

Research Article

Comparative life stages and behavioral characteristics of two Spilomelinae moths with overlapping feeding periods on *Azolla* spp., in the northern Iran

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Abstract: *Diasemiopsis ramburialis* and *Nomophila noctuella* are moths from Spilomelinae (Lepidoptera: Crambidae) with an overlapping feeding period on *Azolla* spp. in northern Iran. Due to some morphological similarities, immature stages often lead to confusion. A five-year study conducted in laboratory and natural environments revealed that, despite these morphological similarities, distinct characteristics in both morphology and behavior can facilitate their primary identification in the field. *Diasemiopsis ramburialis* eggs, and first instar larvae are opaque to pale orange, while those of *N. noctuella* are pale to creamy. Additionally, the last instar larvae of *D. ramburialis* feature orange head capsules with smaller body sizes and tend to pupate near the feeding site. In contrast, *Nomophila noctuella*'s last instar larvae have black head capsules, larger body sizes, and pupate at a distance from the feeding site. Both species exhibit shelter-building behavior, binding *Azolla* spp. leaves together using silken webs to create protective enclosures. However, *D. ramburialis* larvae construct more robust shelters and produce more webs. They primarily remain within these shelters and navigate through web-formed corridors. On the other hand, *N. noctuella* larvae prefer to move along the feeding site margins within corridors formed by bound-up leaves and display a more wandering behavior. The study's findings indicate that *Azolla* spp. serves as the primary host plant for *D. ramburialis*, while *N. noctuella* appears to choose its host plants randomly.

Keywords: Spilomelinae, *Azolla* spp., Behavioral characteristic, Morphological characteristic, larvae

Introduction

Spilomelinae is the largest subfamily of Pyraloidea; it consists of 4026 recorded species within 327 genera and is considered the most diverse subfamily of Crambidae (Landry 2020). The diagnostic characters of Spilomelinae are as follows: head without chaetosemata, male hind wing without

subcostal retinaculum, tympanal organs with a bilobed praecinctorium, projecting fornix tympani, and pointed spinula, male genitalia without gnathos, and corpus bursae of female genitalia usually without rhomboid signum (Minet 1982; Solis and Maes 2003; Regier *et al.*, 2012). This subfamily has numerous cosmopolitan and polyphagous species (Regier *et al.*, 2012; De Prins and De Prins, 2020).

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Considering the wide dispersal and high number of species, members of this subfamily have a wide range of host plants from different families (i.e., Apocynaceae, Araceae, Convolvulaceae, Cucurbitaceae, Euphorbiaceae, Fabaceae, Nyctaginaceae, Oleaceae, Poaceae, Rosaceae, Verbenaceae etc.) (Landry, 2020). In addition, some species such as *Herpetogramma sphingialis* Handfield and Handfield, *Herpetogramma aeglealis* (Walker), *Herpetogramma platycapna* (Meyrick), and *Udea decrepitalis* (Herrich-Schäffer) have been recorded as a fern (*Azolla* spp.) feeders (Lhomme 1935; Kirk 1978; Solis, 2008; Handfield and Handfield, 2011).

Long-term field studies indicated that *Diasemiopsis ramburialis* (Duponchel) and *Nomophila noctuella* (Denis and Schifferrmüller) form Nomophilini tribe of Spilomelinae, feed on *Azolla* spp. in the northern region of Iran with

overlapping feeding periods (Table 1) (Farahpour-Haghani *et al.*, 2016 a, b). Nomophilini are small to medium-sized moths with reticulated wing patterns in many neotropical genera and described by the following genitalia characteristics: The male genitalia have a conical to capitate (uni- to bicapitate) uncus, uncus head naked or with simple and bifurcate chaetae; the valvae are ovate, mainly with a convex costa; the fibula is well developed, straight to arched and emerging from near the costa base (absent in *Diasemiopsis*). In the female genitalia, the corpus bursae have a granulose central area or an elongate signum (longitudinal or transverse in orientation); the colliculum is sclerotized, in *Nomophila* with an apomorphic blind anterolateral evagination (diverticulum sensu Munroe, 1973); the antrum is strongly sclerotized, broad tubular or barrel-shaped (Mally *et al.*, 2019).

Table 1 Fluctuation of *Diasemiopsis ramburialis* and *Nomophila noctuella* on *Azolla* spp. in northern region of Iran.

	January	February	March	April	May	June	July	August	September	October	November	December
<i>D. ramburialis</i>							•	•	•	•	*	
<i>N. noctuella</i>					•	•	•	•	•	*		

•: presence of active larvae and adults on *Azolla* spp.
*: present just in years with mild weather in autumn.

Diasemiopsis was described by Munroe (1957) with *Hydrocampa ramburialis* (Duponchel) as a type species. Only one other species, *Diasemiopsis leodocusalis* (Walker), is assigned to this genus (Nuss *et al.*, 2015). *Diasemiopsis ramburialis* is a cosmopolitan species (De Prins and De Prins, 2020). *Azolla* spp. are the first recorded host plants of this species. *Diasemiopsis leodocusalis* host plant is still unknown (Farahpour-Haghani *et al.*, 2016 a). *Nomophila* was described by Hübner (1825) with *Pyralis hybridalis* (Hübner) as type species. Seventeen identified species were assigned to this genus (Nuss *et al.*, 2015; Gözüaık and Atay, 2016), and *N. noctuella* is mainly spread in the Old World (Martiré and Rochat, 2008). This moth is a polyphagous species associated with numerous host plants such as *Polygonum* spp. (Polygonaceae), *Trifolium* sp. and *Medicago* sp. (Fabaceae), *Zea mays* (Linnaeus), *Triticum* sp., *Stenotaphrum dimidiatum* (L.) (Brongniart) (Poaceae) and

Myriophyllum spp. (Haloragaceae). (Flint, 1922; Smith, 1942).

Behavioral assays are an excellent method for studies of host plant choice and adaptation in herbivorous insects (Knolhoffl and Heckel, 2014). Feeding behavior evaluation influences insect’s fitness (Bernays, 1998), and shelter-making is expected to increase larval survivorship (Cornelissen *et al.*, 2016). Therefore, they are important behavioral processes for insects. Many cases of behavioral assays mainly focused on oviposition and feeding behavior. For example, studies on the timing of oviposition and larval growth by two tephritid fly species concerning host-plant development indicated that they chose different flowering stages of host plant for oviposition (Straw, 1989) or interspecific competition between root-feeding and leaf-galling aphides mediated by host-plant resistance (Moran and Whitham, 1990) and root herbivory affects oviposition and feeding behavior of a foliar herbivore (Anderson *et al.*, 2011).

Azolla spp. are invasive exotic species in Iran. Over the last few years, this obnoxious weed has invaded many wetlands in the northern regions of Iran (JICA, 2005; Delnavaz and Azimi, 2009; Sadegi *et al.*, 2013; Farahpour-Haghani *et al.*, 2016a). In these ecosystems, *Azolla* spp. were in association with other floating aquatic plants (such as *Lemna* spp., *Spirodela polyrhiza*, *Salvinia natans*, etc.) (Mozafarian, 2007; Delnavaz and Azimi, 2009; Golmohammadi *et al.*, 2018; Bidarlord *et al.*, 2019). The *Azolla* invasion puts native species (plants, invertebrates, and even some vertebrate species such as fishes) under pressure. As a result, many native plants were eradicated from the aquatic ecosystems, and insects that feed on these plants faced hunger stress. Under these conditions, they had no choice but to use new plants as a food resource or die (JICA, 2005).

