World Academy of Science, Engineering and Technology
International Journal of Agricultural and Biosystems Engineering
Vol:9, No:8, 2015

Sex Differentiation of Elm Nymphalid (*Nymphalis polychloros* Linnaeus, 1758) on Pupal Stage

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***Abstract*—**This study was conducted to determine sex differentiation of laboratory reared Elm nymphalid (*Nymphalis polychloros* Linnaeus, 1758) by examining the morphological structure of pupal stage. Laboratory colony of elm nymphalid, reared on pear leaves, was used to set up experiments. It was performed with 5 replications having 8 pupae for each replication. Dorsal, ventral and lateral parts of external morphological structures of pupae were examined by Olympus SZX9 stereozoom microscope and photographed. When fully grown, mature larvae wander the highest part of the rearing cage and pupae were formed hanging by cremaster. After completing prepupa stage about 1.5±0.3 days, they all pupated. Pupal stage was completed at 24±1°C about 4.38±1.20 days. Pupal weights were 0.483±0.05 g in females and 0.392±0.08 g (n=40) in males respectively. Pupal emergence rate was 95%, with 22 females and 16 males. Examinations of ventral parts of 8th, 9th, and 10th abdominal segments revealed that anal opening were found at 10th abdominal segment in both sexes, 3 lumps were determined at 9th abdominal segments then the specific opening structure at 8th segment was only found on female pupae.

***Keywords*—**Butterfly, *Nymphalis polychloros*, pupae, sex differentiation.

I. Introduction

THE elm nymphalid, blackleg tortoiseshell or large tortoiseshell, *Nymphalis polychloros* Linnaeus, 1758 (Lepidoptera: *Nymphalidae*) is a butterfly belonging to large family of *Nymphalidae* also commonly known as brush-footed butterflies [1], [2]. According to Harvey, there are 13 subfamilies of the *Nymphalidae* and they are divided into the three tribes Nymphalini, Melitaeini, and Kallimini [1]. *Nymphalis polychloros* (Linnaeus, 1758) is morphologically very close to small tortoiseshell *Nymphalis urticae* and *Nymphalis xanthomelas* in adult appearance [2], [12]. It is known to be native from North Africa and distributed to central and southern Europe, central, western Asia, and North America [3]. It is migrated to Denmark and Southern Sweden, Norway, and Finland. It is now known to be extinct in Britain, even though it is used to be widely distributed throughout England and Wales previously [2], [3]. The reason for its extinction is unknown. *Nymphalis polychloros* is widely distributed in Turkey [4], [5]. The wingspan of the butterfly is 68-72 mm in males and 72-75 mm in females respectively. The forewings are brown, heavily spotted with black and yellowish orange spots on the leading edge of the wing. The edge of the hind wings is black with blue spots [3]. The underside of the wings is dark brown in color. The flight period of *Nymphalis polychloros* is from March to October. Adults emerge in July and/or August and overwinter as adult stage then re-emerge in the early spring. It has one generation a year [3], [4]. The adult butterfly is usually found in woodland and orchards. The butterfly feed on honeydew, nectar sources and damaged fruit sources in orchards. Mated females lay their eggs in a cluster around the terminal twig of the host plant. The eggs are yellow when firstly laid then turn to brown before hatching. The embryonic development takes about 3 weeks. The larva is black with lighter colored strips and it is very spiky. The larvae are gregarious especially in early instars. The larval host plants are Elms (*Ulmus* spp.), Aspen (*Populus tremula*), Birches (*Betula* spp.), Poplars (*Populus* spp.) and Willows (*Salix* spp.) and some orchards trees. The larval development takes about a month [2]-[4].

There are many studies on lepidopteran biology, damage, biological control agents, behavior, artificial rearing, systematics, molecular systematics and managements but not much work found on morphological characters of lepidopteran pupae [5], [6]. It is known to be important to identify males from females for entomological studies especially on artificial rearing; developing sterile insects and many other basic and applied researches such as pesticide screening, determining female fecundity, male and female longevity and biological control [5], [10], [12]-[14].

The aim of the study is to identify external morphological characteristic parts of male and female pupae of *Nymphalis polychloros* to easily distinguish them for further studies.

