



# The cranial apparatus glands of the canthariphilous *Pyrochroa coccinea* (Coleoptera: Pyrochroidae: Pyrochroinae), and their implications in sexual behaviour

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## ABSTRACT

Some Pyrochroidae species are known as “canthariphilous” for their attraction to cantharidin (CTD), a toxic terpene with anti-predatory effects, produced in nature by only two beetle families (Meloidae and Oedemeridae). It has been demonstrated that males of *Neopyrochroa flabellata* ingesting CTD are positively selected by females. Indeed, the compound is re-emitted from a glandular cranial apparatus as secretions that are licked up by females during courtship behaviour, inducing copulation. Herein, we provide the first description of the glands associated to the cranial apparatus of male Pyrochroinae using the European species *Pyrochroa coccinea* as a model. Morphological analyses show that the cranial apparatus consists of a concave pit lined with short setae retaining secretions emitted through numerous glandular pores. Ultrastructural investigations reveal the presence of two different class 3 glands (GLA and GLB), intermixed at the level of the pit but exhibiting distinct features. GLA are mainly characterised by short conducting canals, rounded nuclei and electron-dense vesicles while GLB are characterised by long conducting canals, irregular nuclei, vesicles containing a particulate substance and a multifolded plasma membrane. Observations of sexual behaviour are also reported for *P. coccinea* and compared to *N. flabellata*, confirming the involvement of cranial apparatus secretions in courtship behaviour.

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## 1. Introduction

Semiochemicals are chemical compounds used in communication between organisms of the same species (pheromones) or of different species (allomones if adaptively favourable to the emitter, kairomones if adaptively favourable to the receiver, or synomones if adaptively favourable to both the emitter and the receiver) (Dicke and Sabelis, 1988). These compounds might be either endogenous or exogenous (Reddy and Guerrero, 2004). In some cases, the same compound can act in different ways, depending on the organisms involved in the interaction. This is the case of cantharidin (CTD), a terpene produced in nature only by blister beetles (Coleoptera:

Meloidae) and false blister beetles (Coleoptera: Oedemeridae) (Carrel and Eisner, 1974; Carrel et al., 1986). Due to its high toxicity, CTD acts as a potent feeding deterrent against possible predators and parasites in all phases of beetle development (e.g., Carrel and Eisner, 1974; Smedley et al., 1996; Carrel, 1999). It has been demonstrated that females receive CTD from males as nuptial gift during copulation (especially in blister beetles) and transfer the compound to the eggs (Holz et al., 1994; Nikbakhtzadeh et al., 2007a; Hashimoto et al., 2016), but it is not totally clear if CTD acts as sexual pheromone during courtship behaviour of CTD-producing species (Nikbakhtzadeh et al., 2007b, 2008).

Nevertheless, despite its toxicity and repellent effect, CTD is greatly attractive to some arthropod species known as “canthariphilous species” (Hemp and Dettner, 2001). Some of them can detect CTD-producing beetles and sequester the compound to use it in turn as a defensive chemical (Schütz and Dettner, 1992; Frenzel and Dettner, 1994; Holz et al., 1994; Eisner et al., 1996a, 1996b;

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Molfini et al., 2022).

Canthariphily is particularly common within the two beetle families most closely related to Meloidae: Anthicidae and Pyrochroidae (Hemp and Dettner, 2001; McKenna et al., 2019). In these species, CTD seems to act as a close-range sexual pheromone playing a crucial role in the sexual selection (Schütz and Dettner, 1992; Eisner et al., 1996a; Hemp et al., 1997). Although the role of CTD has only been clearly demonstrated in only a few species, the observed association of CTD with specialised glands localised in different male anatomical structures has led to generalise this assumption for all species with such structures. In Anthicidae, these latter correspond to peculiar elytral notches present in males of several Anthicinae and Notoxinae (e.g., Kejval and Chandler, 2020); while in fire-coloured beetles (Pyrochroidae) they correspond to a peculiar “cranial apparatus” occurring in males of several genera of the subfamily Pyrochroinae (e.g., Young, 2019). In the past, because of its morphological variability, this structure has been called by various terms such as: frontal excavations (Blair, 1914), excavations (Kôno, 1929), cranial pits (Young, 1975), frontal cleft or glandular cleft (Eisner et al., 1996a), until the more inclusive term “cranial apparatus” (Young, 2004). In this work we used the term “cranial apparatus”, referring not only to the excavations but also to the associated external hairs and internal glands.

Although canthariphily was observed in nine species of different genera of Pyrochroinae (*Pyrochroa*, *Pseudopyrochroa*, *Neopyrochroa*, and *Schizotus*) (Young, 1984a, 1984b; Holz et al., 1994; Nardi and Bologna, 2000; Scheffler, 2013; Hashimoto and Hayashi, 2014, 2016; Horiuchi et al., 2018; Molfini et al., 2023), CTD has been clearly demonstrated to act as a close-range sexual pheromone only in *Neopyrochroa flabellata* (Eisner et al., 1996a); while the intersexual transfer of this terpene from male to female and then to eggs was demonstrated in both *N. flabellata* and *Schizotus pectinicornis* (Linnaeus, 1758) (Holz et al., 1994; Eisner et al., 1996b). After CTD ingestion, males of *N. flabellata* can release the terpene both through the reproductive system and the cephalic glands of the cranial apparatus. During courtship, the female samples the male cranial apparatus with her mouthparts and mates preferentially with males that had fed on CTD (Eisner et al., 1996a). This scenario implies the presence of a specific mechanism that allows transporting the ingested CTD to the target structures, though this is still unknown.

