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Defensive behavior associated with secretions from the prosternal paired glands of the larvae of *Heliconius erato phyllis* Fabricius (Lepidoptera, Nymphalidae)[1]

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ABSTRACT. Defensive behavior associated with secretions from the prosternal paired glands of the larvae of *Heliconius erato phyllis* Fabricius (Lepidoptera, Nymphalidae). Our work presents for the first time, the defensive behavior associated with the release of the product of the prosternal paired glands of the larva of *Heliconius erato phyllis* Fabricius, 1775 (Lepidoptera, Nymphalidae, Heliconiinae). The prosternal glands were first described for larvae of *H. erato phyllis*. They are formed by two types of glandular structures: the impair gland and the paired glands. The prosternal glands are located within the conical integumentary sac, which in turn is situated on the individual's prosternum. The main goal of this study is to analyze the existence of any secretion from the prosternal paired glands, and check the action mode of this secretion. The methodology used for chemical analysis of the glands included the aeration and, analysis in gas chromatography and gas chromatography-mass spectrometry. The results show that the prosternal glands do not produce volatiles. Bioassays were conducted with simulated and natural attacks and revealed that the prosternal paired glands produce secretions of defense together with silk produced by labials glands as a defense strategy, described for the first time, against ants. The strategy consists in wrapping the ant with silk threads, the entire wrapped object moved to the end of the body, with the aid of the legs and prolegs, and possibly fixed in a nearby place. Evidence for the existence of a conical integumentary sac in larvae of other species and families of Lepidoptera allows us to propose the possibility of occurrence of prosternal paired glands with defensive function in these other groups as well.

KEYWORDS. Chemical defense; chemical ecology; immature; Insecta.

The defensive behavior of animals is regarded as an evolutionary adaptation, which is defined as an evolutive process that encompasses genetic changes, new phenotypic traits and enhancement of individuals' fitness (Barrows 2000). In particular, Lepidoptera larvae have developed a series of strategic defenses against predators like ants, that often co-occur in its feeding sites (Kaminski *et al.* 2009). These defensive strategies are divided into two classes: primary defenses that prevent the encounter between predator and larvae and secondary defenses that prevent the attack after detection of a potential predator by the larva (Gross 1993). Examples of primary defenses include the construction of shelters and bridges which serves as a mechanical barrier that decreases the likelihood of being located by ant predators, and the chemical camouflage of the larvae that allows it to become undetectable by the ants due to their resemblance to the substrate (Portugal & Trigo 2005). On the other hand, secondary defenses comprise strategies like: biting, struggling, spitting, leaping from the leaf to remain hanged by a thread of silk, and the epidermal specializations. The latter adaptations range from simple bristles to more complex structures in the form of thorn, or even specialized glands that secrete harmful substances to potential predators and parasitoids (Stehr 1987; Salazar & Whitman 2001).

In this study we address a defensive adaptation in Lepidoptera of the genus *Heliconius* Kluk, 1780. The genus *Heliconius* forms one of the most successful groups of neotropical butterflies. They feature several evolutionary adaptations such as Müllerian mimicry, nocturnal aggregation in dormitories (Turner 1981; Salcedo 2011), unpalatability, advantageous mating events (Turner 1981), host plant specificity, wide visual spectrum and large brain (Swihart & Swihart 1970); refined behavior, learning ability and sense of spatial location (Gilbert 1975), nectar and pollen feeding habits which maximizes the longevity, fecundity and fertility of eggs (Gilbert 1972; Dunlap-Pianka *et al.* 1977), non-predation of eggs by larvae relatives (De Nardin & Araújo 2011), among others. *Heliconius erato phyllis* Fabricius, 1775 (Lepidoptera, Nymphalidae, Heliconiinae) is one of the 29 subspecies of *Heliconius erato* Linnaeus, 1758, the most variable species of the genus. It presents a wide distribution in South America, where it occurs from the northeast to the south of Brazil, Bolivia, northeastern Argentina, Uruguay (Kaminski *et al.* 2002), and Paraguay (Holzinger & Holzinger 1994).

