SYNCHRONIZATION OF COINCIDENCES BETWEEN THE LIFE STAGES OF *Pachliopta aristolochiae* AND THE PHENOLOGICAL STAGES OF ITS HOST PLANT *Aristolochia indica*

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Abstract

Reproductive performance and trophic relation between any butterfly and its host plant is very selective. The life cycle of a butterfly entirely depend on its host plant. In the present investigation, the butterfly *Pachliopta aristolochiae* was found to be closely associated with its host plant *Aristolochia indica.* The plant was found to grow synchronously with emergence of the related butterfly's new generation-arrival in the experimental ecosystem. The butterfly was colonized in the laboratory as well as in the natural condition. The adopted colonizing technique has shown that some developmental stages in the host plant were effective in giving high rate of adult production. The feeding potential of the host plant's phonological stages was found to be significant for some of the developmental stages (particularly 3rd and 4th instars larval stages). Sixteen compounds were isolated from the leaves of the host plant and structures of five compounds were characterized which indicated the role of host plant and the feeding potential in the developmental process. Among those five compounds three were the derivatives of Aristolochic acids, namely Cepharanone-A-N-β-D-gluco-5,13"-O, 4"-icosyl-aristolenone (1), Cepharanone-A-N-β-D-gluco-5,13"-O,4"-ethyl-aristolenone (2), and Cepharanone-A-2-hydroxy-N-β-D-gluco-5,13"-O-4"-icosyl-aristolenone (3). This record suggests the presence of high amount of Aristolochic acids in the leaves of the host plant that made the butterfly toxic and unpalatable both in adult and immature stages to its predators. This toxicity may suggest that the presence of warning colouration results in the adults via the biochemical (metabolic) changes in the larval stages.

Key Words: Aristolochic acid, Butterfly, Predator, Rearing, Toxic, Unpalatable.

INTRODUCTION

The common rose butterfly *Pachliopta aristolochiae* (Papilionidae: Lepidoptera) is abundant across South and South East Asia (Wynter-Blyth 1957). According to Bashar (2015) the butterfly is available in Bangladesh. The IUCN status of this species is 'very common' and 'not threatened' in the Indo-Burma hotspot (Collins and Morris 1985).These butterflies can be identified in the field by the presence of sub-marginal row of red lunular markings and a discal row of white spots on upper side of hind wings. The female has a rounder wing contours than the male (Corbet and Pendlebury 2015). The hind wings also bear robust club shaped black tails (Alam *et al.* 2014). The body is black, but the opisthosoma is reddish (Haribal 1992). Like other Lepidopteran insects, *P. aristolochiae* has a life cycle of four different morphological stages. This includes, egg, larva, pupa and adult (Soderstrom *et al.* 1990). The orange-coloured egg is coated unevenly with yellowish material, measures 1-2 mm in diameter (Atluri *et al.* 2012). The egg hatches into an immature larval stage. There are four successive larval instars before the butterfly goes for pupation (Yang *et al.* 2006).The freshly emerged larvae are transparent pale red in colour with faint blackish markings on the body. Their bodies are covered with minute pale yellowish fleshy spines (Barua and Slowik 2007). In the 2nd instar, the body is brownish black bearing 12 pairs of fleshy spines on both dorsal and lateral sides. The larvae grow bigger and their fleshy spines become more conspicuous (Barua and Slowik 2007). The 3rd instar body is velvety black and covered with 12 pairs of fleshy spines. The dorsal spines are brick red while the lateral spines were black with red tips. The 4th instar larvae have a dark velvety black body and the fleshy spines were crimson red in colour at the upper portion and black at the basal half (Alam *et al.* 2014). The chrysalis is

