Variation in hypodermic radular teeth of the snail auger *Hastula cinerea* (Born, 1778) (Gastropoda:Terebridae)

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ABSTRACT

Gastropods of the superfamily Conoidea are present in high diversity in the oceans and are characterized by having modified foregut anatomy and radular morphology. This study provides details on variations in the radula teeth of the species *Hastula cinerea*, which have hypodermic radula teeth of the toxoglossan type and are part of the Terebridae family – inserted in the Conoidea superfamily. *Hastula cinerea* specimens were collected at Flecheiras beach, Traíri, Ceará, Brazil. Scanning Electron Microscopy (SEM) was performed to analyse the radula specificities. Thirty specimens were used between females and males, with different sizes. The total length of the shell and the length of the teeth of all analysed specimens were measured. With the SEM result, more than one radula tooth morphotype was found for the species *H. cinerea*. The pattern of the teeth found is similar to the hypodermic teeth of the group, however, with structural and length differences between smaller and larger individuals. It was possible to observe three radula variations (morphotype-1, morphotype-2 and morphotype-3), 26 related to different sizes of individuals, regardless of sex, configuring a variation in the radula teeth. Therefore, this result brings a contribution that stimulates future research with the functional morphology of *H. cinerea* and others auger snails.

1. Introduction

The radula of gastropods of the superfamily Conoidea is composed of modified teeth, such as the hypodermic-type teeth of the toxoglossan conoideans; the teeth are similar to harpoons with an opening that goes from the radula base to the apex, assisting in the capture of prey and injecting the venom (Taylor and Miller, 1990).

Specifically, the radular apparatus is diversified even within families and genera of conoideans. It may be composed of more than one structure; for example, the radular sac and radular caecum are adjacent, forming part of a single structure—the radular diverticulum—located at the base of the proboscis (Taylor, 1990; Taylor et al., 1993). In the radular sac, there are teeth in formation, whereas in the radular caecum,

there are only mature teeth ready to be used (Taylor and Miller, 1990; Taylor et al., 1993; Imperial et al., 2007; Castelin et al., 2012).

The radular caecum is present in some conoids, such as some genera in family Terebridae. The sublingual pouch found in gastropods with a radular ribbon is considered homologous to the radular caecum (Taylor et al., 1993). A subradular membrane can also be present in many toxoglossan radula; it can persist in mature teeth, with attachment maintained in the radular caecum, as in the family Terebridae (Imperial et al., 2007; Castelin et al., 2012).

The Terebridae, together with the family Conidae, is one of the most diverse of the Conoidea superfamily; there is high plasticity within this taxon, which drives the evolutionary success that leads to the group's speciation (Modica et al., 2020; Vortsepneva et al., 2020). Terebrids are

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distributed in tropical and subtropical oceans; they range from 15 to 150 mm in shell length and are characterised by high, slender, multi-spiral shells (Taylor and Miller, 1990; Castelin et al., 2012; Vortsepneva et al., 2020). Modica et al. (2020) identified 12 types of foregut anatomy in terebrids, based on six characters.

The anatomy of the radular structure and characterisation of the venom of Terebridae species suggests the existence of a strategy similar to that of the family Conidae, which is the most studied in the superfamily Conoidea (Bandel, 1984; Imperial et al., 2007). However, there are occasional differences in the mechanism of envenomation and feeding on the prey, in which terebrids transfer move a tooth to the tip of the proboscis and the venom is injected through this tooth into the prey (Bandel, 1984; Imperial et al., 2007). Thus, the development of the radula and venom apparatus in the superfamily Conoidea is directly related to the evolutionary history of the entire group (Holford et al., 2009; Castelin et al., 2012; Kantor and Puillandre, 2012).

