UNIVERSITAT DE BARCELONA

Antarctic heterobranch molluscs: diving into their challenging ecology, taxonomy, and systematics

Juan Moles Sánchez


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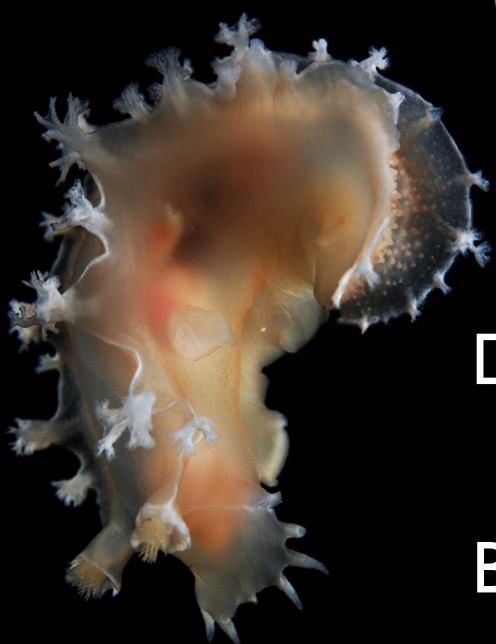
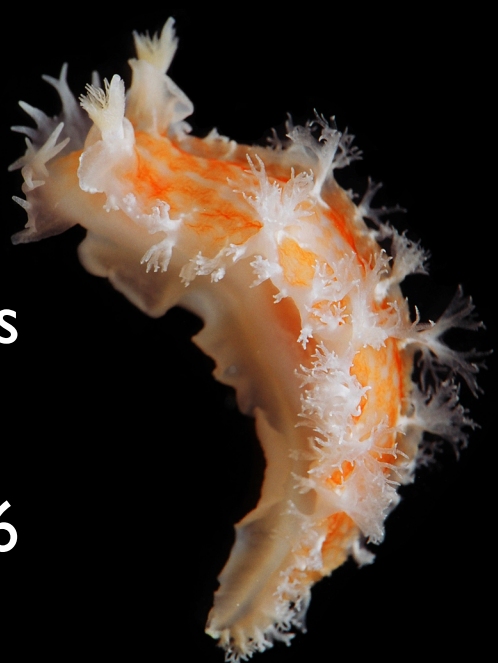

Doctoral Thesis

Juan Moles

Barcelona, 2016

“The truth is rarely pure and never simple.”

Oscar Wilde, The Importance of Being Earnest

Cover pictures: *Tritonia dantarti* of M. Ballesteros, *Charcotia granulosa*, *Doris kerguelenensis*, *Notaeolidia depressa*, *Tritonia challengeriana*, and *Tritoniella belli* of G. Giribet, *Homo sapiens* and *Limacina helicina* of J. Junoy, and *Cuthona crinita*, *Doto carinova*, and *Newnesia joani* of J. Moles

UNIVERSITAT DE BARCELONA

Programa de doctorado
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Departamento de Biología Evolutiva,
Ecología y Ciencias Ambientales

Antarctic heterobranch molluscs: diving into their challenging ecology, taxonomy, and systematics

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La tesis es un proceso de aprendizaje equiparable a una historia de aventuras en la cual las hazañas y proezas del protagonista permiten acontecer su epopeya. En el transcurso de estos años, nuestro aventurero ha conocido a importantes figurantes que han dado forma y color y que participan cuales pilares fundamentales para el logro de esta narración. A diferencia de un relato, éste no tiene su fin, pues la historia prosigue pese a haber depositado su producto final, esta Tesis. Sin lugar a dudas, los mejores frutos han sido las amistades que han permitido al héroe ser lo que hoy en día es. Y es en este apartado donde me permitirán la licencia de vanagloriar a esas personas que han significado y seguirán significando tanto para él.

