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Research Article

Morphology of the Prosternal Glands of *Heliconius erato* (Lepidoptera: Nymphalidae)

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Two types of exocrine glands, located midventrally on the prosternum, are described for the larval stage of *Heliconius erato* (Linnaeus) (Lepidoptera: Nymphalidae). The first type, formed by a single, flat secreting pouch, opens as a transverse slit on the anterior portion of the prosternum. The second, composed of a pair of ellipsoid secreting units, opens laterally by fine ducts on the distal portion of a cone-shaped sac, which is protruded by hemostatic pressure posteriorly between the prothoracic legs. The morphology of these glands is described and illustrated by light, scanning, and transmission electron microscopy. The varied terminologies adopted in the literature for describing these glands are discussed, and we propose a single term, prosternal glands.

1. Introduction

Heliconian butterflies have attracted the attention of biologists for many years, in particular regarding their close association with passion vines, their main host plants in the Neotropics (reviewed in [1–3]). All life stages of these butterflies are supposed to be unpalatable to vertebrates [3, 4]. Several cyanogenic glycosides have been associated with this toxicity, and could be either sequestered or modified from the host plants, or alternatively synthesized *de novo* by the larvae [4, 5]. The existence of specialized larval body structures, if any, where such chemicals are processed is largely unknown.

Chemicals associated with glandular secretions identified for these butterflies have been related to communication at mating [6–9]. The existence of exocrine glands has been reported for the adults, but not for the immature stages of heliconian butterflies. Adult males have modified scent scales (androconia) located on the hind wings [10–12], as well as typical, multicellular exocrine glands within the genitalic valvae [13, 14]. Females have a pair of dorsal abdominal glands on the eighth tergum, which are usually associated with stink clubs (auxiliary glands) that are attached to a lateral fold on the posterior margin of the eighth sternum [10, 12–15]. These abdominal glands were originally presumed to be associated with defense in both sexes [10, 16]. Lately, however, they have also been related to the production (males) and storage and dispersal (females) of antiaphrosidiacs [8, 17, 18].

Prosternal glands are found in the larval stage in certain lepidopteran families, including Nymphalidae [19–22]. There is no consensus regarding their precise position in the larval body, except that they are located midventrally just posterior to the head, on either the cervix or prothorax. The terminology that has been adopted to describe these glands is also inconsistent. They show considerable variation

regarding their glandular units; and the corresponding homologies among lepidopteran families, if any, have not been established [21, 23]. Additionally, their function has been little explored; in some notodontid moths, these glands secrete a fluid of defensive nature [24–26]; and in some riodinid butterflies, they have been recently associated with larval-ant communication [27, 28]. Our observations suggest that they are frequently found in all instars of heliconian butterflies. Their description, which is the main objective of the present paper, is a prerequisite for future studies on the physiology and behavior involving these glandular structures, in order to fully understand their chemistry and function.

Heliconius erato (Linnaeus) (Lepidoptera: Nymphalidae) is one of the most common and well-studied heliconian butterflies in southern Brazil, where it has been used as a model in studies of evolutionary ecology (e.g., [18, 29–32], and references therein). The external morphology of its immature stages has been described in detail elsewhere [33], but the prosternal glands were not included in that study. Here, we describe and illustrate them based upon light, scanning, and transmission electron microscopy. We show that in *H. erato*, these glands are not simple eversible structures located within the integumentary infold, but are a glandular complex consisting of an assemblage of morphologically distinguishable glandular units. In addition, we discuss the limitations of the terminology that has been generally applied to these glands, and propose an appropriate unified term—prosternal glands.

2. Material and Methods

The study was conducted with larvae hatched from eggs collected from a *Heliconius erato phyllis* (Fabricius, 1775) outdoor rearing insectary at the Departamento de Ciências Morfológicas, Instituto de Ciências Básicas da Saúde, Federal University of Rio Grande do Sul (UFRGS), Porto Alegre, RS. The rearing procedures have been described in detail elsewhere [30]. Adults were fed daily with a mixture of commercially available honeybee pollen (AGA), honey (AGA), and distilled water (ratio 2 : 1 : 7). *Passiflora suberosa* (Linnaeus) (Passifloraceae) plants were grown within the insectary for oviposition. Under laboratory conditions, larvae were separately reared on intact *P. suberosa* shoots in bottles of water protected by a fine-mesh cloth [34]. Instars were identified by their head-capsule width [33]. To make sure that molts were not overlooked, larvae were gently marked with small dots of enamel paint (Testor) on the dorsal part of the ninth segment [35].