The species *Hastula cinerea* (Born, 1778), belonging to the family Terebridae, has a pleurembolic proboscis, which is sheltered in the rhynchodeal cavity (Marcus and Marcus, 1960; Taylor, 1990; Simone, 1999), with hypodermic marginal teeth (Marcus and Marcus, 1960; Bandel, 1984; Taylor, 1990; Simone, 1999). *Hastula cinerea* has a long and robust shell that can reach 55 mm in length, colour ranging from cream to dark brown, and albino specimens are found, even if rare (Marcus and Marcus, 1960; Matthews et al., 1975). It is a gonochoric species; females have a female gland and oviduct canal and males possess the penis on the right side of the head (Marcus and Marcus, 1960; Simone, 1999). *Hastula cinerea* inhabits sandy beaches and is active during rising tides. It feeds mainly on polychaetes, making use of its hypodermic teeth containing the toxins (Marcus and Marcus, 1960; Matthews et al., 1975; Simone, 1999; Molina et al., 2013). The distribution of *H. cinerea* is in the Pacific and Amphiatlantic, it includes West Africa, Florida to Brazil (Ceará to Santa Catarina), West Mexico to Peru; It is believed that both the Pacific and West African range is a relatively late expansion of the distribution (Abbott, 1974; Terryn, 2007; Rios, 2009).

Studies point out the therapeutic efficacy of the toxin present in the venom of some toxoglossa radula (e.g., Jones et al. 2001; Puillandre and Holford, 2010; Verdes et al. 2016; Alburae and Mohammed, 2020). Terebrids and conids have peptide toxins that appear to be rich in variety and functionality.

Several studies have been carried out on radular morphology in conoids (e.g., Nybakken and Perron, 1988; Vortsepneva et al., 2019, 2020). However, few studies were conducted on the specificities and radular functions of *H. cinerea*, and no studies were performed on the variations in the radular teeth of the species. Thus, we tested the hypothesis that there is variation in the radular teeth of *H. cinerea* and that, from such variation, we can examine the radular specificities, relating them to shell size and sex of individuals.

2. Materials and methods

2.1. Sampling and laboratory procedure

Specimens of *H. cinerea* (Fig. 1) were collected in the intertidal zone at Flecheiras, a sandy beach in the city of Trairi, Ceará, Brazil (3°13′10.34″S 39°16′23.25″W). In the laboratory, the specimens were separated according to sex. Individuals were, then measured with a digital caliper with an accuracy of 0.01 mm. All specimens were analysed for their shell characteristics and internal anatomy, based mainly on the descriptive study by Marcus and Marcus (1960) and Simone (1999) - discarding the presence of cryptic species. In addition, all specimens studied were taken from the same population.

2.2. Radular preparation for Scanning Electron Microscopy (SEM)

For radular analysis using SEM, 30 specimens of different sizes were

Fig. 1. Specimens of *Hastula cinerea* in different sizes collected at the beach of Flecheiras, Trairi, Ceará, Brazil. Scale bar: 10 mm.

used (16 males and 14 females; 19–48.5 mm in shell length). After measuring the length of the shell, each specimen was dissected, and the proboscis and radular diverticulum (radular sac and radular caecum) were separated (Fig. 2).

The radula dissolution protocol was adapted from Matthews-Cascon et al. (2005). The structures were treated in 10% potassium hydroxide (KOH) to remove all soft tissue, and only the radular teeth remained. The proboscis was dissolved separately from the radular diverticulum to check for the existence of radular teeth ready for immediate use. The sac and radular caecum were dissolved together (Fig. 2). After the soft tissue were dissolved, the radular teeth were separated and washed in distilled
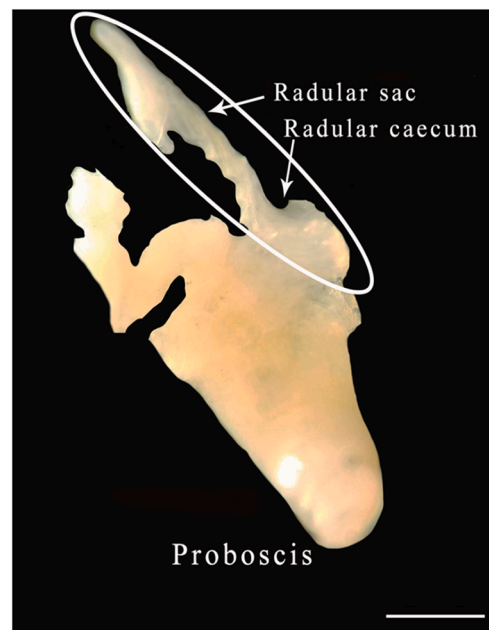

Fig. 2. Proboscis and the highlighted (circle) radular sac and radular caecum of *Hastula cinerea*. Scale bar: 10 mm.

water. Afterwards, the teeth were dehydrated in an increasing alcohol series (70%, 80%, 90%, and 100% P.A., 40 min each).

