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The Diversity of Chemicals of the Defensive Secretions of Five Pterostichine Ground Beetle Species (Coleoptera: Carabidae) and the Morphology of Their Pygidial Glands

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Abstract

The defensive substances of ground beetles are synthesised and stored in a pair of defensive glands, commonly referred to as abdominal or pygidial glands. The chemical composition of the pygidial gland secretions and the morphology of the glands were studied in adults of four species belonging to the tribe Pterostichini: *Poecilus cupreus* Linnaeus, 1758, *Pterostichus melanarius* Illiger, 1798, *P. anthracinus* Illiger, 1798, and *Stomis pumicatus* Panzer, 1796. In addition, *Tapinopterus cognatus* (Dejean, 1831) was examined only with regard to the chemistry of its secretion. The pygidial gland secretions of the five species were investigated using gas chromatography-mass spectrometry (GC-MS). The combined presence of methacrylic acid and tiglic acid was observed in all taxa studied. 2-Tridecanone, which was detected in all species except *P. cupreus*, is reported for the first time in the defensive secretions of representatives of Pterostichini. The pygidial glands of the first four species were examined using both bright-field microscopy (BFM) and nonlinear microscopy (NLM). The examined species were similar in the general appearance of the glands, with the greatest differences observed in *S. pumicatus*.

Keywords: carabid beetles; abdominal glands; Pterostichini; *Stomis*; *Tapinopterus*; allomone

1. Introduction

Beetles (Coleoptera) are the most species-rich order of insects. The remarkable success of beetles in terrestrial habitats is closely linked to their ability to synthesise a wide variety of natural products that regulate complex intra- and interspecific interactions [1,2]. Semiochemicals used to deter predators, often referred to as allomones, are of great importance [3]. Numerous beetle species employ chemical defence mechanisms, and ground beetles (Carabidae) are among the richest in terms of the diversity of allomones produced [4]. The chemical composition of the secretions produced in response to disturbances can vary considerably between species [5]. In all ground beetles, defensive products are synthesised and stored in a pair of defensive glands, commonly referred to as abdominal or pygidial glands. These glands are very similar in general appearance across species. They always comprise four morpho-anatomical units: secretory lobe, main collecting canal, reservoir, and efferent duct [6]. Secretions are released from these glands in three ways: oozing, spraying, or crepitation [7].

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The earliest studies on the diversity of chemicals in the pygidial gland secretions of members of the tribe Pterostichini were conducted for several European representatives [8,9], revealing the presence of methacrylic acid, tiglic acid, decane, undecane, and tridecane [10]. Chemical analyses were also carried out for Australian [11] and Japanese Pterostichini [12]. Nearly three decades passed before the chemistry of the defensive secretions of Pterostichini again became the focus of investigation [13,14]. Recent advances in the semiochemistry of Pterostichini include studies reporting strong antifungal activity [15] and high antitumour potential [16] of the pygidial gland secretion of *Abax parallelepipedus* (Piller & Mitterpacher, 1783). The latest progress in this field was made by Rork et al. [17], who used differential gene expression analyses to investigate the catabolic pathways involved in the synthesis of defensive compounds of *Pterostichus moestus* (Say, 1823).

One of the first investigations into the morphology of the pygidial glands within the tribe Pterostichini was an in-depth study by Forsyth [18]. The paper examines in detail the morphology and ultrastructure of the pygidial glands of *Pterostichus madidus* (Fabricius, 1775). Subsequently, less detailed morphological analyses of several species were conducted [6,13,19]. Kanehisa and Murase [12] presented the first scanning electron microscopy images of the pygidial glands of Pterostichini. In recent years, considerable progress has been made in applying microscopy techniques to study the internal and external ultrastructure of the pygidial glands of ground beetles [20–22]. Later, X-ray phase-contrast synchrotron microtomography [23] and nonlinear microscopy (NLM) [24,25] were used to study the structure of the pygidial glands of Pterostichini.

Despite significant progress in ground beetle chemical ecology, available information remains fragmented, with entire genera still chemically uninvestigated. Greater effort is required to fully understand the capabilities of this diverse group. In this study, we examined the morphology of the pygidial glands and the chemical composition of their defensive secretions in four species of the tribe Pterostichini: *Poecilus cupreus* Linnaeus, 1758, *Pterostichus melanarius* Illiger, 1798, *P. anthracinus* Illiger, 1798, and *Stomis pumicatus* Panzer, 1796. We also investigated the chemical composition of the defensive secretion of *Tapinopterus cognatus* (Dejean, 1831). A brief morphological description of the glands of *P. melanarius* was provided by Forsyth [6], while the pygidial gland secretion of *P. cupreus* was initially chemically analysed by Schildknecht et al. [9]. Given that compounds produced in minor and trace amounts could not have been detected by previously used techniques [9,24,25], a reanalysis of *P. cupreus* is necessary to gain insight into the full potential of the species' defensive capabilities. The other three species of Pterostichini had not previously been examined regarding the morphology of the pygidial glands or the chemical composition of their secretions. Knowledge of the semiochemical features of *P. melanarius* and *P. cupreus*, together with recent findings [24,25], will provide a more comprehensive understanding of the evolution and ecology of their defensive products. Within the tribe, different genera may vary in the defensive chemicals they produce [5]. The genera *Stomis* Clairville, 1806 and *Tapinopterus* Schaum, 1858 have never been investigated in terms of chemical ecology, thereby limiting our knowledge of the evolution and adaptation of the tribe's defensive strategies [5].

Our aims were: (i) to investigate the chemical composition of the pygidial gland secretions of the five pterostichine ground beetle taxa and (ii) to morphologically characterise the pygidial glands of *P. cupreus*, *P. melanarius*, *P. anthracinus*,and *S. pumicatus* using bright-field microscopy (BFM) and NLM.

2. Materials and Methods

2.1. Studied Species

In this study, we examined the following species: *Poecilus cupreus* Linnaeus, 1758, a western Palaearctic species inhabiting various unshaded habitats, fields, steppes and water margins from lowlands to mountains (Figure 1A); *Pterostichus melanarius* Illiger, 1798, a Euro-Siberian species inhabiting fields, meadows and forests from lowlands to mountains (Figure 1B); *P. anthracinus* Illiger, 1798, a western Palaearctic species found in moist, unshaded habitats, meadows near water and forests from lowlands to mountains (Figure 1C); *Stomis pumicatus* Panzer, 1796, a Eurasian species introduced to North America, inhabiting moderately damp and wet habitats (meadows, floodplain forests, river and stream banks) in lowland and mountainous regions (Figure 1D) [26]; and *Tapinopterus cognatus* (Dejean, 1831), a species distributed in the Balkans (Bulgaria, Romania and Serbia) and inhabiting mixed deciduous and coniferous forests, as well as grasslands at high altitudes (Figure 1E) [27]. The first four aforementioned species were investigated in both chemical and morphological aspects. We did not perform a morphological analysis of the glands of *T. cognatus*, as the only available sample was used for chemical analysis, and subsequent searches for additional specimens of this rare species were unsuccessful.