Un joven y orondo Juan se levantaba un fin de semana cualquiera para ir al monte del Montseny o a la playa de Sta. Cristina, daba igual, lo cierto es que disfrutaba con cualquiera de los viajes que organizaban sus padres y su hermano Ramsés cada fin de semana. Juan "El Padre" y Mª Carmen "La Mamá" consiguieron incubar en Juan "El Hijo" la pasión por la naturaleza, la fauna y la flora. Cada logro escolar o descubrimiento personal debe ser reconocido a la tarea de enseñanza de su inseparable familia, sin la cual, el principio y final de este camino no podrían ser escritos. Juan quiere, a su vez, dar una especial mención de reconocimiento a los abuelos cuya sabiduría y felicidad han conseguido moldear el carácter del joven. Desde los higos chumbos que con tanta paciencia recolectaba y pelaba Manuel, a los innumerables momentos risueños y entrañables con Fernando y Mª Carmen, o los tés con galletas que se tomaba con Virginia cuyo recuerdo aún rememora vívidos cual madalena de Proust. Su humilde familia ha producido mella en la personalidad del héroe, su apoyo requeriría una mención especial más allá de la gratitud aquí escrita. Cabe destacar la familia adoptiva constituida por Isabel "The lovely" y Juan "El papichulo" que tan bien han acogido al joven en todo momento cual hijo suyo.

Rondaría el año 2006 cuando nuestro joven y vivaz aventurero se adentraba a los intríngulis de la vida universitaria. En la fortificación que representaba la facultad de Biología, Juan conoció a inseparables compañeras de viaje que han crecido juntos durante la carrera universitaria. Dicha tétrada está representada por Anna "La Viajera", Tamara "La Pequeña", Lídia "La Sensata" y Lidia R "La No-Tan-Sensata". Conjuntamente han compartido grandes viajes a las entrañas acaloradas de Andalucía, han esquiado por los montes helados de los Pirineos, han sobrevivido a los rugientes volcanes de Olot, han combatido los mares enfurecidos de Roses, han conseguido salir airosos de los laberintos de St. Antoni de Vilamajor y han sobrevivido a la dieta hipercalórica de Logroño, entre otras hazañas. A ellas el protagonista les debe innumerables risas y algo de cordura. Que la amistad forjada no la puedan deshacer ni los mares de lava del monte del Destino. Un largo etcétera de personas ha de ser también reconocido, Vane "La Neozelandesa" por su contagiosa risa o Carlos "Chocho" por su amistad durante tantos años de carrera y la pasión compartida para con la fauna y flora, son dos ejemplos destacables.

La época universitaria fue un periodo de adquisición de conocimientos que potenciaron las pasiones naturalistas del joven en cuestión. Más allá de las clases magistrales por profesores como Ramón Mª Masalles, Manuel Ballesteros, Gustavo Llorente, Miquel Arnedo, Josep Mª Ninot y un largo etcétera, la vida durante la carrera obtuvo sus frutos de las colaboraciones en los departamentos de botánica, fisiología vegetal y biología animal. Durante este periodo el ingenuo naturalista obtuvo los conocimientos basales para el desarrollo de su carrera investigadora, adquiriendo a su vez la amistad de muchos investigadores veteranos, a los que Juan debe más de un café. El departamento de biología animal ha sido el refugio del protagonista y donde muchas de las ideas de esta tesis surgieron. Grandes personalidades han compartido risas en este lugar: Lucía, Marcel, Owen, Álex, Fabi, Marta, Jessica, Oriol, Vanina, Marcos, Marc y muchos más a los cuales Juan debe su apoyo al haber compartido tantos años un camino común, el de la tesis.

Nuestro héroe consiguió la conquista de los mares sureños a cargo del barco ELSA y sus tripulantes. Gracias a la asociación CIRCÉ, Juan ha conocido grandes personajes y ha vivido grandes aventuras entre delfines, calderones, ballenas y leviatanes varios. Desde el descubrimiento de la deliciosa comida tarifeña, hasta sumergirse entre cetáceos en las entrañas del Estrecho, a sumergirse literalmente en las entrañas de un pequeño Moby-Dick. Entre los circeños, Pauline "La Rorcual" y Philippe "El Capitán" han sido los franceses de mayor reconocimiento, no sólo por los conocimientos impartidos sobre cetáceos, ni por los viajes a tierras incógnitas en caravana, sino también por su mera amistad. Cabe destacar gente como Ruth "La Panda", Renaud "El Jefe", Carolina "La Pequeña Risueña" y, cómo no, la pequeña familia italiana de Eva, Rocco y su joven promesa Nerea "I Tre Magnifici". Durante las campañas de conquista de los mares gaditanos otras grandes personalidades han de ser reconocidas, tales son Aixa "La Voluntaria", o Isa "La Pajarera" con la cual Juan también descubrió Donosti y sus pinchos.