Subsequently, the teeth remained immersed in 100% P.A. alcohol then mounted on stubs for examination under SEM at the Central Analítica of the Universidade Federal do Ceará (UFC). The radular teeth were positioned horizontally on the stubs and micrographs were performed at a 90° angle.

After SEM analysis, the material was deposited in the malacological collection "Coleç ão Malacológica Prof. Henry Ramos Matthews (CMPHRM)", s é rie B of the UFC (registration number: CMPHRM6584B - CMPHRM6586B).

2.3. Data analysis

After SEM analysis, all teeth were measured (total length–distance between the base and the apex), using their images, in the ImageJ software.

The data were tested for normality (Shapiro-Wilk) and homoscedasticity (Levene test). After being tested, the data did not meet the assumptions of normality and homoscedasticity. Thus, the Mann-Whitney non-parametric test was used to compare the size of radular teeth between sexes, Kruskal-Wallis and Mann-Whitney non-parametric tests were used a posteriori to observe the differences between pairs. Spearman's correlation analysis was performed to observe the relationship between tooth size and shell length of *H. cinerea* individuals, regardless of sex. A logarithmic allometric growth equation, of linearity Log (radular tooth length) versus Log (shell length), was also performed. The tests were performed at a significance level of $\alpha = 0.05$ (Zar, 1999). All analyses were performed using R v.4.0 (R Core Team, 2016).

3. Results

3.1. Morphology of radular teeth

No teeth were found in the proboscis analysed. Therefore, all the teeth observed under SEM came from the radular diverticulum, which includes the radular sac and radular caecum.

We observed variations in the radular teeth, regardless of sex. No difference was found in size of the radular teeth between males and females (Mann–Whitney, z = 0.82, P = 0.41).

Fig. 3. Variation of radular teeth observed in *Hastula cinerea*. A. teeth still attached to the subradular membrane; B. tooth morphotype-1; C. tooth morphotype-2; D. tooth morphotype-3. Scale bars: A = 200 μm; B = 50 μm; C = 200 μm; D = 400 μm.

Radular teeth were observed still attached to the subradular membrane (Fig. 3A). Three variations of radular teeth were discerned and therefore classified into three morphotypes: radular tooth morphotype-1 (M1), radular tooth morphotype-2 (M2), and radular tooth morphotype-3 (M3) (Figs. 3B, 3C, and 3D, respectively). When viewed under an optical microscopy, the morphotypes also followed the patterns observed under SEM. Of the 30 specimens analysed, 17, 3, and 10 presented M1, M2, and M3 teeth, respectively. All specimens showed only one morphotype of radular teeth.

The number of teeth present in each individual was variable, reaching > 20 in some specimens and < 10 in others. The number of teeth also varied among morphotypes, following the order: M1 > M2 > M3, and those of M1 were often found still attached to the subradular membrane. The number of radular teeth analysed was 160 for M1, 15 for M2 and 45 for M3.

