An investigation into the toxicity of tissue extracts from two distinct marine Polychaeta

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ABSTRACT

The present study investigated the potential toxicity of venomous secretions of two polychaetes, *Hediste diversicolor* and *Glycera alba* (Annelida: Phyllodocida). Toxic activity of putative toxins, measured on mussel gills through the Comet assay, revealed higher effects caused by extracts from *H. diversicolor* skin and *G. alba* specialised, jawed proboscis, when compared to control. The results suggest that *H. diversicolor* secretes toxins via skin for protection against predators, contrarily to *G. alba*, who secretes toxins for predation.

Animal poisons and venoms are typically complex mixtures of bioactives such as toxins and enzymes and contain small molecules like peptides and salts. These mixtures are secreted by glandular or subglandular systems of an animal and can cause a pathophysiological injury to other organisms (see for instance Nelsen et al., 2014). These bioactives, through persistent selective pressure between secretor and target organism that ultimately affect toxin efficacy, are effectively evolutionary adaptations for predation, defence and intraspecific competition (Fry et al., 2009). The evolution of these secretions commonly resulted in compounds with extremely high molecular stability plus specificity and potency against their recipients by disrupting physiological, biochemical and molecular processes via binding to specific targets (Fry et al., 2009; King, 2011).

The Polychaeta constitute a polyphyletic group within the Annelida. They are one of the most abundant and diversified group of marine invertebrates, occupying nearly every type of marine habitat from the intertidal to abyssal plains and hydrothermal vents. Albeit recent and comparatively sparse, research on polychaete venoms and toxins already yielded promising results that showcase potential of these marine invertebrates for the bioprospecting of novel bioactives (Rodrigo et al., 2021). Considered as one of the largest Order within the Polychaeta, Phyllodocida represents a group of holopelagic organisms that is distributed through a wide range of environments, spanning from shallow marine substrates to the deep-sea habitats (Bonyadi-Naeini et al., 2017; Díaz Díaz et al., 2017; Ravara et al., 2014). Many Phyllodocida are active predators and some are known to secrete toxins as part of their feeding strategy. It is the case of *Eulalia