We surveyed rice fields and other aquatic ecosystems on local herbivores attacking *Azolla* spp. in Iran. The results indicated that *D. ramburialis* and *N. noctuella* feed on *Azolla* spp. in the northern region of Iran and have overlapping feeding periods (Farahpour-Haghani *et al.*, 2016 a, b). A review of apparent competition, quantitative food webs, and the structure of phytophagous insect communities has indicated that competition's negative effects can influence species' survival (Frank van Veen *et al.*, 2006). Lawton and Strong (1981) reviewed community patterns and competition in folivorous insects and demonstrated that interspecific competition is too rare or impotent to structure communities of insects on plants; however, regularly, species tend to compete most strongly with close relatives. Another review study on interspecific interactions in phytophagous insects indicated that interspecific competition occurred in 76% of interactions (Denno *et al.*, 1995). Therefore, despite the competition, a successful overlapping feeding period of two relative species on the same host plant was a case of adaptation. Evaluating this case would lead to understating more about both species' feeding behavior and changes that would be induced by *Azolla* spp. invasion in the studied areas.

On the other hand, the larvae of both species seemed to be similar and easily confused with each other. In addition, both species have non-aquatic larvae and shelter-making behavior with almost the same patterns, which let them survive on the aquatic host plant and make them more confusing for farmers or anyone unfamiliar with these species. As mentioned, *N. noctuella* is a polyphagous species and a pest for some crops, such as alfalfa, corn, and wheat. All of these species previously recorded as host plants are not aquatic, and *Azolla* spp. are the first recorded aquatic host plants for *N. noctuella*. However, for *D. ramburialis*, *Azolla* spp. are the only recorded host plants worldwide. Therefore, it would be important to recognize both species' different life stages in the field to properly manage *Azolla* spp. in areas where other recorded host plants of *N. noctuella* exist. A brief report on the behavioral characteristics of both species has previously been published (Farahpour-Haghani and Jalaeian, 2021). The specific objectives of this paper are: to compare the morphological characteristics of *D. ramburialis* and *N. noctuella* life stages, to discuss their behavioral characteristics on *Azolla* spp. and to recognize each species during their overlapping feeding period. This study would be a simple and valuable illustrated manual for anyone needing to identify both species in the field.

Materials and Methods

Rearing

To establish laboratory colonies, larvae were collected from *Azolla* species located on waterways and experimental rice fields at the Rice Research Institute of Iran (RRII) (N37° 12' 22", E49° 38' 40", 17 alt.) from September to November 2013 to 2018. In 2013 and 2014, we used 14 cm diameter Petri dishes as rearing and mating chambers. In group-rearing chambers, each petri dish was filled with 40 g *Azolla* spp. and 100 ml of distilled water and covered with same-size Petri dishes. However, for individual rearing and mating chambers, we set up two 6 cm diameter Petri dishes, each filled with 10 ml of distilled water and 5 g of *Azolla* spp., in 14cm diameter Petri dishes to provide more space.

Since 2015, to improve rearing quality, we have used disposable plastic containers (18 × 13 cm, diameter by height) as mating chambers and plastic boxes (24 × 17 × 10 cm, length by width by height) as rearing chambers, each covered with transparent cellophane with small holes in the cellophane for ventilation.

For the mating chamber, we set up three small containers (6 × 5 cm diameter by height), each filled with 50 ml of distilled water in 18 cm diameter chambers. Two small containers were filled with 10 g *Azolla* spp., and in the third container, a sugar cube dissolved. We filled this container with tissue papers and placed it in the mating chamber to provide adults with food supplements. For rearing chambers, boxes were filled with 500ml distilled water and 200 g *Azolla* spp. All chambers were kept at 25–27 °C and 16: 8h (L: D) photoperiod. In mating chambers, a male and female pair were released in each chamber upon emergence. For rearing experiments, first or second-instar larvae were released in rearing chambers (3-6 individuals in each petri dish and 20 individuals in each box). Water and food materials were renewed once every three days.

Morphological and behavioral characteristics studies

Behavioral characteristics of each species were recorded daily during the rearing period, and ten individuals of each life stage from each moth species were chosen each year. In addition, for confirming secondary identification, dissections of both male and female genitalia for each moth were made following Landry (2003) and Lee and Brown (2009). Prepared male genitalia slides were compared with those used for initial identifications (Farahpour-Haghani *et al.*, 2016 a, b). Genitalia structures were examined using Cordero and Baixeras (2015) and Moth Count Dissection Methods Guide (2020)

Two temporary outdoor ponds were prepared in RRII (1.5 × 3.5 × 0.7m) (width, length, depth) from 2013 to 2018, and a monthly sampling program was carried out in 2013 for two years. During the sampling, 20 specimens were collected from natural habitats, each containing about 5 kg *Azolla*

spp., collected by 25 × 20 cm (diameter by height) probe. Specimens were kept in the 28 × 28 × 28 cm pots (width, length, depth) containing ten liters of water in the greenhouse until complete decomposition. In addition, some infested *Azolla* specimens were added to outdoor ponds to maintain natural infestation. Patterns of feeding and shelter-making behavioral characteristics for both species were monitored regularly in collected specimens and outdoor ponds.

Infestation and feeding pattern studies

To study the infestation and feeding pattern, we used Adobe Photoshop CS5 Extended, Version 12.0 (Adobe Systems Incorporated, San Jose, California, USA) to analyze the color range of *Azolla* spp. in infested and control containers. They were photographed within 3-4 days intervals. Leaves colors were analyzed with Photoshop color filters. We used the green range as the index of the uninfected area and yellow, brown, grey, and black as an index of the infested area. All infested areas were marked red, while blue was used for marking the uninfected areas. In addition, we marked larvae pathways with Photoshop in some photos by tracking silken webs.

Results

Eggs

Eggs in both species are almost the same in size, shape, and location and have different colors. They are globular, about 1 mm in diameter, and laid singly or in groups of two or three on *Azolla* spp. leaves or smooth surfaces of other material near the food plant. In *D. ramburialis*, eggs are opaque to pale orange (Fig. 1a) and turn to dark orange or reddish brown before hatching (Fig. 1b). However, in cold weather or under stressful conditions in the laboratory (thermal shock or food lacking), females produced a different type of eggs as well. These eggs were white to opaque and had thick skin with some ridges. These eggs are almost similar to *N. noctuella* eggs. Nevertheless, they did not hatch at room temperature or in the incubator (25- 27 °C). In *N. noctuella* eggs are white to creamy (Fig. 1c) and turn to black or greenish gray before hatching (Fig. 1d).