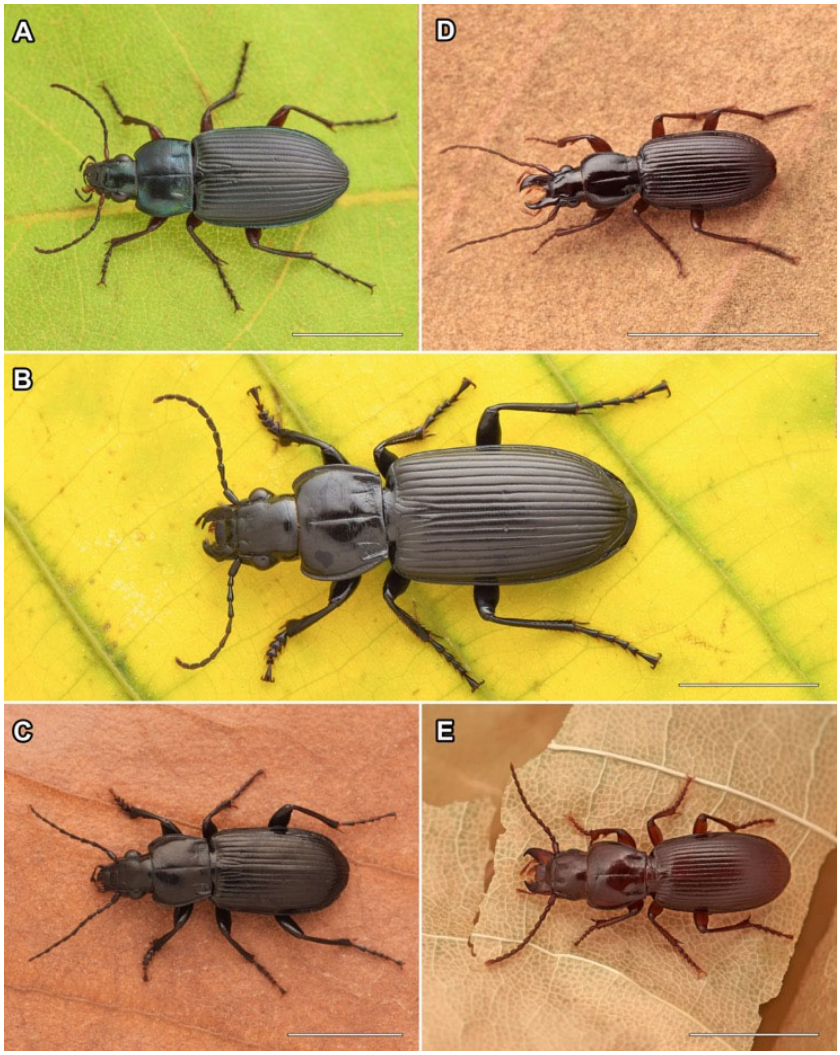


Figure 1. Dorsal view of the habitus of the five ground beetle species studied: (**A**) *Poecilus cupreus*; (**B**) *Pterostichus melanarius*; (**C**) *P. anthracinus*; (**D**) *Stomis pumicatus*; (**E**) *Tapinopterus cognatus*. Photos: N. Vesović. Scales: 0.5 cm.

2.2. Collection and Handling of Ground Beetle Specimens

Adult specimens of four ground beetle species were collected manually at several locations in the city of Belgrade and its surroundings (central Serbia). Forty-five individuals (20 males and 25 females) of *P. cupreus* and three individuals (two males and one female) of *P. melanarius* were collected on 20 May 2023 on the bank of the Tamiš River, near the city of Pančevo (44°52′53.6″ N, 20°36′54.1″ E). Seven individuals (four males and three females) of *P. anthracinus* were collected on 22 June 2023 on the island of Veliko Ratno Ostrvo on the Danube River in Belgrade (44°50′10.8″ N, 20°25′32.0″ E). Nine individuals (three males and six females) of *S. pumicatus* were collected on 3 May 2023 in Sremačka Šuma Forest, Belgrade (44°42′33.4″ N, 20°22′53.9″ E).

Additionally, 11 individuals (seven males and four females) of *T. cognatus* were collected manually on 2 June 2022 on Mt. Čemernik (southeastern Serbia) (42°44′03.3″ N, 22°16′38.5″ E). Samples of all five species were gathered under stones and logs.

The collected beetles were kept in plastic chambers at room temperature and in complete darkness. Water was occasionally sprayed on the surface of the containers to maintain the moisture of the substrate collected at the sampling sites. Earthworms were used as a food source. Photographs of ground beetle specimens were taken using a Nikon D5300 digital camera (Nikon Corporation, Tokyo, Japan) equipped with a Tamron SP Di AF 90 mm F/2.8 macro lens (Tamron Co., Ltd., Saitama, Japan) and a Sigma EM-140 DG ring flash (Sigma Corporation, Kawasaki, Japan) with a homemade light diffuser.

2.3. Chemical Extraction

All collected individuals of five Pterostichini species were milked alive. The beetles were disturbed by pinching their antennae and legs for a few seconds while being held over 12-mL glass vials containing 0.5 mL of dichloromethane (Merck, Darmstadt, Germany) as the solvent. The released secretions were collected directly into the vials. The procedure was conducted in a laboratory at room temperature. The prepared samples were subjected to gas chromatography-mass spectrometry (GC-MS) analysis immediately afterwards to prevent oxidation and degradation of compounds.

2.4. Chemical Analyses

For the gas chromatography (GC) and GC-MS analyses of the pygidial gland secretion extracts, we used a 7890A GC system equipped with a 5975C inert XL EI/CI mass selective detector (MSD) and a flame ionisation detector (FID) (Agilent Technologies, Santa Clara, CA, USA). These were connected by capillary flow technology through a two-way splitter. GC-MS and gas chromatography-flame ionisation detection (GC-FID) analyses were performed in splitless mode (inlet temperature 220 °C, currier gas He, flow 2 mL/min, injection volume 1 μL) on a polar HP-INNOWax capillary column (30 m length, 0.32 mm inner diameter, 0.25 μm film thickness) (Agilent Technologies, Santa Clara, CA, USA). The oven temperature was programmed linearly from 40 to 240 °C at a rate of 10 °C min^{-1}, with a final 10-minute hold. Electron ionisation mass spectra (70 eV) were recorded in the range m/z 35–550.

The NIST AMDIS (Automated Mass Spectral Deconvolution and Identification System) software, version 2.70 (National Institute of Standards and Technology, Gaithersburg, MD, USA), and the commercially available NIST17 (National Institute of Standards and Technology, Gaithersburg, MD, USA) and Wiley07 libraries (Wiley, Hoboken, NJ, USA) were used for mass spectral deconvolution and extraction and library search. In addition, identification of compounds was supported by the calculation of retention indices obtained from *n*-alkane series (C_6–C_{30}) recorded under the same chromatographic conditions.

2.5. Morphological Analysis

Two males and one female of *P. melanarius* and three males and three females of each of the other three morphologically examined species were dissected in 70% ethanol. The pygidial glands were extracted and separated from the surrounding tissues. All morpho-anatomical units of all dissected specimens were measured accurately and photographed. The reservoir was measured from the apical notch to the base and at its widest point. Male and female individuals were measured separately.

2.5.1. Bright-Field Microscopy (BFM)

Imaging of the morphological features of the pygidial glands was performed using a Nikon SMZ800N stereomicroscope (Nikon Corporation, Tokyo, Japan) coupled with a Nikon DS-Fi2 high-definition colour camera head (Nikon Corporation, Tokyo, Japan). Calibration and scaling were carried out with a stand-alone Nikon DS-L3 camera control unit (Nikon Corporation, Tokyo, Japan).

2.5.2. Nonlinear Microscopy (NLM)

Nonlinear microscopy (NLM) was performed using a homemade microscope developed at the Institute of Physics Belgrade, University of Belgrade. For NLM imaging, the morphological structures of the pygidial glands were mounted on microscope slides with glycerol and covered with a slip. The two-photon excitation fluorescence modality of NLM was generated using a femtosecond laser system consisting of a Mira 900-F Ti-sapphire laser (Coherent Inc., Santa Clara, CA, USA), pumped by a Verdi V10 10-W laser (Coherent Inc., Santa Clara, CA, USA). This setup emitted 160-fs pulses at a repetition rate of 76 MHz within a tuning range of 700–1000 nm at 532 nm. Imaging was carried out using a Carl Zeiss Planachromat 20×/0.8 NA microscope objective (Carl Zeiss, Jena, Germany), with the laser beam scanned across the sample by a computer-controlled galvo-scanner unit. The natural autofluorescence of the pygidial glands was detected, with the optimal signal obtained at 840 nm.