M1 is a typical hypodermic-type tooth, in the form of a tube similar to a rolled leaf with overlapping edges. This slender tooth is, from the base to the apex, similar to a needle (Figs. 4A and 4B). M1 has an opening that goes from the base to the apex (Figs. 4A and 4B). The tooth shaft expands outward forming a circular base around the opening (Figs. 4A and 4B, white arrows). The apex resembles the tip of a smooth pointed and triangular in cross-section needle (Figs. 4C and 4D). In the central region, it is possible to see the overlapping edges, as if a leaf were closed (Fig. 4E). M1 has a ligament (long and flexible peduncle) that is the continuation of the base that extends until near the apex (Figs. 4A and 4B, green arrows). An outstanding feature observed is that, generally, these teeth were still attached to the subradular membrane and, some still remained attached to the membrane even after we used KOH to dissolve the soft tissues (Fig. 4F). No denticles were observed in SEM
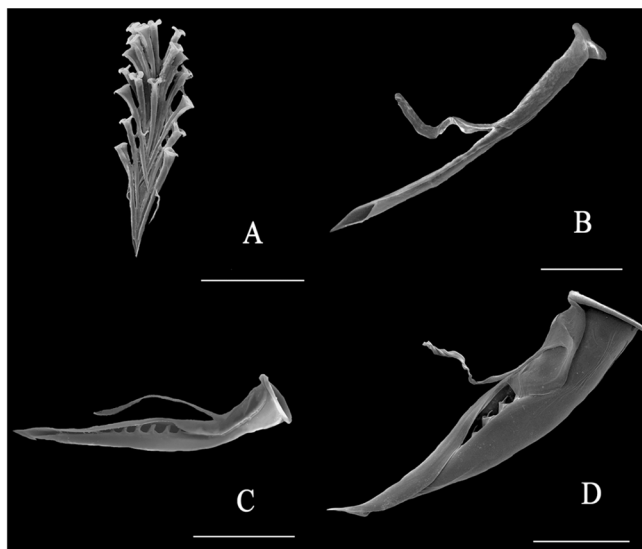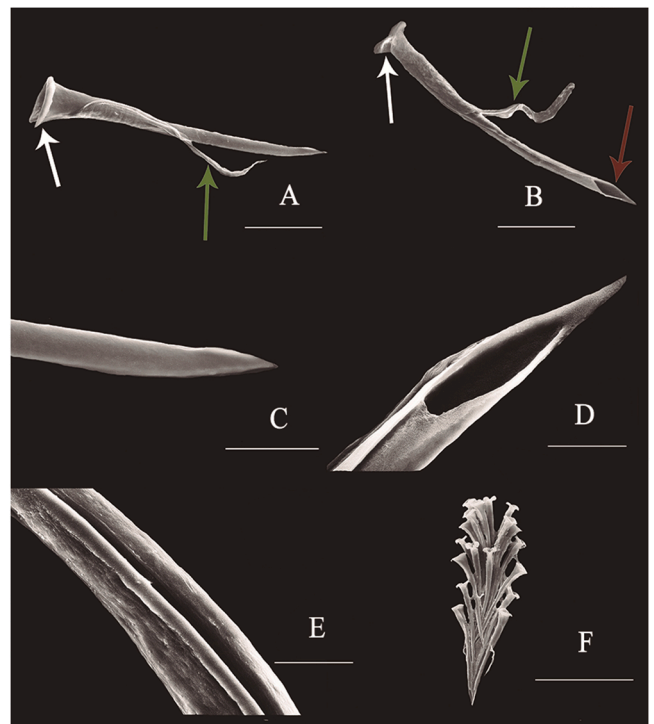

Fig. 4. Morphology of the morphotype-1 tooth of *Hastula cinerea*. A. general view of the tooth; white arrow indicates the base opening, and green arrow points at the ligament. B. general view of the tooth; white arrow indicates the base opening, green arrow points at the ligament, tooth apex is highlighted, and a red arrow indicates the opening. C. apex is barbed, triangular, and smooth; seen from the side opposite to its opening. D. opening of the barbed, triangular, smooth apex. E. central region with overlapping edges. F. teeth attached to the subradular membrane. Scale bar: A = 50 μm; B = 50 μm; C = 20 μm; D = 10 μm; E = 10 μm; F = 200 μm.

analyses, nor were they observed under optical microscopy.

M2 is also a typical hypodermic-type, tube-shaped tooth similar to a curled leaf with overlapping edges; an opening goes from the base to the apex, and the base flares outwards forming a circular border around the opening. Structurally, it differs from M1 (Figs. 5a and 5b) because M2 presents a spike-like apex (pointed and triangular) with a notch (Figs. 5c and 5d, red arrows). In addition, a series of curved and pointed projections is present internally in the central part; these form a serrated structure that can be seen through the longitudinal opening (Fig. 5a, blue arrow; 5e and 5 f). However, similarly to M1, M2 also has a ligament continuation of the base that extends to near the apex (Figs. 5a and 5b, green arrows).