light brown in colour with a mixture of white, orange and dark brown patterned markings on the dorsal side (Barua and Slowik 2007). After pupation, the butterfly emerges from its pupal casing as an adult. As the life cycle passes, all those above stages thoroughly depend on plants (Price *et al.* 1991). The butterflies need 3 categories of plants for their colonization and sustenance- the host plant, the nectar plant, and the shelter/ shade plant (Bashar 2014). The adult feed on different nectar plants and rest on various shelter plants. The other three life stages of butterflies completely depend on their host plants. For *P. aristolochiae*, the breeding females lay eggs singly on the upper and lower surface of leaves and petioles of its host plants (Atluri *et al.* 2012). The larval instars feed on the young leaves, shoots, flowers and fruits of the host plant (Nishida *et al.* 1993). They pupate on the under surface of a leaf or stem of the host plant (Wu *et al.* 2003). In addition to the nourishment and harbor of the immature stages, many host plants of butterfly contain toxic substances that are sequestered by the larvae retaining in the adult (Price *et al.* 1980). This gives them an advantage of protection from the predators as they become unpalatable. Such distastefulness is advertised by using some warning colours like bright red, orange, black or white etc. (Bashar 2014). The butterfly *P. aristolochiae* is a common example of poison-eater butterflies (Kitamura and Imafuku 2010). In Bangladesh, the butterfly has two host plants- *Aristolochia indica* and *Aristolochia tagala* which are closely associated with each other. Field observations revealed that the butterfly *P. aristolochiae* is favoured to the plant *A. indica* (Bashar 2014, 2015). It is known that secondary metabolites of plants can serve as toxicological and behavioural barriers to evolutionary changes in host plant used by phytophagous insects (Bernays and Chapman 2007).The host plant adaptation in a large number of swallowtail butterfly species (Papilionidae) is associated with the host-originated Aristolochic acid derivatives (AAs) (Ehrlich and Raven 1964). Nishida *et al.* (1993) reported that the toxic substances, analogues of Aristolochic acids, secondary metabolites isolated from the genus *Aristolochia*, are characterized as the larval feeding stimulants of the swallowtail butterfly species (Papilionidae). It was demonstrated that Aristolochic acids act as deterrent allomones against vertebrate predators (Nishida 2002). As the Aristolochic acids has been found to be present in all the life stages of the butterfly *P. aristolochiae* and also in the stem of *A. indica* (Che *et al.* 1984), it can be assumed that the Aristolochic acids has been sequestered from the leaves of the plant of *A. indica.*

The present study contemplated to carry out some experiments on the biological and morphological development of different stages of the common rose butterfly and its host plant. The study was also carried out to investigate the chemical found in the leaves of host plant to reveal any possible biochemical synchrony between the *P. aristolochiae* butterflies with the host plant *A. indica.*

MATERIAL AND METHODS

The life cycle of *P. aristolochiae* was observed in laboratory and in nature via colonization technique from January 01, 2015 to May 31, 2016. In the laboratory, the butterflies were kept in a rearing box, covered by a rearing cage measured 32×18×18 cm. The larvae were fed fresh leaves regularly. Water was sprayed over the leaves in a tiny amount so that the leaves do not get dry off due to lack of moisture. The fecal weight, their longevity and their successfulness in molting into the next stage of life was observed and data was recorded. In nature, five plants of *A. indica* were grown from May 2015- April 2016, for 12 months. The plants were fenced by bamboo and net so that no other animals can destroy them. When they reached an average height of ±50 cm, a butterfly was allowed to lay egg on one of them and they were netted again. In this way, in the nature the butterfly immature stage was grown without any interruption of other biotic activities. Data was recorded for the hatching from egg, larval stages molting and development, pupation period etc. In the Zoological Garden, University of Dhaka, 3 experimental rearing cages were made. They were labeled as Cage 1, Cage 2 and Cage 3. The Cage 1 and 3 measured 24×24×24 cm. and the cage 2 measured as 30×18×12 cm. The cages were

covered by net in 3 sides and they were placed upon a sheet so that larvae were safe from the attacks of ant. The cages were shaded by plastic coverings to protect the larvae from rain. Each cage contained a rearing box where the larvae were kept, a water bottle and water soaked cotton to provide the environment proper moisture and sufficient leaves of *A. indica* for their survival. The 1st and 2nd instar larva of *P. aristolochiae* were kept in cage 1, the 3rd and 4th instar larva were kept in cage 2, and the post 4th instar larvae were kept in cage 3 to pupate. In total, around 30 different instars were kept in different cages and they were transferred as they moult into different instars. The larval length, their fecal weight, leaves given per day, their longevity etc. were recorded in a daily basis.