The open source software VolView 3.4 (Kitware, Inc., Clifton Park, NY, USA) was used for three-dimensional visualisation of a set of slices. It employed either volume rendering or maximum intensity projection algorithms. The size of the samples was measured taking into account the field of view of the microscope objective and the laser scanning amplitude.

3. Results

3.1. Diversity of Chemicals of Pygidial Gland Secretions

A total of 24 compounds were detected in the extracts of the pygidial gland secretions of the five Pterostichini species studied. Twenty-one chemicals were identified, while three remained unidentified (Table 1). Methacrylic acid, tiglic acid, and isobutyric acid were present in all samples. The defensive secretion of *P. anthracinus* was characterised by the greatest diversity of chemicals, while the secretion of *T. cognatus* exhibited the lowest diversity of chemicals. We detected no qualitative or quantitative differences between the sexes. Two isomers of an alkene (tricosene) were detected in minor or trace amounts in all examined species except *T. cognatus*.

In the gland secretion of *P. cupreus*, we detected 16 chemical compounds: 11 hydrocarbons (six alkanes, three alkenes, and two dienes) and five carboxylic acids. The dominant compound was methacrylic acid, followed by undecane. All other compounds were present in minor or trace amounts (Tables 1 and S1, Figure 2A).

Table 1. Chemical composition of pygidial gland secretions from five ground beetle taxa analysed by GC-FID and gas chromatography-mass spectrometry (GC-MS).

Peak	Compound	RI [b]	Relative Percentage (%)				
			P. cupreus	*P. melanarius*	*P. anthracinus*	*S. pumicatus*	*T. cognatus*
1	Undecane	1100	31.3	6.7	11.0		
2	Tridecane	1300	T [a]	0.3	0.1		
3	Acetic acid	1482	3.0	3.9	1.8		1.0
4	Propanoic acid	1569	T [a]	0.3	0.4		
5	Isobutyric acid	1590	0.8	0.8	0.9	18.5	7.1
6	Isovaleric acid	1685		0.1		50.6	
7	8-Heptadecene	1697					3.7
8	Methacrylic acid	1741	60.7	73.1	62.0	0.6	34.9
9	2-Tridecanone	1808		0.1	T [a]	3.8	
10	Senecioic acid	1829			0.2		12.2
11	Unidentified compound	1863					6.0
12	Tiglic acid	1899	0.7	14.2	21.3	22.5	35.1
13	2-Pentadecanone	2015				0.3	
14	Heneicosane	2100	0.3	T [a]	0.3	0.7	
15	Tricosane	2300	0.2	0.1	0.1	0.2	
16	Tricosene isomer 1	2311	0.1	0.1	0.6	0.2	
17	Tricosene isomer 2	2339	0.1	T [a]	0.5	0.1	
18	Tricosadiene	2352	0.1			1.2	
19	Unidentified compound	2370			0.5		
20	Pentacosane	2500	0.1		T [a]	0.1	
21	Pentacosene	2517	0.5	0.3	0.1	0.2	
22	Pentacosadiene	2558	1.9				
23	Unidentified compound	2571			T [a]	1.0	
24	Heptacosane	2700	0.2	T [a]	0.2		

T [a] trace amounts (<0.1%). RI [b] retention indices calculated from the GC-MS retention times of a series of *n*-alkanes obtained under the same chromatographic conditions.

In the gland secretion of *P. melanarius*, 15 chemical compounds were isolated: eight hydrocarbons (five alkanes and three alkenes), six carboxylic acids, and one ketone. The dominant compound was methacrylic acid, followed by tiglic acid and undecane (Tables 1 and S1, Figure 2B).

In the extract of the pygidial gland secretion of *P. anthracinus*, 18 compounds were detected: nine hydrocarbons (six alkanes and three alkenes), six carboxylic acids, one ketone, and two unidentified chemicals. The dominant compound was methacrylic acid, followed by tiglic acid and undecane (Tables 1 and S1, Figure 2C).

In the extract of the pygidial gland secretion of *S. pumicatus*, we found 14 chemical compounds: seven hydrocarbons (three alkanes, three alkenes, and one diene), four carboxylic acids, two ketones, and one unidentified chemical. The dominant compound was isovaleric acid, followed by tiglic acid and isobutyric acid (Tables 1 and S1, Figure 2D).

In the extract of the pygidial gland secretion of *T. cognatus*, we found seven chemical compounds: one alkene, five carboxylic acids, and one unidentified chemical. The dominant compound was tiglic acid, followed by methacrylic acid (Tables 1 and S1, Figure 2E).

