmatocytes comprise more than 50% of the hemocytes in circulation and are capable of adhering to surfaces of foreign materials. Spherulocytes are responsible for transportation of cuticular components, while oenocytoids have been suggested to play a role in melanisation of hemolymph. Prohemocytes are known to be stem cells that differentiate into other hemocyte types (Ribiero & Brehé lin, 2006; Lavine & Strand, 2002). Insect hemocytes respond to internal changes during development and to many conditions, such as starvation, wounding, parasitism, diseases, and insecticides (Bhagawati & Mahanta, 2014). For these reasons, we considered it important to characterize the types of hemocytes present and investigate whether different diets affect the relative abundance of hemocytes in these insects.

Since the success of the immune response depends on the number and the types of hemocytes recruited (Russo, Brehelin, & Carton, 2001). Therefore the aim of this study was to characterize the hemocytes of *S. cynthia ricini* larvae using light microscopy and determine the total hemocyte count (THC) and differential hemocyte count (DHC). The hemocyte composition was determined during various developmental stages, and the THC and DHC were compared between the larvae and pupae reared on castor leaves versus an artificial diet.

Methods and Materials

Insect rearing

The larvae of *S. cynthia ricini* were reared under laboratory conditions at 25 ± 2° C with a relative humidity of 65 ± 5%. Newly hatched larvae (n = 30) were randomly selected and individually transferred to the rearing trays using a fine paint brush. They were reared on either a commercial artificial diet (Silkmate L4M) or fresh castor leaves from hatching to the 5th-instar larval stage. The treatments were performed in triplicate. For larvae reared on castor leaves, 1st- and 2nd-instar larvae were fed young leaves cut into small pieces. Middle-aged leaves were fed to 3rd-instar larvae, and mature leaves were fed to 4th- and 5th-instar larvae. For larvae reared on the artificial diet, the food was cut into small pieces and fed to 1st- and 2nd-instar larvae. Larger pieces of artificial diet were fed to 3rd- to 5th-instar larvae. The larvae were fed four times per day except during molting periods. The quantity of food was increased with larval age to meet their increasing nutritional requirements. Beds were cleaned regularly, and mature larvae were transferred to suitable cages to spin cocoons (Deka, Dutta, & Devi, 2011).

Artificial diet

Silkmate L4M (a diet for "polyphagous" silkworm mutants consisting of 4% mulberry leaf powder) was purchased from Nihon Nosan Kogyo Co. (Yokohama, Japan). First, 250 g of Silkmate L4M powder was weighed in a stainless-steel container (14 × 20 × 7.5 cm) and mixed with 750 ml of distilled water. The diet was then steamed for 40 min, mixed thoroughly, placed at room temperature for 1 h, and stored at 4°C until use (Fukuzawa et al., 2004).

Weighing the larva and pupa

The body weights were measured on day 1 for all 3rd- to 5th-instar larvae. Ten larvae from each treatment group were randomly selected and weighed, and the average body weight was calculated. After the insects entered the pupal stage, cocoons of 3-day-old pupae were cut using fine scissors to remove the pupae. The body weights of the pupae were recorded separately.

Light microscopy studies

For the preparation of blood smear slides, hemolymph was collected by either cutting the tip of a proleg of the larvae or stabbing the pupal cuticle. A drop of hemolymph was spread onto glass slide and then dried at room temperature. For staining, air-dried hemolymph smears were fixed in methanol by dipping the slide into methanol 2 times and air dried. Slides were stained with Giemsa stain (Rankem, New Delhi, India; diluted five times with phosphate-buffer saline (PBS) and filtered before use) for 20 min and then rinsed in distilled water. The smear was washed in 0.02% acetic acid followed by rinsing in distilled water. After drying, permanent microscopy slides were prepared using Permount (Fisher Scientific, New Jersey, United States). Hemocytes were examined under a light microscope (Olympus BX51) and photographed with an Olympus BX43.