El joven y audaz estudiante conoció grandes personalidades en el transcurso del máster de Biodiversidad. Qué grandes fueron los viajes a Roses, el Pirineo y Extremadura, donde conoció a gente variopinta, de diversa trayectoria profesional e intereses personales, pero que todas ellas al unísono acabaron por sembrar la semilla del doctorando en nuestro joven héroe. Sin lugar a dudas, Blanca "Tortuguitas" ha sido una hermana, una madre y una gran amiga para Juan. Sus contagiosas risas, interminables cafés con leche y pitillos y filosóficas conversaciones son de alabar. Si la personalidad de Juan se construyera cual edificio, Blanca sería uno de los pilares que la sustentan. Por todo ello el joven le debe el agradecimiento por siempre.

Sin duda la mayor familia científica que el pequeño estudiante de doctorado ha conocido son los Actiquimes o los denominados Conxitos. Durante los inicios, Jenny alias "La Jenny" y Sergi "El Bondadoso" fueron los primeros en enseñar al estudiante las tareas alquímicas de extracción de invertebrados antárticos. A ellos se les deben innumerables momentos antárticos y conocimientos que han permitido forjar al joven

investigador actualmente. Sin duda la mayor aventura fue la primera campaña Antártica en isla Decepción, allí Juan pudo conocer en profundidad al resto de los Conxitos y a su familia antártica. Carlos "El Hippy" fue su compañero de viaje y el primero con el que descubrió las inmensidades del océano austral, así como las enseñanzas de la vida. Laura "La Hiperactiva", aunque ella no lo sepa, su peculiar carácter y actividad frenética han influenciado positivamente en el personaje, más de lo recomendable. Blanca "De Reus" con la que ha descubierto los briozoos y el mundo de la ciencia bajo otro prisma. Cristobo "Cálico Bentónico" ha sido el impulsador del buceo antártico, y al cual el joven héroe le debe una larga retahíla de habilidades. En subsiguientes campañas antárticas nuestro héroe ha compartido inolvidables vivencias con innumerables personajes. Maria "4 Espesies de Peses" fue la joven revelación antártica, con la cual todavía espera compartir vivencias tanto dentro como fuera de Ushuaia. Con Carlos "El Rastas" se han compartido muchas risas y birras. Uri "El Fumeta" con el que se han compartido demasiados pitis a babor del Hespérides, ¿o era estribor?. Patri "La Chiqui" y sus infinitas risas. Juan Junoy "El Nemertinólogo" al que el joven protagonista quiere reconocer su humor, su sabiduría, y su entrañable amistad ¡qué perdure!. Gonzalo "El de Harvard" con el que Juan ha compartido más horas que un reloj en el laboratorio del Hespérides, aprendiendo con cada invertebrado y a cada minuto con la insaciable curiosidad de GG. Lluís Cardona "El Tortuga", se conocían de antes y emprendieron un camino isotópico juntos, han compartido una campaña por todo lo alto y lo que nos rondará moreno!. Por último, este viaje sería infructífero sin la desinteresada ayuda de innumerables militares y científicos antárticos con los que Juan han vivido tantos momentos, a bordo del BIO Las Palmas, Hespérides, la BAE Gabriel de Castilla y la BAC Bernardo O'Higgins. La más sincera gratitud a todos ellos.