Females of *H. erato phyllis* oviposit only on plants of the family Passifloraceae, which suggests a coevolution with their host plants (Benson *et al.* 1976). The passion fruit plants are well known to have extrafloral nectaries on the edge of the leaves and bracts, and they are associated with several ant species (Gilbert 1975; Benson 1976; Smiley 1985, 1986). Despite that the resources provided by plants constitute the main food item of several arboreal ants, many species also behaving as opportunistic predators (Floren *et al.* 2002; Davidson *et al.* 2003) or even having a high degree of specialization (Morais 1994). Since larvae of *H. erato phyllis* feed on the same plant species, they are most likely to be encountered and predated by ants, as well as the eggs (Gilbert 1975; Smiley 1985, 1986). Given this dominance over the foliage it is reasonable to think that ants must have a strong impact on the biology of herbivorous insects.

Defensive strategies in adults and larvae of *H. erato phyllis* include the presence of cyanogenic glycosides in their tissues, which determines the degree of toxicity in adults and makes them unpalatable to vertebrate predators. It is known that the adult produces these substances from aminoacids precursors (Nahrstedt & Davis 1983; Spencer 1988), and these aminoacids are derived from the pollen ingested by them (Gilbert 1991). These compounds could be hijacked or modified from the host plants, and alternatively, synthesized by the larvae (Borges *et al.* 2010). In larvae of *H. erato phyllis*, it is suggested that the presence of thorns along the body prevents the oviposition by parasitoids. The prosternal glands, a recently described trait, may be related to defensive mechanisms as well as odoriferous abdominal glands of adults (Borges *et al.* 2010).

The prosternal exocrine glands of the larva of *H. erato phyllis* are present in the five larval instars and occur in two different types. The first type consists of a pair of ellipsoidal units called 'paired glands'. They are associated with a conical integumentary sac that is projected in the prosternum. It expels a secretion through ducts that open into fine pores on the lateral surfaces of the sac, which is everted by internal hydrostatic pressure. The other type of gland is formed by a single secretion pouch in the anterior portion of prothorax, called 'impair gland'. It opens on the body surface through a transverse slit at the anterior portion of the prosternum. The edges of the slit have several sensilla, which may probably function as proprioceptor. It is also supposed that the prosternal glands may exist in the larva of other species and families due to previous investigations that demonstrate the existence of the conical integumentary sac on the prosternum in these other studied species (Peterson 1962; Osborn & Jaffé 1998; Paluch *et al.* 2001; Furtado & Campos-Neto 2004; Souza *et al.* 2006). A histological analysis of glands and surface area (light microscopy and transmission electron microscopy) demonstrates that the glandular units of the paired glands are composed of simple tall columnar epithelium, and the unit of the impair gland consists of simple low columnar epithelium. Paired glands secretion is amorphous, acidophilic, stored in the secretory units.

The function of these glands may involve a defense mechanism for the larvae, due to observations of changes in its posture when the anterior portion of their body is physically stimulated. The observation of this behavior suggested the hypothesis of involvement in defense against attacks from potential predators (Borges *et al.* 2010). From this scenario, we established the goal to describe and elucidate the function of the paired prosternal glands in larvae of *H. erato phyllis*. In this article we present the results of chemical analysis of aerations, and a description of the behavior related to the use of the prosternal paired glands when the larvae face different threats.