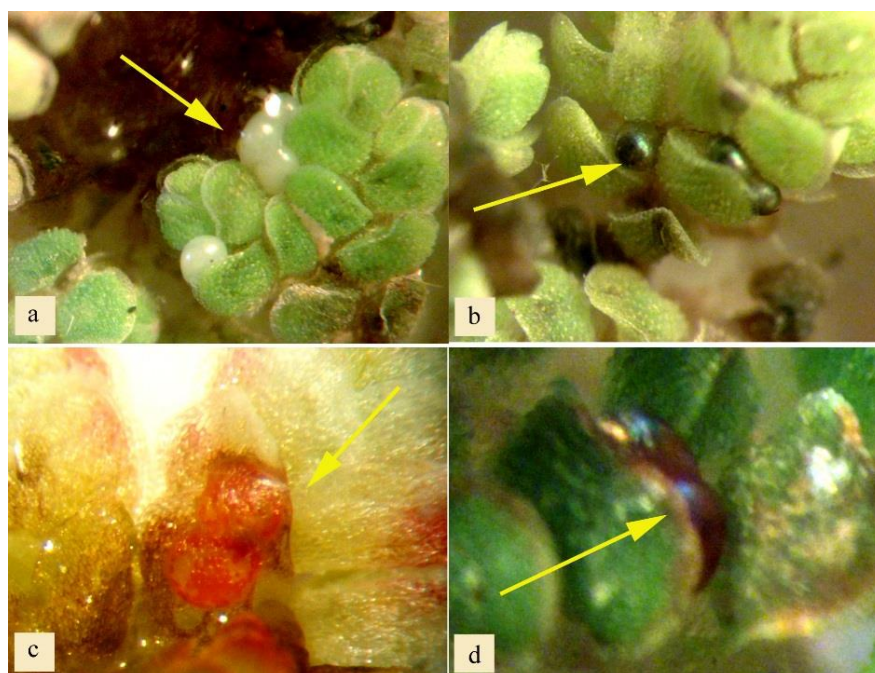


Figure 1 Eggs: a & b: *Nomophila noctuella* eggs, c & d: *Diasemiopsis ramburialis* eggs, a & c: newly laid eggs, b & d: eggs before hatching.

Larvae

Newly hatched larvae of both species are about 1.5 mm long, with black head capsules and sparse setae on the body surface. In *D. ramburialis* newly hatched larvae are dark orange to reddish brown (Fig. 2 c, d), but in *N. noctuella* they are pale to white (Fig. 2a, b).

Second instar larvae of both species are greenish brown with brown head capsule and can not be distinguished from each other due to their small size. Last instar larvae of both species almost look alike as well. However, they have slightly different morphological characteristics. In *N. noctuella* the last instar larvae are greenish brown with black head capsules and about 20-25 mm in length and have sparse seta and distinctive black plates on the body surface (Fig. 3a). In *D. ramburialis*, the last instar larvae have almost the same color, in addition, the sparse seta and distinctive black plates exist on the body surface as well (Fig. 3b). However the head capsule is orange, the body size is slightly smaller (about 18 mm in length), body setae are fewer and shape and size of body plates is somewhat different as

well. Nevertheless, the color of the head capsule is the most distinctive difference.

Pupa

In both species, pupation usually occurred after *Azolla* spp. decomposition, and the pupa color turned from yellowish brown (Fig. 5a, c) to dark brown during (Fig. 5b, d) development. In *D. ramburialis*, the last instar larvae remained on dried *Azolla* spp. and pupation occurred on dried *Azolla* spp. or mud on the sides of the rearing chambers (Fig. 4c). However, in *N. noctuella*, the last instar larvae wandered before pupation for a while and usually climbed on the top of rearing chambers. Therefore, pupation usually occurs on the top of rearing chambers or higher places near the feeding site (Fig. 4a).

N. noctuella larvae make pupal shelter with silken webbings (Fig. 4b) while *D. ramburialis* bound *Azolla* spp. leaves with silken webs to make pupal shelter (Fig. 4d). In addition, *N. noctuella* pupa (Fig. 5a, b) is somewhat larger (9-11 mm) compared with *D. ramburialis* (7-9 mm) (Fig. 5c, d).



Figure 2 First instar larvae: a & b: *Nomophila noctuella* larvae, c & d: *Diasemiopsis ramburialis* larvae, a & c: newly hatched larvae, b & d: larvae making first shelter on *Azolla* leaves.

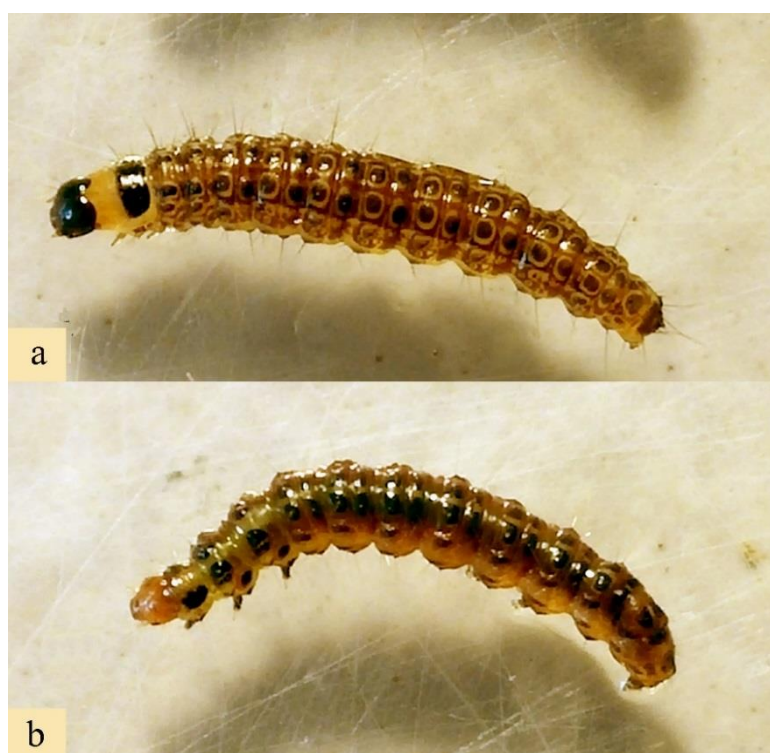


Figure 3 Last instar larvae: a: *Nomophila noctuella*; b: *Diasemiopsis ramburialis*.

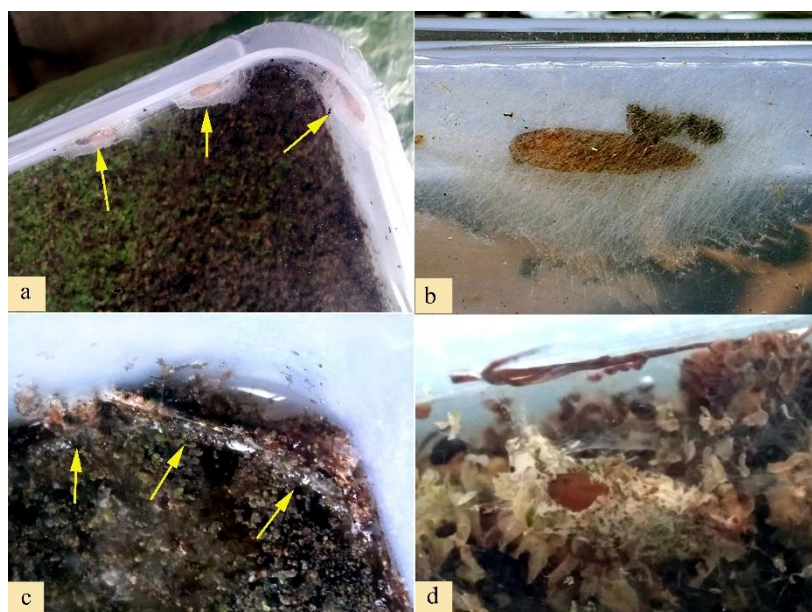


Figure 4 Pupation: a & b: *Nomophila noctuella*, c & d: *Diasemiopsis ramburialis*, a & c: pupation site, b & d: pupal shelter.

