International Journal of Entomology Research
ISSN: 2455-4758; Impact Factor: RJIF 5.24
Received: 27-10-2020; Accepted: 12-11-2020; Published: 27-11-2020
www.entomologyjournals.com
Volume 5; Issue 6; 2020; Page No. 128-133

Oviposition and pupation site preference of *Hypolimnas bolina* L. (Nymphalidae) on *Laportea interrupta* (L.) Chew (Urticaceae) host plant

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Abstract

The preferences on the oviposition and pupation sites are considerably important for maximizing the survival of offspring and reproduction of the butterflies, In this present study, we aimed to investigate the preference of *H. bolina* oviposition site on its host plant namely *Laportea interupta* (Urticaceae). Our study found that the majority of *H. bolina* butterflies preferred to lay the eggs on the underside of leaves (73%) than the upper side, petiole, oviposition mistake, and stems. The young leaves were preferable than the mature leaves, petiole, seedling, and stems. The most frequent type of oviposition of *H. bolina* was in a cluster. Based on the substrate, *H. bolina* pupae were commonly observed on the non-host plant substrate particularly on the living stems. The sites with the height ranged from 31-60 cm above the ground surface were preferable as the place for the pupation. The information about the preferences on the oviposition and pupation sites could be useful for further conservation policies of the butterflies both in situ and ex-situ.

Keywords: *Hypolimnas bolina*, *Laportea interrupta*, oviposition, pupation site preference

1. Introduction

There are about 2500 species of butterflies identified in Indonesia which 600 species were observed in Java and Bali and 1000 species in Sumatra (Soekardi 2007) [40]. A study by Dahelmi *et al.* (2009) [7] found 217 species of butterflies in 10 families in different locations in Sumatra island. Another study by Rusman *et al.* (2016) [37] found 184 species of Papilionidea in Mount Sago, West Sumatra.

The research concerning the life history, ecology and host plants of the butterflies in Sumatra were conducted to the family of Papilionidae (Dahelmi 2002, Dahelmi *et al.*, 2008, Dahelmi & Suwarno, 2014) [5, 6, 8]. While the other families including Nymphalidae remain less explored, life cycle and population dynamic of Acraea *violae* (Andrianti, 2012) [1], life cycle of *Cethosia hypsea* (Dahelmi *et al.* 2017) [9], biology and life table of Doleschallia *bisaltide* (Handayani, 2018) [19]. One of Nymphalidae species namely *Hypolimnas bolina*. This species is a tropical and subtropical nymphalid butterfly that has a widespread distribution throughout Southeast Asia (Clarke & Sheppard 1975) [4]. This species is strongly polyphagous, utilizing at least 28 different host plants (Vane-Wright *et al.* 1977) [47]. The polyphagous great eggfly *Hypolimnas bolina* with the reported larval hosts in the plant families Acanthaceae, Amaranthaceae, Aroidea, Asteraceae, Convolvulaceae, Malvaceae, Portulacaceae, Tiliaceae, and Urticaceae, exhibited host plant preference in the biotope of the Andhra University at Visakhapatnam, South India (Samatha *el al.* 2014) [38].

In Australia, *H. bolina* mostly uses *S. nodiflora* as a host plant (Kemp 1998) [21]. While in Indonesia, there are three common host plants for *H.bolina* such as *Asystasia*, *Sida rhombifolia,* and *Laportea interrupta*. The *Asystasia* and *Sida rhombifolia* are invasive plants. Their associations with *H. bolina* have been previously investigated revealing the ecological and genetical aspects. However, the comprehensive biological information regarding the utilization of *L. interrupta* as a host plant for *H. bolina* remain unavailable. Morphologically, *L. interrupta* is a unique plant species with its stinging hairs on the stems and leaves (Priya and Gopalan 2015) [33]. The hairs could be useful to protect *H. bolina* larvae from the predators. The association between *L. interupta* and *H. bolina* is important to be investigated in further including the aspect of the oviposition and pupation sites preferences.

2. Materials and Methods

2.1. Preparing the host plant and larvae of *H. bolina*

The seedlings of host plant *Laportea interrupta* were collected from the wild in Padang, West Sumatra, and subsequently planted in the plastic bags (25 cm diameter and 35 cm high) at the Biology Department, Andalas University (UNAND), Padang, West Sumatra, Indonesia. All the plants were fertilized with manure.