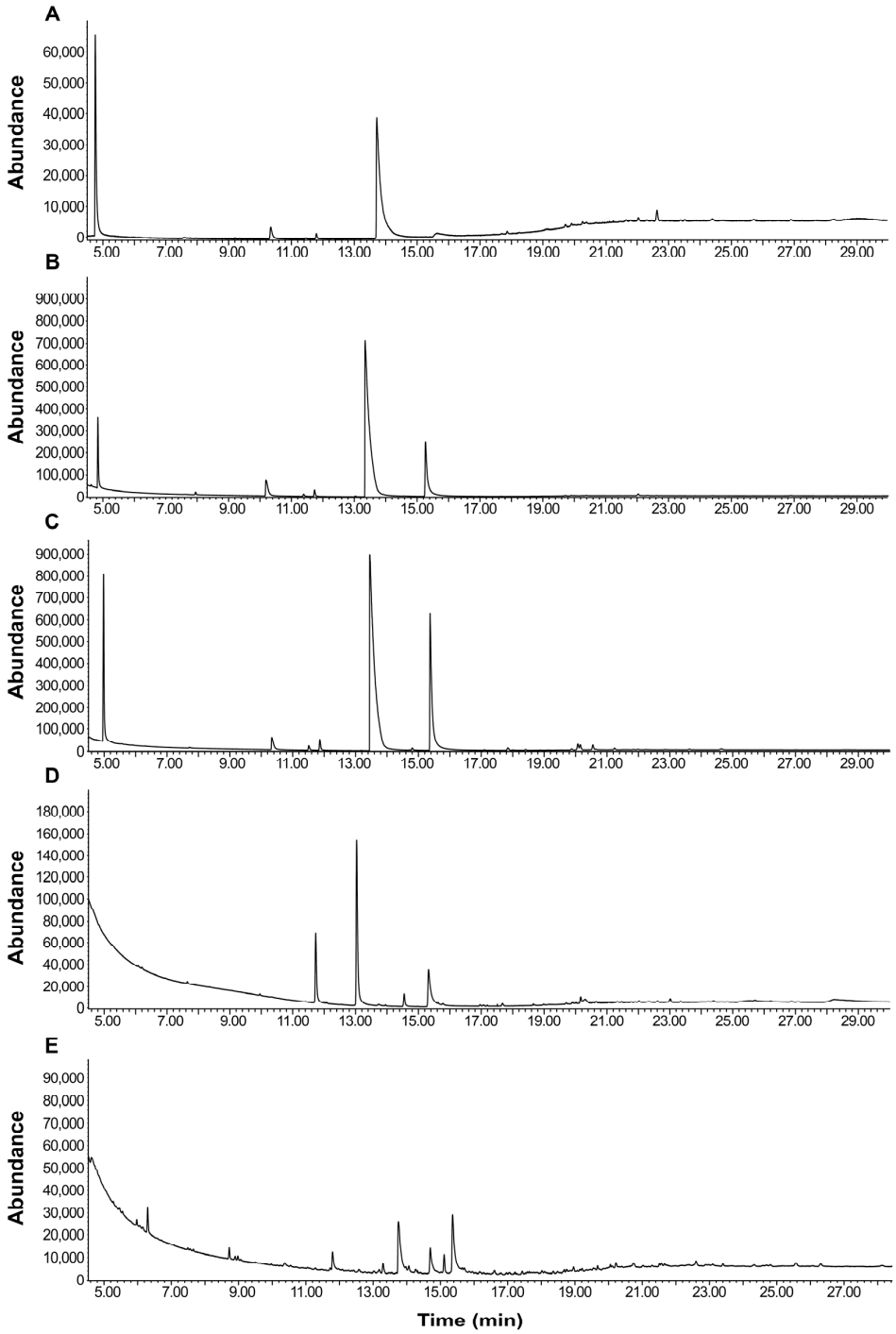


Figure 2. Gas chromatography-flame ionisation detection (GC-FID) chromatograms of dichloromethane extracts of pygidial gland secretions from adults of the five analysed species of Pterostichini: (**A**) *Poecilus cupreus*; (**B**) *Pterostichus melanarius*; (**C**) *P. anthracinus*; (**D**) *Stomis pumicatus*; (**E**) *Tapinopterus cognatus*.

3.2. Morphology of Pygidial Glands

The pygidial glands of the species studied were closely associated with the tracheal system and the Malpighian tubes. The clusters of secretory lobes consisted of a variable number of whitish, spherical or slightly elongated secretory units (Figures 3A, 4A, 5A and 6A). The outer layer of a secretory unit comprised secretory cells arranged radially around the lumen into which their products were released (Figures 3A, 4B, 5B and 6B). The secretions from the secretory units drained into the main collecting canal, which varied in length (Figures 3A,C, 4A,B, 5A,B and 6A,B). The main collecting canal opened

into the distal part of the reservoir (Figure 3B) or into the efferent duct at its basal (Figures 4A and 6A) or median part (Figure 5A). The reservoir was more or less heart-shaped. It gradually transitioned into the efferent duct, at the apical part of which there was an opening valve.

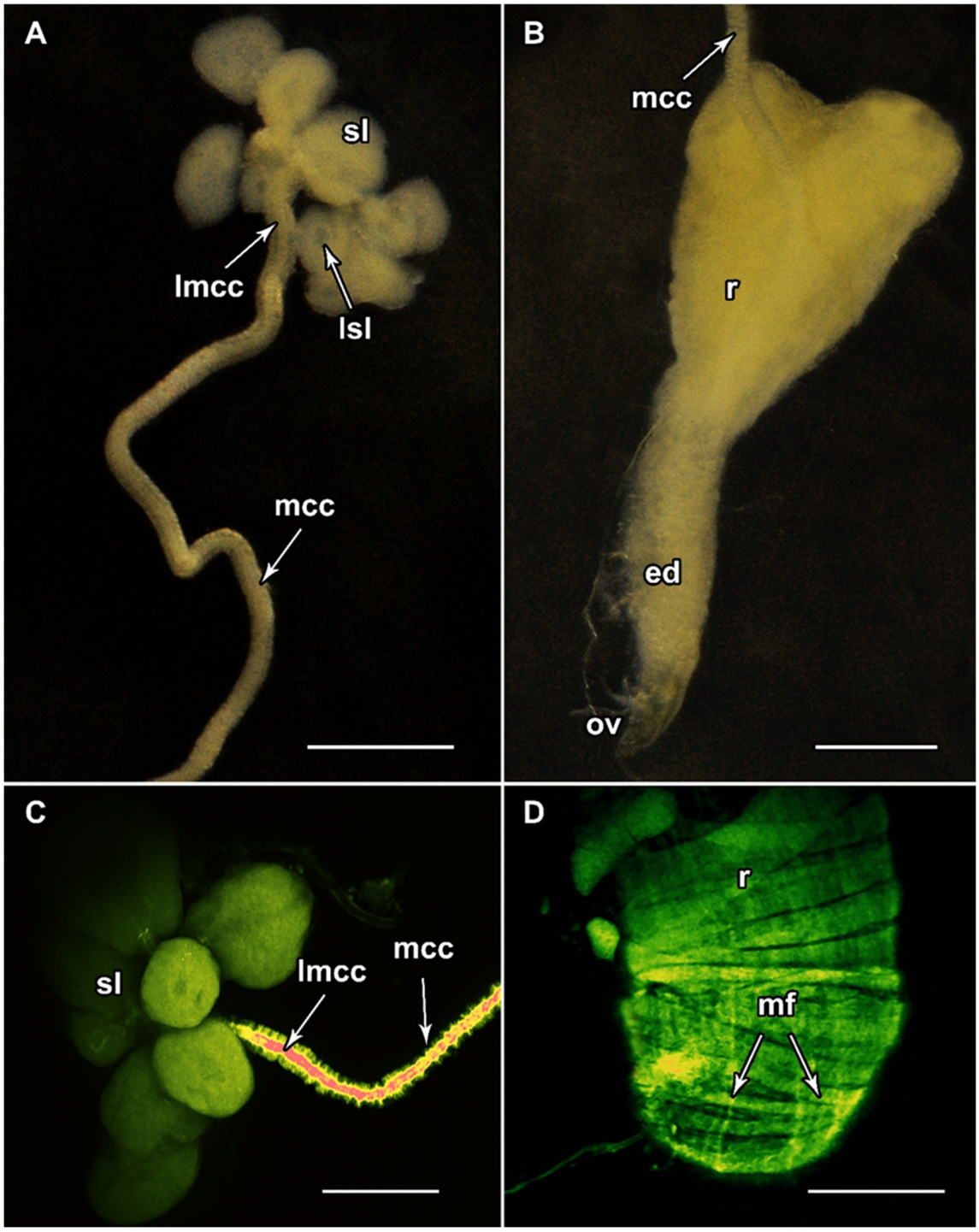


Figure 3. Morphology of the pygidial glands of *Poecilus cupreus* observed with bright-field microscopy (BFM) (**A**,**B**) and nonlinear microscopy (NLM) (**C**,**D**): (**A**) aggregation of secretory lobes; (**B**) reservoir with efferent duct; (**C**) close-up view of secretory lobes; (**D**) close-up view of reservoir. Abbreviations: ed—efferent duct; lmcc—lumen of main collecting canal; lsl—lumen of secretory lobe; mcc—main collecting canal; mf—muscle fibres; ov—opening valve; r—reservoir; sl—secretory lobe. Scales: (**A**,**B**)—250 μm; (**C**,**D**)—100 μm.

