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Research Article

Effect of Artificial Diet on Immature Stage of the Great Eggfly, *Hypolimnas bolina* (Lepidoptera: Nymphalidae)

[1,2]DwiRini Kurnia, [2]Fitri Dahelmi, [2]Henny Herwina and [3]Yaher Wandi

[1]Department of Education Biology, Faculty of Tarbiyah and Teacher Training, State Islamic Institute of Batusangkar (IAIN Batusangkar), Jl. Sudirman No. 137 Lima Kaum Batusangkar, West Sumatra, Indonesia
[2]Department of Biology, Faculty of Mathematics and Natural Sciences, Andalas University, Padang 25163, West Sumatra, Indonesia
[3]Department of Pest and Plant Pathology, Faculty of Agriculture, Andalas University, Padang 25163, West Sumatra, Indonesia

Abstract

Background and Objective: One of the Nymphalidae butterfly species found in West Sumatra in *Hypolimnas bolina*. Currently, research on the artificial diet for the Nymphalidae butterfly is relatively rare in Padang, West Sumatra. The objectives of this study were to analyze the preferences of *H. bolina* larvae, duration of the immature stage and mortality of *H. bolina* in artificial diet treatment. **Materials and Methods:** Some biological aspects of *H. bolina* in corresponding to artificial diet and its effect were investigated in the laboratory. **Results:** The result showed that there was no significant difference in the frequency of visits of the larvae in the two diet treatments namely natural (*Laportea interrupta* leaves) and artificial diets (Sig = 0.289, $p>0.05$) but the duration of the visit of *H. bolina* larvae was significantly different (Sig = 0.000, $p<0.05$). The visit duration of the immature stage of *H. bolina* was significantly different, except the prepupa and pupal stage. There was no mortality of instar larvae and prepupa stage observed in both of the two-diet treatments. However, the mortality of pupae in an artificial diet was 4%. Of the total of 24 individual larvae fed with artificial diet, all of them successfully emerged, consisted of 12 males and 12 females but there was one male with abnormal wings. The average living period in the artificial diet of imago was 14.82 days for males and 16.77 days for a female. The average larval weight was no significant difference (Sig = 0.981, $p>0.05$) but the average pupal weight of the natural diet was slightly higher than the artificial diet. **Conclusion:** The formulation of an artificial diet is suitable for *H. bolina* larvae based on the results of immature mortality and adult emergences. Therefore, the formulation of an artificial diet is suitable for *H. bolina* with its composition almost similar to *L. interrupta* leaves (natural diet).

Key words: Artificial diet, immature stage, larvae, *Hypolimnas bolina*, *Laportea interrupta*

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Corresponding Author: Fitri Dahelmi, Department of Biology, Faculty of Mathematics and Natural Sciences, Andalas University, Padang 25163, West Sumatra, Indonesia Tel: +62- 81266490876

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The phytophagous insect was often specialized in certain species or host groups[1]. As sign stimuli, certain insects employ chemicals found in the host plant. This could be accomplished by using phagostimulatory cells instead of deterrent cells to express the proper receptor[2]. *Hypolimnas bolina* butterflies are classified as polyphagous insects that use several types of host plants. Several plants have been identified as the hosts for *H. bolina* including *Asystasia*, *Sida rhombifolia* and *L. interrupta*. Morphologically, *L. interrupta* has advantages over other host plants, namely the presence of stinging hairs or fine spines on the stems and leaves function as the protector of *H. bolina* larvae from predators[3,4].

Hypolimnas bolina is a member of Nymphalidae family found in West Sumatra and distributed throughout Southeast Asia and Indo-Australia. *H. bolina* is dimorphic and commonly traded since they have the same beautiful colour as Papilionidae butterflies. This butterfly is not included in the 20 butterfly species that are protected under Government Regulation No. 7 of 1999, so there is no regulation prohibiting in the hunting of this species in the wild[5].