Hemocyte characterization

In order to identify hemocyte types, the shape, size, and the cytoplasm constituents of cells were observed. The location and size of nucleus were also recorded as important characters for identification of hemocyte types in present study. (Ribeiro & Brehélin, 2006; Jalali & Salehi, 2008; Huang et al., 2010; Ruiz, Lópezez, Rivas, Sánchez, & Moncada, 2015; Vogelweith, Moret, Monceau, Thiéry, & Moreau, 2016).

Determination of THC

Hemolymph samples were collected from 3-day-old 3rd- to 5th-instar larvae and pupae by cutting their prolegs with microscissors or piercing the cuticle of the pupa. Hemocyte counts were performed individually. The THC was performed by aliquoting diluted hemolymph (300 µl of hemolymph mixed with 300 µl of 20 mM phosphate buffer [pH 6.0] containing 0.37% β-mercaptoethanol) into a Neubauer hemocytometer (HBG, Giessen-Lützellinden, Germany). The THC was expressed as the number of cells per ml of hemolymph.

Determination of DHC

For the DHC, the 3-day-old 3rd-instar larvae, 4th-instar larvae, 5th-instar larvae, and pupae were selected. To determine the DHC, cell categories were quantified for 100 cells chosen from random areas of the stained blood smear. The quantification was performed in triplicate.

Data analysis

A statistical analysis of the data was performed using a one-way analysis of variance (ANOVA; IBM SPSS Statistics 22), followed by a least-significance-difference (LSD) multiple-range test. The significance level was set at 0.05 ($P < 0.05$).

Results

Larval and pupal weight

The weights of 3rd-, 4th-, and 5th-instar larvae gradually increased in later larval stages, and body weights were greatest in 5th-instar larvae fed on the artificial diet. We found no differences between larvae reared on castor leaves and those on the artificial diet among 3rd- to 4th-instar larvae. After the larvae developed to the 5th-instar stage, the body weights of those reared on both diets increased significantly compared to those of 3rd- and 4th-instar larvae. The body weight of 5th-instar larvae reared on the artificial diet was higher than that of larvae reared on castor leaves, which were 3.37 ± 0.24 g and 1.59 ± 0.15 g, respectively (Figure 1A). The body weights of male and female pupae reared on castor leaves were 1.90 ± 0.08 g and 2.40 ± 0.21 g, respectively, whereas the weights of male and female pupae that developed from the larvae reared on artificial diets were 1.75 ± 0.18 g and 2.47 ± 0.07 g, respectively. This indicates that the weights of male and female pupae did not differ between insects fed on castor leaves and those on the artificial diet ($P > 0.05$) (Figure 1B).

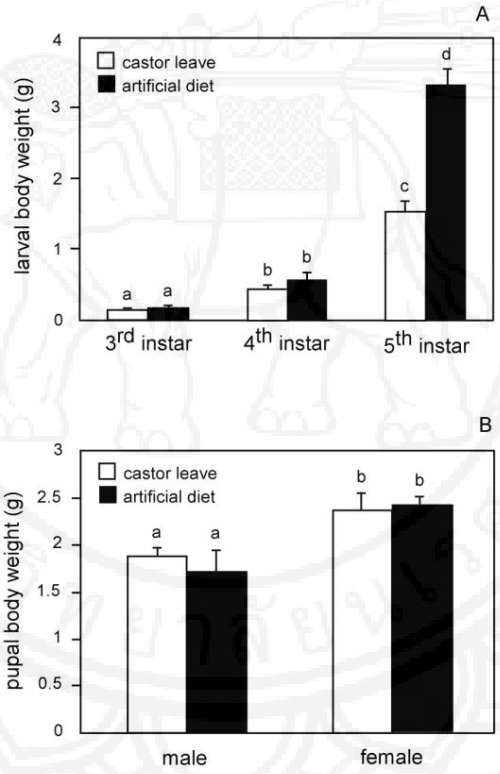

Figure 1 Larval weight (A) and pupal weight (B) of *S. cynthia ricini* fed on castor leaves or an artificial diet. Bars indicate the means of three independent biological replicates with standard deviations (SDs). The letters above the bars indicate significant differences ($P < 0.05$) between *S. cynthia ricini* fed on castor leaves or the artificial diet (A) or between male and female pupae (B).