For the metabolic investigation, leaves were collected from the Zoological Garden. They were extracted, portioned, and then TLC and HPLC were applied to get pure compounds in Centre for Advanced Research in Sciences. NMR of these compounds was taken in BCSIR. All the photos were taken by Sony Cyber shot during the investigation period.

RESULTS AND DISCUSSION

In the Zoological Garden, University of Dhaka, five seeds of *Aristolochia indica* were planted at the beginning of May 2015 (RH: 65). After almost a month those seeds started sprouting and thus the germination of those five plants started. Plant data was recorded twice in a month and their growth rate were also observed.

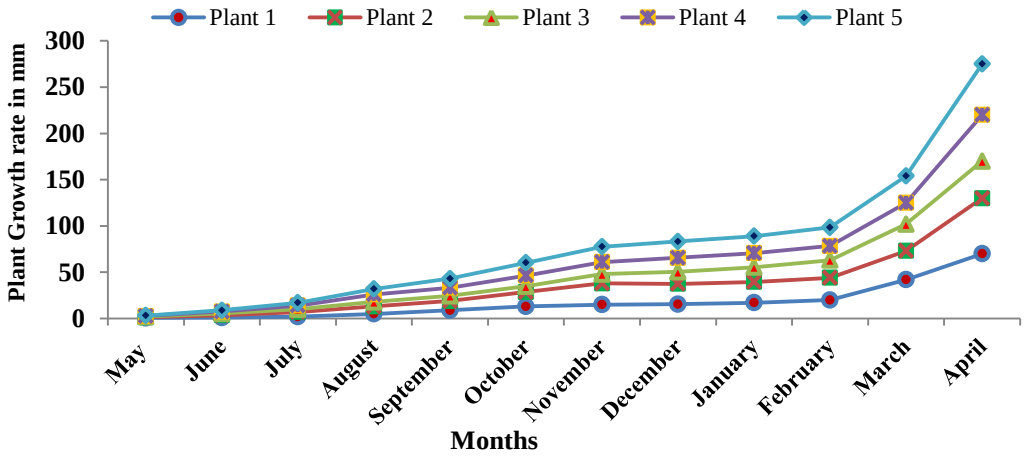

Fig. 1. Growth rate of *Aristolochia indica* in 12 months (both vegetative and pre-reproductive).

During the study period of 12 months it was observed that the plant growth rate was moderate from August to November, the rate became dormant from November to February and rapidly increased in the months March and April for reproduction and fructification. The plant 2 almost increased twice in the March month, other plants' growth rate were also positive. All those 5 plants also grew well in the month April which was the month of new generation of Common Rose butterfly arrival. From the egg laying to the adult emergence, the life stages of the butterfly *P. aristolochiae* were observed completely in natural condition. The host plants that grow in the Zoological Garden were targeted specifically to observe the life cycle of butterfly on it. While egg was laid on a growing plant, the plant area was covered by a net thus no other butterfly was able to lay eggs on it and no caterpillar can infest the plant during data record.

The Fig. 2 shows that, the rate of body length increasing as the larva develops to pupa and then adult. The body length of the butterfly in different stages of life cycle is positively correlated (r= 0.82) with different days and the degree of association between them is very high and low p value indicates

that the relation is very significant and regression value indicates that almost 67% data fits the model. From the graph, the increasing rate of body length increases very rapidly from 2nd to 4th instar and it decreases in the pre-pupal condition. Body length increased almost 13 times.