Questo è il momento di parlare della bella Italia. Dopo la prima stanza di investigazione a Napoli il protagonista della historia arriva a specializzarsi en la chimica dei produtti naturali da prima mano. Sono stati piu da 4 mesi dove Lella e Angelo hanno insegnato come studiare i produtti naturali dei nudibranchi antartici. Il ambiente amichevole al laboratorio è stato grazie a Angela, Jenny, Laura, Francesco e gli altri studenti. Ma la propria immersione italiana dovrebbe essere ringraziata a Luigi "L'Enologo", Amletto "Il Gangster" e Valeria "La Bionda", con que el'aventurero a compartito piso e conosciuto la belleza de il mare, la birra a buon mercato, il cibo straordinario, il buon vino e le buone conversazione.

The second stay of our adventurer was in Bonn, Germany, but we could not expect a fluent German writing or, in fact, any German at all. There, Juan met one of its main advisors, Heike, who turned out to be one of the best collaborators ever. She taught him histological techniques and imparted him an enormous knowledge on heterobranch anatomy and species description. Heike is a good friend and advisor to whom Juan still wants to collaborate in future projects. In Bonn the protagonist also met Vera "The Portuguese" and her family, he owe her good funny moments and a better English pronunciation, as well as a good taste of *paté de sardines*. Among all

other young students at the Museum Juan stablished good relationship with Cora, with whom they shared also nice moments at the beach in southern Spain.

The third short stay is indebted to the Linnean Society of London who granted Juan to stay in the cold and northern Greifswald, Germany. The adventurer shared great moments and parties with his flat mates Leo "The Half-Italian" and Alisa "The Naive". His fellows at the old but cozy research house were like a family for the adventurer, where he discovered how spiders can be interesting and disgusting at the same time. I am talking about Pierick "The Whiteguy", Philip "The Wise", Giulio "Butterfingers", Monika "The Mantophasmatodea", Anya "The Strange", and a long etc. Of course, our young researcher thanks Gabriele for offering him the opportunity of finishing his last two chapters of the thesis as well as for her lovely and ironic humor taste. The whole *spiderteam* deserves a big mention in these acknowledgements.

Y llega el momento de premiar a una figura clave en el transcurso de esta tesis, ella es Ana Riesgo "La Sabia". Aunque los comienzos no fueron agradables, una campaña Antártica unió a los dos personajes forjando una amistad que perduraría las vicisitudes que ambos enfrentarían. Juan ha conseguido afianzarse como investigador en gran parte por el apoyo moral e incondicional de Ana, desde los conocimientos en histología y microscopía electrónica hasta sus aptitudes para la secuenciación y el análisis filogenético. Pero no son todo técnicas, lo más importante han sido las lecciones morales que Juan ha podido adquirir, a veces por medio de escarmientos, de Ana. Tanto en Blanes como en su pisito en Barcelona, desde Nápoles a la Antártida, o desde Madrid a Londres, su unión ha sido fundamental para superar los duros tiempos de tesis. Esperando que dicho lazo no se disuelva nunca.

Y todo esto no sería posible sin la persona que un cierto día depositó confianza en un estudiante de prácticas con innumerables preguntas. La persona que confió en las capacidades del aventurero aun siendo meros primordios y vislumbró su potencial para desarrollar esta historia. Tanto yendo de campaña como desarrollando sus propias ideas sin sesgar su estudiante, las oportunidades concedidas son interminables. Esa persona se merece la más sincera gratitud, sin su apoyo el desarrollo de esta tesis hubiese sido imposible. Aún con diferencias de parecer y algunos escarmientos que otros, el joven protagonista agradece la formación de dicho tándem. Gracias Conxita.

Finalmente, el mundo de nuestro ingenuo investigador se desmoronaría sin el apoyo incesante e incondicional de Joan "El Rizos". Sin duda conocerle ha representado el mayor logro de esta etapa. Las circunstancias han sido duras, las relaciones a distancia no favorecen el buen carácter. Pese a todo, esta relación ha sido clave a la hora de aprender a disfrutar de la vida, meramente siendo feliz con cosas pequeñas: un pequeño yate, una pequeña casa de campo... Fuera bromas, con Joan han descubierto regiones remotas, compartiendo la gastronomía, la cultura y la fauna submarina de muchos lugares. ¡Espero poder descubrir muchos más contigo y compartir un camino investigador paralelo por siempre!