Hemocyte types

The hemocytes of *S. cynthia ricini* could be categorized into five types based on distinctive morphological characteristics using light microscopy (Figure 2). These included prohemocytes (PRs), granulocytes (GRs), plasmatocytes (PLs), oenocytoids (OEs), and spherulocytes (SPs).

PRs were the smallest type of hemocyte, and they were found in the hemolymph. They were round or oval cells of variable sizes (5–25 μm) with a large, round nucleus. The nucleus was typically centrally located and filled most of the cytoplasm. A thin peripheral layer of homogenous cytoplasm surrounded the nucleus (Figure 2A).

GRs were spherical or oval cells of variable sizes (6.25–17.5 μm). The variably sized nucleus was round and centrally located. The cytoplasm was characteristically granular, and the cell membrane was usually articulated (Figure 2B).

PLs were polymorphic cells of variable sizes (5–25 μm). The cytoplasm was abundant and generally agranular or slightly granular. The nucleus was either round or elongated, and it was generally centrally located (Figure 2C).

OEs were oval or spherical cells of widely variable sizes (15–35 μm). The cytoplasm was slightly granular. The nucleus was small, round or elongates and it was generally eccentrically located (Figure 2D).

SPs were ovoid or round cells of variable sizes (12.5–22.5 μm). The nucleus was generally small, round, centrally located, and obscured by the intracytoplasmic spherules. The number of spherules varied from four to six (Figure 2E).

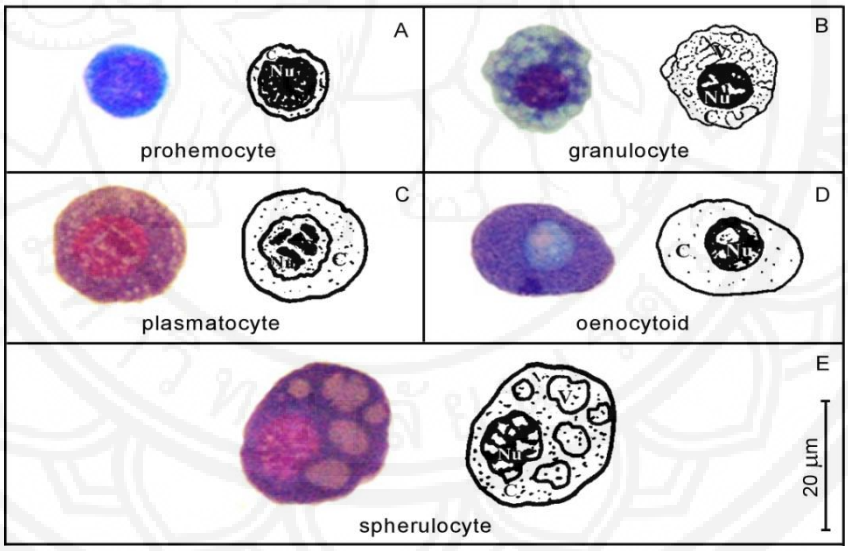

Figure 2 Light microscopy images and annotated drawings of the hemocyte types of *S. cynthia ricini*. Prohemocyte (A); granulocyte (B); plasmatocyte (C); oenocytoid (D); spherulocyte (E). Nu, nucleus; C, cytoplasm; V, vacuoles.