A previous study revealed that *L. interrupta* is preferable as the suitable host plant for the *H. bolina*. However, the existence of *L. interrupta* in nature is strongly influenced by environmental conditions that may affect its nutritional contents and the success of butterfly life. To overcome this condition, *H. bolina* butterflies can be reproduced by providing an artificial diet using *L. interrupta* as additional material. The artificial diet aims to anticipate the shortage of natural food availability[6]. In addition to being nutritional, the diet should encourage insect feeding. Insect-eating may be influenced by diet colour, shape and texture. Most plant species require phagostimulants to begin eating. The addition of plant material selected by the test insect in powder form will facilitate this food (of leaves, fruits, stems, etc.)[7]. One of the most commonly used artificial diet formulas is Morton's formula[8]. The artificial diet can also affect the biology and ecology of the insects being tested[9]. Thus, the study focusing on the effect of artificial diet on insects could provide beneficial information to conserve the butterfly. Based on this, it is necessary to make an artificial diet with *L. interrupta* as an additional ingredient in the breeding process of *H. bolina*. The objectives of this study were to analyze the preferences of *H. bolina* larvae in artificial diet treatment and to analyze the immature stage and mortality of *H. bolina* in the artificial diet.

MATERIALS AND METHODS

Preparation of larvae and host plant of *H. bolina*: The larvae and host plant were prepared according to the method described elsewhere[5]. The emerged butterflies were transferred into the field cage for mass rearing. These works were done from April-September, 2019. Furthermore, the eggs were allowed to hatch and develop into larvae then being subjected to the observations.

Preparation of artificial diet: Artificial diet was prepared according to Morton's formula with a modification[8] Table 1. Accordingly, in the artificial diet for *H. bolina* larvae, the white germ was substituted with red bean as a treatment material. Some of the ingredients of the Morton formula were replaced, namely Methyl P-hydroxybenzoate (anti-bacterial) was replaced with chloramphenicol and aureomycin (anti-fungal) was replaced with sorbic acid. Whereas 10% formaldehyde solution, 4 M potassium hydroxide, 25% acetic acid and 50% choline chloride solution were omitted.

The dry red bean was separately crushed, filtered with an 80-mesh sieve and placed in the plastic container. The leaves of *L. interrupta* were washed and dried at room temperature for 3-5 days. The dried leaves were crushed and kept in a plastic container. All the artificial diet components were weighed separately and placed in an Erlenmeyer tube. The green agar and 250 mL distilled water were boiled for 3-10 min, with slow stirring. When the solution temperature was about 75-85°C, all of the dry components and the red bean powder were added. The warm artificial diet was poured into a plastic container (5×4 cm, diameter×height) up until about 0.5 cm from the bottom of the container. The solution was allowed to cool and stored in the freezer at 4°C. The artificial diet was subsequently brought to room temperature before being used.

Test of larvae preference to artificial diet: The artificial diet was divided into pieces with 1×1×0.5 cm size and placed in a plastic container. One of the first instar larvae was placed between natural diet (P_1 leaves of *L. interrupta*) and artificial diet (P_2), in the centre of the plastic container. The larvae were allowed to freely choose the food as a preference test. This preference test was carried out with 25 replications. Artificial and natural diets were changed with fresh ones daily. The observations were focused on the duration of visit on the diet treatment and frequency of visit. Such preference tests and observations were deployed similarly for the second, third, fourth and fifth instar larvae.

Table 1: Composition of an artificial diet for mass rearing of *H. bolina* larvae

Composition of morton's formula	Amount	Modified artificial diet formula[8]
Soy flour (g)	7.0	7.0
Wheat germ (g)	6.0	-
Red bean (g)	-	6.0
Laportea interrupta dried leaves (g)	1.5	1.5
Yeast (g)	6.0	6.0
Sucrose (g)	3.6	3.6
Wesson salt (g)	1.0	-
Ascorbic acid (g)	0.4	0.4
Potassium sorbate (g)	0.2	0.2
Methyl parahydroxybenzoate (g)	0.15	-
Chloramphenicol	-	0.15
Aureomycine (g)	0.023	-
Sorbic acid	-	0.023
Agar (g)	1.9	1.9
Formaldehyde solution 10%	0.43	-
Potassium hydroxide 4 M	0.8	-
Acetic acid 25%	1.14	-
Choline chloride solution 50%	0.23	-
Distilled water (mL)	To mass	250