The gross morphology of the prosternal gland was studied primarily on fresh material. For dissections, the material was immersed in Ringer's solution and temporarily stained with methylene blue. Specimens previously fixed with Dietrich's fluid and preserved in 75% ethanol were also used. Prothoracic ventral portions (5 per instar) were dissected, cleared in a 10% potassium hydroxide solution (KOH), and slide-mounted in glycerin jelly. The structures were observed under a Leica M125 stereomicroscope, and photographed with an attached Sony DSC-H10 digital camera. An attached ocular grid was used to aid in the drawings.

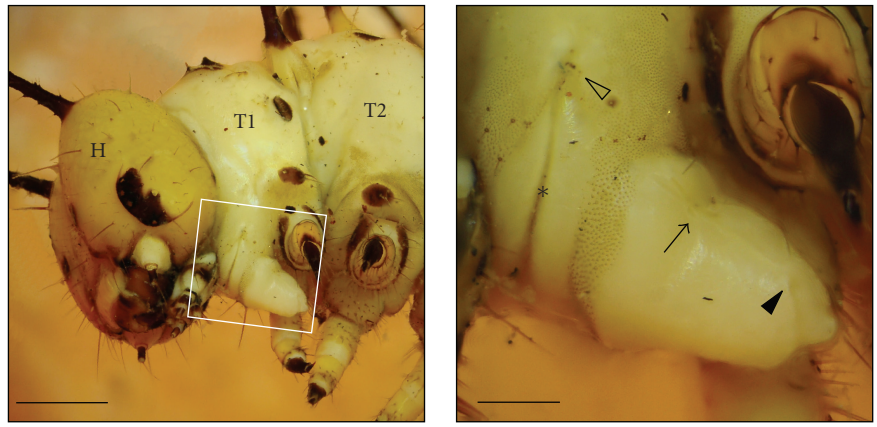
For histological and cellular studies by light microscopy, fresh prothoracic ventral portions ($n = 10$ per instar) were dissected and fixed with Bouin's fluid. For sectioning, a standard paraffin embedding method was employed. Sections 7 μm thick were obtained with a Leica RM2155 microtome. The sections were stained with Gill's hematoxylin and eosin and mounted in Canada balsam.

The integumentary ultrastructure of the prosternal glands was studied at the UFRGS Electron Microscopy Center. For scanning electron microscope analyses, the specimens were dehydrated in a Bal-tec CPD030 critical-point dryer, mounted with double-sided tape on metal stubs, and coated with gold in a Bal-tec SCD050 sputter coater. Specimens were examined and photographed in a JEOL JSM5800 scanning electron microscope. For transmission electron microscopy, the specimens were fixed in a mixture of 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer. Next, the material was washed in the same buffer, postfixed with 1% osmium tetroxide for 1 h, dehydrated in an ascending series of alcohol and acetone, preembedded in epoxy resin and acetone (1 : 1), and finally embedded in epoxy resin (Durcupan ACM, Fluka). The material was then polymerized for 3 days at 68°C. Semithin sections (1 μm) were cut with a Leica UCT ultramicrotome, using glass knives, and stained with 1% toluidine blue in 1% sodium tetraborate. Ultrathin sections (70 nm) were obtained with the same ultramicrotome, employing a diamond knife (Diatome). These sections were stained with 2% uranyl acetate, followed by 1% lead citrate [36]. The ultrathin sections were examined using a JEM 1200 EX II transmission electron microscope.