II. Materials And Methods

The first instars of *Nymphalis polychloros* Linnaeus, 1758, were collected from pear (*Pyrus* sp.) orchards in Bayramiç, Çanakkale province, in early May 2013. The larvae were placed in a plastic container having chiffon lid on and brought to the laboratory. The laboratory colony was established on the natural host plant, pear leaves, daily collected from untreated pear orchards (Figs. 1 (a) and (b)). The larvae were fed freshly cut pear leaves in screen cage. The leaves were replaced daily by transferring all larvae to new leaves until the larvae complete their developments. The colony of *Nymphalis polychloros* was maintained under controlled laboratory conditions at 24±1°C 65% relative humidity and 16:8 h (light: dark) photoperiod.

Nymphalis polychloros larvae developed through five instars in the controlled laboratory conditions. The general appearance of larvae was black, covered with sharp orange

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spikes with white dots over the body (Fig. 2 (a)). The legs and prolegs were brownish to black. The anal prolegs were brown, dark brown to black. When the larva molted, characteristic shape of shedded epidermis (skin) or exuviae was left at the tip of the chrysalis or pupae (Fig. 2 (b)). So, it was still possible to see the visible spikes and all body appearance of larvae after molted (Fig. 2 (b)). In general, the exivua is usually consumed by newly molted butterfly larvae as the first food except for the head capsule but in the case of *Nymphalis polycholos*, this was not possible because of its spiky exivua (Fig 2 (b)).

Fig. 1 Laboratory colony of *Nymphalis polychloros* maintained on pear leaves in the laboratory

Fig. 2 (a) View of young larvae (b) shredded skin or exuviae

When the larvae complete their development and fully grown, they start wandering a place such as to the highest part of the rearing screen cage and first prepupae then pupae were formed. Mature larvae first attached with the cremaster to a stem, a leaf or any other support materials in the plastic screen cage then remained in a crescent shape at about 5-8 hours (Fig. 3 (a)). After that, hanging straight down, they changed into the characteristic chrysalis or pupal shape (Figs. 3 (a), (b)) in a few hours. Pupae were harvested daily in the laboratory, and placed in a Petri dish to set up the experiments. Before setting up the experiments, their length, width measurements and weights were taken.

Fig. 3 (a) View of mature larvae attached with cremaster (b) prepupae and pupation

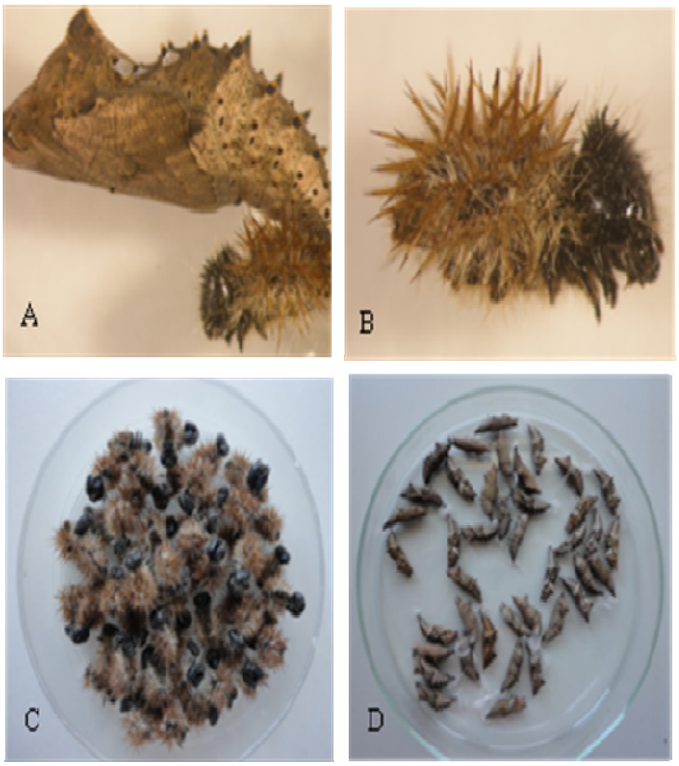

Fig. 4 Pupae with shredded skin (a) close up of shredded skin or exuviae (b) transferred them in a Petri dish separately, (c) shredded shin of mature larvae and (d) a view of *Nymphalis polychloros*

Laboratory colony of *Nymphalis polychloros* was used to set up the experiments. It was performed with 5 replications having 8 pupae for each replication. Dorsal, ventral and lateral parts of external morphological structures of male and female chrysalis or pupae were examined by Olympus SZX9 stereozoom microscope and photographed. Collected pupae and shredded skin (exuviae) were shown in Fig. 4. Pupae were gently separated from shredded skin by hand and placed them

in different Petri dishes for further morphological analyses (Fig. 4).