M3 is also an example of a typical hypodermic-type tooth, shaped like a rolled-up tube with overlapping edges, with an opening from the base to the apex and an outward curved edge forming a circular edge around the opening. However, this morphotype is more robust at the base and gradually tapers to the apex (Figs. 6a and 6b). Similar to M2, the apex resembles a lance (pointed and triangular) with a notch; however, the notch of M3 teeth is larger (Fig. 6c, red arrow) and it has a protuberance (Fig. 6d, yellow arrow). In the central region,

M3 also has an opening with serrations and curved and pointed projections, but these are fully developed (Fig. 6a, blue arrow; 6e and 6 f). It also has a ligament continuation of the base that extends until near the apex, as in the other morphotypes (Figs. 6a and 6b, green arrows). Thus, this morphotype is structurally similar to M2. No tooth of this morphotype was found attached to the subradular membrane.
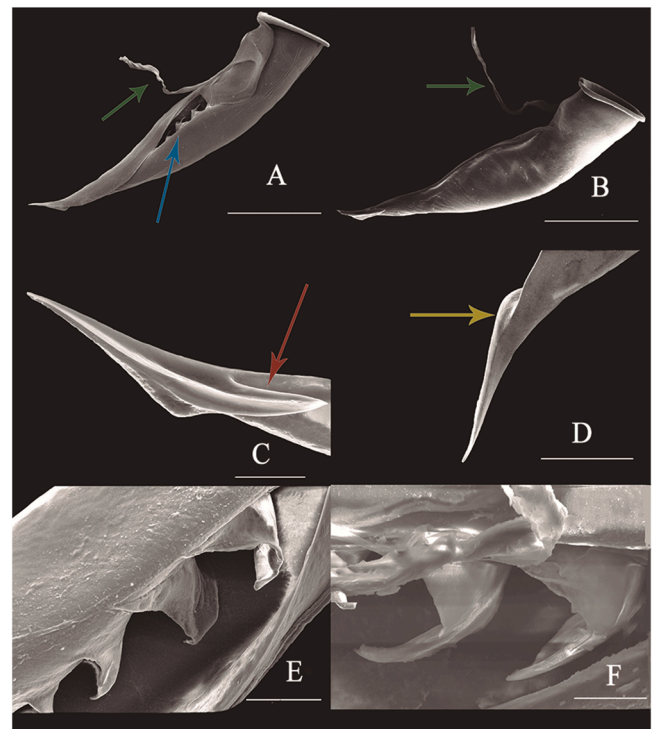

Fig. 6. Morphology of the morphotype-3 tooth of *Hastula cinerea*. A. general view of the tooth, more robust at the base and thinner at the apex; green arrow indicates the ligament, and blue arrow points at the serrations in the central region B. general view of the tooth from another angle; green arrow indicates the ligament. C. barbed and triangular apex; red arrow indicates the notch. D. from another angle, barbed and triangular apex; yellow arrow indicates a protuberance. E. the serrations with curved and pointed projections in the central of the tooth are highlighted. F. serrations with curved and barbed projections in the central of the tooth Scale bar: A = 400 μm; B = 400 μm; C = 50 μm; D = 100 μm; E = 50 μm; F = 50 μm.

3.2. Radular morphometry

M1 teeth showed an average length of 0.177 mm (± 0.01; ranging from 0.150 mm to 0.199 mm). M2 teeth showed an average length of 0.614 mm (± 0.12; ranging from 0.508 mm to 0.794 mm). M3 teeth presented an average length of 1.19 mm (± 0.14; ranging from 0.923 mm to 1.44 mm).

A statistically significant difference was verified in tooth sizes (Kruskal–Wallis test, H = 23.20, P < 0.05) among the morphotypes. From the Mann–Whitney a posteriori test, it was possible to observe that M3 teeth were larger than M1 and M2 (P < 0.05), and M2 teeth were larger than M1 (P < 0.05). The Mann–Whitney a posteriori test showed that the three morphotypes differ in size (Fig. 7).

3.3. Relationship of shell length × radular tooth length

A significant positive correlation was verified between shell length and radular teeth size (Spearman: r = 0.84; p < 0.05). Small animals (19–29.9 mm in shell length) presented M1 or M2 teeth, whereas large animals (30–48.5 mm in shell length), M3 teeth (Fig. 8). A positive allometric growth was found between the radular tooth length (TL) and the shell length (SL) (Allometric equation: TL = 2.59SL - 4.14; r2 = 0.86, P < 0.001).