3. Results

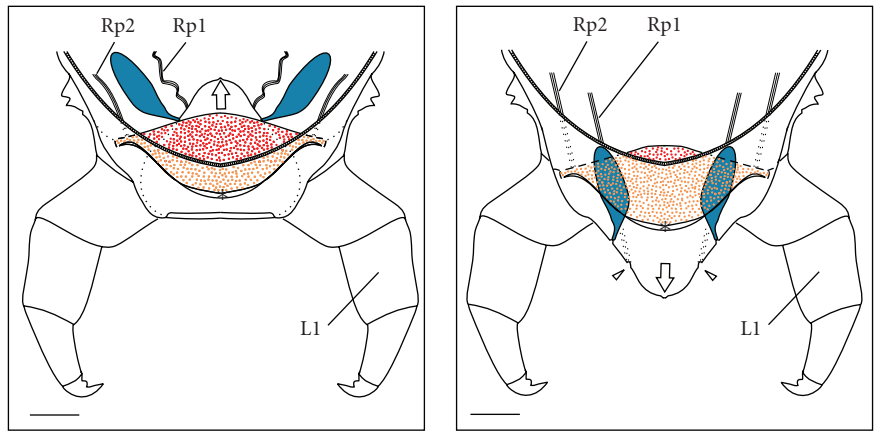
The glands are located ventrally on the prosternum (Figure 1). There are three units and two morphological types of glands, hereinafter called impair and paired glands (Figure 2(a)). The first type, composed of a single, flat secreting pouch, opens as a transverse slit in the anterior portion of the prosternum. The second, composed of a pair of ellipsoid secreting portions, opens laterally through fine ducts in the distal portion of each side of a conical integumentary sac (Figures 1(b) and 2(b)). By hemostatic pressure, the sac can be protruded posteriorly between the prothoracic legs (Figures 1(a) and 2(b)). The sac containing the attached paired glands is inverted and contracted back into the thoracic hemocoele by a pair of retractor muscles (Figure 2, Rp1).

Both types of glands are found in all larval instars, and apparently show negligible changes in shape during ontogeny. Except for the first instar, when they are small, the secretory portion of the impair gland is not everted (Figures 3(c) and 4(c)). The impair gland as a whole is pressed down by hemostatic pressure and pulled up by the action of an additional pair of retractor muscles (Figure 2; Rp2). When



(a) (b)

Figure 1: Latero-external view of the larval prosternal glands of the fifth instar of *Heliconius erato phyllis*, under light microscopy. (a) Midventral location in relation to the body (in rectangle). (b) Lateral view in detail (enlarged rectangle shown in (a)), indicating the openings of the impair (asterisk) and left paired (arrow) glands, and the insertion positions of the corresponding retractor muscles (open and closed arrowheads, respectively). (H) head; (T1) prothorax; (T2) mesothorax. Scale bars = 200, 100 μm, respectively.



(a) (b)

Figure 2: Schematic representation of fifth instar larval prosternal glands of *Heliconius erato phyllis*, from an antero-dorsal internal view, when in situ (a) and during extrusion of the prosternal sac (b). Impair and paired glands are shown in stippled red and solid blue, respectively. Open arrows indicate the direction of the internal hemostatic pressure and the respective movement of the prosternal sac. Impair and paired glandular openings are indicated by one asterisk and open arrowheads, respectively. (L1) prothoracic leg; (Rp1) proximal retractor muscle of the prosternal sac; (Rp2) distal retractor muscle of the prosternal sac. Scale bar = 50 μm.

protruded in the first instar, it appears as a bud, showing a medially located, little-differentiated slit that divides its secretory portion transversely into two lips (Figure 4(c)). The secretory portions of the paired glands are not everted from the sac itself in any instar (Figure 2(b)).

The openings of the paired glands are simple, each appearing from the outside of the everted sac as a small, delicate infold (Figure 1(b)). In contrast, the opening of the impair gland is proportionately large and elaborate. Its margin shows several sensillum-like structures (Figures 4(a) and 4(b); Se), which function remains unknown. In specimens fixed in Dietrich's fluid and preserved in ethanol, the secretion of the impair gland is yellowish, appearing solidified and in considerable amounts as small individual fragments, on the cuticular surface of the secreting epithelium. Under scanning electron microscopy, this secretion appears as small spots that exude from many microcisterns that cover its cuticular surface (Figure 4(d)). The secretion of the paired glands is amorphous and acidophilous, and is stored in their central spherical lumen (Figures 3(e) and 3(f)).