Observation on immature stage and mortality: The one of second larvae instar was placed in a plastic container (diameter = 10 cm, height = 15 cm). A larva only gets one food treatment in each plastic container. The treatments for larvae were natural diet (P_1, leaves of *L. interrupta*) and artificial diet (P_2). This experiment was carried out with 25 replications. The observations were performed until the larvae reached the pupal stage. The number of larvae mortality, pupa development, the sex ratio of adult butterflies emerged and the living period of adult butterflies was subsequently recorded.

Data analysis: The data were analyzed using SPSS and the significant value of difference was tested using t-test[10].

RESULTS

Larvae preference of *H. bolina* on artificial diet: The diet preference test of *H. bolina* larvae indicated that the frequency of visitation of the larvae on artificial diet (P_2) was lesser than the natural (P_1), but statistically insignificant (Sig = 0.289, $p>0.05$). The frequency of larval visits on the diet treatment increases in line with the development of the larval stages. The frequency of visits of first, second, third, fourth and fifth instar larvae on the artificial diet were 1.96, 3.20, 4.88, 5.08 and 6.24 times, respectively and on the natural diet were 3.32, 3.56, 5.32, 7.16 and 12.00 times, respectively. The lowest frequency of visits was during the first instar larvae on an artificial diet (1.96 times) and the highest frequency of visits was during the fifth instar larvae of natural diet (12.00 times). However, the duration of the visit of *H. bolina* larvae in the artificial diet (P_2) was significantly different from the natural diet (P_1) (Sig = 0.000, $p<0.05$). The duration of the visit of first, second, third, fourth and fifth instar larvae on the artificial diet was 12.38, 12.85, 16.11, 47.34 and 25.15 min, respectively and the natural diet were 1274.52, 1478.28, 1266.16, 1268.46 and 1672.32 min, respectively. The lowest duration of the visit was in the first instar larvae of the artificial diet (12.38 min) and the highest was in the fifth instar larvae on a natural diet (1672.32 min). The frequency and duration of the visit of fifth instar larvae are higher than other instar larvae because fifth instar larvae need a lot of nutrients to transform into pupae. The high value of frequency and duration of a visit for each larval instar on the natural diet compared to the artificial diet indicated that the natural diet (host plant) was preferred over the artificial diet as food for *H. bolina* larvae in Table 2.

The artificial diet is also favoured by *H. bolina* larvae in Fig. 1. In Fig. 1a, it is shown that the *H. bolina* larvae were eating the artificial diet, this indicates that the *H. bolina* larvae visited the artificial diet during the observation. Figure 1b shown that larvae climbing on an artificial diet and stay there for a while. This indicates the duration of larval visits on the artificial diet.

Immature stage and mortality: The larval stage is an active feeding and moving phase. The durations of the immature stage of *H. bolina* fed with an artificial diet were significantly different from the natural diet, except for the prepupa and pupal stages. The duration of the second instar larvae of artificial diet (3.96 days) was longer than the natural diet (3.24 days) with the same range in both treatments, namely

