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Studies on the defensive behaviour of *Hypselodoris* species (Gastropoda: Nudibranchia): ultrastructure and chemical analysis of mantle dermal formations (MDFs)

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Abstract. The mantle dermal formations (MDFs) of *Hypselodoris webbi* (D'orbigny, 1839), and *H. cantabrica* (Bouchet and Ortea, 1980) are globular sub-epidermal structures located in the cephalic and caudal regions. Histologically they consist of an accumulation of vacuolate cells surrounded by a basal lamina and an outer muscular capsule. Chemical analysis of *H. webbi* MDFs reveals the presence of high concentrations of longifolin, a well known deterrent furanosesquiterpenoid that had been previously isolated from this species. In the present paper it is demonstrated that the great majority of longifolin accumulated in the mantle of *H. webbi* is stored in the MDF vacuolar cells. This finding strongly suggests that such structures act as chemical weapons against predation, mainly protecting vital organs such as the head, rhinophores and gills.

Introduction

The evolutionary loss of the shell in most opisthobranch gastropods, e.g. *Hypselodoris* species, is correlated with the development of a broad range of sophisticated defensive strategies (Thompson 1960a, Edmunds 1966a, b, Ros 1976, 1977), one of the most effective of which appears to be the utilization of deterrent substances (Thompson 1960a, b, 1983, Edmunds 1968, Marbach and Tsurnamal 1973, Thompson and Colman 1984). An early report suggesting the occurrence of chemical defensive mechanisms in these gastropods showed that the secretions of nudibranchs were distasteful to man (Thompson 1960a). More recently (Hagadone et al. 1979), the mucus secreted by *Phyllidia varicosa* was found to contain terpenoid substances that prove repugnant or toxic to potential predators. Since then similar deterrent metabolites have been isolated from the mantle of many other dorid nudibranchs (for reviews see Schulte and Scheuer 1982, Faulkner and Ghiselin 1983, Faulkner 1984, Cimino et al. 1986).

A high degree of specialization in the use of deterrent chemicals among dorid nudibranchs has been achieved by *Dendrodoris limbata* and *D. grandiflora*, which are capable of synthesizing drimane sesquiterpenoids de novo (Cimino et al. 1983, 1985a, b, 1986). However, these deterrent chemicals are usually obtained from sponges via the diet and are accumulated in the mantle through the digestive gland. To date it has not been reported whether these metabolites are stored preferentially in specific sites of the body, since they have been extracted either from whole individuals or from large fragments of mantle.

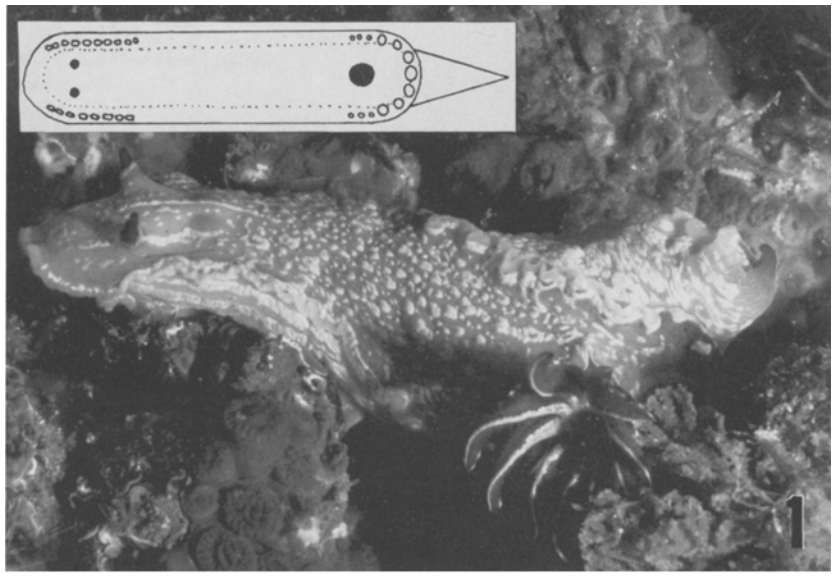
Mantle dermal formations (MDFs), i.e. globular structures located in the mantle of several *Hypselodoris* species, have been suggested to play a defensive role. These structures have a particular unpleasant taste that resembles that of the mucus released by diverse species of *Chromodoris* (García-Gómez et al. 1986). It is thus possible that the MDFs of *Hypselodoris* spp., and probably those of other dorid nudibranchs as well, serve as storage organs containing high concentrations of defensive metabolites. In order to investigate this hypothesis, MDFs were removed from the mantle of *H. webbi* and *H. cantabrica* to study their ultrastructure and chemical composition.