The secreting nature of the two types of gland is clearly shown by the columnar shape of their epithelium cells (Se), which contrasts with the flat cells that form the remaining, nonsecreting epithelium (Ne) of the sac wall (Figure 3). The impair gland is formed by a simple, low-columnar, glandular epithelium. The secretion is expelled directly by the cuticle, through cisterns on its external

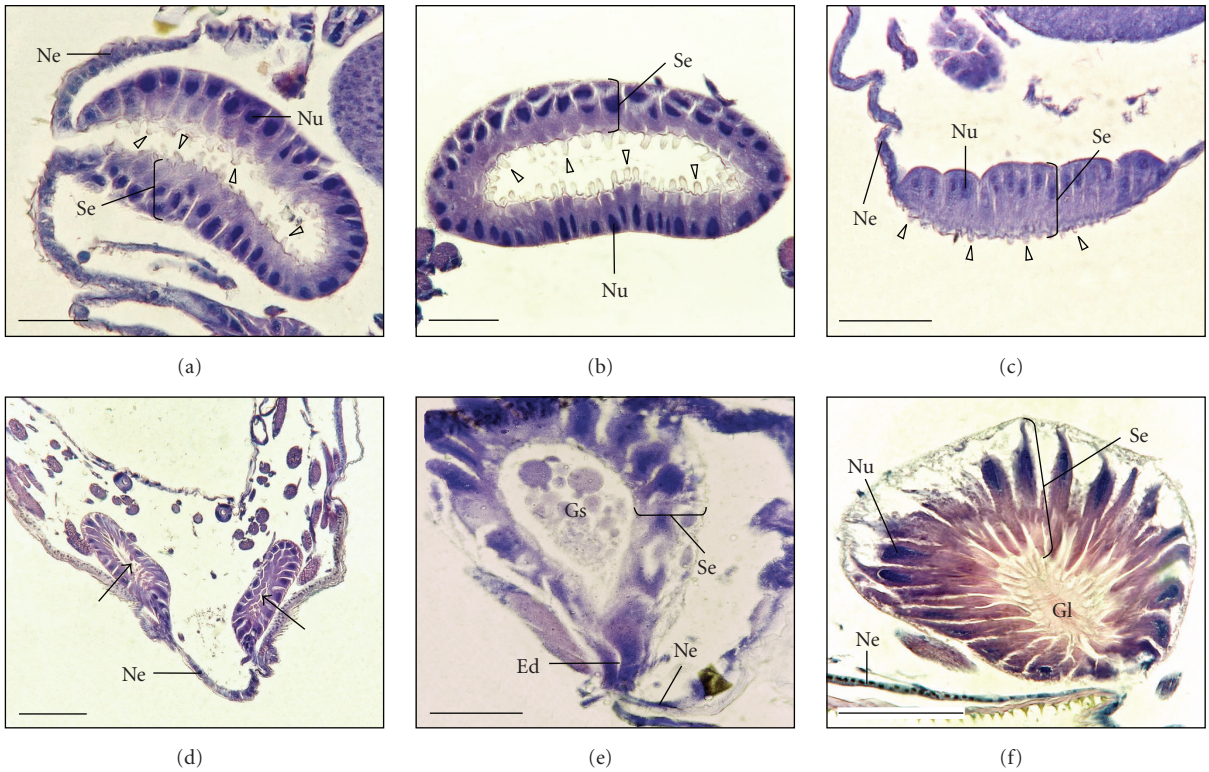


Figure 3: Histological sections of larval prosternal glands of *Heliconius erato phyllis* under light microscopy. (a) impair gland of second instar, longitudinal; (b) impair gland of third instar, cross section; (c) impair gland of first instar, longitudinal; (d) paired glands (arrows) of third instar, longitudinal; (e) paired gland of fourth instar, longitudinal, near the external opening, showing secretion in the lumen; (f) paired gland of fifth instar, cross section, at the middle of the secretory portion, showing the convergent distribution of secreting cells in relation to the lumen. Open arrowheads indicate the excretory cisterns in the impair gland. Ed: excretory duct; Gl: glandular lumen; Gs: glandular secretion; Ne: nonsecretory epithelium; Nu: nucleus; Se: secretory epithelium. Scale bars = 50, 50, 50, 150, 60, and 150 μm respectively.