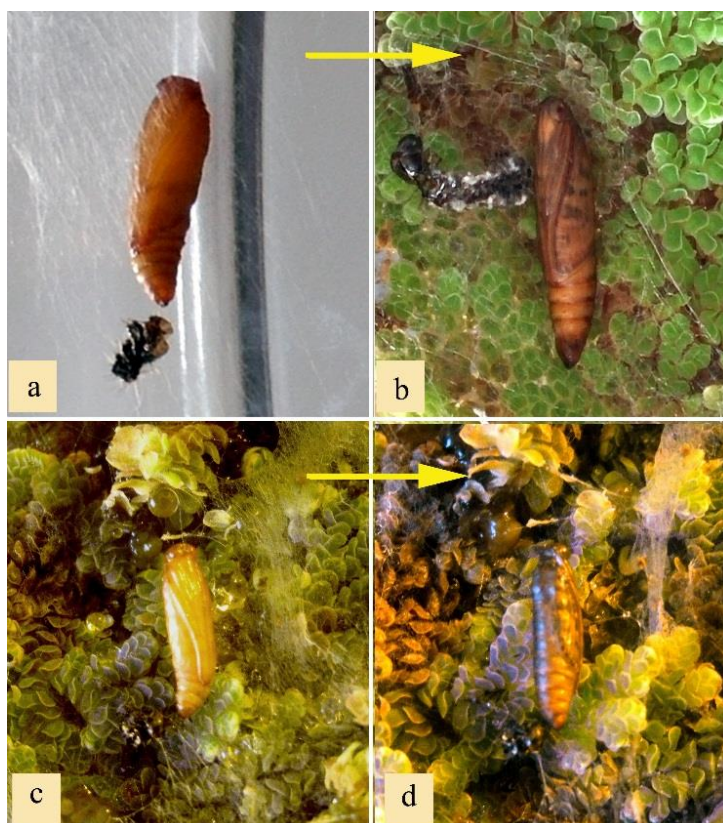


Figure 5 Pupa: a & b: *Nomophila noctuella* pupa, c & d: *Diasemiopsis ramburialis* pupa, a & c: Pupa at the beginning of pupation period, b & d: Pupa at the end of pupation period.

Adults

Adults of both species are completely different in behavioral and morphological characteristics (Fig. 6). Detailed descriptions of adults in both species were given before. However, some behavioral characteristics would be helpful in distinguishing genders for rearing experiments and monitoring. In *D. ramburialis*, adults cannot fold their wings while resting. Therefore, the abdomen would be exposed like many other moths. Male and female could be distinguished based on the morphological characteristics of the abdomen and the form of holding the last abdominal segments while resting in the live specimens. The abdomen in males is narrower and more cylindrical, and most of the time, the last segments of the abdomen are held partly bent up while resting (Fig. 6d). The female abdomen is wider, and they were not able to bend up the last segments of the abdomen (Fig. 6c).

In *N. noctuella* adults, wings are folded on the abdomen while resting due to more flexible axillary sclerites. Therefore, abdominal segments are not exposed, and there are no prominent superficial differences between males and females. However, the male wing pattern (Fig. 6b) seems more distinctive. The body size is somewhat smaller compared with females (Fig. 6a). It has been reported that *N. noctuella*

adults don't feed (Smith, 1942), and *D. ramburialis* adults were not observed feeding in the field or attracting flowers.

Comparative symptoms of damage on host plant

Diasemiopsis ramburialis and *N. noctuella* larvae are phyllophagous and can cause severe damage to their host plants in high-density populations. Two types of damage were observed on infested *Azolla* spp.: Direct damage caused by larvae feeding and indirect damage due to decomposition. Both moth species have non-aquatic larvae, and to feed on *Azolla* spp., newly hatched larvae had to build a shelter by binding over *Azolla* spp. leaves together with silken webs (Fig. 2). In the laboratory, *D. ramburialis* larvae produced more webs than *N. noctuella* (Fig. 7a, b).

Observations indicated that *D. ramburialis* larvae move in corridors made by silken webs among the *Azolla* leaves, and the larvae pathway on *Azolla* spp. were unique and coordinated (Fig. 7b, 8b). They rarely left these corridors, and the main reason for leaving the silken corridors was the high-density population of the larvae. However, pupation always occurred in these shelters (Fig. 4c, d).

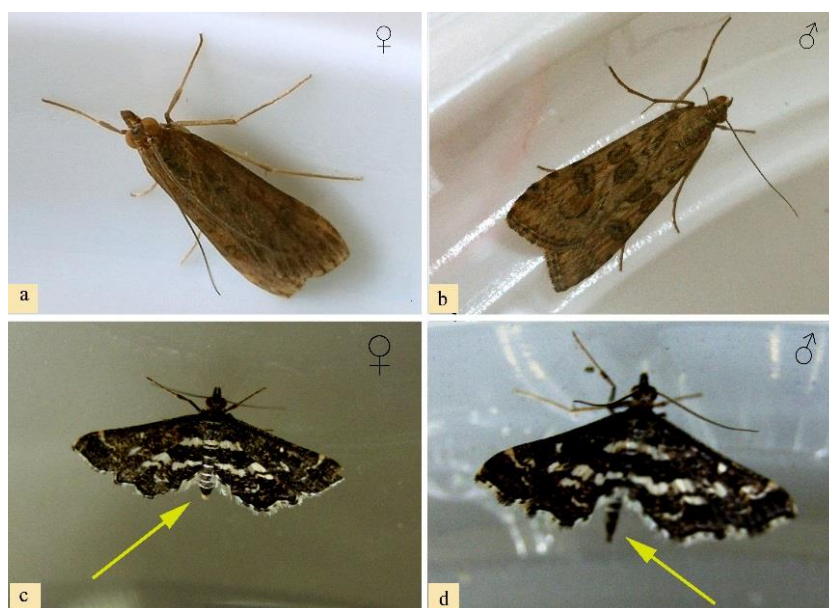


Figure 6 Adults: a & b: *Nomophila noctuella* adult, c&d: *Diasemiopsis ramburialis* adult, a & c: female, b & d: male.

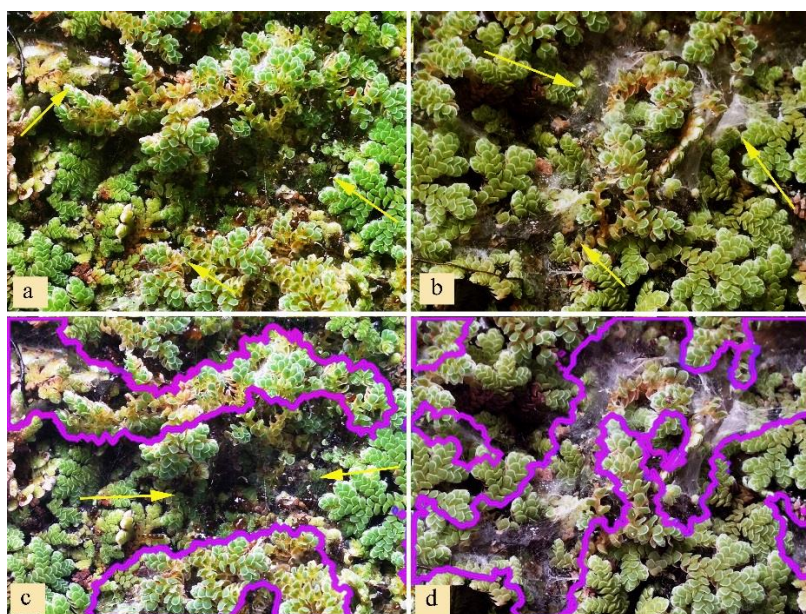


Figure 7 Produced webs in larvae pathway in both species: a & c: *Nomophila noctuella* larvae pathway; b & d: *Diasemiopsis ramburialis* larvae pathway, a & b: Main photo, c & d: Decreased resolution photo of larvae pathway that show amount of webs, Marked areas show the larvae pathway and arrows show areas that are contaminated with larvae frass.