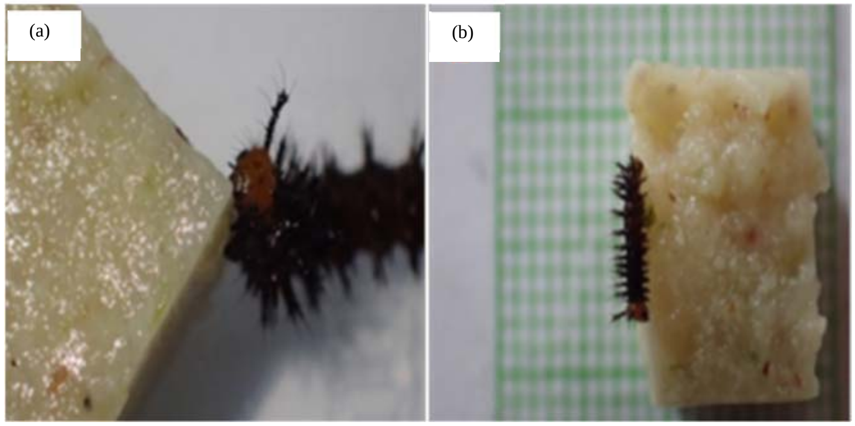

Fig.1(a-b): *Hypolimnas bolina* larvae preference on artificial diet (a) Larvae eat the artificial diet and (b) Larvae visit and stay in an artificial diet

Fig. 2(a-d): Representative photograph of immature stage of *H. bolina* fed with artificial diet (a-b) Larvae, (c) Prepupa and (d) Pupa

Table 2: Larvae preference on diets

	Frequency of visit (times)		Duration of visit (min)	
Instar larvae	P_1	P_2	P_1	P_2
First	3.32	1.96	1274.52^a	12.38^b
Second	3.56	3.20	1478.28^a	12.85^b
Third	5.32	4.88	1266.16^a	16.11^b
Fourth	7.16	5.08	1268.49^a	47.34^b
Fifth	12.00	6.24	1672.32^a	25.15^b

Values followed by different letters in the same line column are significantly different by t-test. P_1: Natural diet (leaves of *L. interrupta*) and P_2: Artificial diet

2-7 days. In the third instar larvae, the duration of artificial diet and natural diet was 4.72 and 3.84 days, respectively. However, the range of these two diets has a slight difference, was 2-9 days and 2-6 days, for both the artificial diet and natural diet, respectively. The fourth instar larvae have the same range as the second instar larvae, both in diet treatments, namely 2-7 days, with the duration were 4.84 and 3.48 days for the artificial and natural diet, respectively. The fifth instar larvae showed a longer duration on the artificial diet (7.88 days) than the natural diet (5.96 days), with a range of 6-9 and 4-9 days for the artificial and natural diet, respectively. The prepupae have the same range between the two-diet treatments, which was only one day each but the duration of the artificial diet prepupa was shorter than the natural diet. The duration of artificial diet prepupae was 0.96 and one day for the natural diet. In the pupa stage, the duration and range of both diet treatments were different and the duration of the artificial diet was shorter than the natural, with values of 7.58 and 7.60 days, respectively. The pupa range of artificial diet was 7-10 days, which is slightly longer than natural diet pupae 6-9 days. There was no mortality of instar larvae and prepupa stage in both diet treatments. However, the mortality of pupa in an artificial diet was 4% with one pupal death. While in the natural diet, the value of pupal mortality was 0%. These results demonstrate that the artificial diet has a good effect on the development and survival of the immature stage in Table 3.

The development of the immature stage of *H. bolina* reared on an artificial diet showed different larval and pupae morphology from the natural diet in Fig. 2. Figure 2a is the second instar larvae after eating the artificial diet. The body colour looks attractive, which is light brown. Figure 2b is a

Fig. 3(a-c): Morphology of *Hypolimnas bolina* imago emerged from the artificial diet (a) Normal wing of male, (b) Normal wing of female and (c) Abnormal wing of male

Table 3: Duration of immature stage and mortality of *H. bolina* fed with artificial diet

	Duration (days)		Range (days)		Mortality (%)	
Immature stage	P_1	P_2	P_1	P_2	P_1	P_2
Second instar	3.24^b	3.96^a	2-7	2-7	0	0
Third instar	3.84^b	4.72^a	2-6	2-9	0	0
Fourth instar	3.48^b	4.84^a	2-7	2-7	0	0
Fifth instar	5.96^b	7.88^a	4-9	6-9	0	0
Prepupa	1.00	0.96	1	1	0	0
Pupa	7.60	7.58	6-9	7-10	0	4