surface (Figures 3(a), 3(b), 3(c), 4(c), and 4(d)). The apical portion of the epithelium secretory cells of this gland shows numerous infoldings (= microvilli) into which the secretion is conducted intracellularly prior to excretion (Figure 5(a)). The nuclei of the secretory cells are elongated and basally located, and contain evident heterochromatin and nucleoli. The distal portion of their cytoplasm shows a well-developed granular endoplasmic reticulum, Golgi apparatus with dilated cisterns, and abundant secretion-containing vesicles (Figures 5(c) and 5(d)). The cuticular surface of the impair gland cisterns is irregular, formed by microtrabeculae that delimit alveoli of variable size and shape (Figure 4(d)). The cisterns decrease in number and increase in size per cell in later instars (Figures 5(a) and 5(b)).

The paired prosternal glands are formed by a high-columnar, glandular epithelium with concentrically arranged cells (Figure 3(f)). The cells have an acidophilous cytoplasm containing a conspicuous, basally located nucleus with evident heterochromatin and nucleoli. An excretory duct (Ed) is formed in these glands (Figure 3(e)), through which the acidophilous secretion is excreted. The excretory ducts are formed by a cubical epithelium, which contrasts with that of the secretory portion of the gland and the flattened part that forms the sac wall as a whole.

4. Discussion

The general morphology of the prosternal glands described herein may not be unique. In the entomological literature, the prosternal glands found in lepidopteran larvae are usually poorly described, as single sacs that are everted by hemostatic pressure (e.g., [19, 20, 37, 38]). Our results clearly showed that they are not located within a single integumentary sac that contains a secreting epithelium that is everted by hemostatic pressure; in other words, the sac is not the gland itself. In *H. erato*, the prosternal glands form a glandular complex, composed of three glandular units of two distinct morphologies. The impair type is located outside the sac, and is not everted. In addition, we demonstrated that although the existing sac itself is eversible, the paired glands located inside are not. Percy and MacDonald [24] arrived at a similar conclusion regarding the internal complexity of these structures in *Schizura concinna* (Notodontidae). However, the two glandular units that are found in this species differ from each other in their general morphology, being interconnected by an interglandular neck, and thus their final product is a mixture of secretions. This is not the case for *H. erato*, where the impair and paired gland types open independently to the outside. Also, their excretions differ