viridis*, an opportunistic predator of rocky intertidal that delivers, in its mucus secretions, a mixture of proteinaceous toxins aiming at immobilising and digesting its prey, as the proboscis is devoid of jaws (Rodrigo et al., 2015; Cuevas et al., 2018). Additionally, the same species is characterized by the productions of pigments whose toxic properties are modulated by light and that likely act as defence against foulants and predators (D'Ambrosio et al., 2020). Also, the transcriptomic profiling of *Glycera dibranchiata*, *G. fallax* and *G. tridactyla* venom glands revealed multiple mRNAs coding for putative venom protein precursors, including neurotoxic peptides, pore-forming proteins and permeabilising enzymes (von Reumont et al., 2014). Supported by these promising discoveries,
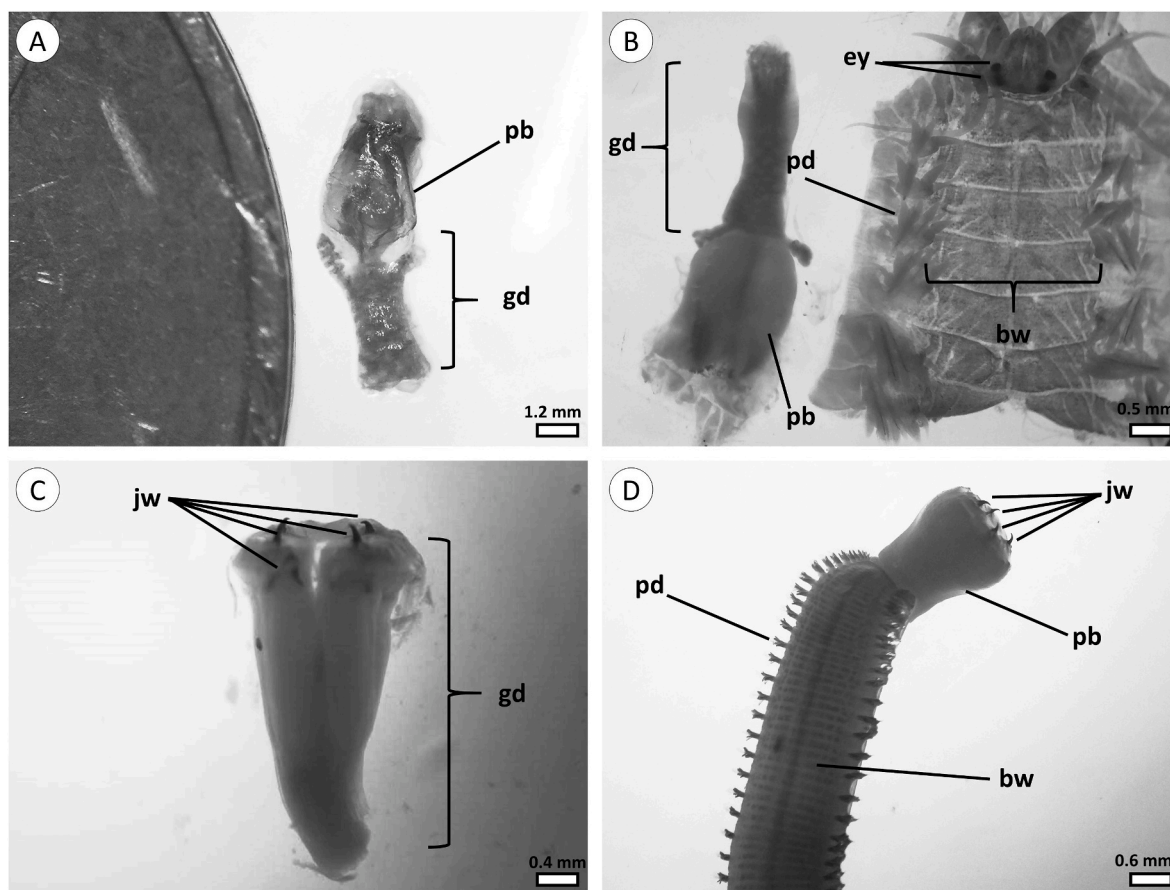
Fig. 1. Anterior anatomy of the Polychaeta *Hediste diversicolor* and *Glycera alba*. (A) Section of *Hediste diversicolor* showing the proboscis (pb) and glands (gd) underlying it (skin removed). (B) *Hediste diversicolor*'s body wall (bw) from the first segments before removing the parapodia (pd) where two pair of eyes (ey) can be identified. At the left but rotated 180°, is the proboscis (pb) and associated glands (gd), after separation from the body wall. The two blackish jaws can be seen inside their respective pouches at the tip of the proboscis (C) *Glycera alba*'s glands (gd) removed from the proboscis, showing the four sacks, each associated to a jaw (jw). (D) *Glycera alba* before microdissection, showing a partially everted proboscis (pb), with the four jaws fully observable (jw), as well as its small parapodia protruding from the body wall (bw).

the present work aimed to comparatively evaluate the toxicity of putative toxins of two distinct Phyllodocida annelids, *Hediste diversicolor* (O. F. Müller, 1776) and *Glycera alba* (O.F. Müller, 1776). *Hediste diversicolor* (Nereididae), is an opportunistic predator that uses its proboscis, an eversible pharynx equipped with two robust jaws, to prey on other invertebrate animals. On the other hand, *G. alba*, a strict carnivore belonging to the Family Glyceridae, a burrower that also uses its proboscis as a predation tool and seems to features four glands connected to slender chitinous jaws, which similarly to other *Glycera*, may secrete a protein-rich venom that should be injected into the prey through the jaws at the time of the attack (Gonçalves and Costa, 2020; von Reumont et al., 2014).