In *N. noctuella* the larvae bind over leaves to make a union layer. However, the pathway was under the *Azolla* leaves. As a result, they produced fewer amounts of the webs, and the larvae pathway was not so distinctive (Figs 7a, b, 8a). Therefore, despite using the same materials for making shelter, each species had specific pathways on the host plant. In addition, despite the low-density population *N. noctuella* larvae wandered on *Azolla* regularly and left frass near the pathways (Fig. 7a, b). Therefore, leaving the shelter in this species seemed to be habitual (Fig. 8a).

Observation of feeding behavior in the laboratory indicated that in *D. ramburialis* infestation seems to be patchy. Infestation started from the specific sites and spread to other areas in time (Fig. 9 b, d). In *N. noctuella*, due to the wandering behavior of larvae, infestations were spread on the entire feeding site (Fig. 9a, c). To confirm this hypothesis, we photographed infestation patterns in each species at 3-4 days intervals (Fig. 10).

Based on the general appearance of the photos, infestation patterns did not seem so

different between the two species. However, when different color filters were used and untouched areas were marked, the infestation pattern became distinctive (Fig. 11).

In *D. ramburialis* there were two distinct areas: untouched areas marked in blue and damaged areas marked in red. However, in *N. noctuella*, in addition to these areas, there were neither blue nor red areas. These areas were not damaged by larvae but were contaminated by larvae frass and turned yellow due to contamination. In addition, the untouched and damaged areas were spread in feeding sites and did not seem to be distinctive. These results confirmed that these species have patchy activity due to the non-wandering behavior of *D. ramburialis*. In *N. noctuella* the larvae wandering behavior and leaving the shelters are habitual.

More behavioral studies indicated *N. noctuella* larvae prefer to move in the feeding site margins (Fig. 12). The behavioral difference of larvae in the two species was the main reason for different pupation sites. In *D. ramburialis* where larvae tend to remain in shelters, pupation occurred on dried *Azolla* spp. leaves or mud near

the feeding site, while the wandering behavior of larvae in *N. noctuella* caused pupation in places far from the feeding sites. As mentioned before, indirect damages on *Azolla* spp. were caused by decomposition. Due to larvae feeding and larvae frass, direct damage caused bacterial and fungal infection on *Azolla* spp. (Fig. 13a, b).

The pathogenic infestation was confirmed by sub-culturing of epiphytic (Fig. 14a, b) and endophytic (Fig. 14c, d) specimens of *Azolla* spp. from before (Fig. 14a, c) and after (Fig. 14 b, d) infestation on PDA in the laboratory.

As a result, at the end of the larvae feeding period, infested *Azolla* spp changed color to greenish gray or black, and due to a higher

amount of web production in *D. ramburialis*, infested sites were covered by silken webs. In addition, saprophagous insects such as Collembola and Scirtidae. (Coleoptera) are attracted to decomposed *Azolla* spp. and feed on reminded materials. However, despite decomposition, the covering layer would not be sunk for a while. Therefore, these species could not wipe the covering layer of *Azolla* spp.. Due to more visible results, most behavioral characteristics were discussed based on the laboratory figures. However, the same behavioral patterns for both species were observed in the collected specimens and outdoor ponds (Fig. 15).

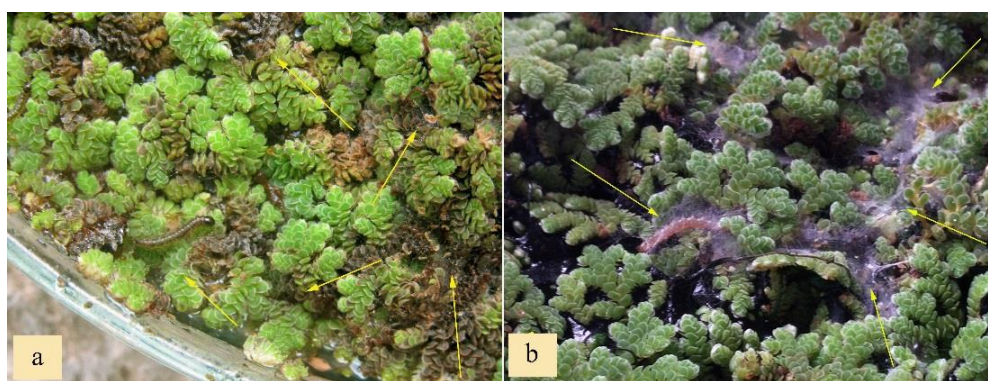


Figure 8 Larvae pathway and shape of shelters in both species: a: *Nomophila noctuella*; b: *Diasemiopsis ramburialis*.

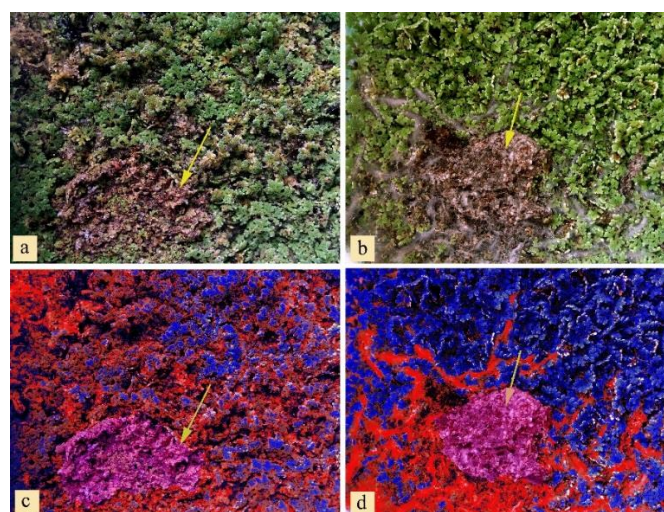


Figure 9 Feeding pattern in both species: a & c: *Nomophila noctuella* feeding pattern; b&d: *Diasemiopsis ramburialis* feeding pattern, a & b: Main photo of feeding pattern, c & d: Photo of feeding pattern with different color layers that mark shape of infestation, violet area shows the origin of infestation; red area shows larvae path way that is contaminated with larvae frass and webs; blue area shows untouched *Azolla*.