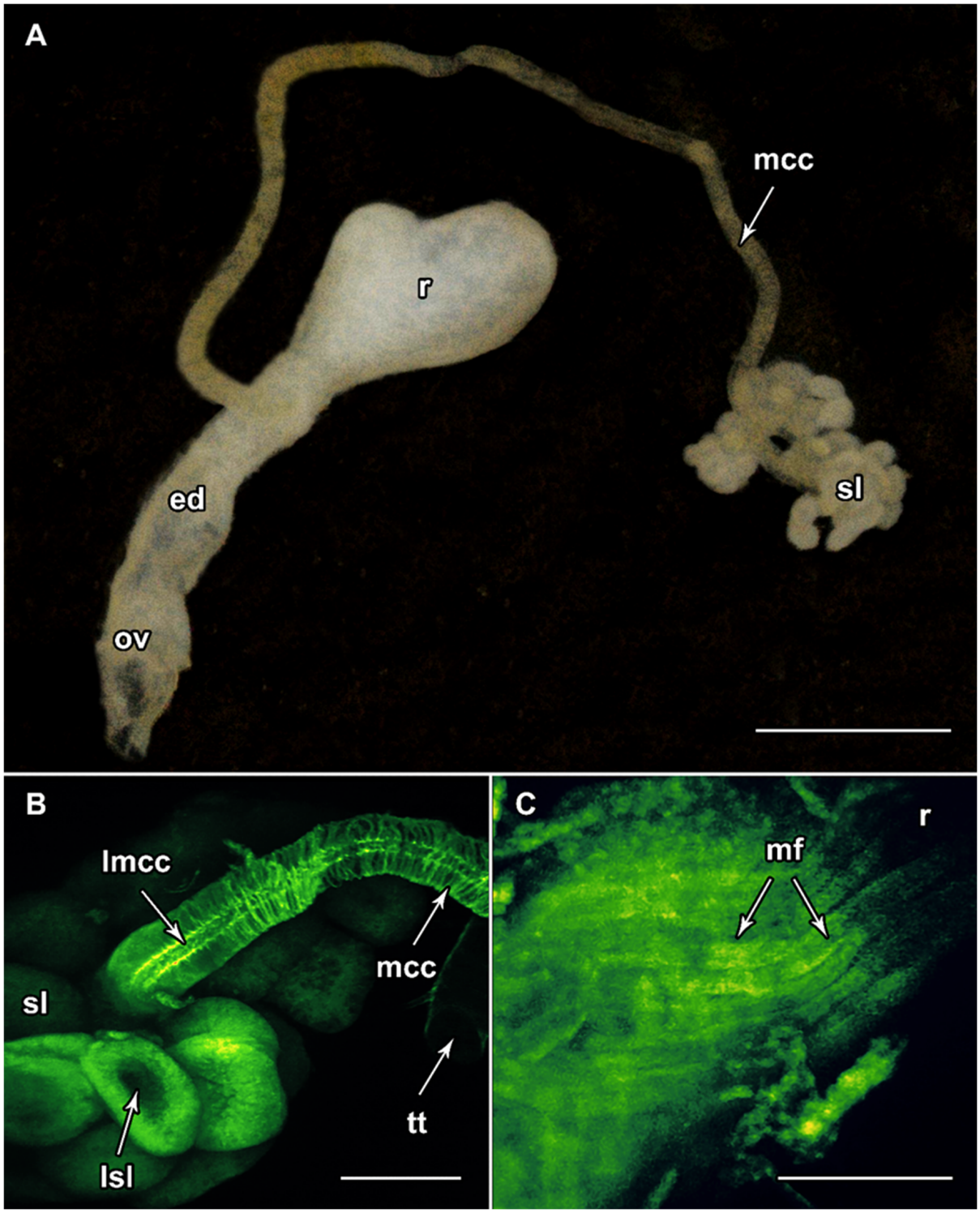


Figure 4. Morphology of the pygidial glands of *Pterostichus melanarius* observed with BFM (**A**) and NLM (**B**,**C**): (**A**) whole pygidial gland apparatus; (**B**) close-up view of secretory lobes and main collecting canal; (**C**) close-up view of muscle wall of reservoir. Abbreviations: ed—efferent duct; lmcc—lumen of main collecting canal; lsl—lumen of secretory lobe; mcc—main collecting canal; mf—muscle fibres; ov—opening valve; r—reservoir; sl—secretory lobe; tt—tracheal tube. Scales: (**A**)—500 µm; (**B**,**C**)—100 µm.

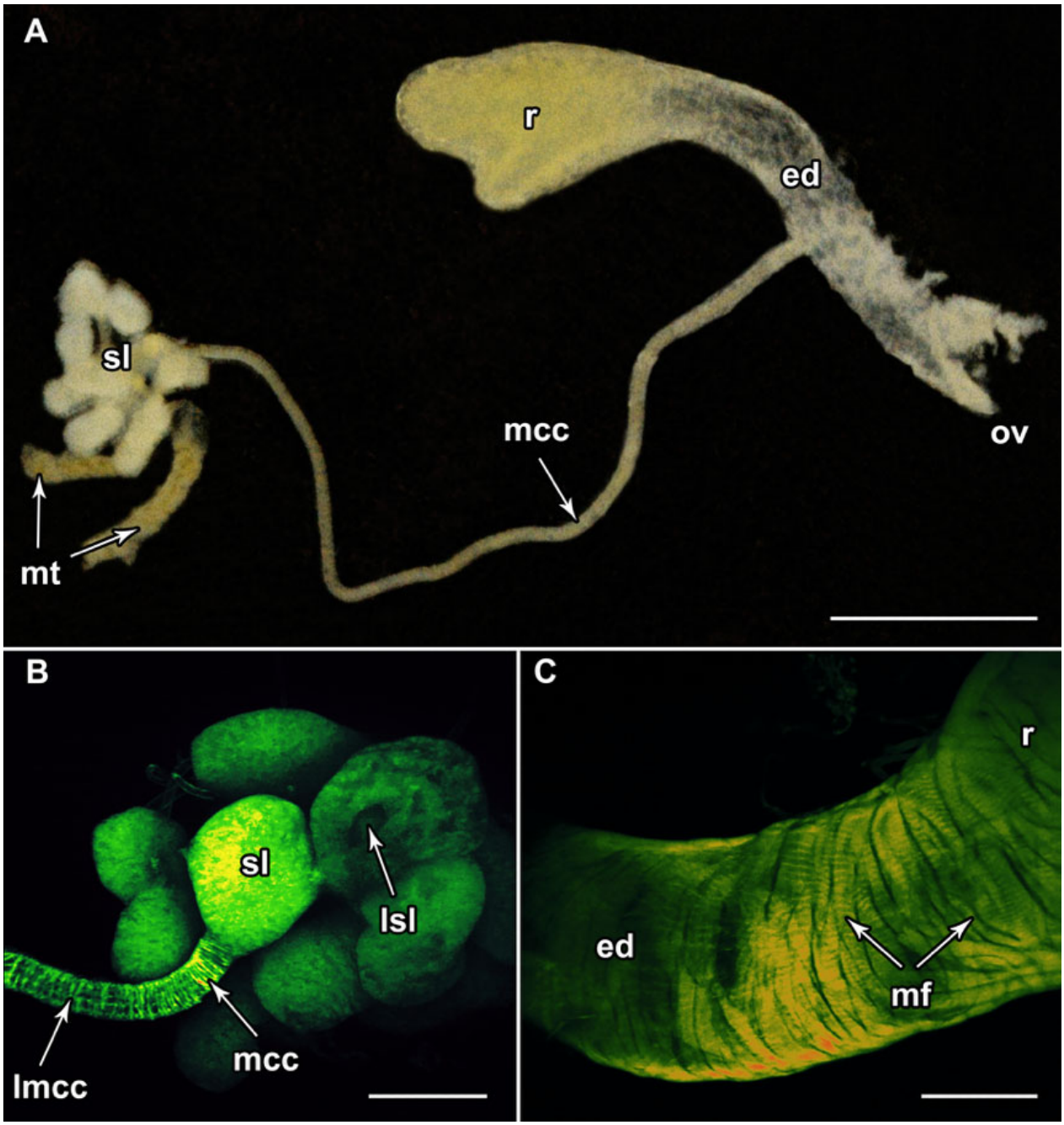


Figure 5. Morphology of the pygidial glands of *Pterostichus anthracinus* observed with BFM (**A**) and NLM (**B**,**C**): (**A**) whole pygidial gland apparatus; (**B**) close-up view of secretory lobes and main collecting canal; (**C**) close-up view of proximal part of efferent duct. Abbreviations: ed—efferent duct; lmcc—lumen of main collecting canal; lsl—lumen of secretory lobe; mcc—main collecting canal; mf—muscle fibres; mt—Malpighian tubes; ov—opening valve; r—reservoir; sl—secretory lobe. Scales: (**A**)—500 µm; (**B**,**C**)—100 µm.