MATERIAL AND METHODS

In order to verify if the prosternal paired glands have a defensive function in the five larval instars, specimens from all instars of *H. erato phyllis* were obtained for analytical experiments, chemical analyzes and behavioral bioassays. We cultivated its host plant *Passiflora edulis* Sims, and their seedlings were transplanted to pots and kept under natural conditions in the open in a reserved area of the *Universidade Federal do Paraná* (UFPR), Curitiba, Paraná, Brazil. Leaves and branches were used as larval food source in the rearing room. Potted plants of *P. edulis* and *Passiflora actinia* Hooker were kept in semi-natural conditions in the insectary built in the same area, whose leaves and branches served as oviposition site for adults. Adults of *H. erato phyllis* used in this study were collected in the cities of Curitiba and Quatro Barras, Paraná, Brazil, and maintained in the insectary that was built in UFPR. Both the renewal of the artificial diet and the collection of the eggs were made daily. The collected eggs were kept in seminatural conditions in a rearing room setting, and the hatched larvae on leaves of *P. edulis* were kept in plastic bottles containing water. The whole setup was wrapped in porous cloth sacs.

We then verified if the prosternal paired glands of the larva released volatiles, and if these volatiles were associated with defensive responses to the threat of potential predators. We assessed the existence of possible volatile compounds extracted in different scenarios, as well as its concentration and chemical composition. The methodology used for chemical analysis of possible volatiles of the prosternal glands included the aeration and analysis in gas chromatography (GC) and gas chromatography-mass spectrometry (GC MS).

The tests were performed in both presence and absence of artificial provocative stimuli. This procedure was then compared in environments with and without the host plant. To explore the presence of volatiles, we built two aeration systems that contained two chambers each, hereafter referred as A and B. The system A was used for the simultaneous collection of volatiles from seedlings of *P. edulis* with *H. erato phyallis* larvae and volatile from seedlings only. The system B enabled the simultaneous collection of volatiles from larvae of *H. erato phyllis* wrapped in porous cloth, and volatile from the porous cloth only. Both chambers have been used for aeration, with adsorbent filter at one end and a constant source of filtered air at the other. Volatiles were collected in time intervals of 24, 48 and 72 hours. In the aeration system A, four and fifth instar larvae received provocative artificial stimuli from bristles of a brush in the dorsal region of the larva on alternate times for 1 hour. The artificial stimuli were maintained until the larva displayed an aggressive behavior. In aeration system B, four larvae individualized by porous cloth were kept in a glass tube during aeration. Fourth and fifth instar larvae were grouped separately. Eighteen samples of volatiles were collected from the first aeration system, and eight samples of volatiles were obtained from the second aeration system. The extracts were then obtained by washing the filter adsorbent with hexane and subsequent concentration (50mL or 50%) for chemical analyzes in gas chromatography.

The chemicals were analyzed in a gas chromatography/mass spectrometry (GC/MS), model Shimadzu QP-2010 Plus equipped with a capillary column RTX-5 (30 mm × 0.25 mm × 0.25 mm) (Restek, Bellefonte, Pennsylvania, USA), with detector flame ionization (FID). The initial column temperature was 50°C, held for three minutes. After this time, the temperature was increased by 7 °C/min until it reached 270°C. This max temperature was then kept constant through a period of 5 minutes.

The use of prosternal paired glands was also analyzed along with behavioral responses to threat scenarios. In the bioassays, we recorded video footage of the behavior of nine larvae in stress situations with artificial provocation stimuli for posterior analysis. We built a setup where a beam of light was projected on the larva of the fifth instar, and filmed it during 30s with camcorder model GZ-HM320 SUB Silver Full HD. A black background was used for contrast. We first used an artificial provocation stimuli that consisted of touch movements on the head of the larvae with a fine brush, as the individual remained in a horizontal position on a leaf of *P. edulis*. In a second scenario, we used specimens of the ant *Tapinoma melanocephalum* Fabricius, 1793 to conduct the behavioral bioassays. The observations of natural provocative stimuli were made by putting all instars in direct contact with the living specimens of *T. melanocephalum*. Workers of *T. melanocephalum* were collected with bait fish in edible oil in the rearing room. This ant species is very generalist, with a diet ranging from sugary food sources to dead arthropods (Osborne *et al.* 1995; Scheurer & Liebig 1998).