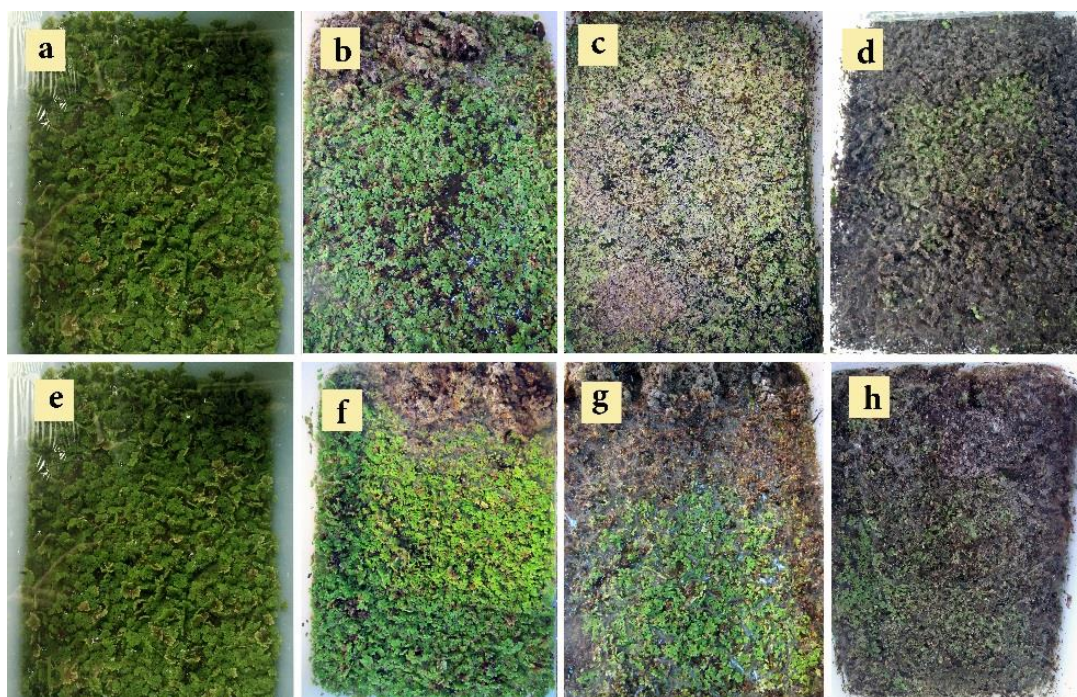


Figure 10 Infestation pattern in both species at 3-4 days intervals: b, c, d: *Nomophila noctuella*, f, g, h: *Diasemiopsis ramburialis*, a & e: un-infested *Azolla* spp., b & f: 4 days after infestation, c & g: 7 days after infestation, d & h: 11 days after infestation.

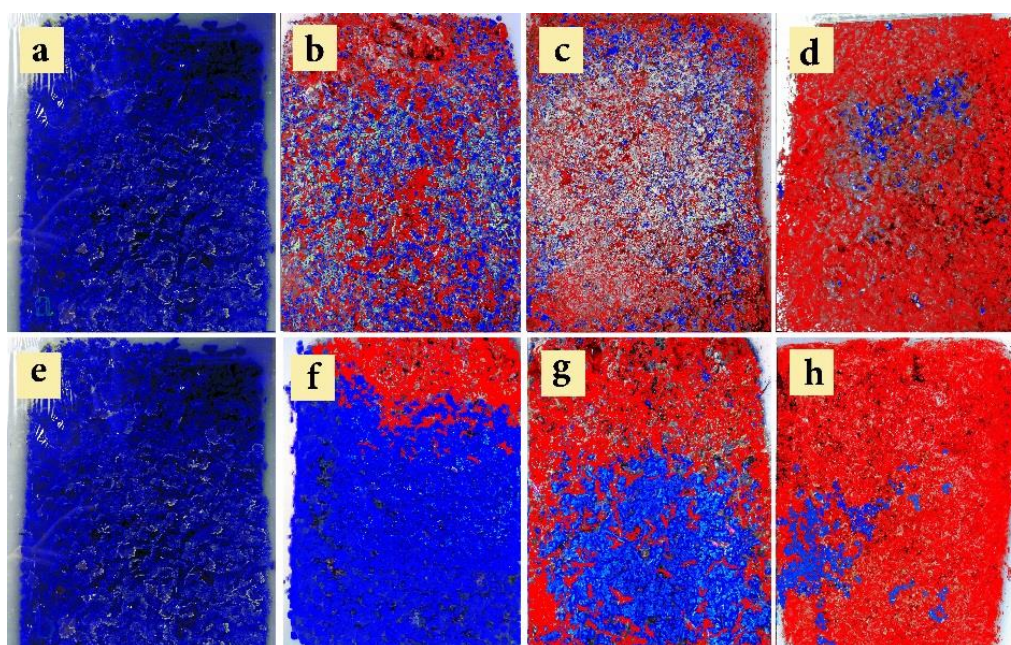


Figure 11 Infestation pattern in both species at 3-4 days intervals using color filters that marked untouched areas with blue and damaged area with red: b, c, d: *Nomophila noctuella*; f, g, h: *Diasemiopsis ramburialis*, a & e: un-infested *Azolla* spp, b & f: 4 days after infestation, c & g: 7 days after infestation, d & h: 11 days after infestation.



Figure 12 *Nomophila noctuella* larvae preference in move and feed in the margins.

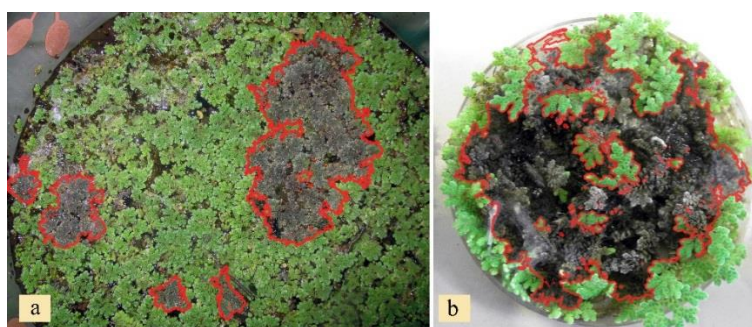


Figure 13 *Azolla* spp. pathogenic infection in feeding sites of both species that marked with red lines: a: *Nomophila noctuella*; b: *Diasemiopsis ramburialis*.

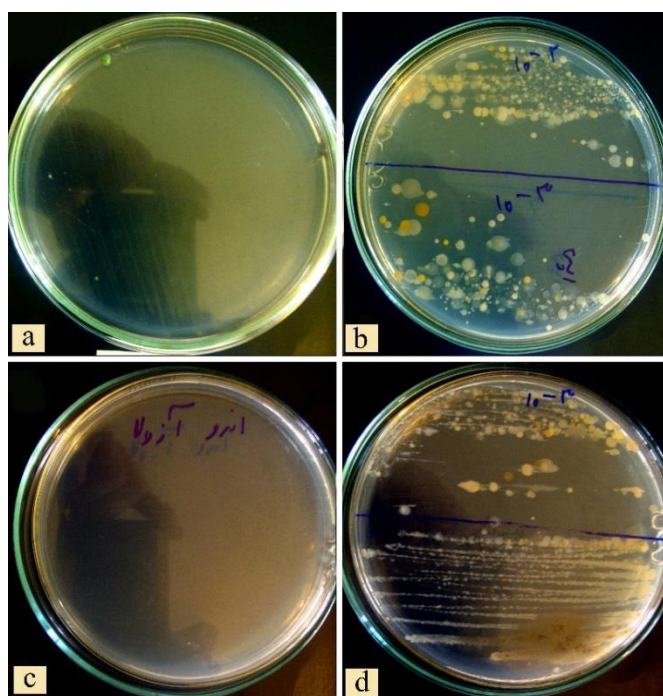


Figure 14 Fungal and bacterial infestation on *Azolla* spp. due to feeding damage of both species: a & b: Epiphytes; c & d: Endophytes, a & c: before feeding; b & d: after feeding.