In *P. cupreus*, 10–15 secretory units formed a cluster (Figure 3A). The diameter of an individual unit was 130–150 µm (Figure 3B). The main collecting canal was approximately 4 mm long, with an outer diameter of 50 µm and a lumen diameter of 20 µm. The apical part of the reservoir was notched medially, giving it a heart-shaped appearance (Figure 3A). Its muscle wall was relatively thick (Figure 3D). The reservoir was 660 µm long and had a maximum width of 510 µm. The efferent duct had a constant width of 180 µm and a length of 600 µm. It had a thick muscle layer (Figure 3B) (Table 2).

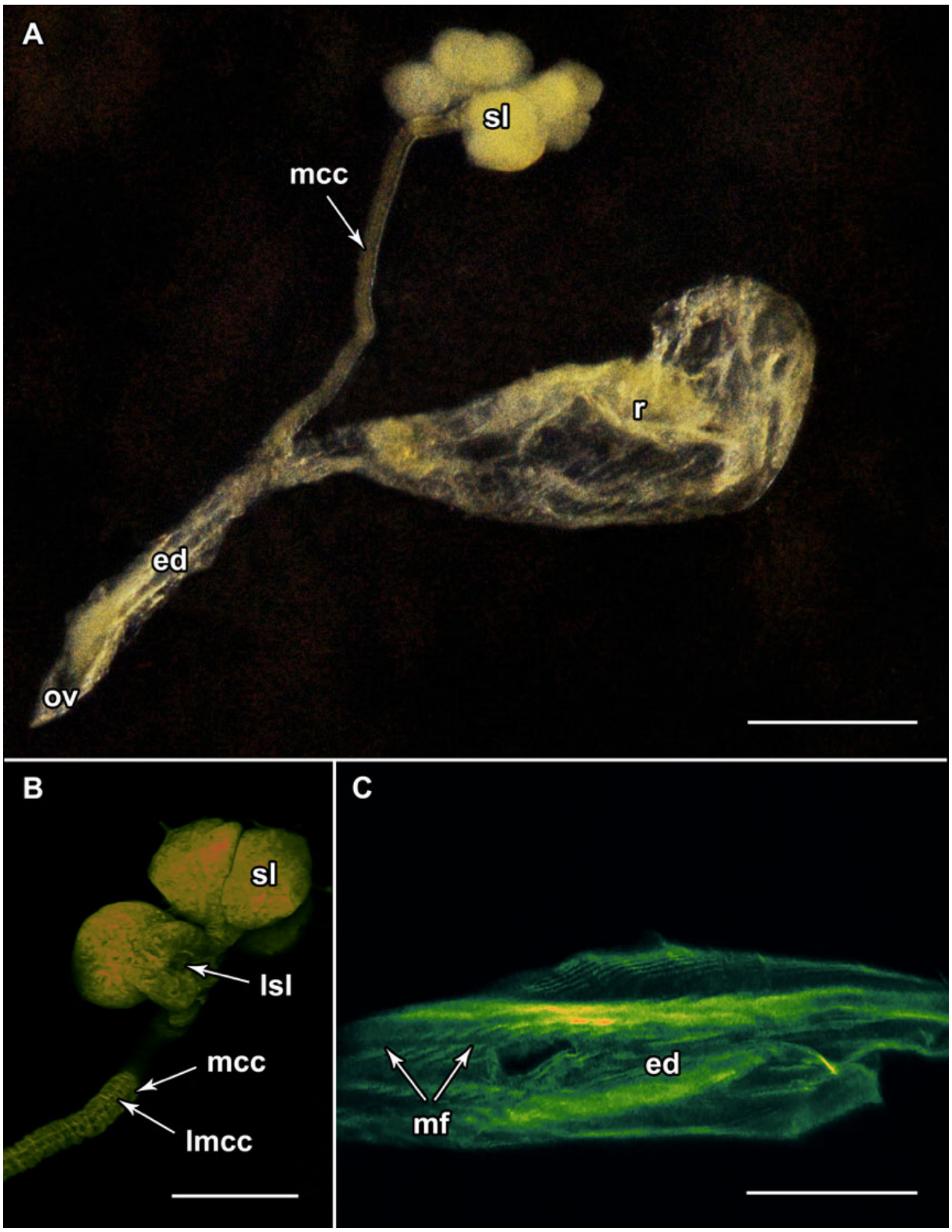


Figure 6. Morphology of the pygidial glands of *Stomis pumicatus* observed with BFM (**A**) and NLM (**B**,**C**): (**A**) whole pygidial gland apparatus; (**B**) close-up view of secretory lobes and main collecting canal; (**C**) close-up view of efferent duct. Abbreviations: ed—efferent duct; lmcc—lumen of main collecting canal; lsl—lumen of secretory lobe; mcc—main collecting canal; mf—muscle fibres; ov—opening valve; r—reservoir; sl—secretory lobe. Scales: (**A**)—250 µm; (**B**)—200 µm; (**C**)—100 µm.

Table 2. Comparative data on the dimensions (in µm) and the number of pygidial gland structures in the four Pterostichini ground beetle species. Abbreviations: ED—efferent duct; MCC—main collecting canal; R—reservoir; SL—secretory lobe.

Species	Gland Structure							
	SL		MCC		R		ED	
	Number	Diameter	Length	Diameter	Length	Width	Length	Diameter
P. cupreus	10–15	130–150	4000	50	660	510	600	180
P. melanarius	20–25	90–120	2630	50	460	560	880	160–200
P. anthracinus	15–20	190–230	2520	70	390	470	1000	170
S. pumicatus	7–10	70–80	610	30	810	270	510	80–120

In *P. melanarius*, 20–25 secretory units constituted a cluster. A single unit had a diameter of 90–120 µm. The secretory units were grouped in several clearly separated aggregations (Figure 4A). The main collecting canal was about 2.63 mm long, with an outer diameter of 50 µm and a lumen diameter of 10 µm. The reservoir was more or less heart-shaped, with the apical notch dividing its anterior part into two unequal portions (Figure 4A). The reservoir was 460 µm long and had a maximum width of 560 µm. The muscle layer was well-developed (Figure 4C). The efferent duct was 880 µm long, with a width varying from 160 to 200 µm along its length (Table 2).

In *P. anthracinus*, 15–20 secretory units formed a cluster (Figure 5A). The diameter of a single unit varied between 190 and 230 µm (Figure 5B). The main collecting canal was about 2.52 mm long, with an outer diameter of 70 µm and a lumen diameter of 20 µm. The reservoir was more or less heart-shaped and unevenly divided into two parts by the apical notch (Figure 5A). The muscle layer was relatively thin and semitransparent. The reservoir was 390 µm long and 470 µm wide at its maximum. The efferent duct was 1 mm long and 170 µm wide. Its muscle wall was thin and semitransparent, with muscle fibres extending transversely (Figure 5A,C) (Table 2).

In *S. pumicatus*, a cluster consisted of 7–10 secretory units (Figure 6A). A single secretory unit was 70–80 µm in diameter (Figure 6B). The main collecting canal was 610 µm long, with an outer diameter of 30 µm and a lumen diameter of 8 µm. The reservoir was sac-shaped (Figure 6A). The constriction was laterally displaced and could only be observed in the turgescent state, giving the reservoir a more or less kidney-shaped form. The extremely thin muscle wall was transparent. The reservoir measured 810 µm in length and 270 µm in width. The efferent duct was 510 µm long and 80–120 µm wide, with muscle fibres extending transversely (Figure 6C) (Table 2).