The larvae of *H. bolina* were collected in October 2017 from *L. interrupta* plant in Halaban, Payakumbuh, West Sumatra. Then the larvae were reared in the laboratory using a screen cage (with 30 x 30 x 30 cm of size) until the emerging stage. The emerged *H. bolina* adults were subsequently transferred to a field cage (6 x 5 x 5 m) to facilitate the mating and oviposition. The flowers of *Lantana* sp., *Euphorbia* sp., *Ixora* sp., and *Clerodendrum japonicum* were provided as food sources for the adults of *H. bolina*.

2.2. Oviposition preference
The plants of *L. interupta* were placed in the field cage, positioned one meter apart. The plants were at least 50 cm away from the wall of the captivity. Furthermore, two pairs (consisted of two females and two males) of newly emerged *H. bolina* were introduced into the cage. The numbers of eggs laid by the females on each host plant were recorded daily until the females died.

2.3. Pupation site preference
Once larva selected its pupation site, the position was immediately marked then being observed daily until the stage of newly emerged butterflies. Furthermore, the data including the location of pupation, pupa condition, and the height above the ground were recorded.

2.4. Data analysis
The data of oviposition and pupation sites preference of *H. bolina* on the host plant were analyzed using a one-way analysis of variance (ANOVA) by SPSS software version 25 (George and Mallery 2019) [15].

3. Results
3.1. The oviposition preference
The observation on the oviposition preference was carried out in the field cage with eight females of *H. bolina* and *L. interupta* as the host plant. In this study, the activities of *H. bolina*

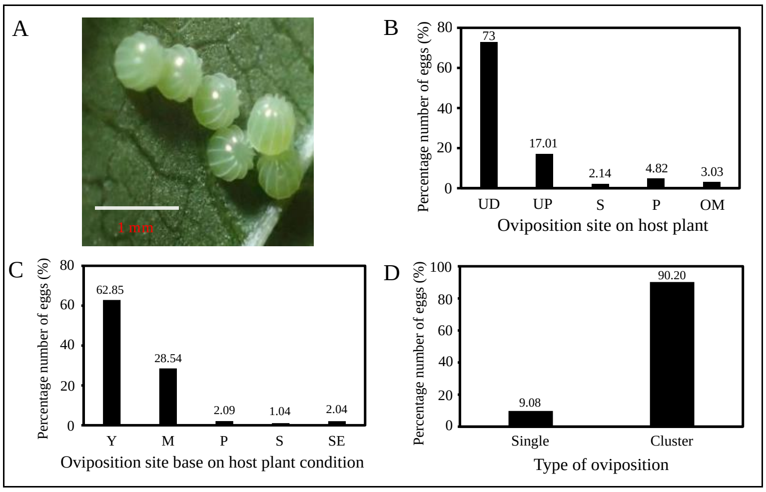

Fig 1: The Oviposition preference of *H. bolina.* (A). The representative photograph of *H. bolina eggs*; (B-D) The percentage of eggs laid on the host plant based on (B) oviposition site, (C) host plant characteristics, (D) type of oviposition. (UD = underside, UP = upper side, S = stem, P = petiole, OM = oviposition mistake, Y = young leaves, M = mature, SE = seedling)

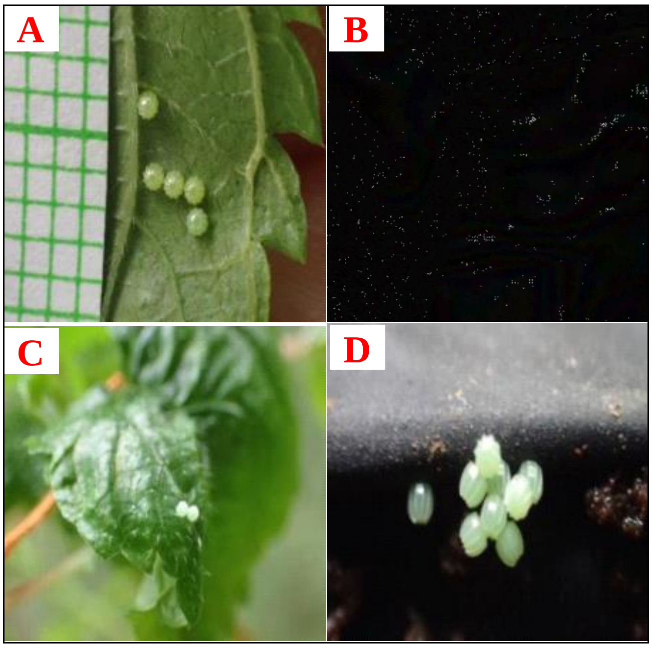

Fig 2: The oviposition site of *H. bolina* on the host plant *L. interupta*, (A) underside, (B) petiole, (C) upper side, (D) oviposition mistake