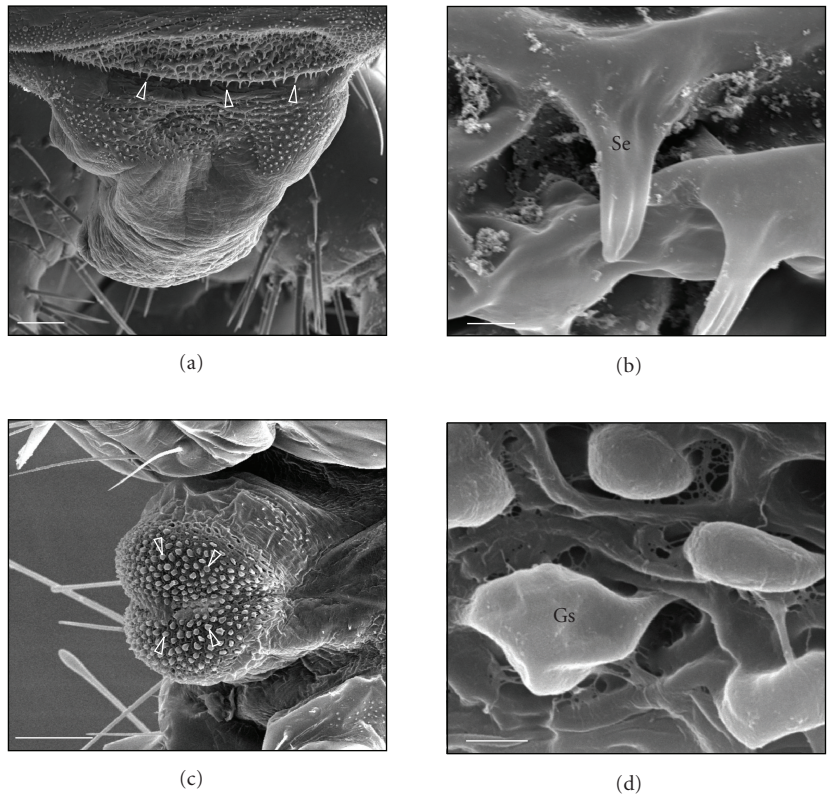


Figure 4: Impair prosternal gland of *Heliconius erato phyllis* under scanning electron microscopy. (a) Detail of the transverse opening slit in a fifth instar, anterior view; (b) sensilla on the opening margin (indicated by open arrowheads in (a)); (c) detail of a protruded gland in a first instar, latero-ventral view; (d) cisterns in detail, showing exudation (indicated by arrowheads in (c)). Gs: glandular secretion; Se: sensillum. Scale bars: 100, 5, 100, 2 μm, respectively.

in physical consistency and color, and probably also in the amount of secretion produced. The existence of noneversible units of the prosternal glands was previously detected in *Spodoptera frugiperda* (Noctuidae) [39]. Again, when the general description given for this species is compared with that of the present paper, it is clear that although the glands are also located in the prosternum, the tubular glands described are not homologous to the prosternal glands of *H. erato phyllis,* where such tubules are absent. These aspects should be taken into account in the search for homologies among the prosternal glands of different lepidopteran taxa, and equally importantly, in identifying their secretions. In the case of *H. erato phyllis*, the existence of differences in chemical constitution between the secretions of the impair and paired glands is very likely, since they differ in color and physical consistency.

The function of the prosternal glands also remains unknown for *H. erato phyllis*. They might be involved in defense, as previously suggested for notodontids [24–26]. Larvae of *H. erato phyllis* are solitary feeders, behave aggressively toward other heliconian larvae, and are cannibalistic [1, 40, 41]. The early stages of heliconians in general are preyed upon by ants, against which they have developed complex defense mechanisms [42–44]. When the anterior portion of its body is gently touched, the larva of *H. erato phyllis* assumes a defensive posture, moving its head and elevating its front legs, and protruding the sac containing the paired prosternal glands.

At the microscopic level, the gland cells studied here are similar to those described for *S. concinna* [24] and *Abananote hylonome* (Nymphalidae) [26]. They fit into type I in the classification of Noirot and Quennedey [45, 46], where the gland cells are in direct contact with the cuticle. We found no perforations in the cuticular layers of cells of the impair type, and therefore we hypothesize that the secretion diffuses through the cuticle, as in the defensive glands of many other insects [24, 45]. The presence of microvilli on the apical surface of their cells, together with the abundant secretion vesicles, lends further support to this suggestion. Microvilli facilitate transport of secretions from the basal portion of the cell into the cuticle. In the case of *H. erato phyllis*, transport might be facilitated in the central area of the cisterns, where the corresponding cuticular layer is thinner and the secretion accumulates on the glandular surface.

Revised Terminology. Several terms have been used more or less interchangeably to identify the glands described herein, including "cervical" [21, 27, 28], "neck" [26], "ventral" [37], "thoracic" [24, 38], "prothoracic" [25, 47, 48], "eversible" [19, 20] gland(s), and "adenosma" [21, 49]; and also in combination (e.g., "ventral prothoracic" [50]). "Cervical"