Hediste diversicolor (≈30 mm total length and weighting ≈90 mg each) were manually-collected from the estuarine intertidal mud flat in Alcochete bay, Portugal (38°45′28.7″N; 8°56′35.3″W). *Glycera alba* (≈29 mm total length and weighting ≈60 mg each) were manually-collected from the sandy-muddy intertidal flat at Seixal bay, Portugal (38°38′40.7″N; 9°06′07.8″W). For the toxicological assays, *Mytilus* sp. (≈2.5 cm shell length) were collected from a clean rocky intertidal area in Costa da Caparica beach (38°38′52.1″N; 9°14′48.6″W). Following a brief period of acclimatisation (c.a. one week), the worms (approximately 30 individuals of each species) were microdissected by separating the head from the trunk through a single incision at the base of the peristomium, then carefully removing the skin ensuring that all organs underneath stayed unscathed. The target organs were selected based on the predicted site of toxin secretion, namely the glands adjacent to the

jaw pouches of each species' proboscis. Portions of the body wall of either worm were also harvested from the trunk as reference organ to enable between-tissue comparisons. Body wall samples include epidermis and the underlying musculature. Particularly for *H. diversicolor*, a portion of the proboscis comprising the two jaws pouches and underlying secretory glands was excised (Fig. 1A and B). In turn, the area of glands within the proboscis was elected as main target. These glands were noted to be composed of four sacks (presumably toxin reservoirs), each gland being individually associated to a chitinous jaw (Fig. 1C and D).

Tissue samples were then homogenised in cold Dulbecco's phosphate-buffered saline (PBS, 4 °C) and centrifugated to precipitate insoluble cell debris. Then, the clear supernatant was collected, and total protein was quantified using a Nanodrop 1000 spectrophotometer (Thermo Fisher Waltham, MA, USA) at 280 nm. Mussel (*Mytilus* sp.) gills were the target organs chosen to compare *ex vivo* the toxicity of extracts between species and organs. Mussel demibranchs ($n = 3$ per treatment) were exposed to the extracts (diluted in PBS) to obtain a normalised concentration of 1 mg total protein mL^{-1}. Exposure lasted 15 min (in cold). The assays also included controls, i.e., gills treated with PBS only ($n = 4$). Damage to DNA was evaluated in mussel gills exposed to the different extracts using an adaptation of the alkaline single cell gel electrophoresis (Comet) assay for solid tissue described by Martins and Costa (2020). This assay is based on the extent of DNA migration during electrophoresis, which is directly dependent on DNA relaxation following damage present in the nucleoids that remain embedded in
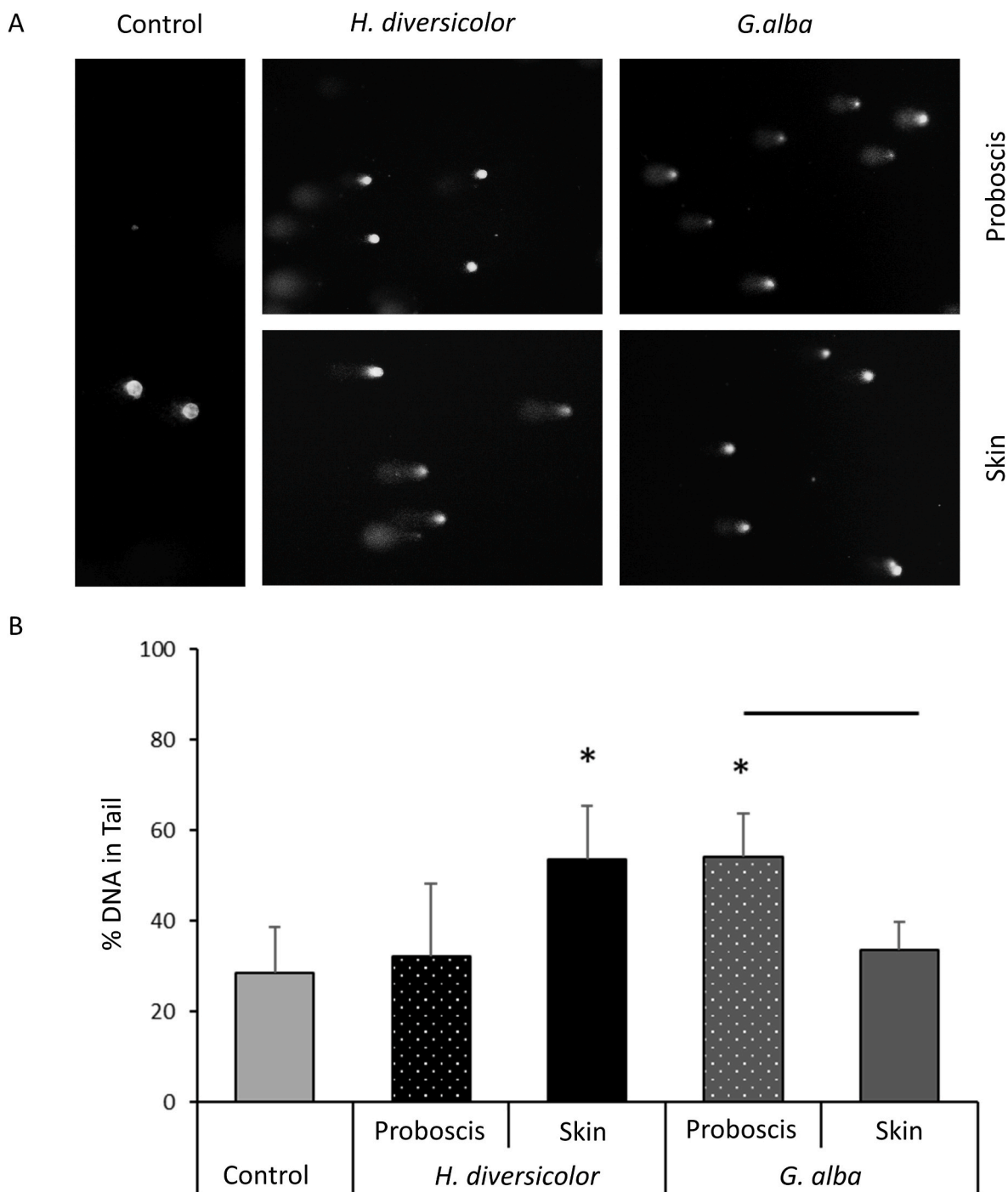
Fig. 2. Comet assay results from the gills of mussels exposed *ex vivo* to crude protein extracts of *H. diversicolor* and *G. alba* skin and proboscis A) Representative Comet fields from each tested condition, 200× magnification. B) % DNA in Tail, expressed as means + standard deviation. * and bar indicate significant differences to the control (gills treated with PBS only) and between organs, respectively (Student's *t*-test, *p* < 0.05).