Occurred throughout the day from 07.00 am to 05.00 pm including the oviposition activity on the host plant *L. interupta*. Morphologically, the eggs of *H. bolina* were green and clear with the longitudinal lines on the side except on the top of the egg. The average eggs diameter was 0.648 ± 0.070 mm (Figure 1A), with the average number of eggs of *H. bolina* was 251 eggs for each individual of female. Based on the data of oviposition preference (Figure 1B and Figure 2), it was found that *H. bolina* preferred to lay their eggs on the underside of leaves (73%) followed by the upper side (17.01%), petiole (4.82%), and the stems (2.14%). The females of *H. bolina* also laid their eggs as the oviposition mistake (3.03%) in which the eggs were not laid on the host plants. The number of eggs laid on the underside was the highest one and significantly different ($P < 0.05$) as compared with other position, including the upper side, petiole, oviposition mistake, and the stems.

Fig 3: The type of oviposition site of *H. bolina* eggs on *L. interupta*, (A) single; (B) cluster

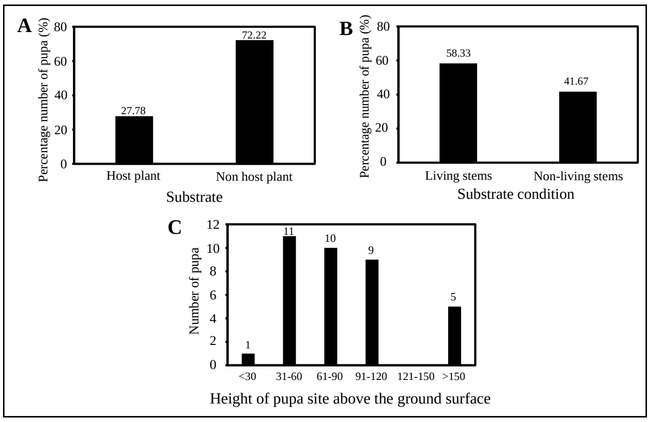

Fig 4: The pupation site preference of *H. bolina* on the host plant *L. interrupta*. (A-B) The percentage of pupation sites preference based on the substrate and substrate condition; (C) The number of pupae based on the height (cm) of the site above the ground.

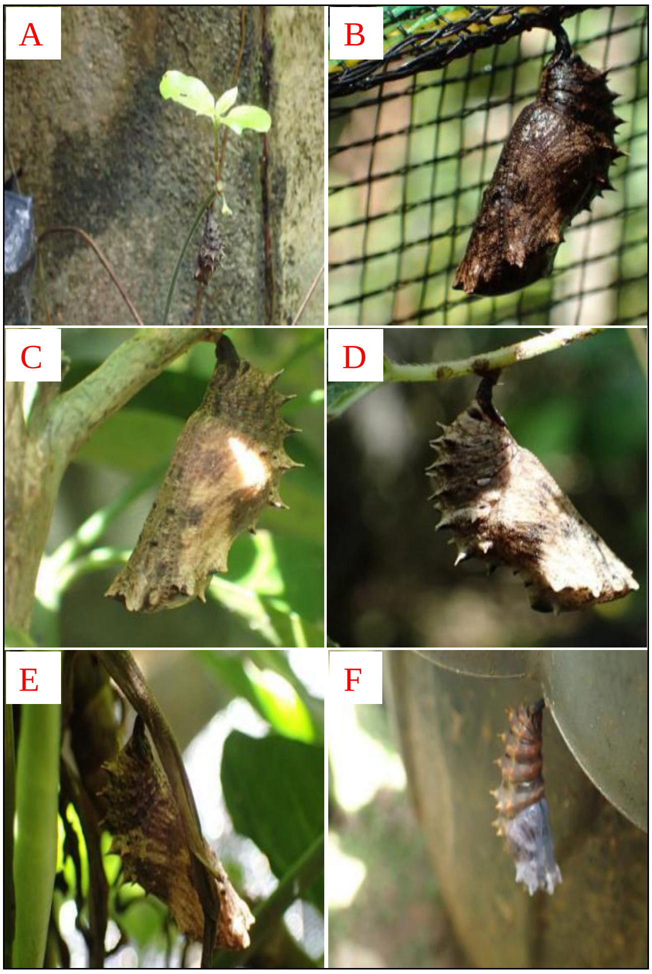

Fig 5: The pupation site preference of *H. bolina*. (A) Non-host plant stem; (B) Cage; (C) Non-host plant branch; (D) Host plant branch; (E) Dead leaf of non-host plant; (F) Pot.