All the European species of *Pyrochroa* are known to be canthariphilous as attracted to blister beetles: *Pyrochroa coccinea* (Linnaeus, 1761) has been observed feeding on individuals of *Meloe brevicollis* Panzer, 1793, *Meloe proscarabaeus* Linnaeus, 1758, and *Meloe violaceus* (Marshall, 1802) (Lückmann, 1999; Lückmann and Niehuis, 2009; Scheffler, 2013); *Pyrochroa serraticornis* (Scopoli, 1763) was observed on *M. violaceus* and *M. proscarabaeus* (Bologna and Havelka, 1985; Nardi and Bologna, 2000), and *Pyrochroa bifoveata* Molfini, Mancini & Bologna, 2022 was observed on *Meloe cicatricosus* Leach, 1815 (Molfini et al., 2023) and probably on *Meloe violaceus* and *M. proscarabaeus* (in fact reports of *P. serraticornis* in Lückmann and Niehuis, 2009 could be attributed to the recently described cryptic species *P. bifoveata*).

Past studies have mainly focused on either the presence/absence of the cranial apparatus, on its external shape, and on the functional and chemical aspects of its secretions (Eisner et al., 1996a). Conversely, fine morphology and ultrastructural details of the glands associated with the cranial apparatus have never been investigated in any species of Pyrochroinae.

In the present study we describe for the first time the anatomy and fine morphology of the cranial apparatus glands of a fire-coloured beetle, analysing as a model the European species *P. coccinea*. Additionally, in light of the documented canthariphily of the species (Lückmann, 1999; Lückmann and Niehuis, 2009;

Scheffler, 2013), we assess the presence of ultrastructural features of the frontal glands that might be suggestive of CTD uptake from haemolymph. Furthermore, behavioural observations performed in captivity, demonstrate for the second time that the cranial apparatus is involved in the courtship behaviour of Pyrochroinae.

## 2. Material and methods

### 2.1. Examined material

Adult males of *P. coccinea* were collected on flowers and leaves of riparian Apiaceae and Urticaceae within deciduous riparian forests. A total of six males were collected: four near the banks of the Cremera river (Italy, Latium, Roma, Formello, 42.253442 N, 12.064791 E) on May 4th, 2020, and two near the banks of the Biedano stream (Italy, Latium, Viterbo, Barbarano Romano, 42.102447 N, 12.396114 E) on May 18th, 2020. Samples collected in different locations showed no morphological differences in the cephalic glands according to the used methods (see below).

Additionally, mature larvae of *P. coccinea* were collected in an urban natural area by searching beneath bark of broadleaved trees in February and March 2021 (Italy, Latium, Roma, Riserva Naturale dell'Insugherata, 41.957778 N, 12.432222 E). Once morphologically identified (Molfini et al., 2021; Molfini and Bologna, 2023), the larvae were individually reared in Petri dishes (90 × 20 mm) on their natural substrate until adult eclosion to conduct courtship observations.

### 2.2. Light microscopy

For histological analysis, the same resin-embedded specimens prepared for electron microscopy (as described below) were cut into 1 µm thin sections using a glass knife on an Ultracut T ultramicrotome (Leica Microsystems, Vienna, Austria). The resulting semi-thin sections were stained with 1 % toluidine blue and were observed and photographed with a BX51 optical microscope (Olympus, Tokyo, Japan) equipped with an OM-D E-M5 digital camera (Olympus, Tokyo, Japan).

### 2.3. Scanning electron microscopy

Four males of *P. coccinea* were analysed by scanning electron microscopy to investigate the external morphology of the cranial apparatus and the fine morphology of its glands. After being sacrificed, one specimen was left intact while the others were dissected as follows under a SZX16 stereo microscope (Olympus, Tokyo, Japan). Heads were cut and mouthparts were removed using a combination of fine scissors and forceps, two heads were further cut longitudinally into halves. Samples were then placed in boiling KOH until complete soft tissue digestion. Subsequently, the lower part of the cephalic capsule was removed with scissors while head components covering and concealing the glands (e.g. tracheal tubes) were carefully removed with forceps, seeking not to compromise gland integrity. Dissected specimens were dehydrated in a series of ethanol at increasing concentrations (30 %, 50 %, 70 %, 85 %, 95 %, 15 min each and 100 % for 1 h) and critical point dried using a CPD 030 unit (BalTec, Balzers, Liechtenstein). Dehydrated samples were mounted on the aluminium stub using double-sided conductive adhesive carbon disks, gold coated using a K550 sputter coater (Emittech, Kent, UK) and finally examined under a Gemini 300 field emission SEM (Carl Zeiss AG, Jena, Germany) at the electron microscopy laboratory of Roma Tre University (LIME, Roma, Italy). Microphotographs were acquired detecting secondary electrons and using a voltage between 1 and 5 kV.

#### 2.4. Focused ion beam/scanning electron microscopy

Two male specimens of *P. coccinea* were anaesthetised with CO<sub>2</sub>, their heads were quickly removed under a SZX51 stereo microscope (Olympus, Tokyo, Japan) and immediately immersed in a mixture of 2.5 % glutaraldehyde and 2 % paraformaldehyde dissolved in 0.1 M cacodylate buffer (pH 7.4) for 12 h at 4 °C. Subsequently, while maintaining a temperature of 4 °C, the samples were rinsed in the aforementioned buffer, post-fixed in 1 % osmium tetroxide for 90 min and en-bloc stained with UranylLess (Electron Microscopy Sciences, Hatfield, PA, USA) for 90 min. Finally, the heads were dehydrated in a graded series of ethanol (70 %, 85 %, 95 %, 30 min each and 100 % for 2 h), embedded in epoxy resin (Sigma–Aldrich, Burlington, MA, USA) and polymerised for 72 h at 60 °C. Sequential slices, approximately 15–20 µm thick, were produced from the resin-embedded specimens at the level of the cranial apparatus by means of a glass knife mounted on an Ultracut T ultramicrotome (Leica Microsystems, Vienna, Austria). These thick slices were mounted to aluminium stubs with double-sided conductive adhesive carbon disks, coated with a thin layer (30 nm) of gold using a K550 sputter coater (Emithech, Kent, UK), and analysed with a Dual Beam (FIB/SEM) Helios Nanolab 600 (FEI Company, Hillsboro, USA) at the LIME laboratory following the “Slice&Mill” method (Di Giulio and Muzzi, 2018), which proved successful for the analysis of several insect tissues (Muzzi and Di Giulio, 2019).