agarose after cell lysis (Tice et al., 2000). Normality and homoscedasticity of data were assessed by the Shapiro–Wilk sand Levene's tests, respectively. Comparisons between exposure to extracts and controls were done with the Student's *t*-test. All statistics were computed using R 3.6 (Ihaka and Gentleman, 1996). A significance threshold of 0.05 was considered for all analyses.

The crude extracts from skin and proboscis of *H. diversicolor* and *G. alba* caused DNA strand breakage in mussel gill cells (Fig. 2A). Whereas extracts from *H. diversicolor* proboscis (glandular region) caused a similar response in mussel gills (≈32% DNA in tail) comparative to control (≈28% DNA in ail); DNA damage in gill cells exposed to

extracts from the skin (body wall) of the same species attained 55% DNA in tail, yielding significantly higher DNA damage to control (Student's *t*-test, *p* < 0.05). Contrarily, exposure to extracts from *G. alba* proboscis caused the highest DNA damage in mussel gills, c. a. 55% relatively to control (Student's *t*-test, *p* < 0.05). Whereas only a mean of ≈33% % DNA in tail were obtained in cells exposed to extracts from the skin of this species (Fig. 2B).

The current work showed differential toxicity of extracts from two Polychaeta and, moreover, between distinct toxicity of extracts from the two organs of either species, the proboscis and body wall. The higher toxicity of *H. diversicolor* extracts from the body wall, comparatively to