Toda esta historia ha sido sazonada por innumerables cantantes retro, compositores clásicos y de jazz, los cuales han sonado cual banda sonora diaria a lo largo de estos años. Cabe destacar a Supertramp, Miles Davis, Philip Glass y un larguísimo etcétera, a los cuales el ya-no-tan-joven protagonista quiere agradecer su don musical.

A todos vosotros, gracias.

CONTENTS

General Introduction and Objectives

GENERAL INTRODUCTION

Terra Australis Incognita

Antarctica is the coldest, driest and windiest continent on Earth. Situated in the Southern Pole it extents 14 million km^2 (*ca.* twice Australia's extension), and it is surrounded by the **Southern Ocean** (SO). Officially, Antarctica was first sighted in 1820 by the Russian expedition of FG von Bellingshausen and M Lazarev. However, speculation about a *Terra Australis Incognita* dates back to antiquity, where several sailors such as the Spanish Gabriel de Castilla (1603) or James Cook (1773) are claimed to be the first to sight the white continent. This remote land was once connected to Gondwanaland until the final breakup during the Early Cenozoic, ~25 million years ago (Mya), allowing the opening and widening of the two Antarctic gateways, *i.e.*, Tasmanian and Drake Passages (Scher & Martin, 2006). This separation allowed the formation of the **Antarctic Circumpolar Current** (ACC) and the establishment of its strongest eastward-flowing jet, the **Polar Front** (PF; see Figure 1) (Clarke *et al.*, 2005). The ACC is a thermal and hydrographic barrier which hampers marine organisms' dispersion from North to South at the SO (Barker & Thomas, 2004). The establishment of the ACC led to the isolation of the Antarctic continent, allowing benthic species to co-evolve in habitats characterized by low and relatively stable temperatures and extreme seasonality of primary production (Clarke, 1992; Dayton *et al.*, 1994; Clarke *et al.*, 2004). Cold waters condition a slow growth rate, longevity, and delayed age of maturity in invertebrates (Clarke, 2003). Prolonged developmental times coupled to short periods of primary production led Thorson (1936) to discuss why there is a depletion of taxa with planktonic larvae in polar waters (known as **Thorson's rule**). Still, planktonic development is a common strategy in SO shallow-water fauna, since they are more likely to colonize unoccupied areas destroyed by iceberg scouring (Smale *et al.*, 2008). The PF promotes the dispersal of marine organisms – larvae and/or adults – from West to East around Antarctica allowing the **circum-Antarctic** distribution of several taxa (Fell, 1962; Olbers *et al.*, 2004). However, the generalization that many Antarctic species are circumpolar has been recently challenged by broad-scale studies on population genetics, suggesting instead that much unrecognized diversity and genetic structure exists in the Antarctic biota (Hemery *et al.*, 2012; Wilson *et al.*, 2013). Overall, the combination of geographical isolation and climate change has led to a rich marine Antarctic biota with a high number of **endemic taxa** (Brandt & Gutt, 2011). Total species richness of macrozoobenthic organisms inhabiting the Antarctic continental shelf have been estimated to comprise between 11,000 and 17,000 species, of which over 8,800 are presently known and described (Griffiths, 2010; De Broyer *et al.*, 2011). Despite the present knowledge, data on marine biodiversity is still lacking for most regions of the SO (Kaiser *et al.*, 2013). This needs to be addressed urgently to identify biological responses to predicted environmental changes in Antarctica.