THC

The results clearly showed that the THC of larvae reared on the artificial diet was lowest during the 3rd- to 5th-instar stages (Figure 3). Comparing these two groups, the THC in larvae reared on castor leaves was greater than in larvae reared on the artificial diet (about 8.5-, 3.6-, and 3.8-fold higher in 3rd-, 4th-, and 5th-instar larvae, respectively) (Figure 3A). The THC in male pupae on the artificial diet was lower than the castor leaf-

fed group, whereas there was no significant difference in THC for the female pupae in the two groups ($P > 0.05$) (Figure 3B).

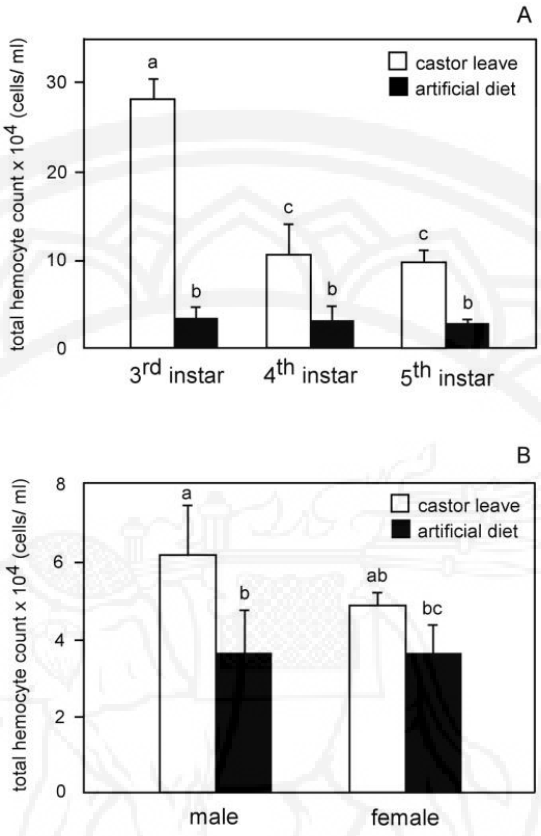

Figure 3 THCs in larvae (A) and pupae (B) of *S. cynthia ricini* fed on castor leaves or the artificial diet. Bars indicate the means of three independent biological replicates with standard deviations (SDs). The letters above the bars indicate significant differences ($P < 0.05$) between *S. cynthia ricini* fed on castor leaves and the artificial diet (A) or between male and female pupae (B).

DHC

The DHC profile of insects reared on castor leaves or the artificial diet showed a similar pattern. In 3rd- to 5th-instar larvae, PLs were dominant, followed by GRs, PRs, and SPs. Meanwhile, OEs were present only in 5th- instar larvae. In 5th-instar larvae, the percentage of PLs in larvae reared on the artificial diet was higher than in larvae reared on castor leaves (63.73 ± 3.71 and 54.97 ± 2.89%, respectively). In contrast, PRs and SPs in 5th-instar larvae reared on castor leaves were higher than in larvae reared on the artificial diet. The percentage of PRs in larvae reared on castor leaves or the artificial diet were 1.32 ± 0.68 and 0.93 ± 0.37%, respectively. For SPs, the percentage was 8.0 ± 2.49 and 4.92 ± 0.66% for larvae reared on castor leaves or the artificial diet, respectively (Figure 4A). In contrast, the percentage of PLs in female pupae in the castor leaf-fed group was higher than in pupae fed the artificial diet (82.13 ± 3.10 and 72.15 ± 0.93%, respectively). For female pupae, a greater proportion of GRs were observed in artificial diet group than in the castor leaf-fed group (25.08 ± 0.63 and 14.62 ± 3.27%, respectively). Interestingly, PRs were not observed

in pupae (either male or female) fed the artificial diet. However, PRs were observed at a lower proportion in the castor leaf-fed group for both males and females (0.18 ± 0.1 and 0.36 ± 0.23%, respectively) (Figure 4B).