We found no differences between the pygidial gland structures of males and females of the same species in terms of dimensions, shape, or number. The secretory lobes showed the greatest variation in measured diameter. This variability was more pronounced within individual beetles than between different specimens.

4. Discussion

4.1. Chemical Survey

The great diversity of carboxylic acids in the analysed ground beetles corresponds to that recorded in other Pterostichini [24,25]. Carboxylic acids are produced by species of numerous ground beetle tribes as an integral part of their pygidial gland secretions [5]. These are short-chain, volatile compounds with a pungent odour that have been shown to deter various invertebrate and vertebrate predators [28,29]. Although ground beetles produce quantities of carboxylic acids that can be lethal to other insects, it is generally

assumed that the irritant effect of a much smaller quantity provides sufficient protection against these predators [30].

Our work showed that methacrylic acid and tiglic acid were present in all five species examined. Both compounds are common in the defensive secretions of Pterostichini and usually occur together. The use of the methacrylic acid/tiglic acid pair for defence has evolved in numerous groups of ground beetles [5]. The former acid appears to be consistently present in these groups and is typically accompanied by a smaller amount of tiglic acid [29]. Methacrylic acid was the predominant compound in the three Pterostichini species examined (*P. cupreus*, *P. melanarius*, and *P. anthracinus*), which is consistent with the literature [29]. However, only a small amount of methacrylic acid was detected in *S. pumicatus*. A similar situation was previously observed in another pterostichine, *Molops montenegrinus* (Apfelbeck, 1890), where methacrylic acid was present in smaller quantity. In contrast, the dominant compounds in these two species were isovaleric acid (50.6% in *S. pumicatus*) and 2-methylbutyric acid (31.25% in *M. montenegrinus*), respectively [24]. While tiglic acid was the second most dominant compound in *P. anthracinus* and *P. melanarius*, it was only a minor component of the secretion in *P. cupreus*. Furthermore, *Pterostichus niger* (Schaller, 1783) and *M. montenegrinus* are known to lack tiglic acid [24,25]. In contrast, in *S. pumicatus*, the concentration of tiglic acid was much higher than that of methacrylic acid, whereas in the secretion of *T. cognatus*, the relative percentages of the two acids were almost equal, with a slight predominance of tiglic acid. The predominance of tiglic acid over methacrylic acid is rare and occurs only under controlled laboratory conditions [14,28]. Isoleucine, an essential dietary amino acid, is known for its role in the synthesis of tiglic acid [14]. Methacrylic acid is synthesised from valine, another essential amino acid [17,31]. This suggests that changes in the ground beetle diet can significantly affect the production of these acids [14,17,31]. This could explain the occasional absence of tiglic acid and the variation in the concentration of the two acids in the defensive secretions of these beetles. However, we cannot exclude the possibility of differences between the genera in this regard.

The dominant compound in the secretion of *S. pumicatus* was isovaleric acid. It was also a minor constituent in the extract of *P. melanarius*. This compound is generally rare in the defensive secretions of Pterostichini. So far, it has been detected in *Pterostichus sulcitarsis* Morawitz, 1863 [32] and *P. californicus* (Dejean, 1828) [14]. Isobutyric acid was another dominant compound in the secretion of *S. pumicatus* and, along with methacrylic acid and tiglic acid, was found in all studied species. This acid is recorded in many recently analysed species of Pterostichini [5]. Acetic acid and propionic acid are occasionally detected as minor constituents in the pygidial gland secretions of Pterostichini. The former chemical was present in all samples except *S. pumicatus*, while the latter was absent in *T. cognatus*. Senecioic acid is another less abundant acid that accounted for a small proportion of secretions [24]. It was present only in the extracts of *P. anthracinus* and *T. cognatus*. Within the genus *Pterostichus* Bonelli, 1810, this acid was recorded in the secretions of *Pterostichus melas* (Creutzer, 1799) and *P. californicus* [14,24].

With regard to the distribution of minor secretion components, it is important to note that modern techniques have proven to be far more effective for detecting these constituents [14,24,25]. As most acids considered rare have been reported mainly in recent studies and are present in low amounts, it may be assumed that their distribution in the defensive secretions of ground beetles is actually greater than it appears. Although the precursors and metabolic pathways for some carboxylic acids produced by the pygidial glands are known, the full biochemical capabilities of ground beetle species remain unknown. Vesović et al. [33] demonstrated that distant populations of the same ground beetle species differ in pygidial gland secretion composition. However, it is unclear whether the observed differences are genetic or environmental. The first option is highly unlikely,

given the generally conservative distribution of major secretion components in the subfamilies and the inconsistency in the distribution of minor secretion components, even among closely related species within the family [5,34]. Environmental factors such as different food sources or temperature fluctuations may account for the observed differences. However, there are very few studies to date that have examined the effects of these factors on changes in defensive secretion mixtures in ground beetles. Wheeler et al. [28] demonstrated that some changes occur when beetle diet is controlled. The same study showed that small variations in temperature did not affect secretion production. Nevertheless, it would be instructive to investigate the effects of more diverse food sources and greater temperature fluctuations on the pygidial gland products of ground beetles. It has also been suggested that the breeding season may influence the chemical composition of secretions in some ground beetle species [35,36].

Ketones have previously been detected in Pterostichini. 2-Pentadecanone, which we recorded in *S. pumicatus*, was previously found in species of the genera *Loxandrus* LeConte 1852 and *Oxycrepis* Reiche, 1843 [7,13]. 2-Tridecanone, which we detected in all species in the current study except *P. cupreus*, is reported here for the first time in the defensive secretions of representatives of the tribe Pterostichini. Undecane and tridecane, which we detected in the extracts of *P. cupreus*, *P. anthracinus*, and *P. melanarius*, are medium-chain alkanes commonly found in Pterostichini and are often accompanied by various carboxylic acids [5]. Interestingly, none of these alkanes were found in the secretion of *S. pumicatus*. It is assumed that both ketones and medium-chain alkanes serve as penetration enhancers [29,37], although a dual function of the latter chemicals (especially undecane) was suspected in earlier studies [38]. Furthermore, tridecane is known to promote the residual action of irritants in some pentatomid bugs, so a similar role in the pygidial gland secretions of ground beetles should not be excluded without further examination [29]. Schildknecht [10] detected the combined presence of three alkanes (decane, undecane, and tridecane) in six Pterostichini species, some of which have been recently reanalysed [24,25], but none of the latter samples contained all three alkanes. The gland secretion of *P. melas* contained undecane and tridecane [24], while that of *P. niger* contained only undecane [25]. The defensive secretion of *P. cupreus* tested here lacked decane. These variations could indicate geographical differences in the chemical composition of the defensive secretions of these ground beetle taxa. The extract of *T. cognatus* lacked alkanes, but it was the only sample that contained an alkene (8-heptadecene). In the present work, several long-chain hydrocarbons were recorded. These compounds are occasionally detected in different ground beetle species [34,39]. Their origin is questionable, as these compounds are typically present in the cuticle of many insects, including ground beetles [40]. Almost all of the long-chain hydrocarbons detected in the sample of *P. cupreus* were also found in the cuticle of the same species [40]. It is possible that these compounds actually originate from the cuticle, but their role in the defence of these beetles remains questionable.