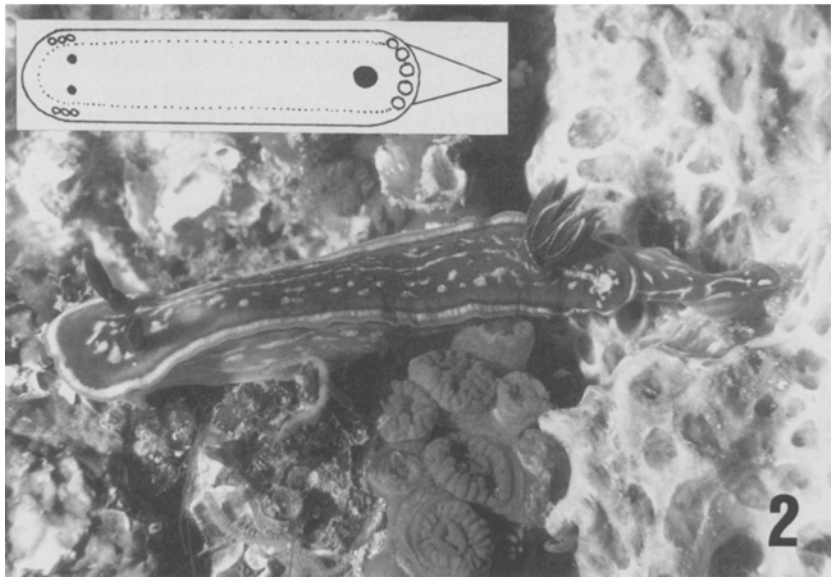
Materials and methods

Hypselodoris webbi (D'orbigny, 1839) and *H. cantabrica* (Bouchet and Ortea, 1980) specimens were collected from the Straits of Gibraltar, either from the coast of Algeciras (Cádiz, Southern Iberian Peninsula) or Ceuta (Northern Africa). Specimens were transported to the laboratory and kept in aerated sea water for 1 to 6 d at room temperature. For the anatomical study, specimens were preserved by first freezing in seawater and then transfer to 4% formaldehyde in seawater.

Ultrastructure

MDFs were removed from living *Hypselodoris webbi* and *H. cantabrica* specimens and fixed for 3 h in 2.5% glutaraldehyde in





Figs. 1 and 2. *Hypselodoris* spp. Photograph and inset diagram showing location of mantle dermal formations. **Fig. 1.** *M. webbi*, ×1.5; **Fig. 2.** *M. cantabrica*, ×2.2

0.1 *M* Millonig's phosphate buffer (pH 7.3) supplemented with 7% sucrose at 4 °C. Subsequently, samples were washed in buffer, post-fixed with 1% osmium tetroxide for 2 h at 4 °C, dehydrated through a series of acetones and embedded in epoxy resin according to Spurr (1969).

Semi-thin sections were cut on an LKB III ultramicrotome and stained with toluidine blue. Ultrathin sections were mounted on copper grids, stained with uranyl acetate and lead citrate, and viewed in a Siemens transmission electron microscope (TEM).