4. Discussion

This study is the first to identify the morphological variation in radular teeth in *H. cinerea*.
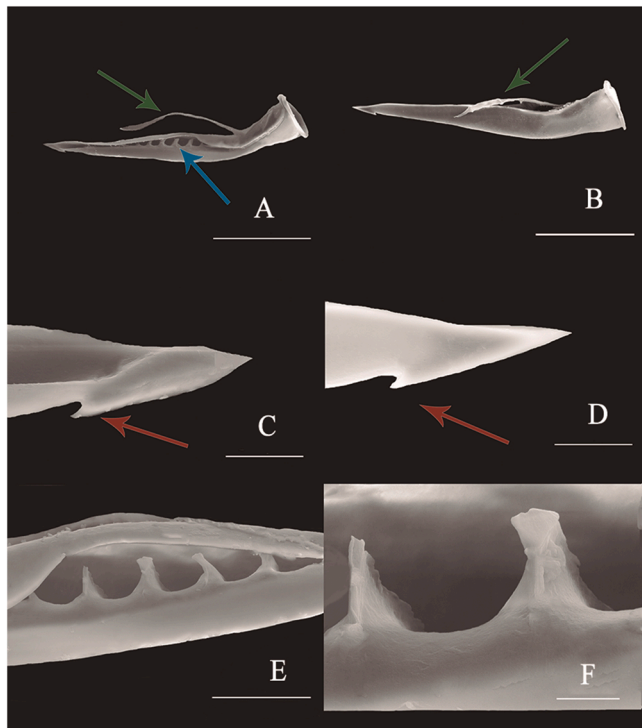

Fig. 5. Morphology of the morphotype-2 tooth of *Hastula cinerea*. A. general view of the tooth; green arrow indicates the ligament, and blue arrow points at the serrations of the central region. B. general view of the tooth from another angle; green arrow indicates the ligament. C. barbed and triangular apex with visible opening; red arrow indicates the notch. D. from another angle, barbed and triangular apex; red arrow indicates the notch without the presence of a protuberance. E. the serrations with initially curved and pointed projection, in the central of the tooth is highlighted. F. serrations with short curved and pointed projections in the centre of the tooth. Scale bar: A = 200 μm; B = 200 μm; C = 20 μm; D = 20 μm; E = 50 μm; F = 10 μm.
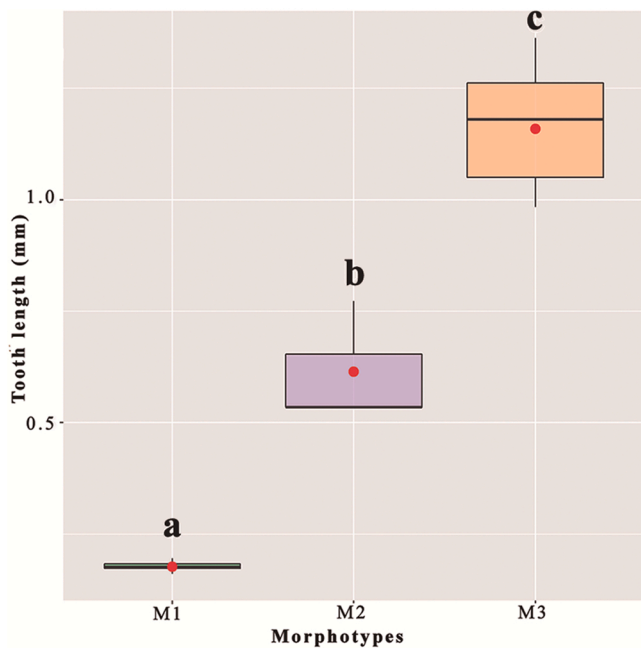
Fig. 7. Average length of the three tooth morphotypes of *Hastula cinerea*. Different letters indicate a statistically significant difference (p < 0.05)—a posteriori Mann–Whitney test.
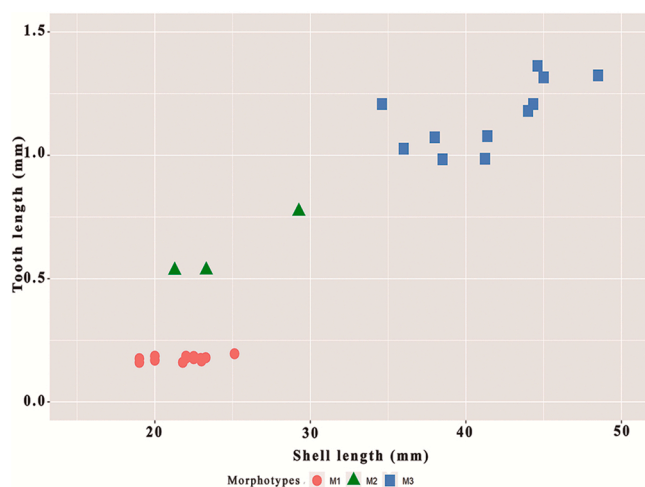