Schildknecht et al. [9] were the first to analyse the chemical composition of the pygidial gland secretion of *P. cupreus* and its congener *P. lepidus* Leske, 1785. The study reported the presence of methacrylic acid, tiglic acid, and unidentified alkanes in the former species, which were later identified as decane, undecane, and tridecane [10]. Except for decane, the same components were detected in the same species in the current study, along with 12 additional compounds new to the genus *Poecilus* Bonelli, 1810, but present in other genera of the tribe Pterostichini [5]. Two additional congeners were chemically analysed: *Poecilus fortipes* (Chaudoir, 1850) and *P. versicolor* (Sturm, 1824) [12]. Their dominant compounds were identified as methacrylic acid and tiglic acid, while the minor constituents of the extracts were intentionally omitted [12], limiting the possibility of comparison between the species. Based on the available evidence [12], the defensive products of the genus

Poecilus are consistent with those of other Pterostichini. The lower relative percentage of tiglic acid in *P. cupreus* may be due to the specific metabolic status of the individuals examined or may represent a common feature of the species. Further analysis is required to confirm either hypothesis. In previous literature, Schildknecht et al. [9] did not report the concentration of this acid in the two *Poecilus* species (*P. cupreus* and *P. lepidus*), while Kanehisa and Murase [12] reported a relative percentage of tiglic acid of 4% in the secretions of *P. versicolor* and *P. fortipes*, which is higher than in *P. cupreus*, but lower than the typical concentration of this acid in other Pterostichini [14,24,25].

The pygidial gland secretions of *P. melanarius* and *P. anthracinus* contained a similar mixture of carboxylic acids. The former species lacked senecioic acid, while the latter lacked isovaleric acid. Comparison of the pygidial gland secretion extracts of *P. anthracinus* and its recently studied relative, *Pterostichus nigrita* (Paykull, 1790) [24], revealed a similar composition of active compounds. *Pterostichus anthracinus* lacked 2-methylbutyric acid, whereas *P. nigrita* lacked senecioic acid and 2-tridecanone [24]. The recorded relative percentages were also almost identical. However, it is highly unlikely that any of these features are relevant for chemotaxonomy. Individuals of both species were collected from moist soil on a river bank [24] and may have been exposed to similar ecological conditions, which could also affect secretion content. All recorded compounds were occasionally detected in other taxa of Pterostichini [5]. Furthermore, the metabolic pathways for the synthesis of all acids found here have not been fully elucidated, but it is known that some are diet-dependent [14,28,31]. Geographically-dependent variation in defensive secretion has so far only been suspected in Pterostichini, but has been demonstrated in some other ground beetles [33].

The relationship between pygidial gland morphology and the chemical nature of their secretions has long been established [12]. The contractions of the reservoir muscle wall are responsible for discharging the secretions of the species investigated in this paper. Species with well-developed musculature release secretions by spraying, while those with a thin muscle layer typically ooze them out [7]. During handling, *S. pumicatus* emitted a markedly more pungent and longer-lasting odour than the other examined species. Our chemical analyses revealed that this effect is primarily due to the high proportion of isovaleric acid [41], the dominant component of its pygidial secretion mixture. Such a persistent odour may compensate for the smaller size of the glands and the weaker gland musculature, which together limit the beetle's ability to spray the secretion at a distance or to aim at a target. In contrast, methacrylic acid, the typical dominant compound in many Pterostichini with thick reservoir musculature [24], has a less penetrating odour, but elicits a stronger burning sensation upon skin contact [42]. These differences suggest that the defensive strategy is closely linked to the chemical nature of the secretion. Methacrylic acid is more effective for long-range defence, as it can be sprayed away from the body [11], minimising the risk of self-damage. In contrast, the slowly volatile isovaleric acid is oozed over the beetle's body [11], producing a sharp and persistent odour that repels predators at close range while causing minimal damage to the beetle itself.

4.2. Morphological Survey

The greatest difference in the morphology of the pygidial glands among the four Pterostichini species examined was observed in *S. pumicatus*. The pygidial glands of the other three species were similar in general appearance, which is consistent with the literature data [6,18,19,24,25].

According to the available evidence [5,6], pygidial glands in representatives of the genus *Poecilus* appear quite similar to those of species in the genus *Pterostichus*. However, the reservoirs of *S. pumicatus* clearly differed from those of the other Pterostichini.

Interestingly, we detected 20–25 secretory units in *P. melanarius*, whereas Forsyth [6] reported 13 secretory units in the same species. Overall, the number of secretory units varied slightly among the species studied. The smallest number of secretory units was recorded in *S. pumicatus* (7–10), while the greatest was observed in *P. melanarius* (20–25). However, the number of secretory lobes in both species falls within the usual range for European representatives of the tribe (8–25) [6]. Kanehisa and Shiraga [42] reported an even larger number of secretory units per gland in Japanese Pterostichini (20–50), but without precise details of the species concerned. Taking body size into account [26], the species studied here had smaller secretory units than some previously examined [24,25]. The main collecting canal was longest in *P. cupreus*, while it was shorter in the two *Pterostichus* species studied (*P. melanarius* and *P. anthracinus*), similar to previously investigated congeners [24]. The greatest length of the main collecting canal in a *Pterostichus* species has so far been found in *P. niger* [25]. The reservoir of all species examined in this study, except *S. pumicatus*, had a uniform appearance common to representatives of the tribe Pterostichini [6,18,19,24,25]. Species of this tribe are known to have a triangular to heart-shaped reservoir, depending on the depth of the apical notch [18,25]. In some species, the top of the reservoir is almost completely flat, while in others, it is clearly divided into two parts [24,25]. The two *Pterostichus* species studied here had a similar, well-defined apical notch, which was visible from the centre, dividing the apical part of the reservoir unevenly. A similar situation was observed in *P. melas* [24]. The reservoir of Pterostichini is typically equipped with more or less thick muscle layers that extend mainly in the longitudinal direction [18,24]. Our study has shown that the appearance of the reservoirs of the tested species of the genera *Poecilus* and *Pterostichus* is consistent with the literature data. *Pterostichus anthracinus* had a slightly thinner muscle layer, which made its reservoir slightly transparent, similar to its relative *P. nigrita* [24]. In *S. pumicatus*, the reservoir wall was characterised by an extremely thin and transparent muscle wall, which is not common for representatives of the tribe [6]. Another deviation from the rule is the position of the depression of the reservoir in this species, which, unlike other Pterostichini, was visible laterally.

The average secretion output varies between ground beetle species and is related to the average body size of the individuals. Larger species and conspecifics of ground beetles have bigger reservoirs, so the amount of secretion they discharge is greater [15,30]. It has not been established that the shape of the pygidial glands and their parts affects the quantity of discharged secretion, however, the shape of the secretory lobes is related to the main chemical components of the secretions they produce [12,43].

5. Conclusions

The deterrent compounds in the pygidial gland secretions of the ground beetles studied in this work are qualitatively similar to those of other Carabidae. However, the quantitative differences observed, especially in the secretions of *S. pumicatus* and *T. cognatus*, may represent unique features of the genera in question. This highlights the importance of the proportion of secretion compounds when considering intergeneric differences, as well as environmental factors and the biochemical pathways underlying the observed differences. Further analysis of pygidial gland secretions from all genera of the tribe Pterostichini, with emphasis on those that have been little studied chemoecologically to date, is required to elucidate the diversity of their chemical compounds. One idea for future studies is to quantify not only the percentage of compounds within pygidial gland secretion mixtures, but also the amount of secretion and its individual chemical components. This is useful for determining whether there are variations between species, as well as within the chemical components of their secretion mixtures.

The two *Pterostichus* species examined did not differ significantly in the general appearance of the pygidial glands. Minor morphological differences were evident in the number of secretory lobes and the size of the glands in relation to the body size. As our study provides the first data on the morphology of the pygidial glands in species of the genera *Poecilus* and *Stomis*, it is not yet possible to draw comprehensive conclusions about the pygidial gland structure typical of these genera.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d18020112/s1, Table S1. Chemical composition of pygidial gland secretions from five ground beetle taxa analysed by GC-FID and GC-MS, with spectral data for the 10 most abundant ions.

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