The external morphology and behaviors were studied primarily on fresh material. The drawings followed the standard procedures of Borges *et al.* (2010) and Vegliante and Hasenfuss (2011). All illustrations were made with the aid of a stereoscopic microscope coupled to a camera lucida.

RESULTS

Airings. The analysis of the chromatograms shows that in aeration system A it was collected only the plant's volatiles, and in the aeration system B it was collected only volatiles from the porous cloth. These results indicate that no defensive volatiles are produced by the larvae.

Bioassays – Behavioral Experiments

Direct observations and video footage revealed that the prosternal paired glands act together with the labial silk glands. The product of glandular secretion is a jet, which initially is suggested to be in a liquid state. These glands seem to promote a chemical-mechanical defense that is used as a strategy of active defense against predators, specifically ants. In the rearing room it was found that workers of the ant *T. melanocephalum* preyed on eggs, larvae and weakened adults with reduced flight space.

Responses to artificial stimuli. All larval instars displayed the same behavioral sequence of events when artificially stimulated. The description of the response of fifth instar larva to artificial provocation happen as follows: the larva initially detects the potential predator, and then it folds its thorax several times with regular movements. The legs remain free and it turns its head towards the disturbing subject. In this initial stage the larva has slow movements and also tries to move through the substrate. If the attack movements persist, the larva becomes increasingly active by displaying more incisive movements of its thorax. After a few minutes of constant attack it is possible to observe that the conical integumentary sac inflates (Fig. 1A). The sac is presented as a protrusion, similar in color to the head capsule, and its length is subequal to the length of the fore legs. After that, four different possible outcomes of behavior are observed. In some occasions, the larvae moved through the substrate while maintaining the inflated sac. On a second possible outcome, it was observed the larvae directing jets of glandular secretion from pores of the prosternal paired glands. In the observation of the film images it was noted that the secretion of both pores are eliminated at the same direction of the brush's bristles, which is probably interpreted as a potential predator (Fig. 1B).

In a third possible response, the larva releases silk from their labial glands toward the predator during the artificial attack (Fig. 2A). The larva positioned itself with a lifted thorax and loose legs, and then moves the thorax in an attempt to hit the target (Fig. 2B). The jets are directed to the base of the object (Fig. 2C). After a few minutes of constant artificial attacks and intense defensive behavior of the larva, it folds its thorax back to its maximum in order to gain momentum. Soon after the larva delivers a sharp blow with the head toward the artificial predator (Fig. 2D) while still releasing secretion from labial glands and possibly also from the prosternal paired glands.

In a fourth behavior outcome, the larva resists initially to the threat. Then, it raises its thorax and directs its head to the fake predator, to regurgitate and bite the threating object.

Responses to natural stimulation

When the ant *T. melanocephalum* was used as a natural stimulus, we observed that all larval instars showed the same sequence of behavioral events described for the artificial