#### 2.5. Courtship observation settings

Once pupated, specimens collected as larvae were moved to new Petri dishes kept moist by a wet cotton wad. After eclosion, two males and two females were maintained individually on a diet of 10 % sucrose solution for five days. Then, a coverslip containing *ad libitum* (50 mg) crystalline CTD was offered to each male (Eisner et al., 1996a). After 1 h, each CTD-fed male was moved in an open Petri dish with a female, and interactions between sexes were recorded.

### 3. Results

#### 3.1. Fine morphology and ultrastructure of the cranial apparatus

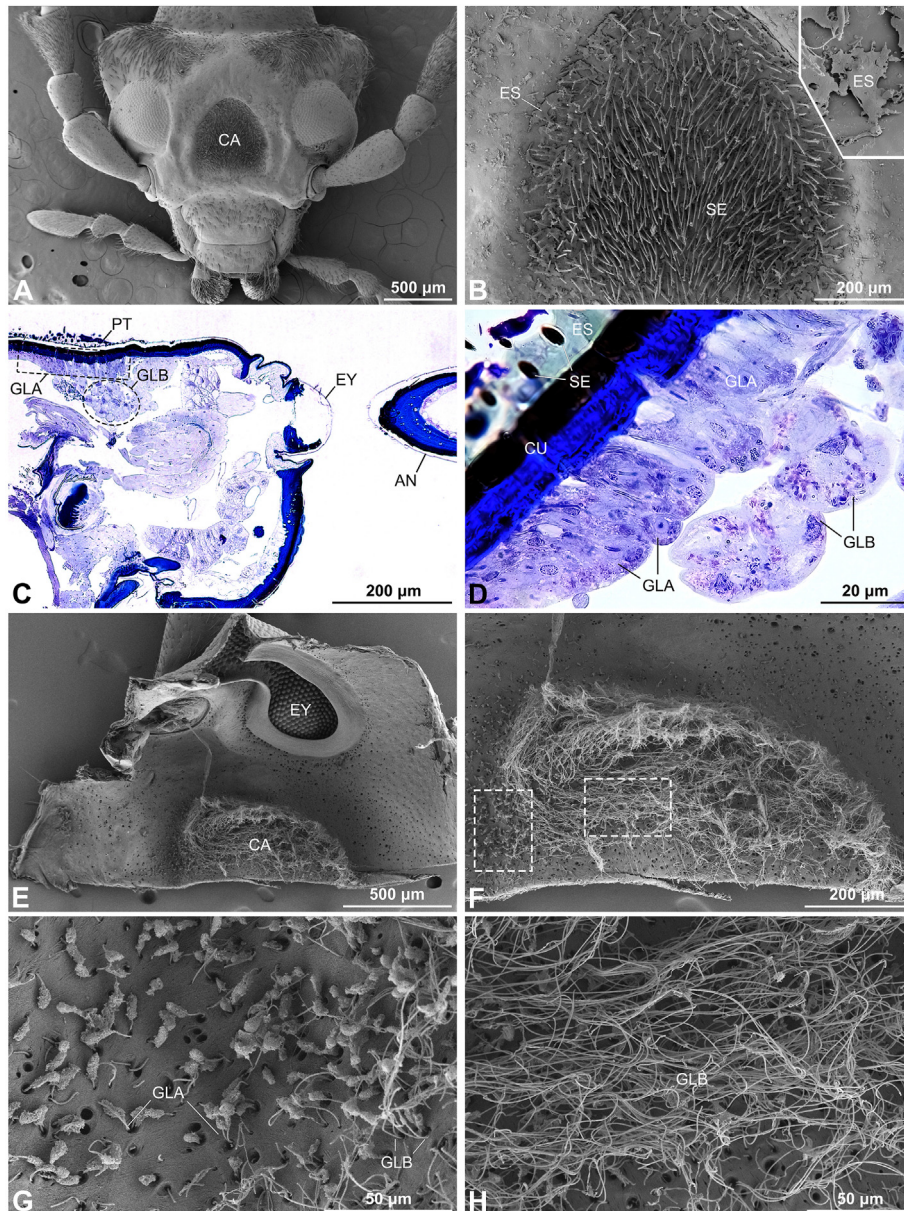
Males of *P. coccinea* have a cranial apparatus in the frontal region that consists of a single pit, located at the interocular level, distinctly impressed and of moderate depth (Fig. 1). The cranial apparatus shows a dense array of evenly sized setae, forwardly decumbent in the central region of the pit and suberected in its marginal areas (Fig. 1A and B). The analysis of the KOH digested heads (Figs. 1 and 2) shows the occurrence of a multitude of cuticular ducts entering and passing through the cuticular wall of the cranial apparatus (Fig. 1E and F). In the area immediately underneath the pit, two different types of cuticular ducts can be distinguished, both referable to the conducting canal of an ectodermal gland of class 3 and thus representing the component responsible for conveying the substances produced by the associated secretory cells. The first type of gland, Gl.A, is characterised by short canals with a length between 8 and 20 µm (Figs. 1G, Fig. 2A–D); while the second one, Gl.B, has distinctly longer canals ranging from 120 to 160 µm (Figs. 1H, Fig. 2E–G). The short canals usually penetrate the cuticular wall individually at the level of round cuticular perforations with a diameter of 1–2 microns and only rarely insert in groups that are at most limited to only 2 or 3 units (Fig. 2A and B). The long canals, on the other hand, insert into the cuticle in clusters of 4–6 units at the level of rounded perforations measuring 4–6 µm in diameter, and only rarely occur individually within smaller rounded perforations of approximately 1 µm in diameter (Fig. 2E and F). Both types of conducting canals