Chemical analysis

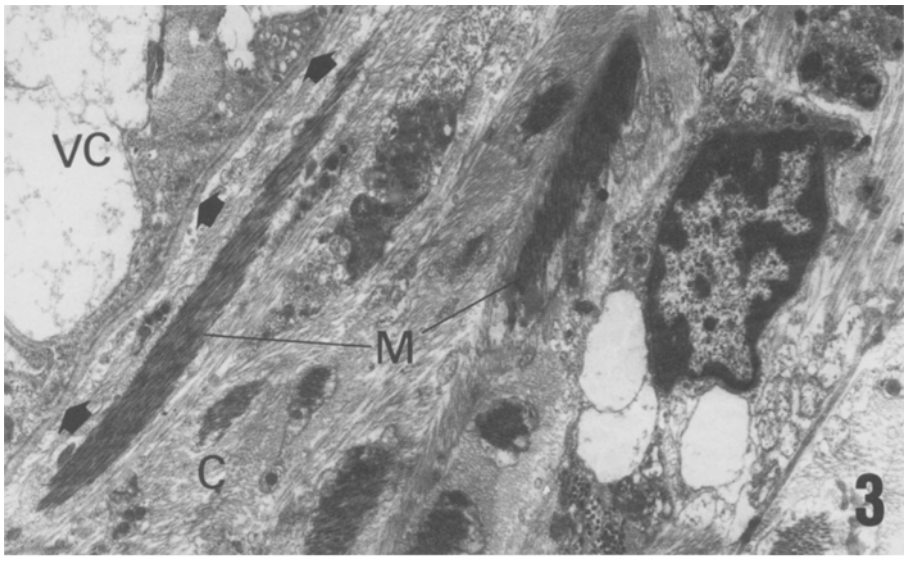
For MDFs analysis *Hypselodoris webbi* were used. This species was chosen because its large size allows for easy isolation and handling of the MDFs. MDFs from three specimens were separated in vivo from the mantle and extracted for 1 mo with acetone. Prior to extraction, MDFs were burst with forceps in order to facilitate solubilization of their contents. Fragments of mantle devoid of MDFs were also extracted with acetone.

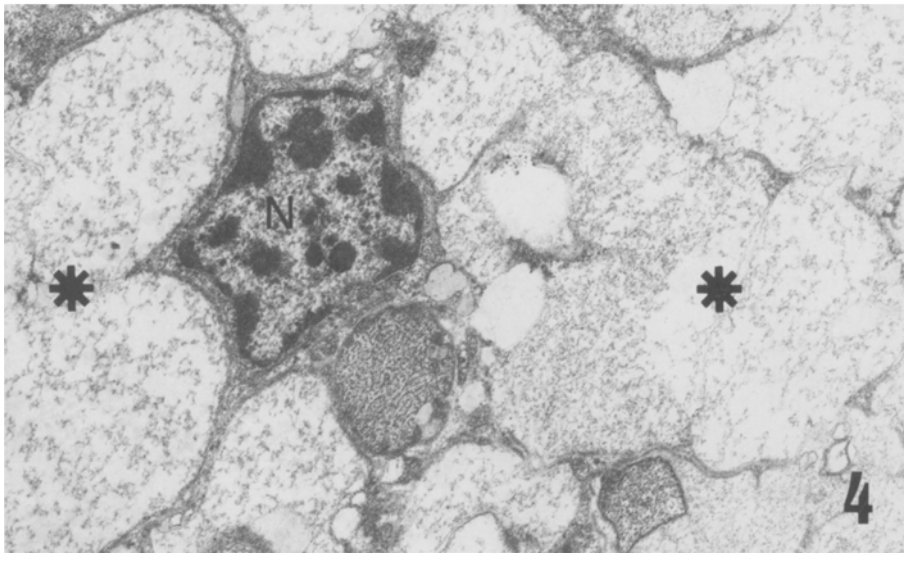
Acetone extracts were analysed by thin layer chromatography (TLC; precoated Merck Kieselgel 60 F 254 plates) and fractionated by column chromatography on Merck Kieselgel 60 powder (0.063 to 0.200 mm). Light petroleum was used in both chromatographic systems. The ^{1}H-NMR (nuclear magnetic resonance) spectrum was measured in $CDCl_3$ on a WM 500 Bruker spectrometer; TMS was used as internal reference. Mass spectra were taken on a Kratos MS 50 instrument.