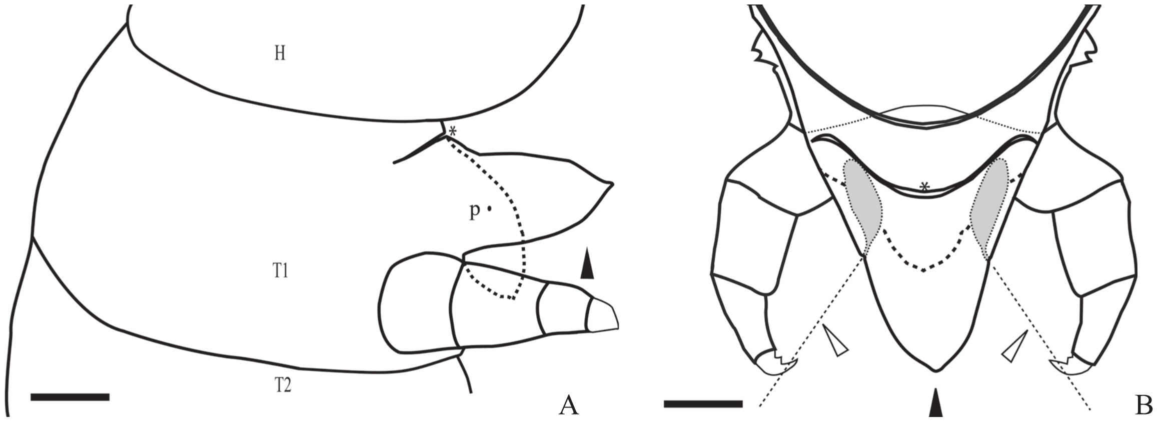

Fig. 1. Schematic representation of fifth instar prosternal glands of *Heliconius erato phyllis*. A. Lateral view of the prosternal sac during extrusion; fixed sac (dotted line) and sac in natural extrusion (closed arrowhead); head (H); prothorax (T1); mesothorax (T2), paired glandular opening (p), impair glandular opening (*). B. Antero-dorsal internal view of the prosternal sac during natural extrusion; fixed sac (dotted line) and sac in natural extrusion (closed arrowhead); prosternal paired glands (solid gray), impair glandular opening (*). The direction of the secretion release during artificial and natural attack is indicated by the open arrowhead. Scale bar = 50µm.