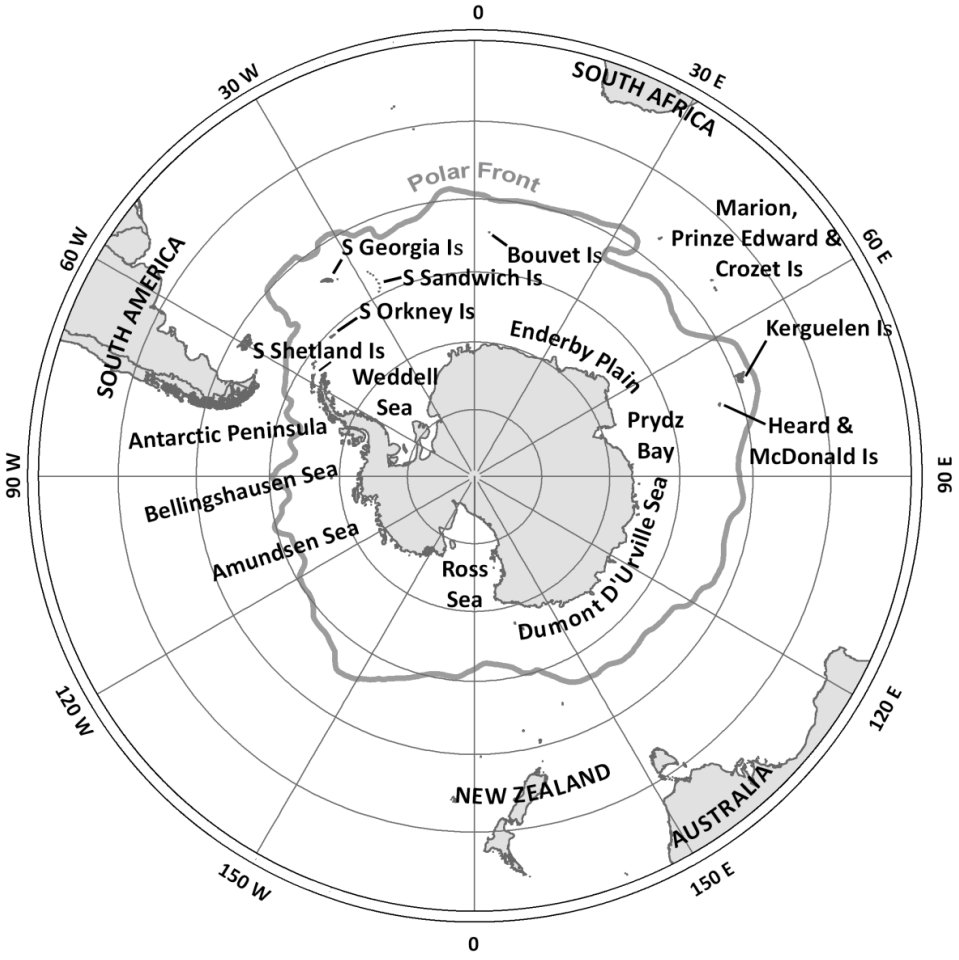


Figure 1. Map of the Southern Ocean and adjacent waters showing the main Antarctic regions. Polar Front delineated in grey. *Source: Moles* et al.*, 2015a*

Antarctic marine benthos

The structure of Antarctic benthic communities is the result of long- and short-term events which have shaped the ecosystem until present. Among the long-term events, Milankovitch cycles, known as Earth's orbit shifts, caused changes in Earth's climate patterns during the early Cenozoic, starting 65 Mya ago (Zachos *et al.*, 2001). Such changes resulted in long-term **glacial and interglacial periods** on which dramatic biodiversity changes occurred. This includes the massive **extinction** of taxonomic groups such as some pelagic and benthic top predators, and a reduction in the richness of groups such as bivalve molluscs, teleost fishes, and decapods (Clarke, 1983; Aronson *et al.*, 2007). Several taxa such as nothotenid fishes, gastropods, isopods, amphipods, and pycnogonids coped with the Antarctic harsh circumstances and **radiated,** favoured by the decrease of such predators and/or competitors (Clarke & Johnston, 2003; Clarke *et al.*, 2004; Thatje *et al.*, 2005). Shelf fauna was completely impoverished by grounded ice masses during glacial maxima, inducing the sheltering migration into marine sheltered oasis (polynyas) and deep-sea waters (Thatje *et al.*, 2005, 2008). Vertical migrations may explain the wide bathymetric tolerance of several taxa (Brey *et al.*, 1996). In fact, comprehensive surveys of benthic **deep-sea** reflect a high diversity