have a constant diameter of approximately 1 µm and are tubular along their entire length, from their insertion into the cuticular wall up to their base, corresponding to the permeable receiving canal. In the short canals it is common to observe partially undigested cellular material encircling part of the receiving canal and corresponding to the end apparatus remnants (Fig. 2A, C). The medial region of the pit shows both types of canals interspersed, with a predominance of the longer ones. Conversely, in the restricted region of the pit, located in correspondence to the anterior part of the frons, only shorter canals are found (Fig. 1D, F).

Histological and ultrastructural analyses (Fig. 1B and C, Figs. 3 and 4) confirm that the two different types of cuticular ducts detected in the cranial apparatus can effectively be ascribed to two different types of ectodermal glands of class 3, in accordance with the standard classification by Noirod and Quenedey (1974, 1991). These consist of bicellular units made up of a larger secretory cell and a duct forming cell that carries the secretions externally.

The secretory cells of Gl.A, associated with shorter canals, are columnar in shape and are arranged in a compact layer, tightly adherent to the inner cuticular layer of the pit (Fig. 1C and D, Fig. 3A). These cells are elongated, with a length of roughly 30 µm and a width of 9 µm. A single rounded nucleus of approximately 4 µm in diameter is located in the basal region of the cell (Figs. 1C and 3A), while a large extracellular cavity (varying in length from 8 to 20 µm) can be observed in its apical region (Fig. 3A, B and D). The extracellular cavity is usually surrounded by several slightly electron-dense vesicles and is filled with many elongated microvilli that are moderately crowded together (Fig. 3A–D). The receiving canal appears tubular, in accordance with SEM observations (Fig. 3B). Both the receiving and conducting canals frequently contain a substance with an electron density similar to that of the aforementioned vesicles surrounding the end apparatus (Fig. 3B, D). In close vicinity to the extracellular cavity, a considerable number of elongated mitochondria can be found, while multivesicular bodies are only rarely detected (Fig. 3B–E). Within the cytoplasm, there are also frequent rounded inclusions characterised by a strongly electron-dense multi-chambered pattern (Fig. 3A, F and G). Flattened stacks of rough endoplasmic reticulum, although scattered throughout the cytoplasm, appear to be preferentially localised near the nucleus and towards the basal region of the cell where Golgi apparatus with expanded cisternae is also frequently encountered (Fig. 3A, F).

The secretory cells of Gl.B, associated with the longer canals, are rounded or ovoid in shape and freely suspended in the haemolymph (Fig. 1C and D Fig. 4). These cells have a length of approximately 20 µm and a width of 16 µm. Within their cytoplasm there are several conspicuous inclusions, distinguished by an electron-lucent or slightly opaque matrix and highly electron-dense fibrillar components, appressed to each other (Fig. 4A, B and H). The nucleus is slightly irregular in shape with subtly indented boundaries, eccentrically positioned in the cell and is typically 6–8 µm in length (Fig. 1D; 4B). A wide extracellular cavity (approximately 10–15 µm in size) can be located in different areas of the cell and is surrounded by a large number of mitochondria and numerous secretory vesicles containing an electron-dense particulate dispersed in an electron-lucid matrix (Fig. 4B–E). The extracellular cavity is crowded with numerous, long, and slender microvilli that, tightly packed together, constitute a dense and intricate network enveloping the receiving canal (Fig. 4B and C). This last is tubular in shape and shows an evident cuticle perforation (Fig. 4F). In some micrographs it is possible to observe two segments of the receiving canal in the same cross section, suggesting a slight curvature of these structures within the cavity (Fig. 4C) as observed also in SEM investigation (Fig. 2G). Cuticular canals often contain substances reminiscent of the particulate content of the vesicles produced by



**Fig. 1.** *P. coccinea* male head. A: cranial apparatus (CA) from dorsal view at SEM; B: close-up of the cranial apparatus showing the setae (SE) lining the surface of the pit and the encrusted substance (ES), more evident in the top right inset; C: histological transverse semithin section of the head at the level of the cranial apparatus, the eye (EY) and the antenna (AN), showing the two types of glands below the pit (PT). Glands type A (GLA) are lining the cuticle while glands type B (GLB) are positioned deeper within the head capsule; D: close-up of the cranial apparatus in histological section, showing the thickness of the cuticle (CU), the setae (SE) with the encrusted substance (ES) at their base and the two types of glands (GLA and GLB); E–H: longitudinal section of head capsule at the level of the cranial apparatus from internal view, after digestion with KOH, showing the different length of the conducting canals in GLA and GLB imaged at SEM.