the proboscis where specialised venom glands involved in predation, as in *Glycera*, could be expected, supports the original reports by Linke (1939) and Harley (1950) stating that this errant annelid displays a broad span of unspecific feeding mechanisms. Indeed, as noted by these authors, *H. diversicolor* is an opportunistic scavenger that has been recorded to feed on small animals, macroalgae, detritus and even as even act as a filter-feeder. The evolution of such diversified feeding ecology is therefore unlikely to have involved organs specialised in the production and delivery of toxins targeting preferential prey. It must be noted that the proboscis is the essential organ used for sensing and feeding by Polychaeta, with basic morphoanatomy and function (i.e., feeding and sensing) being well conserved among the Class (Dales, 1962). Notwithstanding, specific adaptations may include the secretion of substances to facilitate trapping, capture or digestion of food items such as mucins, toxins and enzymes. However, *H. diversicolor* lack of such specialisation should offer adaptative leverage for an omnivore like *H. diversicolor*, which can, nonetheless, benefit from the skin secretion noxious substances as a means of defence. Therefore, our findings support the idea that toxin-bearing secretions produced by this annelid should be mainly for protection, and possibly ascribed to the skin. Indeed, higher toxicity of body wall secretions can be an effective strategy to protect the animal from predators such as crabs and other Polychaeta as it roams exposed through the intertidal. In fact, the secretion of toxins by the skin of Polychaeta for protection have already been identified in some species, such as *Lumbriconereis heteropoda* (Lumbrineridae). This marine annelid features an integumentary neurotoxin that is a ganglionic blocker particularly lethal to insects (Dales, 1962). This neurotoxin is a not proteinaceous but rather a secondary metabolite and it was isolated for the first time by Nitta (1934), who named it nereistoxin. This compound, authorised for commercialisation in a few countries as the pesticide thiocyclam (3/4-*N,N*-dimethylamino-1,2- dithiolane) acts as repellent against prey and also protects the worm from oxidation and exposure to UV (Okaichi and Hashimoto, 1962).

In turn, the higher toxicity secretions from *G. alba*'s proboscis, indicated by the higher DNA damage on *Mytilus* gill cells may indicate an evolutionary investment in the specialisation of *Glycera albas* predatory behaviour, which is coherent with previous the early observations on the feeding strategies of this Polychaeta, which seems to be an active hunter that ambushes moving prey from its burrow, being unresponsive to dead animals (see Ockelmann and Vahl, 1970), unlike *Hediste*. A striking adaptive feature for a burrowing predator of muddy habitats is the highly developed system of serial receptors and peripheral nerve cells on *Glycera*'s prostomium, which is responsible for the vibration-sensing in its immediate surroundings (Stolte, 1932). In addition, the sweeping movement of the proboscis and the four chitinous jaws is responsible for the precision physical attack by this predator, which is reinforced by previous studies reporting *Glycera* sp.'s jaws high hardness, stiffness and resistance to abrasion. Our findings suggest that *G. alba* also releases a chemical secretion that is likely delivered by its jaws, as a mean to stun or injure its prey. It must be noted, though, that the production of effective amounts of specific-acting bioactives is a considerable metabolic investment that must be compensated by providing *Glycera* with a furtive and efficient attack. Indeed, many glycerid worms are long known to secrete toxins that are administered by injection through jaws and recent advances in molecular tools such as RNA-Seq have been revealing a wide range of proteinaceous substances in their venoms. Indeed, transcriptomic analyses of *G. alba*'s congeners *G. dibranchiata, G. fallax* and *G. tridactyla* already revealed a wide diversity of putative toxin-encoding genes, including neurotoxins, permeabilising agents like matrix-directed enzymes and other common venom elements (see for instance the dedicated review by von Reumont et al., 2014). Altogether, the present findings on the toxicity of extracts from *H. diversicolor* and *G. alba*'s, indicating unspecified cell and molecular damage, suggest the secretion of noxious substances for different purposes, the first for defence, the latter as a further refinement of its specialised feeding strategy. Full chemical and toxicological characterisation are needed in the future, implying isolation, identification and understanding the mode-of-action of the major bioreactive compounds.

Ethical statement

The authors declare no conflict of interest.

CrediT author statement

Conceptualization, M.D. and P.M.C.; laboratory methodology, M.D., I.R, C.M., data analysis, M.D., I.R.; writing—original draft preparation, M.D. I. R, and P.M.C.; writing—review and editing M.D. P.M.C. C.M.; supervision, P.M.C.; project administration, P.M.C. All authors have read and agreed to the published version of the manuscript.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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