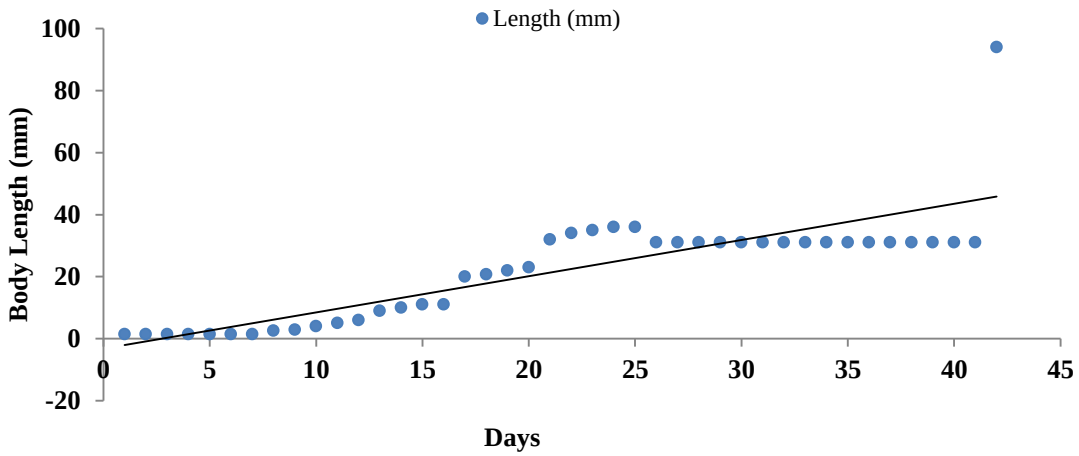

Fig. 2. Day-wise body length of different stages of the butterfly's life cycle in natural condition.

In the laboratory experiment, four 1st instar, six 2nd instar and four 3rd instar, that is, in total 14 larval instars were kept in an experimental cage for observation. During the observation the larval instars moulted to the next instars and in this way the life cycles were observed.

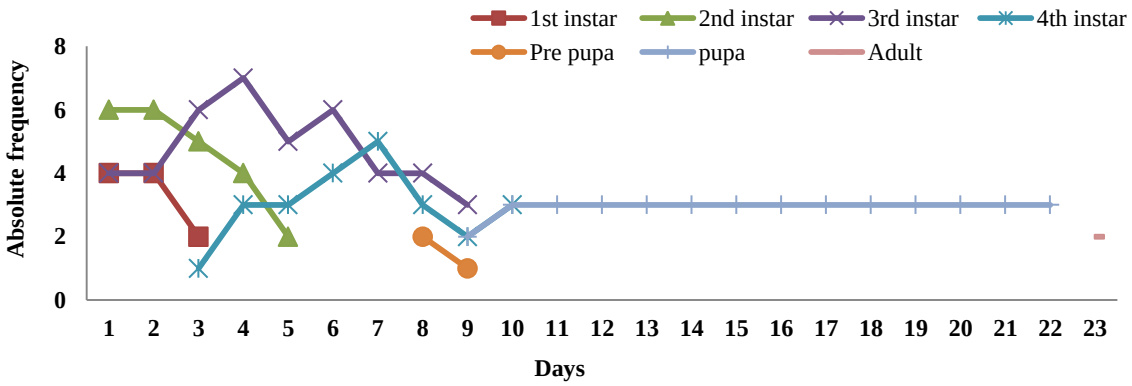

Fig. 3. Daily changes in the frequency of larval instars while reared in the laboratory condition.

In the Fig. 3, decline of a larval instar slope refers that it is transferring itself to the next stage. For example declination in the 1st instar frequency indicates the transformation of the larva to second instar and so on. On the other hand, acceleration of a slope simply refers to the fact that the present stage is getting more instars from the previous one. For example when the 2nd instar slope declines, the 3rd instar slope inclines as more larvae are becoming 3rd instar from 2nd instar. The graph also indicates less survivability from the pre-pupal phase. This may refer to the fact that the laboratory rearing wasn't completely successful. The work needs more sophistication and more experience on the control of abiotic factors.