III. Results and Discussion

Field collected and laboratory reared *Nymphalis polycholos* were examined for their external pupal characters under stereo zoom microscope. The mature larvae of *Nymphalis polychloros* were completed their development and pupated in the laboratory after completing prepupal stage about 1.5±0.3 days. Pupae or chrysalis were initially soft and grayish to light brown then became brown in color and marked with golden visible spots, having spiky unique appearance (Fig. 5). The grayish patches with black dots on the dorsal side of pupae were distinguished characters (Fig. 5 (a)). Pupae were approximately 4.3±0.2 cm in length and 1.2±0.1 cm in width (Fig. 5).

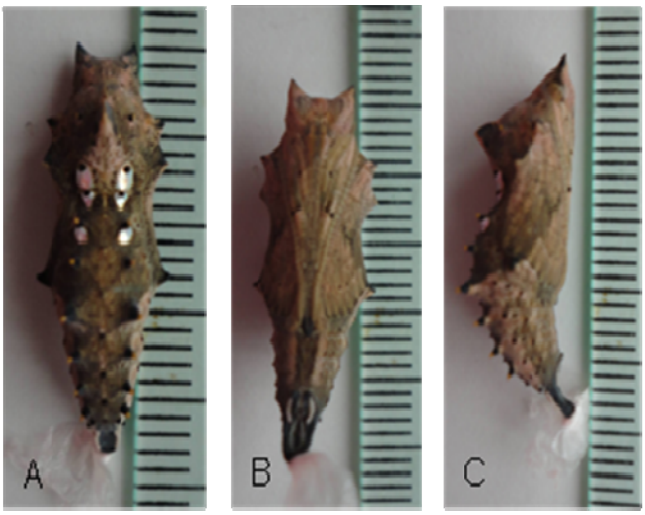

Fig. 5 Pupal stages of *Nymphalis polychloros* (a) dorsal, (b) ventral (c) lateral view

Pupal stage was completed at 24±1°C about 4.38±1.20 days in the laboratory. Pupal weights were 0.483±0.05 g in females and 0.392±0.08 g (n=40) in males respectively (Fig. 5).

In order to distinguish specific male and/or female characters of *Nymphalis polychloros*, ventral parts of the eighth, ninth, tenth abdominal segments of pupae were examined under the stereozoom microscope and photographed.

In total, 40 pupae were examined in this study. The pupal abdomen of *Nymphalis polychloros* was consisted of 10 segments, with the 10th segment bearing the cremaster by which pupae attached to a support material. Examining of the ventral parts of pupae showed that the male genital opening was located in the middle of 9th abdominal segment as 3 visible lumps and indicated as the first red arrow (Figs. 6 (a) and (b)). There was no sign and/or any specific structure in the 8th abdominal segment of male as shown in Fig. 6. The anal opening or slit was located on 10th abdominal segment in males shown as second red arrow (Fig. 6 (b)).

The female genital opening or slit was located between the 8th and 9th abdominal segment (Figs. 7 (a) and b). In the eighth segment, a longitudinal ridge or suture was present and indicated as the first red arrow. This structure was only found on female pupae (Fig. 7 (b)). It was also hard to see because of the dark brown color of pupae. The anal opening or slit was located on 10th abdominal segment, as was the cremaster in females.