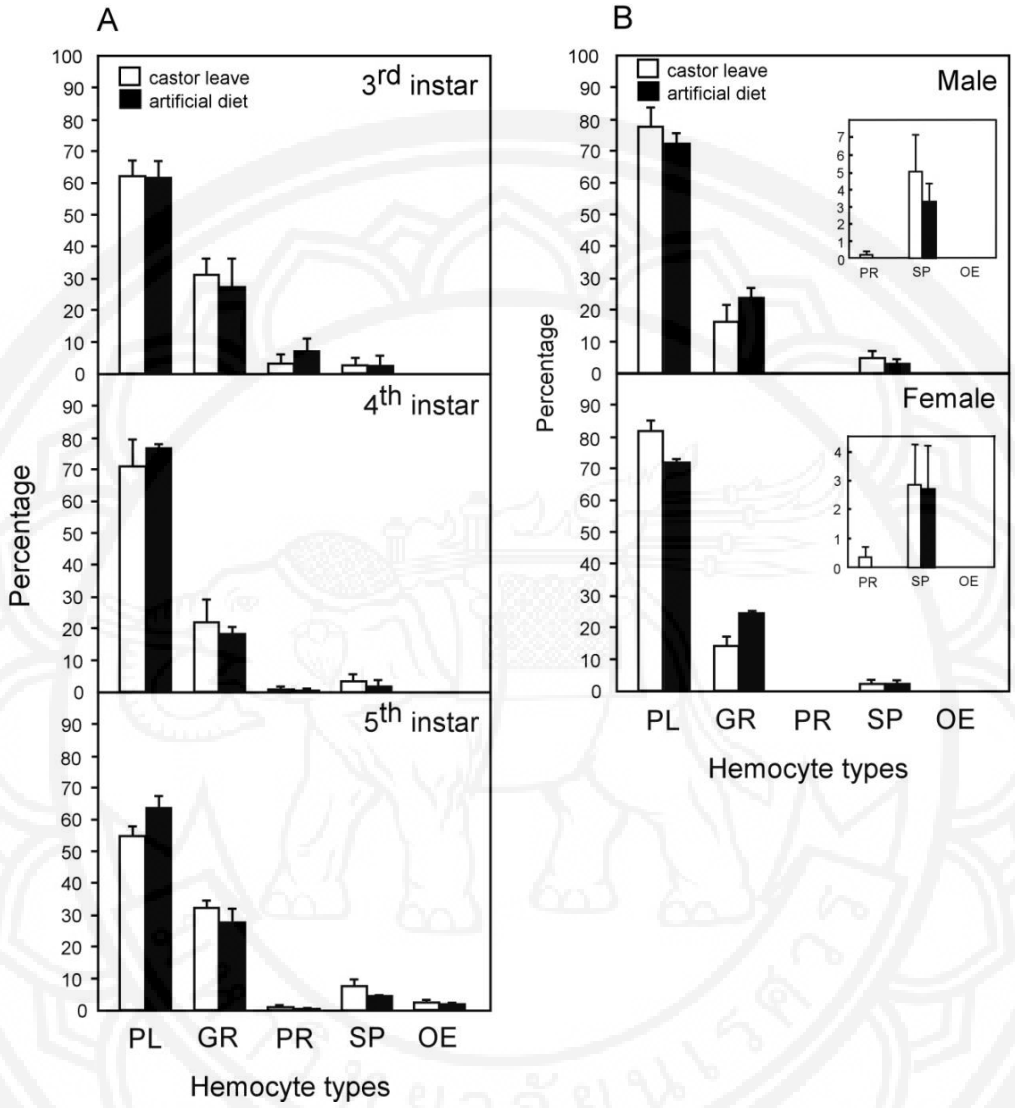

Figure 4 DHCs in larvae (A) and pupae (B) of *S. cynthia ricini* fed on castor leaves or the artificial diet. PL, plasmatocyte; GR, granulocytes; PR, prohemocyte; SP, spherulocyte; OE, oenocytoid.