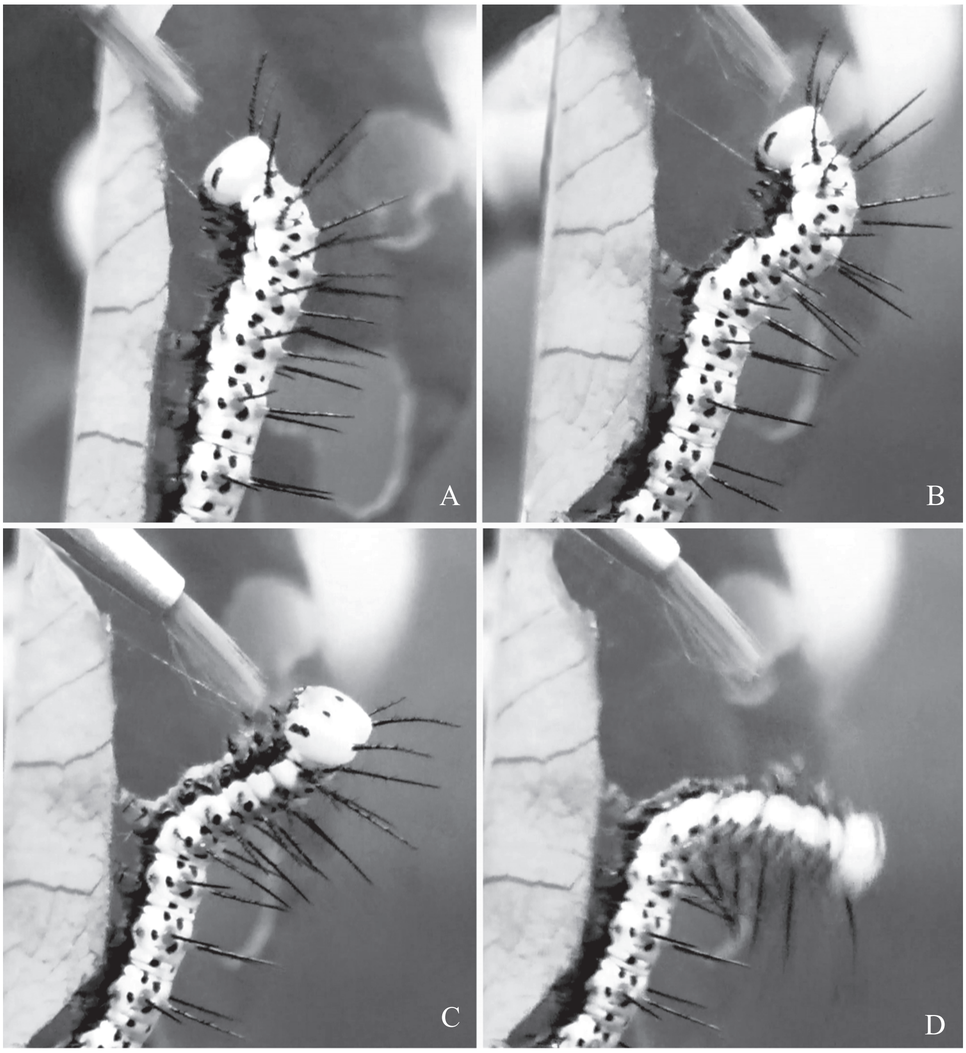

Fig. 2. Fifth instar larva of *Heliconius erato phyllis* when threatened by artificial stimuli. A. The larva releases silk threads from its labial glands to the base of the object. B. The larva raises its thorax and moves it around the target. C. The larva directs jets of the prosternal gland secretions and silk threads to the base of the object. D. The larva raises its thorax, bends it backwards, and then releases a strike in direction of the object. It also releases silk threads and possibly secretion of the prosternal paired glands.

stimulus. However, one larva of the third instar and one in the fourth instar also showed an additional behavior. After the display of the sequence of behavioral events described above, they approached the predator and with the aid of the legs wrapped the ant with threads of secretion forming a small sphere. Then the sphere was conducted with the legs and prolegs towards the last larval segment (Fig. 3).

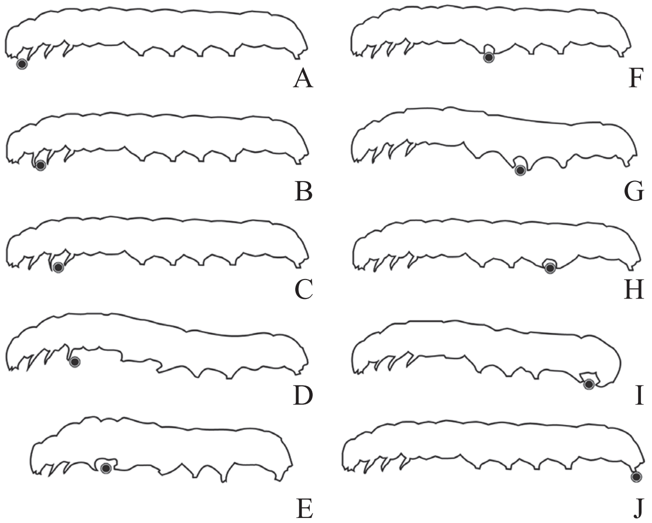

Fig. 3. Illustration of the behavior of fifth instar larva of *Heliconius erato phyllis* after facing a natural predator. The picture represents the larva conducting the silk sphere containing a worker ant with the help of its legs and prolegs towards the last larval segment (based in Vegliante & Hasenfuss 2011).