3.2. Pupa site preference

The pupae of *H. bolina* were frequently observed on the non-host plants (72.22%) than the host plants (27.78%) (Figure 4A). Based on the host plant conditions, the percentage of pupation site preference of *H. bolina* was higher on the living stems (58.33%) than non-living stems (41.67%) (Figure 4B). Moreover, based on the height of the pupation site, the pupae of *H. bolina* were frequently found on the height ranged from 31-120 cm above the ground instead of the lower or higher sites (Figure 4C).

In our study, *H. bolina* preferred to pupate on the host plant and non-host plant substrates. Some pupae were also found particularly on the non-living object including the wall of the cage (Figure 5B), non-host plant branch (Figure 5C), dead leaf of non-host plant (Figure 5E) and Pot (Figure 5F).

4. Discussion

The butterflies in the suborder of Rhopalocera, including *H. bolina*, are the diurnal insects. As a heterothermic animal, *H. bolina* requires the external heat to maintain its body temperature. This external heat is obtained from the ambient temperature. Therefore, an increased ambient temperature might directly cause an increase in the metabolic rate of the body (Schowalter 2011) [39]. In this study, we observed that the activity of *H. bolina* butterfly occurred throughout the day, starting from 7 am to 5 pm. Peggie and Amir (2006) [30]. Described that the butterfly is generally active on a bright, warm, and quiet daytime at 09.00 - 15.00. The common activity of butterfly is to fly from one flower to another to suck the nectar as food. The other activities are mating and oviposition. Such activities could be observed in the morning, while it will rapidly decrease during the day. However, it has been reported that some species of butterflies actively fly during the day and apparently prefer the open fields. These activities could be influenced by the weather conditions such as temperature, humidity, and light intensity (McDonald and Nijhout 2000) [25].

In this present study, our investigation was firstly focused on the oviposition activity starting after the mating process. It revealed the tendency of females to choose the place for depositing their eggs. The female butterfly will lay the eggs in a suitable position and habitat for an optimum development of larvae. It is critical for the survival ship of the offspring of polyphagous and oligophagous butterflies (Renwick and Chew 1994) [35].. The butterfly oviposition preference is strongly influenced by the characters of the host plant including the morphological appearences and the content of chemical compounds (Konstantopoulou *et al*. 2002) [22]. The chemical and physical stimulations emitted by the host plant allow the butterfly to identify the suitable host plant for oviposition (Miller and Strickler 1984) [26].

In laying their eggs on the host plant, the butterflies may choose several locations as their oviposition sites. In our current observation, it was found that the most preferred oviposition site for *H. bolina* was on the underside of the leaf (73%). The underside position is considerably preferable to protect the eggs from the predator attacks, parasitoids, and weather changes. The selection of the correct oviposition site by the female butterflies is crucial for the survival of their offspring. The oviposition preference is a complex behavior of the females. Various sensory inputs guide this process (Carrasco *et al*. 2015; Finch and Collier 2000) [2,13].. A previous study observed that the females of *M. Leda* (Nymphalidae) also prefer to lay their eggs on the underside of the leaf (Molleman *et al*. 2020) [27]. Likewise, *Graphium Agamemnon* prefers to put the egg on the underside of leaves instead the upper side (Fitriana *et al*. 2016) [14]. Another study found that the females of several species of Papilionidea choose the soft bottom of the sheet in a shady and moist location as a place to lay the eggs (Rajeswari and Jeyabalan 2017) [34]. The butterfly of *Pieris brassicae* also lays the eggs on the bottom of the leaves of the family Brassicaceae (Little *et al*. 2007) [23]. The oviposition behavior of *Papilio polytes* and some species of Papilionidae also exhibits the same pattern. The oviposition preference of the butterflies on the underside instead of the upper side of the host plant leaf as reported by various studies (Murakami *et al*. 2003; Xuishan *et al.* 2006; Heinz 2008; Suwarno *et al*. 2018) [28, 49, 20, 44] could be a crucial strategy to protect the eggs from the predatory attacks and high rainfall (Pencoe and Lynch 1982; Suwarno 2010) [31, 43].