Fig. 8. Spearman correlation between shell and teeth length of *Hastula cinerea* specimens (r = 0.84; p < 0.05). The red circles represent morphotype-1 teeth, the green triangles represent morphotype-2 teeth, and the blue squares represent morphotype-3 teeth.

This variation is considering the different sizes of individuals. There was no radular difference between the sex. The teeth of specimens of the genus *Hastula* are a marginal hypodermic type connected to a thin, reduced membrane and often with multiple perforations in the tooth walls (Fedosov et al., 2019). However, these perforations were not found in *H. cinerea*. In this study, we observed teeth still attached to the radular membrane. Tthe subradular membrane, it is present in *H. hectica* (Linnaeus, 1758) and *H. cinerea*, and its integrity persists even after the dissolution of the radular sac or radular caecum, after which the teeth remain attached to the membrane (Imperial et al., 2007; Castelin et al., 2012). However, during radular growth, the teeth in the radular caecum are transferred from the oldest region of the membrane to the tip of the proboscis where they remain ready for deployment (Imperial et al., 2007).

Three morphotypes were found (M1, M2 and M3), two of these (M1 and M2) were characteristic of individuals with short shell lengths, whereas M3 was characteristic of those with a larger shell. M2, as it presented similar—but clearly reduced—structures to those of M3, is a morphologically intermediary form in the transformation from M1 to M3. Thus, the pattern of teeth found for *H. cinerea* in this study is similar to the hypodermic-type teeth described for the group (e.g., Bandel, 1984; Nybakken and Perron, 1988; Taylor, 1990; Simone, 1999; Imperial et al., 2007; Castelin et al., 2012; Vortsepneva et al., 2019, 2020). However, *H. cinerea* radular teeth present structural and length differences within the species, discriminated in the three morphotypes described in this study.

Marcus and Marcus (1960) a described *H. cinerea* tooth similar to the M2 found in the present study; the similarity was mainly due to the detailed tooth size, which was 0.54 mm. Bandel (1984) described the radula of specimens of the genus *Terebra*, among them that of *H. cinerea* [as *Terebra cinerea* (Born, 1778)]. The description includes characteristics such as a small splinter at the tooth apex. Considering the descriptions and the images provided in that study, the teeth described by Bandel resemble M2. Following the same pattern, Taylor (1990) mentioned that some species of the genus *Hastula* have the simplest and most complete form of teeth. Among the cited species were *H. penicilata* (Hinds, 1844), *H. salleana* (Deshayes, 1859), and *H. cinerea*, which had robust teeth with a barbed tip, similar to a harpoon and a broad base—resembling M2 and M3. Observing the images and part of the description of the study published by Simone (1999), the author may have observed a tooth similar to M1; however, the tooth length described was 650 μm, indicating tooth in transformation—M2. However, these previous studies do not mention the variation in radular teeth in *H. cinerea*.

The very detailed SEM images are essential for diagnosing and recognising subtle differences in structures such as the radula. When well detailed, such analyses can confirm or exclude pre-visualised differences, which could have happened in the studies mentioned above. Therefore, it is clear the importance of analysing a large number of specimens of different sizes, previewing them under optical microscopy and capturing SEM images of the structure from several angles. SEM analysis assists the identification of new morphological characteristics and details that facilitate species identification (Kownacki et al., (2015)).

