

# First steps in morphological analysis of the of the reproductive system of *Doto pinnatifida* (Montagu, 1804)

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## Aims

The genus *Doto* Oken, 1815 (Mollusca, Opisthobranchia) is currently composed by 90 species (Bouchet & Gofas, 2017). The differentiation between species is mainly made according to external characters (coloration, number and shape of cerata, shape of the rhinophoral sheath) (Ortea & Urgorri, 1978).

Opisthobranchia are hermafrodite, and the anatomy of its reproductive system is an important feature used to distinguish among species (Thompson, 1976). It is usually studied through techniques of classical histology, although in the last years some authors (Candás *et al.*, 2016; Moles, 2016) have already made an approach to its study through micro-computed tomography (micro-CT).

The aim of this work is to make a comparative study of the anatomy of the reproductive system of *Doto pinnatifida* (Montagu, 1804) through the use of micro-CT, in order to establish if there are any morphological features that could be considered as intraspecific differences. Moreover, morphometric parameters were calculated to determine if there is a relation between the size of the animal and the development of the reproductive system.

## Method

Specimens with lengths between 0.60 and 10 mm were studied. After being collected, they were kept in 70° ethanol. Subsequently, they were dehydrated in consecutive ethanol baths (80°, 90° and 96°) and stained with 1% iodine in 96° ethanol for three days. They were then washed with 96° ethanol, dried with hexamethyldisilazane (HMDS) for 2 hours and left to dry overnight (Alba Tercedor & Tocino, 2011; Faulwetter *et al.*, 2013; Candás *et al.*, 2016).

Scannings were performed with a Skyscan 1172 system using the following parameters: 55 kv, 165 µA, and no filter was used. Samples were rotated 360°. The X-ray projection images obtained during scanning were reconstructed with the software NRecon. The sections obtained were processed with the softwares CTAn and DataViewer. 3D representations were obtained with the softwares CTVox and Amira 5.3.3. Morphometric parameters (specimen length, vas deferens length, ampulla length, ampulla maximum width, genital atrium-bursa copulatrix distance, bursa copulatrix diameter) were calculated with DataViewer and Amira 5.3.3.

## Results

The reproductive system of *Doto pinnatifida* is located in the anterior part of the animal. It can be distinguished three parts: masculine, feminine, and a third part formed by the ovotestis and the ampulla. The masculine part is composed by a vas deferens (which widens forming the prostate) and a penis, which opens in the genital atrium anteriorly to the vagina (fig. 1).

The feminine part consists in an oviduct which opens into receptaculum seminis; this latter leads distally into the vagina. There is also a bursa copulatrix and several glands (responsible for adding protective and nutritive layers to the egg-masses), which end in the atrium (fig. 1).