Values followed by different letters in the same line column are significantly different by t-test. P_1: Natural diet (leaves of *L. interrupta*) and P_2: Artificial diet

Table 4: Number of newly emerged adults of *H. bolina* under two diet treatments

	Number of emerged butterflies			
Diet treatment	Male	Female	Total	Description
P_1	12	13	25	All butterflies had normal wing
P_2	12	12	24	11 normal males, 12 normal females, 1 abnormal male

P_1: Natural diet (leaves of *L. interrupta*) and P_2: Artificial diet

fourth instar larva after moulting. The spines on the body of the artificial diet larvae look attractive in colour. The body of the larva is black but the head and spines are cream-coloured and pink mouth. Figure 2c is the prepupa stage, where the prepupa is seen hanging on the provided twig. After the prepupal stage ends, the final molting will occur to transform into a pupa. Figure 2d is the pupa stage of the artificial diet. In this phase, the pupa appears to have a brown colour on the whole body, which is different from the natural diet pupa which is brown with grey on the wings.

The number of emergence butterflies from the natural diet (*L. interrupta* leaf) was 25 individuals, consisting of 12 males and 13 females. All of the emergence butterflies from *L. interrupta* leaves had normal wings. 24 butterflies emerged from the artificial diet, consisting of 12 males and 12 females but there was one male with abnormal wings in Table 4.

The morphology of *H. bolina* imago emerged from the artificial diet in Fig. 3. Figure 3a showed a male butterfly emerging from an artificial diet treatment. This male has the

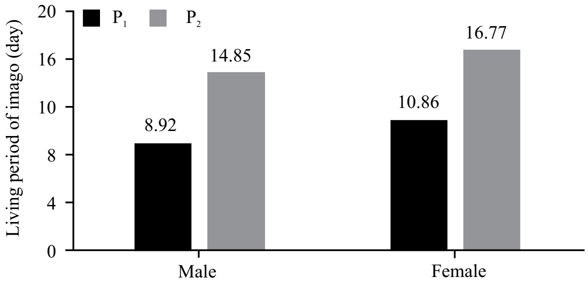

Fig. 4: Living period of imago *H. bolina* emerged from two diet treatments

P_1: Natural diet (leaves of *L. interrupta*) and P_2: Artificial diet

Table 5: Mean weight of the immature stage of *H. bolina* under diet treatments

	Mean weight (g)	
Immature stage	P_1	P_2
Second instar	0.03	0.03
Third instar	0.08	0.08
Fourth instar	0.20	0.22
Fifth instar	0.69	0.69
Pupa (male)	0.75	0.67
Pupa (female)	0.77	0.74

P_1: Natural diet (leaves of *L. interrupta*) and P_2: Artificial diet

same colour as the male from the natural diet. Males have a black dorsal wing surface with three pairs of white spots (two on the forewings, one on the hind wings) surrounded by iridescent blue/purple. Because the scales on the wing are oriented to reflect light, the colour can appear to change. A diagonal white band runs across both wings and the ventral wing surface is black with white spots. Males' wings have a black underside with a diagonal white band and a curved arc of white spots on each wing. Figure 3b is a female butterfly emerging from an artificial diet. This colour is one of the colour variations in the female *H. bolina*. There was no difference in the colour of the female butterflies that emerged from the artificial diet with the natural diet. Females are similar but also have orange markings on each forewing and their colouration is much more varied. The underside of the female's wings is brown with a diagonal white band and an arc of curved white spots on each wing. Figure 3c shown the abnormal male that emerged from the artificial diet. It has the same colour as the normal male but the male has twisted wings on both wings, especially on the hind wings. Even though the wings are twisted, this male can fly even though it's not like a normal butterfly.