Previous studies have shown intraspecific variations in radular teeth, such as ontogenetic differences and tooth formation and maturation for other species of the superfamily Conoidea, mainly for family Conidae (e. g., Nybakken and Perron, 1988; Vortsepneva et al., 2019, 2020). A study on *Conus magus* (Linnaeus, 1758) found an ontogenetic difference in radular teeth; adult teeth were larger and more complex than those of young specimens (also the presence of a tooth in transition). Variations were found also in the foregut, in which the radular sac had a different shape and size (Nybakken and Perron, 1988). There are also recent studies on the process of tooth formation and maturation in Conoidea emphasising the transformations in the shape of marginal teeth, which occur during different life stages; thus, these studies confirm the existence of ontogenetic differences for the superfamily (Vortsepneva et al., 2019, 2020). In the present study, a variation in *H. cinerea* was observed, when considering different tooth sizes.

Many studies on the toxoglossa radula describe the shape of teeth by relating them to the way of life of organisms (e.g., Nybakken and Perron, 1988; Taylor et al., 1993; Imperial et al., 2007). Conoideans gastropods with a hypodermic radula have several teeth ready for immediate use, including multiple teeth that can be used on a single prey, such as those of the genus *Conus* (Imperial et al., 2007). In contrast, the genus *Hastula* have teeth in the cecum attached to the membrane, hidden and supported on the base of the proboscis; during the poisoning of the prey, only a small part of the teeth protrudes, not penetrating deeply into the body of the prey (Imperial et al., 2007).

The structure of a tooth is related to its functionality, such as the most needle-like tip ready to penetrate the prey, as well as the presence

of barbs or serrations that secures the tooth in the prey until the entire venom is injected and the predator can feed on the paralysed prey (Kohn et al., 1972). *Hastula cinerea* feeds on polychaetes (Marcus and Marcus, 1960). In this context, the morphotypes found in this study may be related to *H. cinerea* eating habits, as the specimens can capture different polychaetes in different stages of life. Bandel (1984) reported that structural differences in teeth are related to the eating habits of organisms, exemplifying different species of *Conus* and their various prey (fish, polychaetes, and other molluscs). Some studies cite the relationship between the eating habits of molluscs and ontogenetic dimorphism (e.g., Nybakken and Perron, 1988). Imperial et al. (2007) mentioned that the link between the diet and *Hastula* radula morphology remained tenuous, and the authors supposed that this connection would remain fragile. Thus, the radular variation found in the present study reinforces the hypothesis proposed by these authors.

The adaptation may also be related to the venom potential, as to paralyse some prey (e.g., fish and polychaetes), organisms with toxoglossa radula may need a larger amount of venom (Nybakken and Perron, 1988; Castelin et al., 2012). Some studies detail differences found in the venom potential within superfamily Conoidea (Imperial et al., 2007; Holford et al., 2009; Castelin et al., 2012; Kantor and Puillandre, 2012; Gorson et al., 2021).

Gorson et al. (2021) suggests a strong correlation between the venom gland and the amplitude of the terebrid diet, proposing a review of the definition of the conoid venom complexity in relation to prey capture. These authors also affirm that it is essential to build robust theories that relate the evolution of venom and neglected species. Our research makes an important contribution to this construction, as it includes an anatomical feature that is easy to study, such as radular anatomy, and an ecological feature that has not been explored in previous studies, which is radular variation.

This research contributes to the anatomical and morphological study of the group of conoids with toxoglossa radula, especially for the genus *Hastula,* as we described the radular variation in *H. cinerea.* It is essential that anatomical investigations use tools such as SEM together with analyses in optical microscopy, and with specimens of different sizes, so that no details of the structures that favour studies of populations at different life stages are lost. Our results suggest an intrinsic relationship between eating habits and/or the potential of the toxin present in the venom of *H. cinerea* at different life stages, which may be proven in future investigations on the functional morphology of the species.

Declaration of Competing Interest

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.

Data Availability

Data will be made available on request.

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