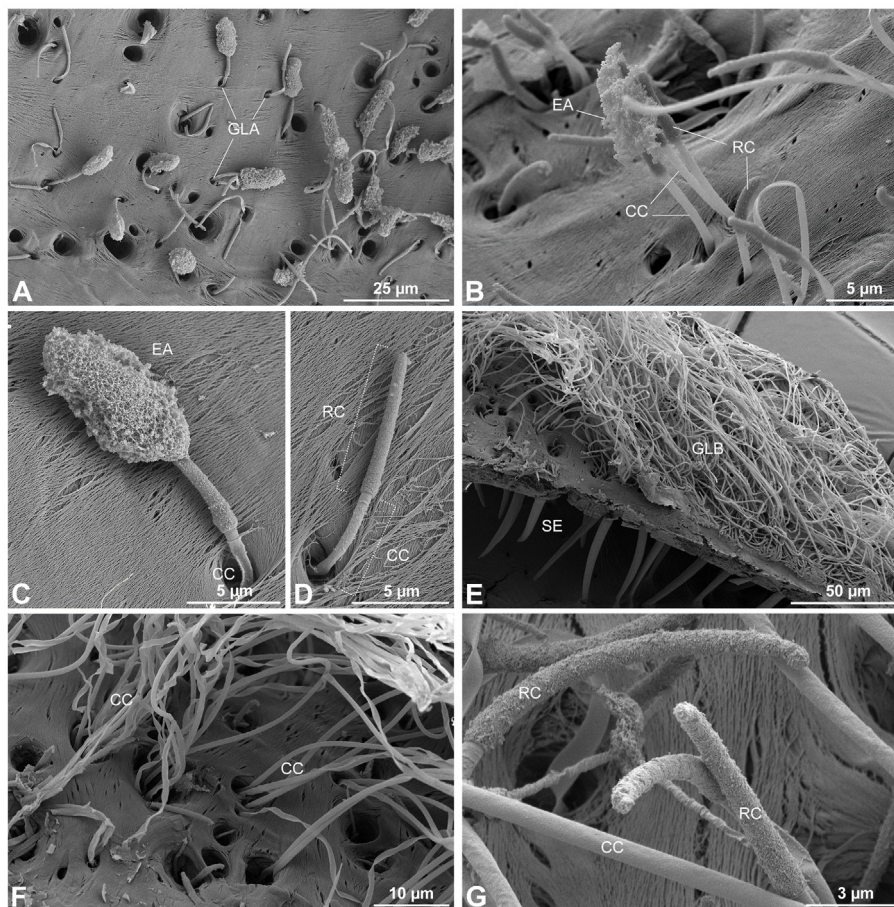
the secretory cells (Fig. 4C, F). Although limited in number, the cytoplasm also contains small electronlucent vesicles (Fig. 4E). At the cell boundaries, a strong invagination of the plasma membrane can be observed, consisting of a series of folds that cause a tight packing of the plasma membrane and only leave reduced spaces between the folds (Fig. 4G). Close to these regions are often found numerous mitochondria, slightly larger in size than the organelles located in the rest of the cytoplasm, and with well-developed cristae (Fig. 4G).

The secretions produced by the glands of the cranial apparatus, once released at the pit level, can be equally visualised through SEM, histological and ultrastructural analyses as a substance with an encrusted appearance that can withstand the aggressive

treatments required for sample preparation (e.g. chemical fixation, dehydration, KOH maceration) (Fig. 1B, D and Fig. S1).

### 3.2. Courtship behaviour of *P. coccinea*

The phases of courtship behaviour are based on observations of two couples, in which the males had ingested CTD. Both the couples showed similar behavioural patterns after being introduced in the experimental arena (Fig. 5, Video S1). After a short settling period, the male moves toward the female, orienting itself face-to-face. After which they approach each other mutually antennating. The male orients the antennae laterally, presenting the cranial apparatus to the female and she quickly samples it with her mouthparts



**Fig. 2.** *P. coccinea* cranial apparatus from internal view at SEM, after digestion with KOH, showing details of the two types of glands. A–D: glands type A (GLA) showing the end apparatus remnants (EA), the receiving canal (RC), and the short conducting canal (CC) inserting singly in the cuticle; E–G: glands type B (GLB) underlying the setae (SE) of the pit; note the curvature of the receiving canals (RC), the great length of the conductive canals (CC) of GLB and their insertion in clusters inside the cuticle.

(Fig. 5A). Then, the male climbs the female and starts mating (Fig. 5B and C). During copulation, the male firmly holds the female body at the level of the pronotum and elytra (respectively with hind- and mid-legs) and pushes his open mandibles onto the pronotum (Fig. 5C and D). After copulation, the male positions itself frontally to the female, presenting his cranial apparatus insistently for several minutes (Fig. 5E and F). In this post-copulatory stage, the female repeatedly samples the cranial apparatus, possibly ingesting the secretion.

Supplementary video related to this article can be found at <https://doi.org/10.1016/j.asd.2023.101316>