Discussion

The results of our study clearly showed that the body weight of 5th-instar larvae reared on an artificial diet was about two-fold higher than that of larvae reared on castor leaves. This supports a recent study in *S. cynthia ricini* that an artificial diet (Silkmate L4M) affects the lengths of the larval and pupal periods, the weight of the posterior silkgland, the THC, total hemolymph protein concentration, total lipid concentration, and α-amylase activity (Tungjitwitayakul & Tatun, 2017). Likewise, a study of the silkworm *B. mori* revealed that insects reared on the artificial diet during larval stages were of lower quality than those fed mulberry leaves. This is also reflected in many other biological properties, such as the filament quality of cocoons, the survival rate of young

larvae, and overall resistance to diseases caused by bacteria and viruses (Kataoka & Imai, 1986; Zhou et al., 2008; Vogelweith et al., 2016).

In this study, we identified PR, PL, GR, OE, and SP hemocytes based on their morphological features. The sizes and other characteristics of each type of hemocyte of *S. cynthia ricini* in this study were similar to the hemocytes found in other Lepidopteran insects, such as *Ephestia kuehniella*, *Plutella xylostella*, *Ectomoyelois ceratoniae*, *Hyphantria cunea,* and *B. mori* (Strand & Pech, 1995; Huang et al., 2010; Khosravi, Jalali, & Ghasemi, 2012; Ajamhassani, Jalali, Zibaee, Askary, & Farsi, 2013; Tan et al., 2013; Ghasemi, Moharramipour, & Sendi, 2014).

It has been reported that the THC varies with the developmental stage and physiological condition of the insects. The THC of *S. cynthia ricini* reared on castor leaves showed a significant decrease in the number of hemocytes during larval and pupal development. During larval development, the THC of the velvetbean caterpillar *Anticarsia gemmatalis* also displays a significant decrease in the number of hemocytes (Andrade, Negreiro, Gregó rio, Moscardi, & Falleiros, 2003). In contrast, the THC of *S. cynthia ricini* larvae reared on an artificial diet remained at a low level during the early larval stage (3^{rd}-instar), and this persisted throughout the pupal stage. For the same developmental stages, the number of hemocyte in *S. cynthia ricini* larvae reared on castor leaves was higher than that of larvae reared on the artificial diet. The results clearly showed that the abundance of hemocytes was influenced by the larval diet. Larvae reared on castor leaves were probably in much better condition, which might have enabled them to rapidly produce more hemocytes in response to an immune challenge (Vogelweith et al., 2016).

The relative abundance of different types of hemocytes is not constant, which has been presumed to be related to the feeding and molting activities of the insects (Mahalingam & Muralirangan, 1998). Our results for the DHC showed that larval diet (artificial) had little impact on the abundance of five hemocyte types in 3^{rd}- and 4^{th}-instar larvae of *S. cynthia ricini*. However, PLs and SPs in 5^{th}-instar larvae were affected by the artificial diet, resulting in a decrease in SPs and an increase in PLs. This suggested that during the last instar, the larvae were sensitive to changes in nutrition or the composition of the diet.

Among five classes of hemocyte in *S. cynthia ricini*, PLs and GRs were the most abundant. This was similar to the findings of Takahashi and Enomoto (1995), who found that late 5^{th}-instar larvae of *S. cynthia ricini* have more abundant PLs and GRs compared to PRs and OEs. PLs and GRs in larvae of *E. kuehniella*, *G. mellonella,* and *Manduca sexta* have an important role for the cellular immune response (Tojo, Naganuma, Arakawa, & Yokoo, 2000; Ling & Yu, 2006; Ghasemi et al., 2014). Generally, these comprise about 50% of the hemocytes in insect. The percentage of PLs and GRs is higher than that of OEs and SPs, most likely because they can differentiate into OEs and SPs (Han, Lee, Kim, Wago, & Yoe, 1998).