Results

Location and size of MDFs

The MDFs of *Hypselodoris* spp. are small spherical structures that can be easily observed through the mantle skin because of their opaque white coloration. Although the location of MDFs in the European *Hypselodoris* species is not constant, in both *H. webbi* and *H. cantabrica* they are situated in the cephalic region, at both sides of the rhinophores, and in the rear region of the mantle, close to the branchial ring (Figs. 1 and 2). The number of MDFs in a species varies from one specimen to another, and is apparently correlated to some extent with its' size. This





Figs. 3 and 4. *Hypselodoris spp.* Transmission electron micrographs of *H. cantabrica* mantle dermal formations. **Fig. 3.** Muscular capsule. Muscle fibres (M) are embedded in an abundant collagenous extracellular matrix (C). The basal lamina (arrows) separates this layer from the outer vacuolar cells (VC); × 6 000. **Fig. 4.** Portion of a peripheral vacuolar cell showing numerous vacuoles enclosing an amorphous content. Some of them appear to coalesce into larger vacuoles (asterisks). The nucleus (N) is central; × 11 000

variability seems to be more marked in the anterior MDFs.

Whereas in adult *Hypselodoris webbi* the MDF size ranges from 1000 (anterior MDFs) to 2000 μm (posterior MDFs), in *H. cantabrica* the MDFs measure between 600 (anterior MDFs) and 1400 μm (posterior MDFs). In the former they are more numerous (15 to 18 anterior MDFs and 14 to 20 posterior MDFs) than in the latter (3 to 4 anterior MDFs and 7 posterior MDFs).

Data on the anatomy of MDFs in all the European species of chromodorid nudibranchs based on the study of 126 specimens were given previously (García-Gómez et al. 1986).

Anatomy and ultrastructure

The MDFs are embedded in the subepidermal connective tissue. They consist of an outer thick capsule enclosing an accumulation of large vacuolar cells. The capsule is formed by a great number of muscle fibres oriented in all directions and some connective cells containing dense granules. Abundant collagen fibres are present in the extracellular matrix between muscle fibres (Fig. 3).

Underlying the muscular layer is a basal lamina that encloses the vacuolar cells. While in *Hypselodoris cantabrica* the basal lamina is readily visible (Fig. 3), in *H. webbi* it is difficult to detect, probably because it is masked by the surrounding collagen fibres. The peripheral cells of the MDFs rest on the basal lamina and show a considerable number of cytoplasmic vacuoles filled with an amorphous content (Fig. 4). The central cells possess fewer and larger vacuoles, most of the content of which appears to be removed along with the acetone solutions used in the tissue processing, especially in samples where the post-fixation step is omitted. Some of these cells show a unique "big" vacuole surrounded by a cytoplasmic ring containing the nucleus. In MDFs post-fixed with osmium tetroxide and stained with toluidine blue a pale vacuolar content of varying intensity is observed in semithin sections. Under the electron microscope the vacuolar content does not appear evenly distributed