Beside the predominant preference on the underside of the host plant leaf, our study also found that a small number of *H. bolina* laid the eggs on the upper side of the sheet (17.01%). The female butterfly will deposit the eggs on the host plant or nearby. Then, after hatching, the newly hatched larvae will firstly eat the egg chorion as a source of nutrients. Furthermore, the larvae will eat the leaves where the eggs placed. The female deliberately prefers to lay the eggs on the leaves to facilitate the new hatching larvae in finding their food easily (Zalucki *et al*. 2002) [50]. Besides the oviposition preference on the underside and upper side, we also observed that a few females of *H. bolina* deposited the eggs on the other sites including petiole, oviposition mistake, and stem. Although the females have a great ability in oviposition, particularly on the determination of the potential host plant, the error in depositing their egg also could be occurred (Chew and Robin 1984) [3]. *H. bolina* also laid their egg on the non-host plant. This case is known as an oviposition mistake that could be observed on the other plants or non-living objects. The non-host plants selected by *H. bolina* to lay their eggs were citrus (*Citrus* sp.), *Lantana* sp., and the *Euphorbia* sp. While the non-living objects included the polybags, hanging labels, rocks, and deadwood. Another study reported that *Oeneis jutta* butterflies (Nymphalidae) always lay their eggs on the stem of *Betula nana*, but their larva will feed subsequently on *Eriophorum* spp. leaves as the host plant. Several species of Nymphalidae in the subfamily of Satyrinae, such as *Lasiommata megera*, *Hipparchia semele*, *Coenonympha pamphilus*, *C. arcania*, and *Maniola jurtina* often lay their eggs on the grass blades that is also a suitable food of the larvae. However, some of these species also lay their eggs on the dead plant materials. In such oviposition mistake condition, the newly hatched larvae will move immediately to find the plants nearby as their food sources (Wiklund 1984) [48].

Our study observed that the preference of oviposition site of *H. bolina* was also associated with the host plant conditions. Accordingly, *H. bolina* prefererred to laying eggs on the young leaves (62.85%) (Figure 1C). Such preference could be due to the fact that the young leaves have a higher nutritional content then the other parts of the plant. Among the essential nutrients are nitrogen and water. An adequate nutrition content is essential for the larvae of *H. bolina*. Hence, the selection of the right ovipositional site by the female butterfly of *H. bolina* is vital for hereditary survival and contributes to maximize the reproduction of *H. bolina* (Nylin *et al*. 1996; Zalucki *et al*. 2002; Doak *et al*. 2006) [29, 50, 11]. Previous study also revealed that *Graphium Agamemnon* preferred the young leaves to lay the eggs (Fitriana *et al*. 2016) [14]. In addition to the common preference on the young leaves, our study also found that *H. bolina* deposited the eggs on the mature leaves (28.54%), petiole (2.09%), seedlings (2.04%), and stem (1.04%).

In depositing the eggs on the host plant, *H. bolina* preferred placing the eggs on a cluster type rather than a single type, with the percentage number of eggs 90.20% and 9.80%, respectively (Figure 1D, 3). The cluster type is the deposit of eggs in a group consisting of several eggs about 2 to 4 or more at once oviposition activity. Otherwise, the single type is consisted a single egg only. The majority of butterflies deposit their eggs in a single type, however some individuals also may lay their eggs on a cluster. The differences in the types of oviposition may occur between the sympatric species that use the same hostplant. However, the cluster type never be predominant among individuals within the same species at any geographical regions. This phenomenon is thought to be a part of the improvement of the reproductive tactics in several butterfly families (Stamp 1980) [41]. The butterflies in some specific groups such as Nymphalidae, Pieridae, and *Acraea* prefer laying eggs in the cluster type, but this type is rarely observed in Papilionidae, Satyrinae, Danainae, Riodinidae, and Hesperiidae (Stamp 1980) [41]. The butterfly *P. rapae* prefers putting eggs on a single type that could be due to the limited time available for the oviposition (Tabashnik 1983) [46]. Unlikely, *Diaphania indica* laid their egg in a cluster type with an average of 3 to 5 eggs per plant (Gharaei *et al.* 2019) [16]. Tabashnik (1987) [46] reported that the non-host plants are more preferable as the place of oviposition with a single type than cluster type. The various plant structures such as leaf hairs, trichomes, leaf waxes, and leaf toughness also affect the preference of butterfly in determining their host plant (Hagstrum and Subramanyam 2010) [18].