Studies of some insects noted that PRs can be differentiated to other cell types (Gupta, 1991). In *S. cynthia ricini*, population of PRs in 3^{rd}-instar larvae is larger than that of the 4^{th}- and 5^{th}-instar larvae and pupae. The decrease in PR numbers coincided with the increase in PLs, SPs, and OEs in penultimate and last instar larvae, which suggests that their differentiation probably provides a sufficient amount of PLs and OEs at those timepoints. It has been shown that 43% of PRs in *B. mori* developed to PLs, GRs, and SPs (Yamashita & Iwabuchi, 2001).

In general, the small proportion of OEs in larvae has been reported in many other insects, such as *G. mellonella*, *Spodoptera litura*, *D. saccharalis*, and *Lacanobia oleracea* (Shapiro, 1979; Kurihara, Shimazu, & Wago, 1992; Richards & Edwards, 1999; Falleiros, Bombonata, & Gregorio, 2003). It is known that OEs are

involved in the production of prophenoloxidase (Lavine & Strand, 2002), one of the most important enzymes in humoral immunity. Moreover, they are also involved in various biological responses, such as apoptosis. This might explain why the relative abundance of OEs in *S. cynthia ricini* larvae are greatest prior to the larval-pupal transformation.

Schmitz et al. (2012) reported that SPs are involved in the coagulation process shortly after hemolymph collection, and they participate in energy storage and lipid transport (Vogelweith et al., 2016). The results of our study clearly showed that in 5th-instar larvae, the amount of OEs and SPs was affected by the larval diet. The larvae reared on the artificial diet may have a reduced immune response compared to the larvae reared on castor leaves.

Nutrition is now recognized as a critical factor in immune defense and resistance to pathogens (Ponton, Wilson, Cotter, Raubenheimer, & Simpson, 2011; Vogelweith et al., 2016). Experimental studies of insects have revealed that food deprivation affects immune responsiveness (Siva-Jothy & Thompson, 2002; Yang, Ruuhola, Haviola, & Rantala, 2008) and changes the expression of several genes involved in the immune system (Pletcher et al., 2002). A proteomic analysis of *B. mori* reared on either fresh mulberry leaves or an artificial diet identified eight proteins involved in silkworm innate immunity, including Nuecin, P50, PGRP, *β*-*N*-acetylglucosamidase, and four isoforms of Gloverin-like proteins (Zhou et al., 2008). There were also two antibacterial peptides (Nuecin and Gloverin-like proteins) identified in silkworms fed on an artificial diet, suggesting that the different diets alter the expression of proteins related to the immune system. Furthermore, changes in innate immunity reduce the resistance to specific pathogens in the silkworms fed on an artificial diet.

In addition to the malnutrition of *S. cynthia ricini* fed on the artificial diet, Silkmate L4M contains 4% mulberry leaf powder, which includes dried mulberry latex. Konno et al. (2006) reported that mulberry leaves (*Morus* spp.) are highly toxic to larvae of *M. brassicae* and *S. cynthia ricini* that are non-mulberry feeder. This may be caused by the materials in the latex of mulberry. There have been reported that mulberry produced secondary metabolites and sugar-mimicking alkaloids in the latex including 1, 4-dideoxy-1, 4-imino-D-arabinol (D-AB-1) and 1-deoxynojirimycin (DNJ). These molecules acts as defense chemicals that protect this plant from various phytophagous insects. In addition, D-AB-1 and DNJ were fed to *S. cynthia ricini* by mixing these compounds with the artificial diet, which reduced insect body weight and decreased the activity of sugar metabolism enzymes, including sucrase and trehalase (Hirayama et al., 2007). However, the effects of sugar-mimicking alkaloids on insect immunity need to be examined in the future.

Conclusion and Suggestions

In this study, a comparison of *S. cynthia ricini* larvae reared on castor leaves versus an artificial diet revealed that the artificial diet affected the larval body weight, THC, and DHC. This artificial diet may not suitable for rearing eri silkworm for laboratory use. Development of artificial diet for eri silkworm using their host plants as main ingredient is needed in order to eliminate some toxicity from mulberry leaves.

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