#### 4. Discussion

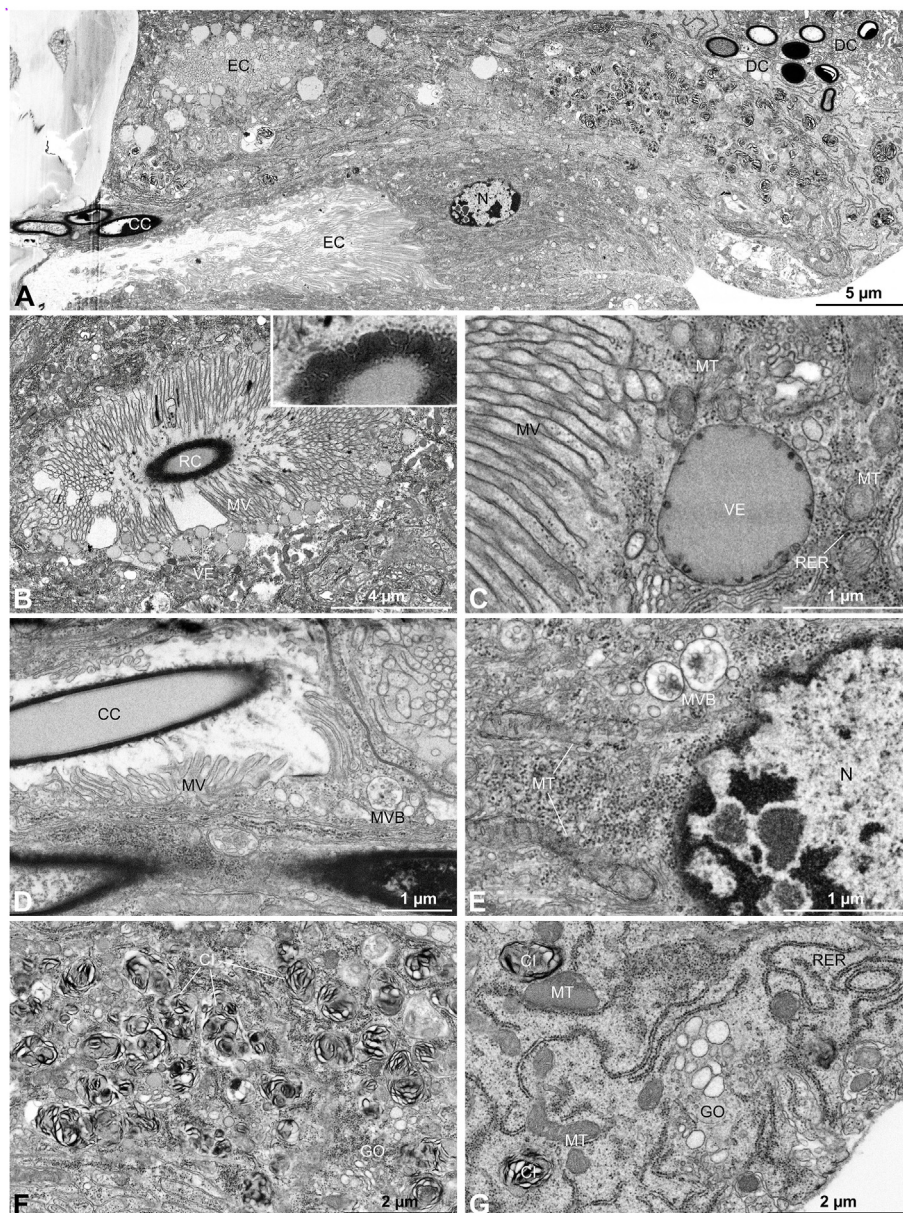
The cranial apparatus has been reported in males of 11 of 16 genera of Pyrochroinae and can vary from a single shallow depression between the eyes (*Pyrochroa* Geoffroy, 1762, *Eupyrochroa* Blair, 1914, and *Himalapyrochroa* Young, 2004), two paired pits behind (*Schizotus* Newman, 1838 and *Hemidendroides* Ferrari, 1869) or between the eyes (*Neopyrochroa* Blair, 1914, *Phyllocladus* Blair, 1914; *Pseudodendroides* Blair, 1914; *Pseudopyrochroa* Pic, 1906; *Dendroidopsis* Young, 2004), to a swollen frontal shelf in the frontoclypeal region (*Frontodendroidopsis* Young, 2004; Young, 2004a; b, 2009, 2014).

The present paper provides the first description of the cranial apparatus of *P. coccinea*, focusing on the fine morphology and ultrastructure of the associated glands. Morphological features

presented therein, although limited to a single species, could also be representative of other Pyrochroinae as, according to Young (2005), the complex cranial apparatus should be regarded as a derived character shared by several related Pyrochroinae genera. The cranial apparatus in *P. coccinea* is composed of a single pit, in contrast to other species characterised by two depressions. It has a concave shape that, together with the presence of a dense cluster of short setae, allows the retention of secretions. As evidenced by the presence of persistent encrustations at the base of the pit, the substances concentrated in the pit are, at least partially, non-volatile. The production of a consistent and dense secretion seems to be in line with the observed female behaviour of sampling the cranial apparatus with the mouthparts before and after mating (Fig. 5, Video S1).

It is interesting to note that the presence of frontal glands is apparently uncommon among coleoptera and is typically involved in interspecific interactions, as in myrmecophilous beetles (Di Giulio et al., 2009; Mynhardt and Wenzel, 2010; Moore et al., 2022). In contrast, in Pyrochroinae (Eisner et al., 1996a) and in some other families such as Galerucinae (Mohamedsaid and Furth, 2011), frontal glands can be regarded as male secondary sexual characters involved in short-distance courtship behaviour.