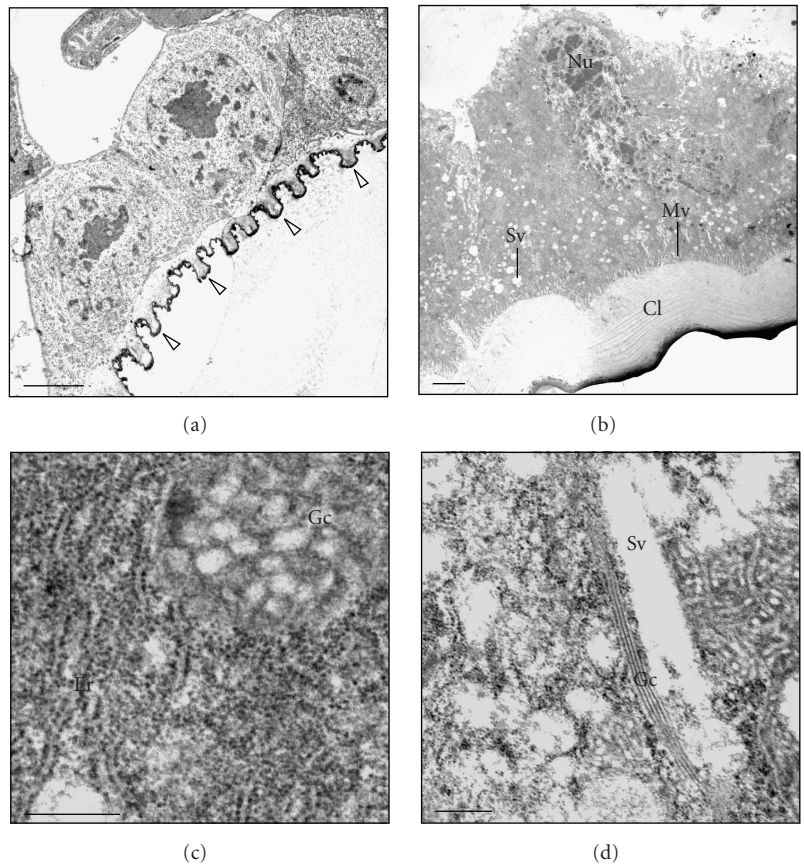


Figure 5: Transmission electron micrographs of the impair prosternal gland of *Heliconius erato phyllis*. (a) longitudinal section of secreting cells, showing irregular external sculpture of cuticle (open arrowheads) in a first instar; (b) longitudinal section of secreting cells of a fifth instar, with basal nucleus, several vesicles containing secretion in the cytoplasm, and abundant microvilli associated with the cuticular layers; (c) detail of the cytoplasm of cells from a fifth instar, showing well-developed rough endoplasmic reticulum and Golgi complex; (d) detail of the cytoplasm of cells from a fifth instar, showing numerous secretion vesicles. Cl: cuticular layers; Er: rough endoplasmic reticulum; Gc: Golgi complex; Nu: nucleus; Mv: microvilli; Sv: vesicle containing secretion. Scale bars: 2, 2, 0.4, 0.4 μm, respectively.

and "neck" are not appropriate to describe these glands, because they are situated within integumentary infolds that are located midventrally, not on the cervix (= neck, the membranous region located between the head and the prothorax [51]), but rather on the prothorax. "Ventral", "thoracic", and "prothoracic" are ambiguous, leading to confusion regarding the specific body site where these structures are located in relation to the body tagmata, thoracic segments, and prothoracic sclerites, respectively. In particular, the usage of "prothoracic" may lead to confusion with the "osmeterium" glands [52], which are also located on the prothorax, but dorsally on the tergum (= pronotum). Moreover, this term has been traditionally adopted in the entomological literature for the endocrine glands involved with hormone secretion (ecdysone), which are located on the same thoracic segment [53–55]. The term "eversible" is also inappropriate, because, as described in this paper, the secretory units of the glands themselves are not always everted. "Adenosma" suggests a gaseous nature of their secretion and associates the sense of smell with it (in Greek: adeno = gland; osma = odor), which cannot be generalized for all situations, for example in the case described herein. The use of the composite term "ventral prothoracic" is redundant (= prosternal). Thus, we propose "prosternal glands" as best suited to describe this complex assemblage of glandular units (as a broad definition, *sensu* Noirot and Quennedey [42]). For *H. erato*, we propose the lexicon paired and impair prosternal glands to demarcate the two types. The term prosternal glands relates them to the prothoracic sternum (= prosternum [56]), the body site where they are in fact located. Also, this term does not imply any particular number or shape of their secretory units, nor the chemical nature and function of their secretion.

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