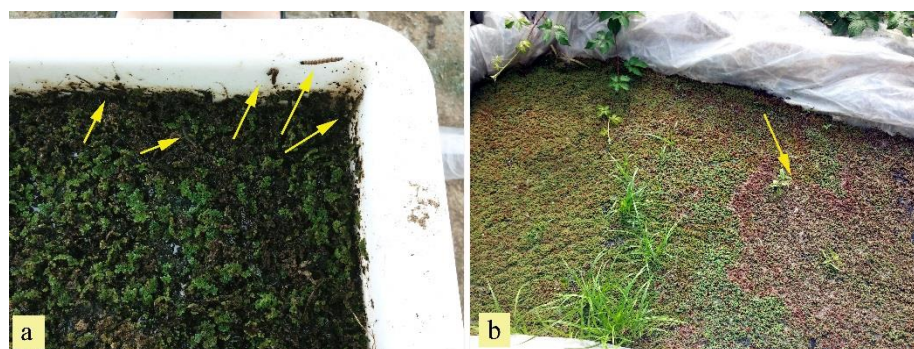


Figure 15 Behavioral characteristic of both species in field and collected specimens: a: marginal path- way of larvae in *Nomophila noctuella* in collected specimens; b: patchy activity of *Diasemiopsis ramburialis* in outdoor pond.

Discussion

Azolla spp. were introduced in 1986 in the northern region of Iran. During almost four decades, they have covered many aquatic ecosystems such as stagnant waters, ponds, ditches, canals, or paddy fields in this area (Delnavaz and Azimi, 2009). It is generally accepted that biological invasions have major ecosystem impacts, and some ecologists consider biological invasion the second leading cause of biodiversity loss (Pimentel *et al.*, 2000; Vitousek *et al.*, 1996). Hyrcanian climatic conditions, in addition to the rich fauna and flora of the northern region of Iran, have caused complex host plant-herbivore interactions for hundreds and thousands of years (Akhani *et al.*, 2010; Hoekstra *et al.*, 2010). *Azolla* spp. invasion, put many migratory or native species under hunger stress due to malformed and asymmetric environmental conditions. Therefore, to survive, many of these species had to consume *Azolla* spp.

It has been demonstrated that *D. ramburialis* and *N. noctuella*, are two species from the same tribe with overlapping activity periods in the northern regions of Iran, which feed on *Azolla* spp. Due to the non-aquatic activity of both species' larvae and shelter-making behavior, they almost have the same feeding pattern. However, these studies show that with different morphological characteristics of each life stage, the shape of the shelter, and specific behavioral characteristics, they could be perfectly different from each other. Concealed feeding in insects is

an adaptive behavior for the amelioration of harsh conditions, manipulation of host plant quality, and protection from natural enemies (Lill and Marquis, 2007; Cornelissen *et al.*, 2016). The construction of shelters is expected to increase insect survivorship (e. g., caterpillars) and might influence other organisms of the community through non-trophic direct and indirect effects when the shelter is co-occupied or occupied after abandonment (Fukui, 2001; Cornelissen *et al.*, 2016).

Diasemiopsis ramburialis is a cosmopolitan migratory species with immature stages and feeding activity on *Azolla* spp. remained uncovered for years. *Azolla* spp. are the first recorded host plants for this species worldwide. Since *D. leodocusalis* (Walker) host plant is still unknown, this would be the first host plant record for *Diasemiopsis* genus (Farahpour-Haghani *et al.*, 2016a). Studies indicated that the shelters made by *D. ramburialis* larvae on *Azolla* spp. are firm and well-formed. Considering non-aquatic larvae of this species, making such shelters on the aquatic host plant protects the larvae from being drowned. Therefore, this would be an adaptive behavior in response to feeding on aquatic host plants. Non-wandering behavior of the larvae and pupation in concealed sites that protects the larvae and pupa from predators in aquatic ecosystems could be considered as other beneficial adaptive behaviors in the same way (Farahpour-Haghani and Jalaeian, 2021). It has been demonstrated that insects adapt with their host plant and mediate their feeding behavior and strategy in

response to host plant chemical components and morphological characteristics (Bernays 1998; Knolhoff and Heckel, 2014). Therefore, these results indicate that *D. ramburialis* is perfectly adapted to feed on the *Azolla* spp..

Unlike *D. ramburialis*, *N. noctuella* is a polyphagous species mainly spread in the Old World (De Prins and De Prins, 2020). This moth can feed on many host plants, and its feeding behavior on *Azolla* spp. indicates that it is not so adapted to feed on aquatic plants. As mentioned, the shelters that this moth larvae make on *Azolla* spp. are not so strong. Therefore the larvae chose to move near the margins. In addition, the wandering behavior of the larvae is not beneficial in terms of concealed activity, which protects immature stages from predators. However, due to larvae polyphagy and a high abundance of *Azolla* spp. this species chose to feed on the new host plant (Farahpour-Haghani and Jalaeian, 2021). In addition to *D. ramburialis* and *N. noctuella*, other fern-feeding groups of moths from Heliothelinae (Amsel 1961) in Crambidae (Mally *et al.*, 2017). Therefore, feeding on *Azolla* spp. in *N. noctuella* could also be based on phylogenetic connections. Some other non-aquatic caterpillars from other families have been found, which were feeding on *Azolla* spp. occasionally in the northern region of Iran. However, they could not complete an entire generation in *Azolla* spp. Therefore *D. ramburialis* and *N. noctuella* are the only non-aquatic moths that feed on *Azolla* spp. in these areas. Since climatic and environmental conditions of these regions caused an overlapping feeding period for these species, they have to compete with each other for food and resources. Competition is a key factor affecting the structural bases of animals and insect communities, and the negative effects of competition can influence species' survival (Lawton and Strong, 1981; Frank van Veen *et al.*, 2006). Phytophagous insects are more likely to compete if they are closely related, introduced, sessile, aggregative, feed on discrete resources, and feed on forbs or grasses (Denno *et al.*, 1995). Therefore, most species

with overlapping activity periods should have some methods that let them avoid competition; in the case of *D. ramburialis* and *N. noctuella*, the behavioral differences of both species let them coexist on the same host plant. Compared with *N. noctuella*, the patchy activity of *D. ramburialis* lets the larvae feed on *Azolla* spp. surface and non-wandering behavior of this species keeps larvae near each other, while *N. noctuella* larvae move and feed in margins. Therefore behavioral differences of these species let them feed on the same host plant simultaneously while avoiding severe competition. Choosing a different path or site on the same host plant is one of the most used methods in insect-plant interactions to avoid competition (Anderson *et al.*, 2011). For instance, *Hayhurstia atriplicis* (Linnaeus) and *Pemphigus betae* (Doane) (Homoptera: Aphididae) chose different sites (root and shoot) on *Chenopodium* spp. (Linnaeus) (Amaranthaceae) to avoid direct competition (Moran and Whitham 1990) or *Euura lasiolepi* (Smith) (Hymenoptera: Tenthredinidae) on *Salix lasiolepi* (Bentham 1857) (Salicaceae) form gall on stems while three other species of the same genus induce gall on petioles (Fritz and Price 1990).