During larval rearing in the laboratory, the first five days show high rate of leaf consumption in relation to given foods. This was due to the presence of highest number of larvae in experiment. Also molting of different caterpillars from one instar to another was rapid in those days which need more feeding. After five days, it slowed down as the number of larvae decreased gradually and prior to

pupation, the feeding potential almost ceased. In the day 4, 14 larvae consumed five leaves giving fecal removal of 0.8 g and in the day 5, 10 larvae had the highest consumption of six leaves giving fecal disposal of 0.7 g (Fig. 4).

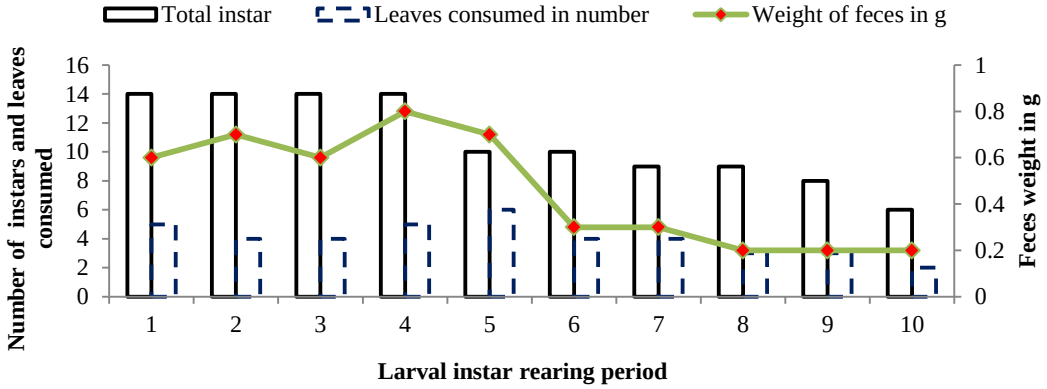

Fig. 4. Daily rate of consumption and fecal excretion of leaves by caterpillars of *Pachliopta aristolochiae* in the laboratory.

In Culture process of natural colonization in the Zoological Garden, the first and second instars were kept in experimental cage 1, the third and fourth instars were kept in experimental cage 2 and the post fourth instars, prior to pupation, pre-pupae and pupae were kept in experimental cage 3. Through the process, a synchrony was observed while collecting the data. The larvae preferring the same type of food were kept in the same cage. For example 1st and 2nd instar larvae preferred the tender leaves thus they were kept together and the 3rd and 4th instars preferred almost every kind of leaves and twigs. In this way a true colonization was established between the same types of larvae.

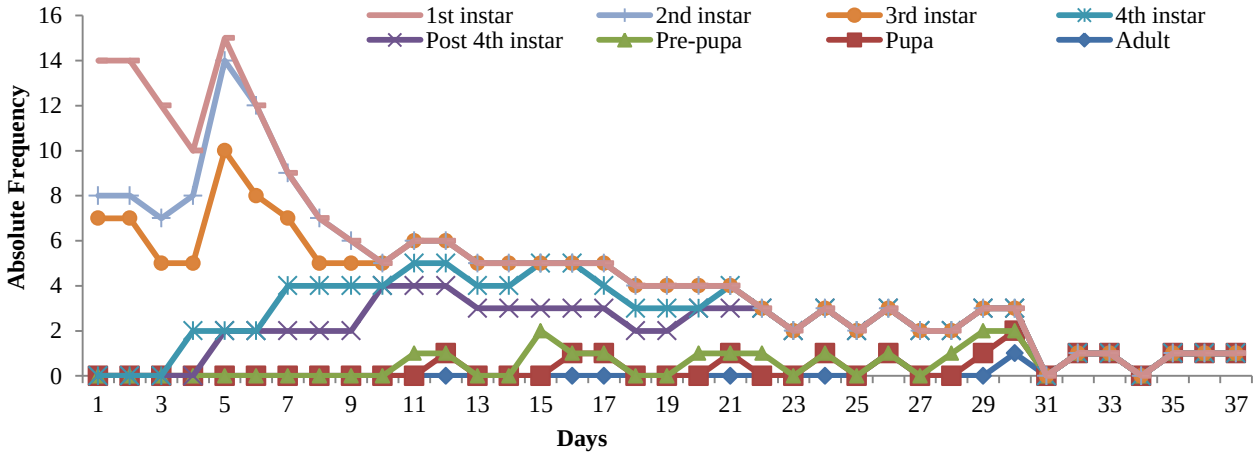

Fig. 5. Day-wise frequency of occurrence of the different stages of butterfly observed during the process of natural colonization in the Zoological Garden, Curzon Hall campus, University of Dhaka.