The life spans of butterflies in the two-diet treatments were varied. The average living period butterfly which reared in *L. interrupta* leaves diet for males was 8.92 days and the female was 10.86 days with a range of 1-15 and 6-16 days, respectively. In artificial diet, the average living period of male imago 14.85 days and female imago 16.77 days with a range of 12-23 and 7-26 days, respectively in Fig. 4.

Effect of artificial diet on *H. bolina* larvae: Artificial diet is essential in the rearing system of entomological research. The effect of an artificial diet on insect growth can be determined by measuring the mean weight of the immature stage. There were no differences between the mean weight of larva in the two-diet treatment (Sig = 0.981, $p>0.05$). The mean weight of the second, third and fifth instar larvae in both diet treatments, namely 0.03, 0.08 and 0.69 g, respectively. However, the mean weight of the fourth instar larvae on the artificial diet was higher (0.22 g) than the natural diet (0.20 g). The mean pupal weight of the natural diet was slightly higher than the artificial diet in both male and female pupa but statistically insignificant. The mean pupal weight in the natural diet were 0.75, 0.77 and 0.67, 0.74 g in the artificial diet for male and female pupal, respectively (Table 5).

DISCUSSION

Feeding preferences are very important for the survival of insects as well as for their adaptation. However, there was less information about determining feeding preferences in insects including in *H. bolina* butterfly. Based on the selection of host plants, phytophagous insect food preferences are grouped into monophagous, oligophagous and polyphagous. Lepidoptera is a group of phytophagous insects with the highest diversity of food preferences. The preference of Lepidoptera or butterflies to plants is the tendency or attraction of butterflies to plants. Butterflies use plants as perch, food plants or host plants.

Current laboratory experiments revealed that *H. bolina* larvae visited the control more frequently than the artificial diet treatment. Likewise, the duration and frequency of visit *H. bolina* larvae in control were longer than the artificial diet. This could be due to that *L. interrupta* leaves contains the nutrients needed by *H. bolina* larvae.

Larvae can accept or reject an artificial diet for a variety of factors, including its nutritional value, texture or structure, cohesiveness and phagostimulants[11,12]. Lyophilized host plant components were used as a phagostimulant and around 10% of the dry elements from the tested diet were added to the lepidopteran artificial diets just to induce the larvae to start eating it[6,13]. Nutrients are environmental elements that combine the physiology and ecology of an animal and intersect them. Nutrition refers more generally to the intake and processing of substances that fuel the energy of the body and its structural growth, maintenances and reproductive demands[14]. Proteins or free amino acids from which the organism makes its proteins and carbohydrates, which provide energy and can be used to make lipids are the two most important nutrients. The balance of phagostimulatory and deterrent inputs governs phytophagous insect feeding[2].

Based on observations of the duration of the larval stage in the diet treatment, it was found that the larval stage in the artificial diet was longer than the host plant, both in terms of the average duration of each larval stage and the range of days required for each larval stage. Larvae that fed on highly nutritious foliage increased their growth rates and showed a shorter development period[15]. The larvae's growth can also be affected by differences in the chemical composition of the host plant (e.g., secondary metabolites)[16].

From the observations, it was also found that there was no larval mortality in the two-diet treatments. It indicates that the larval survival rates in both diet treatments were high. Likewise, with the prepupa stage in the two-diet, there was no mortality for prepupa in both treatments. This was also observed in the pupal stage of the host plant, where there was no mortality in pupal stage. Whereas at the pupa stage with artificial diet, only one pupa was found to be dead (percentage of mortality was 4%).