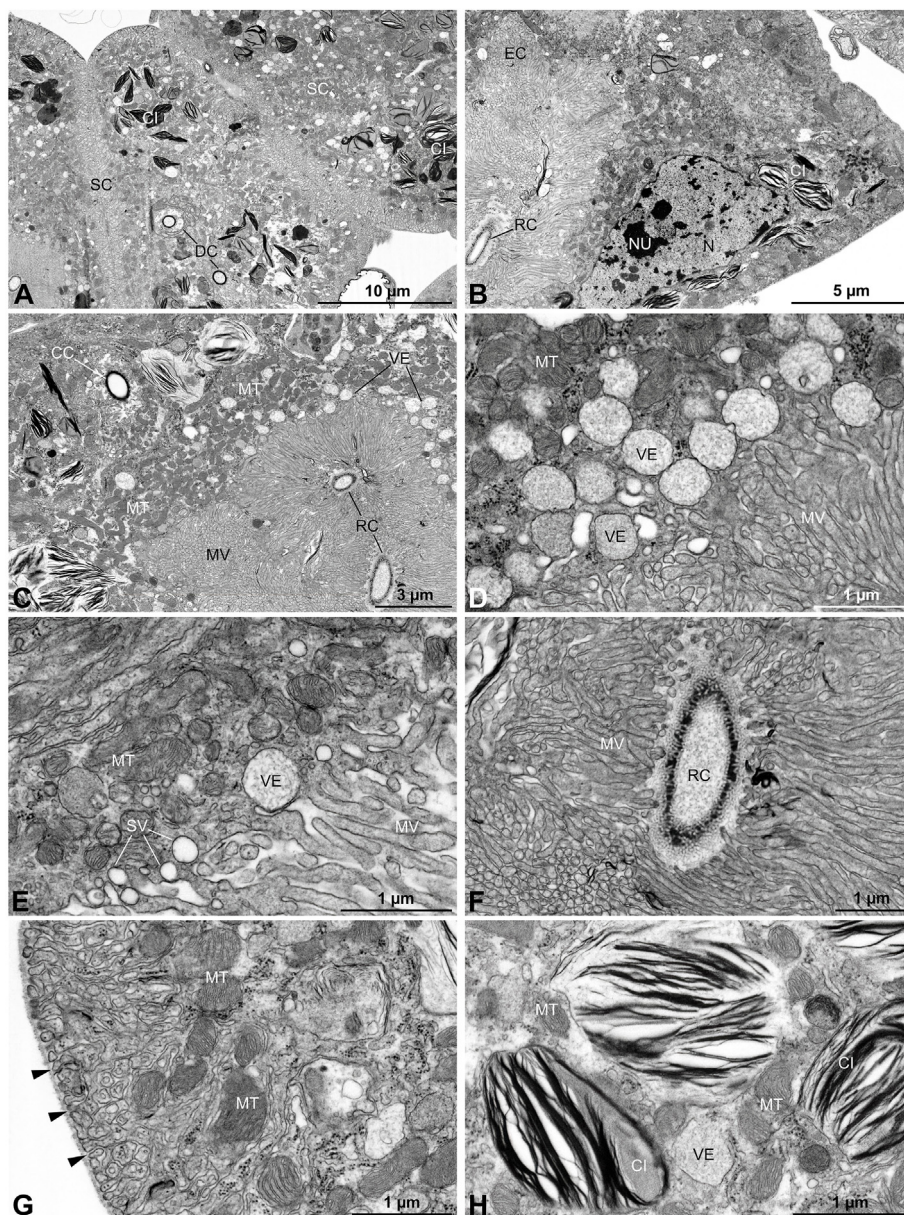
Considering what has been documented for *N. flabellata* (Eisner et al., 1996a), we hypothesise that emission and concentration of CTD during the courtship behaviour, at the level of cranial apparatus, could be mediated by the associated glands also in other Pyrochroinae species, including *P. coccinea*. The cranial apparatus is



**Fig. 3.** *P. coccinea* ultrastructure of type A glands imaged at FIB/SEM. A: longitudinal section of an entire GLA, showing the suboval nucleus (N), the extracellular cavity (EC) and the conducting canal (CC); note the presence of duct cells of type B glands (DC) on the right side of the micrograph, with their clustered conducting canals; B: extracellular cavity of GLA in cross section, showing the receiving canal (RC) surrounded by microvilli (MV) and the vesicles inside the gland cell; on top right window a close-up of the receiving canal showing the micropores; C: details of the microvilli (MV), the mitochondria (MT), the rough endoplasmic reticulum (RER), and the electron-dense vesicle (VE); D–E: details of the conducting canal (CC), microvilli (MV), mitochondria (MT), multivesicular bodies (MVB) and nucleus (N); F: cytoplasmic inclusions (CI) inside a GLA gland cell; G: close-up of the cytoplasmic inclusions (CI) and Golgi apparatus (GO) surrounded by rough endoplasmic reticulum (RER) and mitochondria (MT).

more complex than expected due to the presence of two different types of intermixed class 3 glands (Noirot and Quennedey, 1974, 1991), releasing the produced substances directly at the level of the pit and without the use of an internal cuticular reservoir. The two gland types, defined as GLA and GLB, differ significantly in many morphological and ultrastructural features such as: secretory cell shape (columnar in GLA, rounded in GLB); conducting canal length (short in GLA, long GLB); kind of vesicles and their content (multivesicular bodies and electron-dense vesicles in GLA, small electron-lucid vesicles and vesicles containing particulate substances in GLB); cellular inclusions (multi-chambered in GLA, fibrillar in GLB); plasma membrane folding (infrequent and scarcely pronounced in GLA, frequent with tight folds in GLB). These dissimilarities suggest

that GLA and GLB probably produce two different compounds and it is therefore reasonable to assume that only one of them is involved in CTD transfer while the other could be implied in favouring the adhesion of the secretions or, alternatively, it could be involved in the emission of volatile or non-volatile pheromonal attractive compounds, other than CTD. From this perspective, we believe that the most suitable candidate for CTD release is GLB, as the membrane multifolding could be indicative of active CTD adsorption from the haemolymph. In fact, despite the different embryonic derivation, a similar membrane folding has been observed in the male accessory glands of several Meloidae species in which the concentration and release of CTD are well established (Muzzi et al., 2020, 2022). The molecular mechanism that bypasses