During the oviposition activity, the female butterflies have a strategy in depositing the eggs on the underside of the leaf. This strategy is also reported in *Byasa impediens* butterflies that spread their eggs on the bottom of the leaves with no more than seven eggs on each sheet (Xiushan *et al*. 2006) [49]. This oviposition strategy is deployed by the female butterfly in order to reduce the probability of being attacked by the natural predators of the eggs and larvae. This strategy is thought to spread the risk that could reduce the rate of premature death thereby increasing of adult survival ship (Den Boer 1968) [10].

Our investigation on the pupation site preference of *H. bolina* was started from the prepupa stage. The prepupa is a stage right before the formation of the pupa. In this stage, as the final instar of larva (fifth instar), the larvae will prepare themselves to become a pupa in which they will be immobile, stop eating, and start to secrete green vomit. Prior to this stage, the fifth instar larvae will seek a place that is a safe and protected site from the predators, parasitoids, and exposure of weather changes, to hang and pupate (Gueratto *et al*. 2019) [17]. The sites selected for pupation could be on the twig, host plant branches, in another plant, or other non-living objects. Figure 4A shows that the pupae of *H. bolina* were more frequently found on the non-host plant than on the host plant. The non-host plant substrates were substrates for the pupation of *H. bolina* consisting of other plants, polybags, cage, flower pots, and the dead materials of plants. Otherwise, the host plant substrates were the substrates consisting of the branch or the stem of the host plant. Another study described that the green pupae of species *Iphiclides podalirius* prefer the host plant as the pupation site including the branches and the underside of the leaves. In contrast, the brown pupae of this species are frequently found on the non-host plants, such as grass stems, weed stalks, woody stems, and on the underside of the living leaves (Stefanescu, 2004) [42].

The percentage of pupation sites of *H. Bolina*, based on substrate conditions, was higher on the non-living stem than living stem. The non-living stem included the branches of a dead plant, cage, polybags, and pots. Otherwise, the living-stem was a site for pupation consisted of living parts of plants such as host plant branches, other plant branches, and leaves of other plants. In *Iphiclides podalirius*, the brown pupae usually hang on the underside of dead leaves and underside of rock (Stefanescu 2004) [42]. *Papilio glaucus* L. pupates on the site near the natural ground and on the bottom of the blue surface, but *Battus philenor* (L.) and *Eurytides marcellus* (Cramer) prefer to pupate both on the yellow and blue surfaces (Marshall *et al*. 2005) [24].

The preference of pupa above the ground level is an evolutionary adaptation of the butterfly species against the predators like small mammals and avian (Stefanescu 2004) [42]. Another study reported that the pupae of *Iphiclides podalirius* also prefer the pupation sites on various height above the ground. In this case, the green pupae prefer the height ranged from 1-180 cm above the ground surface while the brown pupae prefer the lower position particularly at the height ranged from 1-35 cm above the ground (Stefanescu 2004) [42]. An observation on the species of *Phalanta phalantha aethiopica* revealed that most of the larvae climb vertically up the wall then pupate under the ledges and eaves. Most of them also crawl away from the sun and climb up the walls of a house to pupate. Moreover, the larvae do not ascend on the rough surfaces such as tree trunks (Phipps 1968) [32].

5. Conclusion

This present study revealed that *H. bolina* butterflies preferred to lay the eggs on the underside of the leaves particularly the young leaves of their host plant *L. interupta*. The female of *H. bolina* also preferred to lay the eggs in a cluster type instead of single type. In determining the pupation site, *H. bolina* tended to prefer a non-host plant with living stem substrate conditions. The height pupation site preferred by *H. Bolina* was in the range of 31-60 cm from the ground. The selection of the pupation site could be beneficial to protect the pupa against the predators and unfavorable environmental conditions. The strategies for a variety of oviposition and pupation sites preferences of *H. bolina* are essential for the survival rate of this species.

6. Acknowledgements

We thank the authority of IAIN Batusangkar, West Sumatra, for the supports. The Ministry of Religious Affairs financially supported this research (Grant No. 1966 Fiscal Year 2019; 8th April 2019).

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