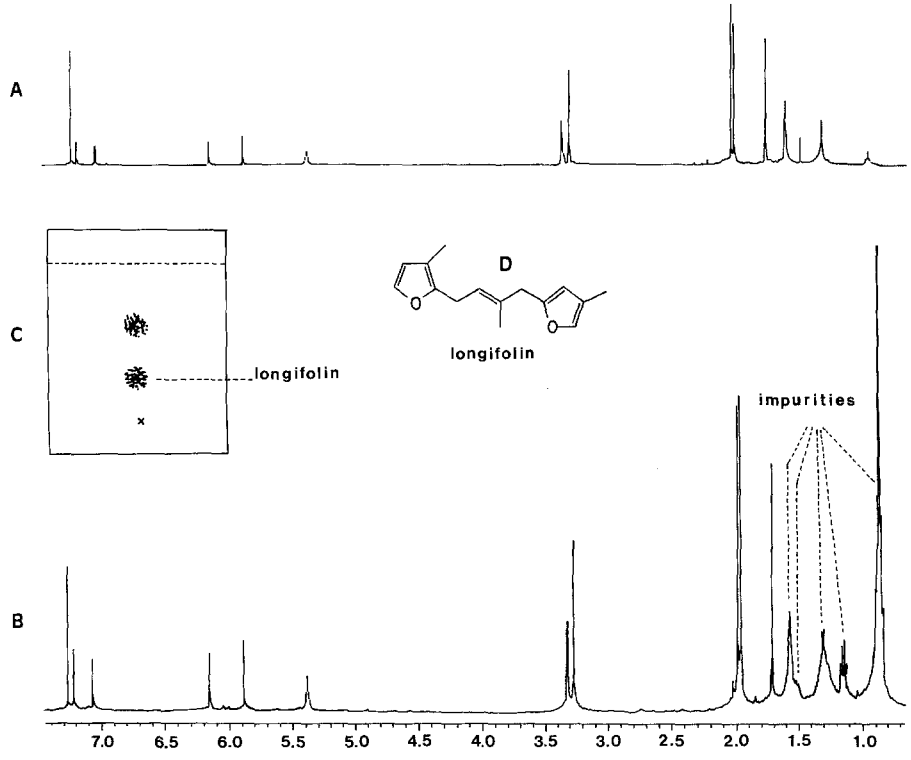


Fig. 5. (A) ^{1}H-NMR spectrum of longifolin from *Hyperselodoris webbi*. (B) ^{1}H-MNR spectrum of longifolin from the Mediterranean sponge *Pleraplysilla spinifera*. (C) Thin layer chromatography of acetone extracts from *H. webbi* mantle dermal formations. (D) Structure of longifolin

throughout the vacuole. The appearance of this substance, as well as the extension to which it is confined in the vacuole vary considerably. These features are presumably the result of artifacts due to tissue processing and in normal conditions such a substance probably occupies the total vacuolar volume.

Ultrastructural observations suggest that in the MDF cells the vacuolar size increases as a result of coalescence of small vacuoles into larger ones (Fig. 4). These events appear to occur from the edge to the centre of the MDF.

The MDF cell cytoplasm surrounding the vacuoles is poor in organelles and possesses free ribosomes, vesicular cisternae of smooth endoplasmic reticulum and occasionally (in *Hypselodoris webbi*) lipid droplets associated in clusters that stain dark with osmium and toluidine blue.

The general organization of the MDFs in *Hypselodoris webbi* and *H. cantabrica* is similar to that of the other European *Hypselodoris* species. However, it differs in some respects from the histological arrangement observed in *Chromodoris* MDFs (García-Gómez et al. 1986). Nevertheless, in both *Hypselodoris* and *Chromodoris* species the ultrastructure of the MDF cells is apparently similar.

Chemical analysis

Acetone extracts (35 mg) from the MDFs (dry weight after treatment with acetone = 20 mg) showed in thin layer chromatography (light petroleum) the presence of two compounds (R_f = 0.4 and 0.3) sensitive to Erlich reagent (Fig. 5C). The more polar component was isolated (8 mg) by column chromatography (light petroleum) (Table 1), while the second, highly unstable, one was recovered in amounts too small to be analysed by chemical methods.

Table 1. *Hypselodoris webbi*. Results of the chemical analysis of the acetone extracts obtained from mantle dermal formations (MDFs) and fragments of mantle devoid of these structures. Dry weight (dry wt) data after extraction with acetone

Fragment	Dry wt (mg)	Extract (mg)	Metabolites
Mantle	200	22	Longifolin (2 mg)
MDFs	20	35	Longifolin (8 mg)

The ^{1}H-NMR spectrum (Fig. 5A) led to identifying the structure as longifolin (Fig. 5D). This identification was confirmed by HREIMS measurements.

The same chromatographic procedure on the acetone extract from the mantle devoid of MDFs (dry weight = 200 mg) led to only 2 mg of longifolin (Table 1).

Discussion

Among the diverse mechanisms of defence present in opisthobranchs, the utilization of deterrent chemical substances is particularly effective. In some opisthobranchs acid secretions are well known to make predatory fish reject these as food (Thompson 1960b). Recently, other repulsive substances, usually terpenoids, have been isolated from many opisthobranch species and shown to have deterrent properties. These substances accumulate at high concentrations in such organs as the digestive

gland and mantle (Cimino et al. 1981, 1982) and are typical of doridaceans, although they may be also encountered in dendronotaceans (Ayer and Anderson 1983).