From the Fig. 5, it can be seen that the frequency of 1st instar was high at the beginning of colonization, through the time the frequency of first instar larvae declined and the second instar larvae rises. Whereas, in a different experimental cage 2, during the same period, the frequency of 3rd instar larvae was accelerating, as the time passes the fourth instar larvae and 2nd instar larvae took its place and 3rd instar larvae frequency declined. In the same way, post 4th instar larvae frequency slowly declined as the pre-pupal and pupal frequency arose. Finally, with the time, adult butterflies emerged from pupal casing and this indicates that the natural colonization process was successfully cultured.

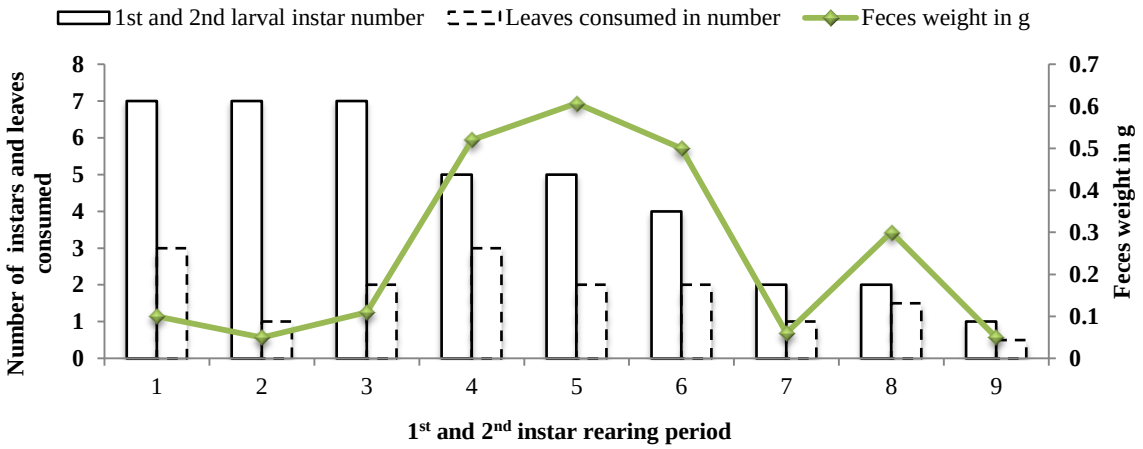

Fig. 6. Daily rate of consumption and fecal excretion of leaves by 1st and 2nd, and 3rd and 4th instar larvae of *Pachliopta aristolochiae* in the process of natural colonization.

The 1st and 2nd instar larvae in the process of natural colonization held in experimental cage 1 for nine days. In the first 3 days of culture, the number of leaves consumption and the fecal weight seems low as the 1st instar larvae fed less amount of food compared to other instars. After molting to 2nd instar, their feeding rate increased and the rate decreased again during molting. The lowest amount of fecal disposal was observed to be 0.05g in the day 2 and day 9 by seven and one instar, respectively (Fig 6). The highest amount of consumption was by 7 instars in day 1 and by 5 instars on day 4 (Fig 6). On day 5 highest amount of fecal secretion was recorded to be 0.607g (Fig 6). The chart shows the relation between the larval number and their feeding potential.