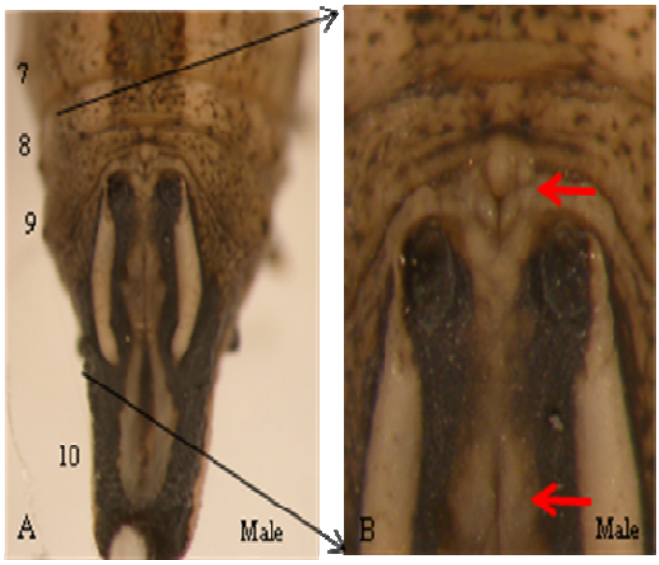

Fig. 6 A ventral view of 8th, 9th, and 10th abdominal segments of *Nymphalis polychloros* male pupa (♂) (a) and close up (b)

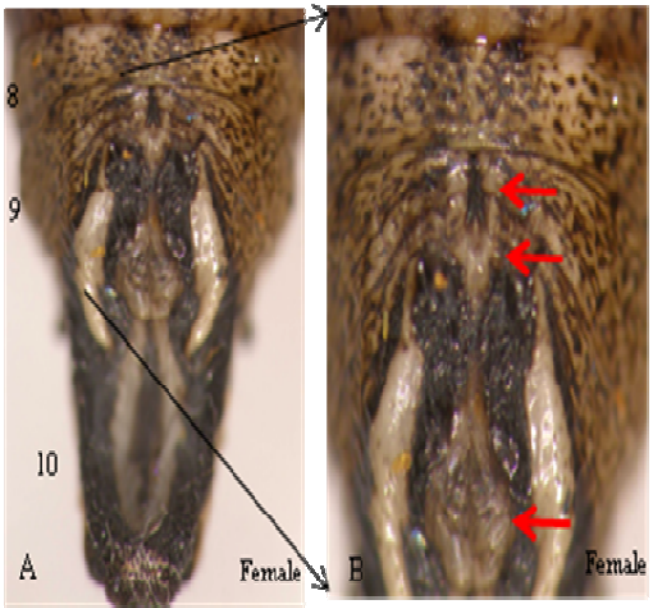

Fig. 7 A ventral view of 8th, 9th, and 10th abdominal segments of *Nymphalis polychloros* female pupa (♀) (a) and close up (b)

Pupal developments were followed up by adulthood (Fig. 8). Fig. 8 showed the newly emerged adult of *Nymphalis polychloros*. Emergence rate was 95%, with 22 females and 16 males. The presence of this suture provided easy and

accurate distinguished character for the sex differentiation of *Nymphalis polychloros* pupae (Figs. 6 and 7).

In this study, pupal sexual characters were determined to separate female and male pupae of *Nymphalis polychloros*. In total, 40 lived pupae were examined. Sex determination from pupal stage using external abdominal structures was 100% consistent in separating pupae. So, now it will be possible to identify male and/or female pupae of *Nymphalis polychloros* by using visual and external characters described in this study.

Fig. 8 The adult stage of *Nymphalis polychloros* (a) underside and (b) upperside of the wings

Some morphological structures have already been reported to determine the sex of the lepidopteran pupae [7]-[12]. Even though, the reported characters are similar but they are not exactly the same in all lepidopteran species. Besides, identification of adult male and female lepidopterans are easier and possible by using the catalogs based on wing patterns, determination of lepidopteran pupal sex requires special training, experience and visual information. Presented study indicates that by using detailed information given here, it is now possible to determine male and female pupal characters of *Nymphalis polychloros*.

IV. Conclusions

Morphological details in the external structures of *Nymphalis polychloros* Linnaeus, 1758 pupae were determined in the presented study. By using absence and presence of suture presented on the eighth abdominal segment, male and/or female pupae can be identified easily and accurately. The photographs were clearly indicated the specific parts on the pupae of *Nymphalis polychloros*.

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