Chemical analysis of *Hypselodoris webbi* MDFs supports the hypothesis that these structures act as defensive organs, since they accumulate longifolin, a furanosesquiterpenoid of well-known deterrent properties (Cimino et al. 1982, 1986) that was first isolated (Hayashi et al. 1972) from the terrestrial plant *Actinodaphne longifolia*. Although longifolin had been previously identified in the mantle of *H. webbi* (quoted as *Glossodoris valenciennesi*) (Cimino et al. 1982, 1986), it was unknown whether such a substance accumulated preferentially in specific sites. This seems evident from our observations which show that MDF vacuolar-cells store much higher concentrations of longifolin than the rest of the mantle.

Many of the mantle metabolites used as defence allomones by dorid nudibranchs have been traced to the sponges they consume and from which they are thought to be obtained (Hagadone et al. 1979, Hochlowski et al. 1982, Faulkner and Ghiselin 1983, Faulkner 1984, Cimino et al. 1986). Frequently, the defence metabolite of a dorid nudibranch has been identified in a particular sponge, but a predator-prey relationship between the two has not been determined. Such species of nudibranchs are believed to obtain the active principles from unknown dietary sources (Hochlowski and Faulkner 1981, Faulkner 1984, Okuda and Scheuer 1985). For instance, the furanosesquiterpene longifolin, isolated from the mantle and digestive gland of *Hypselodoris webbi*, had been previously obtained from the sponge *Pleraplysilla spinifera* (Cimino et al. 1975). Both marine organisms are found together in the Gulf of Naples, Italy, which would suggest a possible predator-prey relationship. However, the chemical composition of *P. spinifera* is characterized by many other metabolites that are absent in *H. webbi*. Because of this it has been suggested that longifolin in *H. webbi* is derived from feeding on an unknown sponge. Nevertheless, until now *H. webbi* has been found far from prey containing longifolin and the finding of this metabolite in two populations of the same species living in different localities (Gulf of Naples and Straits of Gibraltar) suggests two possibilities with regard to the origin of longifolin in this species: (a) the nudibranch is able to synthesize de novo its own chemical defence; (b) the mollusc can select, in different geographical areas, sponges containing the same kind of metabolites. Of course, confirmation of the dietary origin of longifolin in *H. webbi* would require a systematic screening of the sponges living in the same area.

The MDFs of *Hypselodoris* species do not open to the mantle surface, as stated by Rudman (1984) in his diagnosis of the genus, but are entirely surrounded by a thick capsule. The fact that the peripheral MDF vacuolar cells rest on a basal lamina appears to indicate an epithelial character and suggests that the MDFs could originate in the same manner as Edmunds (1968) suggests for the subepidermal multicellular acid glands in *Discodoris* and *Anisodoris* species. According to Edmunds, initially scattered epidermal cells first concentrated into pockets, thus multiplying their defensive effectiveness, and finally sank into the connective tissue.

The presence of structures accumulating deterrent metabolites would provide an effective strategy of defence. If a potential predator would reach to bite the mantle, the MDFs would rupture releasing unpleasant substances causing the predator to cease its attack.

The secretion of waste metabolites by mantle glands or their accumulation in isolated structures of mantle might have a selective basis because such substances could be used as weapons against predators.

The principles or their precursors contained in the MDF must pass to the vacuolar cells through the muscular capsule and basal lamina. The metabolites would selectively accumulate in vacuoles that would increase in volume and coalesce to form one large vacuole that occupies the greater part of the cytoplasm. The increase in size undergone by the MDFs as the individual grows may be due to the progressive accumulation of material, which is stored in vacuolar cells.

It is concluded that *Hypselodoris webbi* is chemically protected against predation by accumulation of the deterrent longifolin in the mantle and, more particularly, in MDFs. The great anatomical and histological similarity of MDFs in all European *Hypselodoris* species that possess such structures (García-Gómez et al. 1986) leads one to suggest that all share the same defensive strategy. Ros (1977) assumed that the species of *Hypselodoris* and *Chromodoris* studied by him, although lacking acid secretions, produced some kind of repulsive substance. Thus, he suggested, for Iberian chromodorids, the occurrence of aposematic circles that would correspond to a Müllerian mimicry.

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