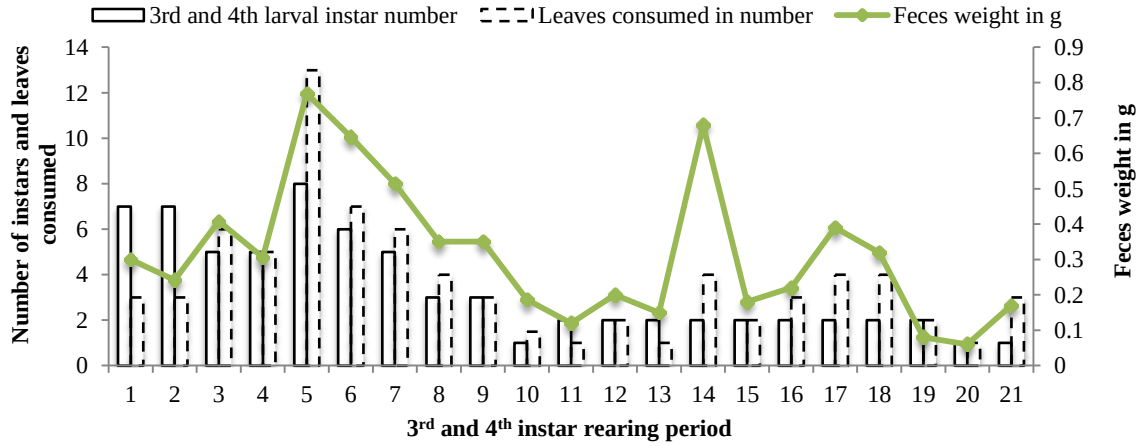

Fig. 7. Daily rate of consumption and fecal excretion of leaves by 3rd and 4th instar larvae of *Pachliopta aristolochiae* in the process of natural colonization.

Larvae in the 3rd and 4th instars duration are rapid and voracious feeders. They were found to consume sometimes even more number of leaves than the totality of the number of larvae in the experimental cage 2. The total process of natural colonization of different 3rd and 4th instar experimental larvae took 21 days in total. The experimental 8 larvae ensemble (during the stage of 3rd-4th instar-stage) devoured 13 leaves only on the 5th day of culture. After that, their fecal record was measured 0.769g (Fig. 7). The lowest number of leaves consumed was one by a single larva on the 20th day, showing the fecal record of 0.05g (Fig. 7). This chart shows relation to the number of leaf consumption and fecal disposition by the 3rd and 4th instar larvae, respectively.

Generally butterfly-larvae feed on the host leaves scattered but in the case of *P. aristolochiae*, they were found to feed on gregarious habit like the moth larvae of the family Noctuidae (Fig. 8A). This occurs in the earlier instar stages (1^{st}-3^{rd} instar), but in the ultimate and penultimate larval instar(s) towards the pupation while feeding voraciously, they are found on scattered feeding habit (Fig. 8B). The voracious food habit of the ultimate and penultimate stages of the insects attain pest status in the forest areas where their host plant population is scarce. This happens particularly in the sub-tropical forest ecosystem in the country like Bangladesh (Alam *et al.* 2014).

A B C

Fig. 8. Different feeding stages of larvae. A. Gregarious feeding of 2^{nd} and 3^{rd} instar-stage larvae; B. and C. Voracious feeding of larvae on plant leaves and twigs in the penultimate and ultimate stage, respectively .

Sixteen compounds were isolated and five compounds were elucidated among them to be asseverating work. They are namely E1b:Cepharanone-A-N-β-D-gluco-5, 13"-O, 4"-icosyl-aristolenone, E1d: Cepharanone-A-2-hydroxy-N-β-D-gluco-5,13"-O-4"-icosyl-aristolenone, B6: Cepharanone-A-N-β-D-gluco-5, 13"-O, 4"-ethyl-aristolenone, D(3-6) d: 5-butyl-4-methoxy-1,2,3-trimethyl-dibenzo[de,g] quinolone-7-one and D(3-6) j: 1-Ethyl-2, 3, 4, 5, 6-pentamethyl-cyclohexane. Compound E1b, E1d and B6 are the derivatives of aristolochic acids which imply the fact that the plant leaves of *A. indica* possesses aristolochic acids that can be metabolized by the plant eater larval stages of *P. aristolochiae*. Primary investigations have suggested that the larval and adult warning colouration is due to the chemical metabolites which are sequestered from edible parts of the host plant.

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