It is indicated that *N. noctuella* overwinters as larvae in heavy silken cases at or below the ground surface (Flint 1922). Therefore, this species cannot overwinter on *Azolla* spp. and must find a suitable site for overwintering. So, despite the negative effect of the wandering behavior of the larvae on concealed feeding activity, this behavior would be a beneficial factor in finding suitable sites for feeding and overwintering. In contrast, the overwintering life stage is not reported in *D. ramburialis*, and we failed to find the overwintering life stage of this species in the natural habitats. However, as mentioned before, under the stressful conditions in the laboratory, the female produced a different type of eggs. Since these eggs did not hatch at room temperature or in the incubator, we assumed these eggs must be in the overwintering life stage of this species that needed to be incubated in cold

weather for a defined period to prepare for hatching.

Some insects that feed on the same host plant tend to mediate their life stages time zone to avoid competition with other insects. For example *Tephritis bardanae* (Schrank) and *Cerajocera tussilaginis* (Fabricius) (Diptera: Tephritidae) chose different times of oviposition on *Arctium minus* (Hill) (Bernhardi) (Compositae) to avoid competition between larvae (Straw 1989) or root feeding by the wireworm, *Agriotes lineatus* (Linnaeus) (Coleoptera: Elateridae), influences oviposition decisions and larval leaving rate of an aboveground herbivore, *Spodoptera littoralis* (Boisduval) (Lepidoptera, Noctuidae) (Anderson *et al.*, 2011). Since *N. noctuella* overwinters as the larvae in other sites, this species gets active much sooner than *D. ramburialis*, which lets it build a high-density population. However, considering the better shelter-making behavior and concealed activity of *D. ramburialis*, this species can survive on *Azolla* spp. better. As a result, despite the later appearance of *D. ramburialis*, both species have a balanced population density at the end of summer on *Azolla* spp.. Therefore, the difference in overwintering life stages could be another factor, letting both species avoid competition.

As mentioned, shelter-making behavior might influence other community organisms through non-trophic direct and indirect effects when the shelter is co-occupied or occupied after abandonment. Therefore, this behavior places leaf and stem shelter-builders within the context of ecosystem engineering (Fukui, 2001). In the case of *D. ramburialis* and *N. noctuella*, fungi and bacteria that grow on larvae frass or Collembola and Coleoptera that attack decomposed materials are species that get the advantage of these moth larvae activity on *Azolla* spp. These species seem harmless for the moths' larvae, but wiping the covering layer of *Azolla* spp. is essential. Decomposed materials are not helpful for the moth larvae, and females would not lay eggs on these materials. Since the larvae could not wipe the

covering layer of *Azolla* spp., these species let the new layer regrow the next session by decomposing the covering layer. Therefore, these species' activities also benefit the moths' larvae.

Conclusion

Azolla spp. are invasive aquatic species, and due to poor management, they get to invade aquatic ecosystems of the northern region of Iran. Unbalanced and non-stable environmental conditions put local species under stress and cause new interactions between local and exotic species. *Diasemiopsis ramburialis* and *N. noctuella* are two micromoths from Crambidae found feeding on *Azolla* spp. under such environmental conditions. We described both species' biology on the new host plant separately; however, their overlapping feeding period, similarities between immature stages of both species and even some general behavioral characteristics were confusing. Since *N. noctuella* was recorded as a polyphagous pest species, it was felt necessary to properly distinguish it from *D. ramburialis*. Further studies indicated some morphological characteristics and specific behaviors that would be useful for the primary identification of each species in the field. In addition, by observing some behavioral characteristics, we assumed that *D. ramburialis* is more adapted to feed on *Azolla* spp. so these species could be the main host plants for this moth. This hypothesis is not confirmed yet; however, it is sure that *D. ramburialis* and *N. noctuella* are important biotic resistance factors for *Azolla* spp. in the northern region of Iran.

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Author Contributions

Material preparation: AFH, MJ; Data collection: AFH, MJ; Analysis: AFH, MJ; Writing the first draft of the manuscript: AFH; Conceptualization: AFH, MJ; Methodology: AFH, MJ; Software: AFH; Validation: AFH, MJ; Formal Analysis: AFH, MJ; Investigation: AFH; Resources: AFH, MJ; Data Curation: AFH; Writing – Original Draft Preparation: AFH; Writing – Review & Editing: AFH, MJ; Visualization: AFH, MJ; Supervision: AFH, MJ; Project Administration: AFH, MJ; Funding Acquisition: AFH, MJ.

Conflicts of Interest

The authors declare no conflict of interest.

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مقایسه چرخه زندگی و ویژگی‌های رفتاری دو گونه شبپره *Spilomelinae* با دوره تغذیه هم‌زمان روی گونه‌های آذولا در شمال ایران

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چکیده: *Diasemiopsis ramburialis* و *Nomophila noctuella* دو شبپره از زیرخانواده *Spilomelinae* روی گونه‌های آذولا فعال بوده و در منطقه شمال ایران دوره تغذیه آن‌ها با یکدیگر همپوشانی دارد. به دلیل برخی شباهت‌های مورفولوژیکی، مراحل نابالغ به راحتی اشتباه گرفته می‌شوند. مطالعات پنج‌ساله در آزمایشگاه و زیستگاه‌های طبیعی نشان داد که علی‌رغم شباهت‌های موجود بین مراحل نابالغ، ویژگی‌های مورفولوژیکی و رفتاری خاصی در هر گونه وجود دارد که برای شناسایی اولیه در مزرعه مفید است. تخم‌های *D. ramburialis* و لاروهای سن اول خاکی تا نارنجی کم‌رنگ هستند درحالی‌که در *N. noctuella* تخم‌ها و لاروهای سن اول بی‌رنگ یا کرم رنگ هستند. کپسول سر لارو سن آخر در *D. ramburialis* نارنجی است، اندازه بدن کوچکتر است و شفیره شدن در نزدیکی محل تغذیه رخ می‌دهد. در *N. noctuella* لاروهای سن آخر دارای کپسول سر سیاه هستند، اندازه بدن در آن‌ها بزرگتر است و شفیرگی دور از محل تغذیه انجام می‌شود. لارو هر دو گونه دارای عادت لانه‌سازی هستند و با چسباندن برگ‌های آذولا به یکدیگر با استفاده از نوارهای ابریشمی پناهگاه درست می‌کنند. اما لاروهای *D. ramburialis* پناهگاه مستحکمی می‌سازند و تارهای بیشتری تولید می‌کنند. علاوه بر این، بیشتر اوقات در پناهگاه می‌مانند و بین دالان‌هایی که از رشته‌های ابریشمی ساخته شده‌اند حرکت می‌کنند. لاروهای *N. noctuella* ترجیح می‌دهند در حاشیه محل تغذیه در دالان‌هایی که از برگ‌های چسبانده شده آذولا ساخته شده حرکت کنند و اغلب رفتار سرگردانی دارند. این نتایج نشان داد که گونه‌های آذولا احتمالاً میزبان اصلی برای *D. ramburialis* هستند درحالی‌که *N. noctuella* آن‌ها را به طور تصادفی به عنوان میزبان انتخاب کرده است.

واژگان کلیدی: *Spilomelinae*، *Azolla* spp.، ویژگی‌های رفتاری، ویژگی‌های مورفولوژیکی، لارو