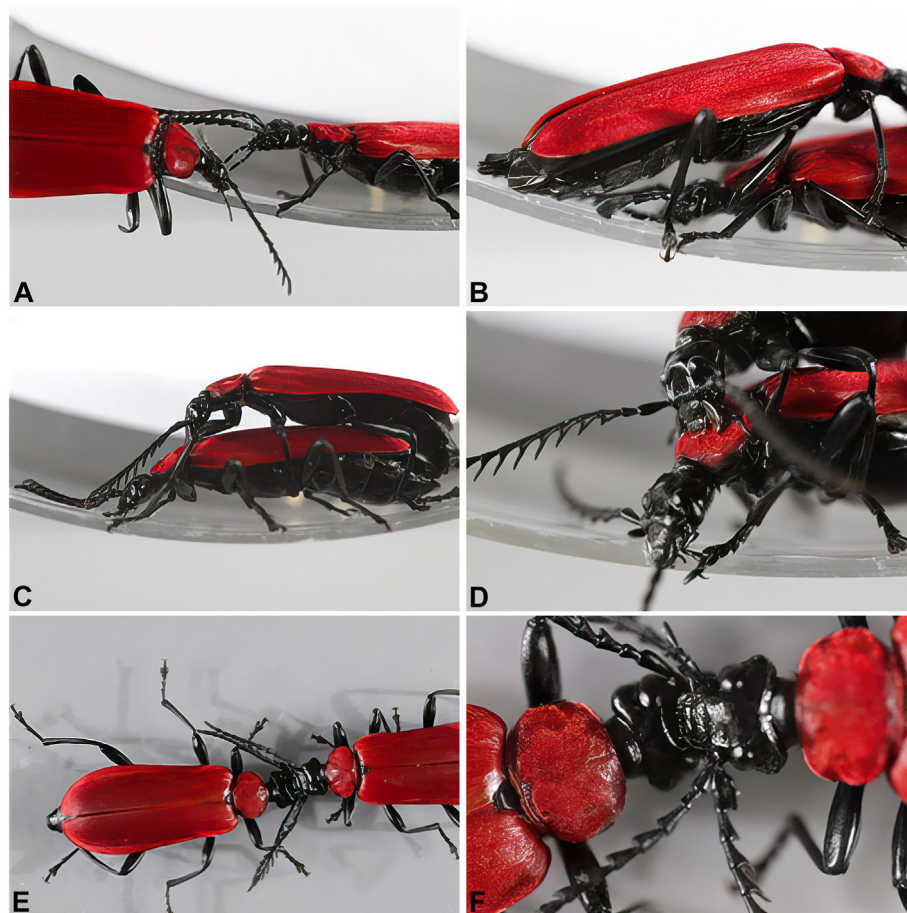


**Fig. 4.** *P. coccinea* ultrastructure of type B glands imaged at FIB/SEM. A: overview of the secretory cells (SC) and duct cells (DC) in type B glands showing the presence of numerous cytoplasmic inclusions (CI); B: secretory cell details illustrating the irregularly shaped nucleus (N) with an evident nucleolus (NU) and the broad extracellular cavity (EC) hosting the receiving canal (RC); C: Secretory cell cytoplasm showing a well developed extracellular cavity filled with densely packed microvilli (MV) surrounding the curved receiving canal (RC) and conducting canal (CC). Surrounding the extracellular cavity there are abundant mitochondria (MT) and vesicles (VE). D–E: close-up of the extracellular cavity periphery showing mitochondria (MT), vesicles containing particulate substance (VE) and smaller electronlucent vesicles (SV); F: receiving canal (RC) surrounded by packed microvilli (MV) and filled with particulate substance; G: plasma membrane invaginations (indicated by arrowheads) with abundant mitochondria (MT) in its vicinity; H: Details of cytoplasmic inclusions (CI), mitochondria (MT) and vesicles (VE).

the toxicity of CTD and allows its trafficking throughout the different districts of meloid body is currently unknown, however, the morphological similarities regarding the presence of membrane folding in *P. coccinea* could be indicative of an analogous process of terpene sequestration from the haemolymph.

Although the cranial apparatus has been observed in males of most genera of Pyrochroinae (Young, 2004a, 2004b, 2009, 2014), to date, its involvement in the courtship behaviour of the fire-coloured beetles has only been reported in *N. flabellata* (Eisner et al., 1996a). Overall, our observations on the courtship behaviour of *P. coccinea* were consistent with those conducted on *N. flabellata* (Eisner et al., 1996a) concerning pre-copula sampling of

the cranial apparatus. The fact that, after copulation the male presents the cranial apparatus to the female and that the female continues testing this structure, is an apparently unusual post-copulatory behaviour. In fact, in insects, although nuptial gifts may be consumed by females after copulation, they are typically offered by males before or during copulation (Omkar and Mishra, 2022). The specific post-copulatory behaviour we observed could, however, fall within the mechanisms of “mate guarding” (post-copulatory sexual selection) (Omkar and Mishra, 2022). Although our experimental design did not allow us to state whether male *P. coccinea* secretes CTD in their cranial apparatus, or whether the consumption of CTD leads to positive selection of males from



**Fig. 5.** Frames of the courtship sequence of *Pyrochroa coccinea*. A: male presenting cranial apparatus to the female; B: male climbing the female; C: copulation; D: detail of the male mandibles pushed against the female pronotum; E–F: post-copula behaviour in which the female repeatedly samples the male cranial apparatus.

females (as observed in *N. flabellata*; Eisner et al., 1996a), the canthariphilous behaviour (Lückmann, 1999; Lückmann and Niehuis, 2009; Scheffler, 2013), and the similarities with the courtship behaviour of *N. flabellata*, suggest that CTD could be secreted in the male cranial apparatus. Further chemical investigations would, in any case, be required to solve the issues concerning CTD release in *P. coccinea*.

Possible insights regarding the study of canthariphily in Pyrochroidae could include extending chemical, behavioural, and ultrastructural analyses to other species representative of the diversity of the genera, thus including species with different development of the cranial apparatus, and even species without this structure that could be equally instructive to understand the evolution of this structure in Pyrochroidae.

#### CRediT authorship contribution statement

Marco Molfini: Conceptualization, Methodology, Investigation, Resources, Writing - Original Draft, Review & Editing. Maurizio Muzzi: Conceptualization, Methodology, Investigation, Resources, Writing - Original Draft, Review & Editing. Emiliano Mancini: Conceptualization, Writing - Review & Editing. Marco Alberto Bologna: Conceptualization, Resources, Writing - Review & Editing, Supervision, Funding acquisition. Andrea Di Giulio: Conceptualization, Methodology, Investigation, Resources, Writing - Original Draft, Review & Editing, Supervision, Funding acquisition.

#### Declaration of competing interest

All the authors declare that they have read and approved this final version of the manuscript and also declare they have no competing interests.

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#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.asd.2023.101316>.

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