DISCUSSION

In this paper we proved the defensive behavior related to the use of prosternal paired glands in larvae of *Heliconius erato phyllis*. The prosternal glands are located within the conical integumentary sac, which in turn is situated on the individual's prosternum (Borges *et al.* 2010). To the best of our knowledge, there are no studies in other species attesting the existence of an internal gland within this structure. Therefore, in these cases we will reference only the conical integumentary sac. The conical integumentary sac is a very common structure in species of the family Nymphalidae (Peterson 1962; Stehr 1987; Miller 1991), and has a scattered systematic distribution in other groups like Yponomeutidae, Noctuoidea, Hesperiidae and Papilionoidea (Bourgogne 1951; Peterson 1962; Miller 1991; Devries & Martinez 1993). It is important to consider that the structure displays morphological plasticity in different groups, which in turn can be associated with a variety of physiological and adaptive functions as well (Vegliante & Hasenfuss 2011). In *Caligopsis seleucida* Hewitson, 1877 (Nymphalidae, Brassolinae), the conical integumentary sac is shaped as a red projection in front of the prothoracic legs (Furtado & Campos-Neto 2004). Particularly in Yponomeutidae, the structure is not protruded and opens on a tapered protrusion of the movable body wall (Povel & Beckers 1982) with a pair of muscles placed near the opening (Schaffer 1889). In other families it is an invagination of the body wall around a hole, which forms a vestibule. It can be everted by an increase in the hydrostatic pressure and retracted by the action of muscles originated in the head or in the segment T1 (Detwiler 1922; Marti & Rogers 1988). In some Noctuoidea and Nymphalidae, there are extrinsic muscles inserted in the structure (Klemensiewicz 1883; Marti & Rogers 1988) which can be actively everted by some Nymphalidae (Fanfani & Dazzini 1989). The chemical analyses that resulted from the experiments shows that the prosternal glands of *H. erato phyllis* do not produce volatiles, even when the individual in threatened. The elimination of impair gland secretion was also not observed. In other Nymphalidae species, the conical integumentary sac diffuses volatiles repellents to ants and unpleasant to human olfaction (Devries & Martinez 1993; Osborn & Jaffé 1998; Osborn *et al*. 1999). On the other hand, some representatives of the family Rodinidae use it for communication with symbiotic ants (Devries *et al*. 2004; Kaminski 2008).

In Lepidoptera larvae, the chemical composition of the secretions of the conical integumentary sac is diverse. It consists of carboxylic acids in Nymphalidae (Osborn & Jaffé 1998) or formic acid as already described in Notodontidae and Noctuidae (Marti & Rogers 1988; Nakamura 1998). In these families they can also be composed by alcohols and esters (Hallberg & Poppy 2003) or single-chain hydrocarbons and terpenes (Severson *et al.* 1991). In Heliconiinae, chemical analysis in larvae of *Dione juno* Cramer, 1779 and *Abananote hylonome* Doubleday, 1844 demonstrated the existence of defensive compounds such as acetic, linoleic and palmitic acids (Osborn & Jaffé 1998).

The observed behaviors of this study demonstrates for the first time that specifically the prosternal paired glands in *H. erato phyllis* have clearly a defensive function. This is evidenced when the larva inflates its conical integumentary sac when threatened, and then secretes against the target through the pores of the glands if the threat persists. The same pattern of behavior related to the use of the conical integumentary sac has also been described in other species of Notodontidae, Nymphalidae and Noctuidae (Klemensiewicz 1883; Detwiler 1922; Percy & Macdonald 1979; Marti & Rogers 1988; Fanfani & Dazzini 1989; Osborn *et al*. 1999). It is also known that the secretion of the conical integumentary sac is added to the silk that form the cocoons of *Cerura vinula* Linnaeus, 1758 (Notodontidae) (Byers & Hinks 1976). Our field and laboratory observations suggest that all larval instars of *H. erato phyllis* exhibit similar behavioral responses to both artificial and natural attacks. The glandular units of the prosternal paired glands can also operate in combination with the silk glands of the head. However, the success of the strategy depends on different factors like the larval instar, its physiology, size, and the number of predators. It is very likely that the emission of a discharge of silk toward the base of the object that disturbs the larva suggests an initial goal of restricting movements of the predators. The strike with the head over the immobilized predator can also facilitate the next step that consists in wrapping the prey with the secreted threads along with the aid of the legs. The conduction of the wrapped enemy toward the last larval segment through movements of the legs to the prolegs suggests that the sphere containing the immobilized predator is likely tied in a place nearby with additional threads produced by the larva's spinnerets.

According to the classification of Gross (1993), prosternal paired glands are secondary defense strategies that prevent the attack after the detection of a potential predator by the larvae. Evidence for the existence of a conical integumentary sac in larvae of other species and families of Lepidoptera allows us to propose the possibility of occurrence of prosternal paired glands with defensive function in these other groups as well. Furthermore, in these cases the possibility of the adoption of similar defensive behavior in also very plausible. We look forward for future studies where addressing this topic may help elucidate these new assumptions.

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