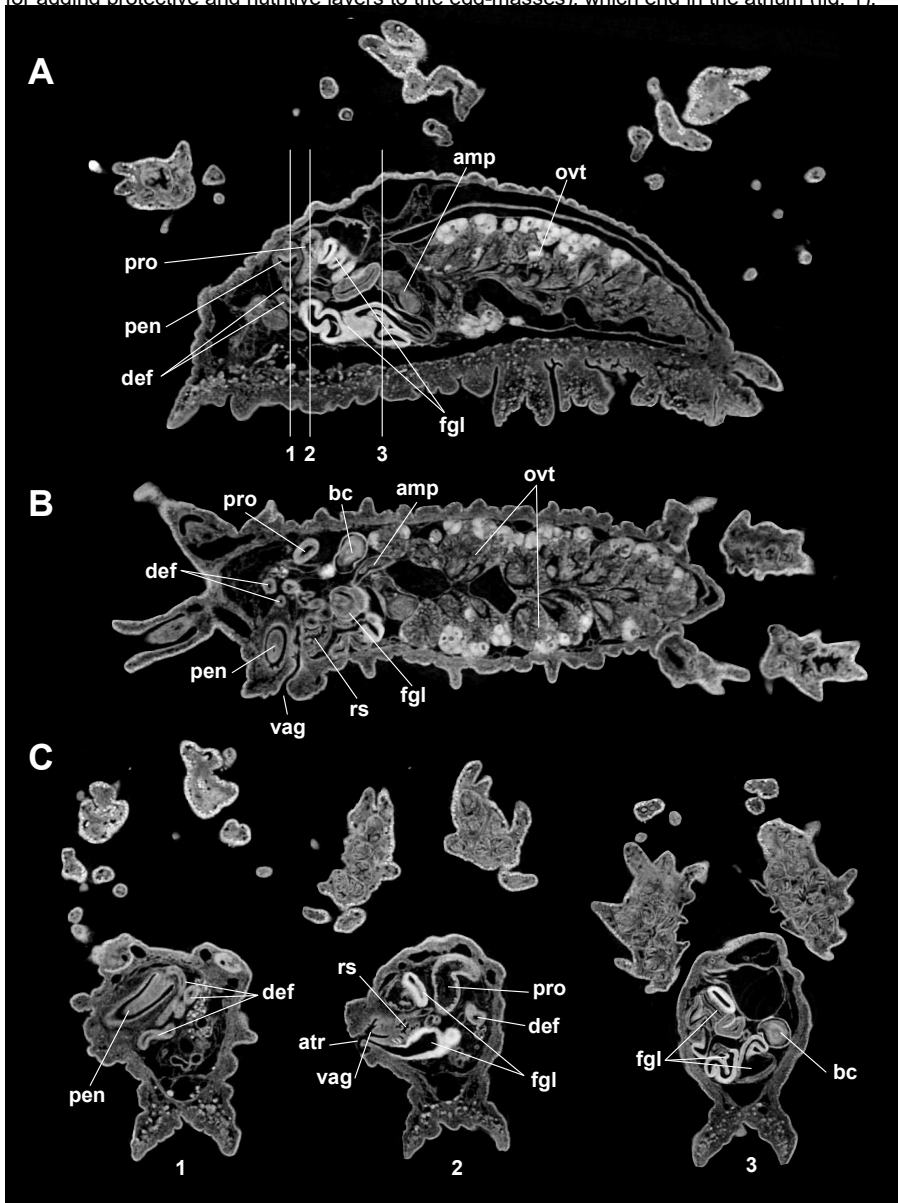


Figure 1: *Doto pinnatifida*, adult (4.08 mm length). A: 2D sagittal section; B: 2D frontal section; C: 2D transverse sections. Abbreviations: amp: ampulla; atr: genital atrium; bc: bursa copulatrix; def: vas deferens; fgl: feminine gland; ovt: ovotestis; pen: penis; pro: prostate; rs: receptaculum seminis; vag: vagina.

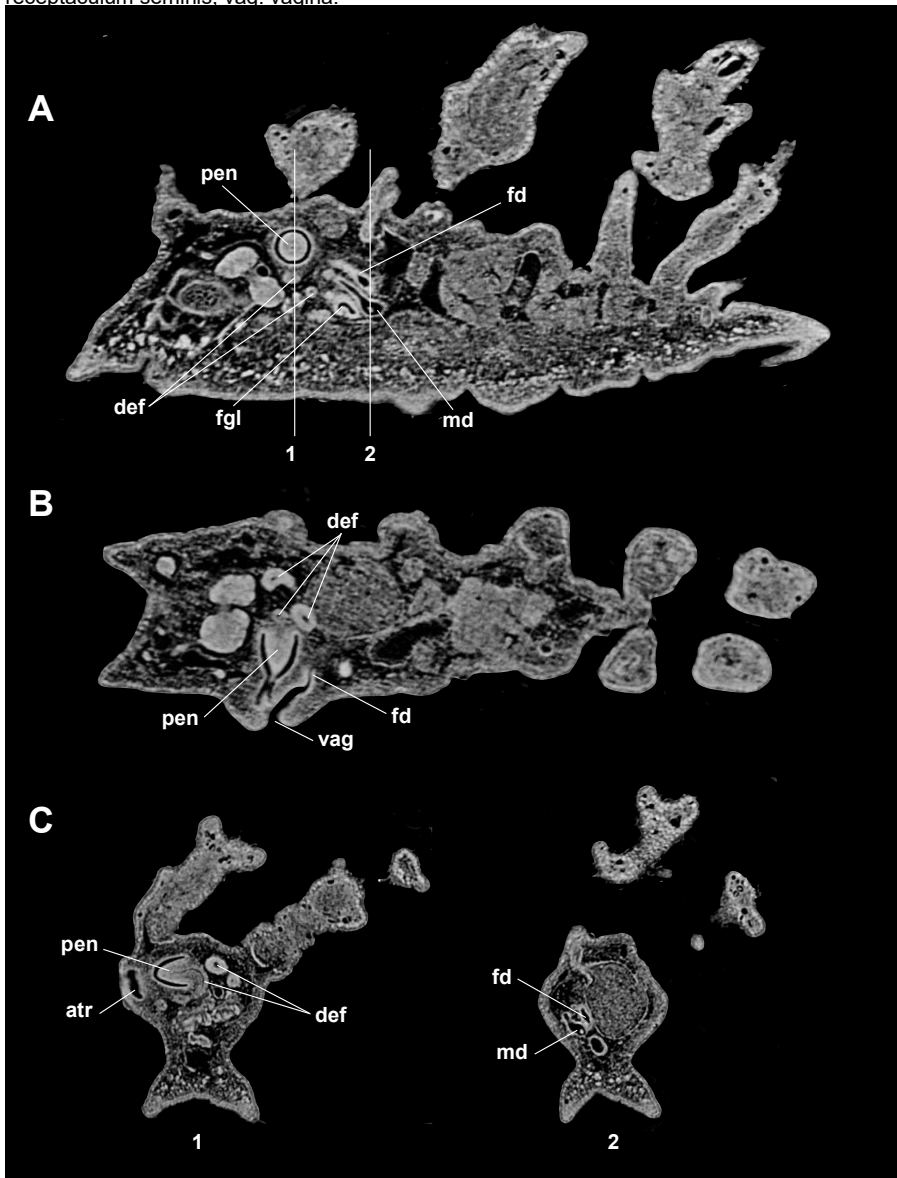


Figure 2: *Doto pinnatifida*, juvenile (1.43 mm). A: 2D sagittal section; B: 2D frontal section; C: 2D transverse sections. Abbreviations: atr: genital atrium; def: vas deferens; fgl: feminine gland; fd: feminine duct; md: masculine duct; pen: penis; vag: vagina.