Survival of larvae *Spodoptera exigua* (Hubner) using artificial diet also significant from the natural diet was 96.67% (larvae) and 86.11% (pupa). The larval and pupal periods of those fed with artificial diet were 15.7±0.84 and 6.73±0.51 days respectively, whereas, those fed with natural diet were 18.65±0.83 and 8.5±0.52 day's, respectively[17]. *Idea leuconoe clara* (Butler) reared with the artificial diet, had a lower survival rate during every life stage and had a longer developmental period. There was no change in appearance or colour between groups even though up to 60% of the adult wings in the experimental group were twisted and unable to fly[18]. *Helicoverpa armigera* larvae fed on several host plants (pea, bean) and an artificial diet had a shorter lifespan. The pea and artificial diets had the lowest mortality rates. There were no significant variations in pupal duration between larval fed on host plants and artificial diet. The average pupal mortality percentages were 45.16% for the host plant and 7.27% for the artificial, respectively[19]. Survival of *Duponchelia fovealis* Zeller larvae was highest in those fed an artificial diet. The artificial diet is appropriate for the continuous rearing of *D. fovealis* in the laboratory[20].

Twenty four butterflies emerged from the artificial diet, consisting of 11 males with normal wings, one male with abnormal wings (twisted) and all 12 females with normal wings. The butterflies that emerged from the host plant were 25, consisting of 12 males and 13 females, all with normal wings. The living period of butterflies that emerged from the artificial diet was longer than host plant treatment, for both males and females. The average living period of males in host plant treatment was 8.92 days and the number of females was 10.86 days. Meanwhile, the average living period of males in the artificial diet was 14.82 days and the female was 16.77 days.

The lack of linseed oil in the artificial diet used in the recent study was thought to be responsible for the debilitating wings phenomenon in *H. bolina*. The absence of linseed oil in an artificial diet with Morton's formula also caused the crippling wing in *T. helena* males[8]. Although an artificial diet has sufficient nutrition, the absence of volatile oil and secondary metabolites will influence the development of the target insect[21]. In diets containing 30% leaf powder of *M. japonica* and *M. tomentosa*, the rate of adult emergence of *Parantica sita* (Kollar) was high (100 and 97%) and most adults appeared healthy. An artificial diet based on *M. japonica* appears to be the most practical for mass rearing of *P. sita*[22]. In the present research, it was demonstrated that *H. bolina* larvae fed with artificial diet were capable of developing to male and female butterflies with normal wings.

The artificial diet could influence the growth of butterfly larvae. The effect of an artificial diet on insect growth can be determined by measuring the mean weight. In this research, it was found that the mean weight of second instar larvae, third and fifth in both diet treatments was the same, while the weight of fourth instar larvae in the artificial diet was slightly heavier than the host plant treatment. Based on the measurement of the pupal weight, there were differences in pupal weight in the two-diet treatments, where the mean pupal weight of artificial diet was lower than host plant treatment, for both males and female pupa.

There were highly significant differences in *H. armigera* pupal weights. Pupal weights ranged from 2.80-3.30 g for different host plants and 3.25 g for artificial diet[19]. Pupal weights, adult weights and larval development rates all increased as nutrition levels raised. Furthermore, pupal and adult weights differed significantly between host plant species. The availability of plant nutrients influenced pupal weights as well, however, this varied according to the host plant. Additionally, the host plant species had an impact on the effect of plant nutrient availability on larval growth rates[15].

Many factors influence larval feeding on artificial diets, including nutritional proportion and balance, moisture level and diet texture[14]. The inclusion of some host plant components in artificial diets frequently enhances growth, survival, fertility and may act as required stimulants for oviposition and effective rearing[6].

CONCLUSION

The formulation of an artificial diet is suitable for *H. bolina* larvae based on the results of immature mortality and adult emergences. Artificial diet affects the development of *H. bolina* butterflies, where the colour of the larvae looks more attractive and the butterflies that hatch can live longer than a natural diet. Artificial diet affects the growth rate of butterflies so that it can be used for the development of *H. bolina* in the rearing cage.

SIGNIFICANCE STATEMENT

This study provides important information regarding the growth and relative condition factors of *H. bolina* that are reared by artificial diet. This study could also be extended to other species of butterflies, with some alteration to the nutrient requirements of the species involved as well as the use of specific food plants. This study indicated that more than 90% of the *H. bolina* larvae successfully emerged as adults if individually reared on an artificial diet. The artificial diet would be potentially useful as the diet for rearing several other economically important species of Lepidoptera.

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