The third part comprises an ampulla connected with the ovotestis. The ampulla is also connected to masculine and feminine parts through gonoduct.

The reproductive system can be readily observed in 2D and 3D images in the studied specimens. All the features are easily developed in those animals equal or larger than 1.43 mm. In the smaller ones, structures like the penis or the bursa copulatrix are not completely formed, and others like prostate are hardly seen.

The measurements of the linear structures (that is, bursa copulatrix and ampulla) are measured in 2D sections (transverse, frontal and sagittal) by DataViewer. However, it is impossible to measure the length of ducts with this software; for this purpose we use the software Amira.

It is observed that bursa copulatrix and ampulla are located in the right part of the animal in adults. However, there are not such structures in individuals equal or smaller than 1.43 mm length (fig. 2); the ends of the feminine and masculine ducts are found in the left side of the animal, next to the feminine glands (fig. 2C).

Penis is observed in specimens equal or longer than 1.12 mm length.

Feminine and hermaphrodite parts are not distinguished in animals shorter than 0.70 mm. It is only observed a short duct that opens to the outside; because of its wall thickness, this duct should be a rudimentary vas deferens.

According to the results obtained, we can conclude that *D. pinnatifida* reaches its sexual maturity from aprox. 1.4 mm individual length.

## Conclusion

This study shows the development of the reproductive system of the nudibranch *D. pinnatifida* from juvenile stages to adults, by means of micro-computed tomography. We can conclude that there is a general trend, the larger the animal the larger the structures measured. Moreover, we have not observed any intraspecific differences.

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The images of the sections obtained are of high quality. The level of detail achieved with this technique is good, when comparing them with histological sections. The use of the micro-CT in the study of the internal anatomy of *Doto* species has been previously tested, also with great results (Candás *et al.*, 2014; Moles *et al.*, 2016).

Micro-CT results in a great tool for this kind of research. It allows us to study a big number of samples in a relatively short period of time, comparing with classical histology. Besides, we can obtain sections in three planes (transverse, frontal and sagittal) from one sample, which is impossible by histological techniques. It also allows measuring some structures quite easy.

Furthermore, we can use the obtained sections in other reconstruction softwares like Amira. Micro-CT sections have the advantage that they are already aligned (alignment must be performed manually in classical histology); this means reducing time and labour. Unfortunately, DataViewer does not allow us to measure the length of ducts; this problem is solved by measuring them in the Amira 3D reconstructions.

## References:

1. Alba-Tercedor J, Sánchez-Tocino L, "The use of the SkyScan 1172 high-resolution micro-CT to elucidate if the spicules of the sea slugs (Mollusca: Nudibranchia, Opisthobranchia) have a structural or a defensive function", SkyScan Users Meeting 2011, 113- 121, 2011.

2. Bouchet P, Gofas S, "*Doto* Oken, 1815", In: MolluscaBase (2017). Accessed through: World Register of Marine Species at <http://www.marinespecies.org/aphia.php?p=taxdetails&id=137916> on 2017-03-16, 2017.
3. Candás M, Díaz-Agras G, Urgorri V, "The use of micro-CT for the study of the internal anatomy of sea slugs (Opisthobranchia, Nudibranchia, Dotidae)", SkyScan Users Meeting 2014, 241- 243, 2014.
4. Candás M, Díaz-Agras G, Abad M, Barrio L, Cunha X, Pedrouzo L, Señarís MP, Tato R, García-Álvarez Ó, Urgorri V, "Application of microCT in the study of the anatomy of small marine molluscs", Microscopy and Analysis, 30(2), S8- S11, 2016.
5. Faulwetter S, Daillanis T, Vasileiadou A, Arvanitidis C, "Contrast enhancing techniques for the application of micro-CT in marine biodiversity studies", Microscopy and Analysis, 27(2): S4-S7, 2013.
6. Moles J, Wägele H, Ballesteros M, Pujals Á, Uhl G, Ávila C, "The end of the cold loneliness: 3D comparison between *Doto antarctica* and a new sympatric species of *Doto* (Heterobranchia: Nudibranchia)", PlosOne, 11(7): e0157941, 2016.
7. Ortea JA, Urgorri V, "El género *Doto* Oken, 1815 en el Norte y Noroeste de España", Boletín de la Estación Central de Ecología, ICONA, 7(14): 73-92, 1978.
8. Thompson, TE, "Biology of opisthobranch molluscs. Volume 1", The Ray Society, London, 207 pp, 1976.