in the SO (Brandt *et al.*, 2007). On the other hand, current short-term seasonal and spatial variations from anchor and sea ice contribute to the patchiness of benthic communities in the Antarctic continental shelf (Raguá-Gil *et al.*, 2004). Nevertheless, the Antarctic marine benthic communities below the limit of the anchor ice and ice scour (3–400 m depth) are mainly influenced by **biotic factors** (Dayton *et al.*, 1974; Orejas *et al.*, 2000), which constitute important driving forces in controlling population structure (Pawlik, 2012). This leads to a continental shelf characterized by the presence of diverse, well-structured benthic communities, dominated by eurybathic suspension feeders and mobile fauna (see Figure 2; Dayton *et al.*, 1974; Gili *et al.*, 2006). Among these, echinoderms are the dominant mobile megafaunal taxa in terms of abundance and diversity (Griffiths, 2010; Moles *et al.*, 2015a), and have a predominant role in structuring benthic communities (Dayton *et al.*, 1974; Clarke & Johnston, 2003).

Among Antarctic echinoderms, the sea star ***Odontaster validus*** Koehler, 1906 is one of the most abundant species on the shallow Antarctic shelf, where it exerts considerable predatory pressure on benthic assemblages (Dayton *et al.*, 1994). It is considered a model predator for repellence assays because of its generalist and opportunistic feeding habits (McClintock *et al.*, 1990; Avila *et al.*, 2000; Moles *et al.*, 2015b). Consistent with the high predation pressure exerted by this keystone asteroid, recent studies have demonstrated the presence of feeding repellents in crude organic extracts of most taxonomic groups of Antarctic invertebrates (Avila *et al.*, 2008; McClintock *et al.*, 2010; Taboada *et al.*, 2013; Figuerola *et al.*, 2013; Moles *et al.*, 2015b). Overall, Antarctic benthic ecosystems are presently characterized by environmental stability (Dayton *et al.*, 1974), and accordingly, effective **defence** mechanisms come to be crucial for the survival of the species.

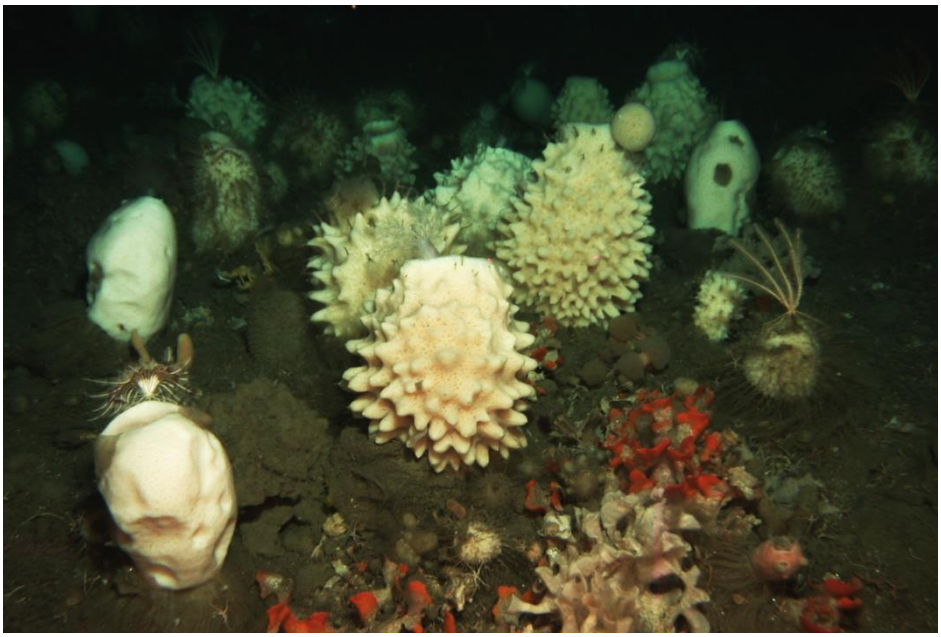


Figure 2. Benthic community of suspension-feeders from the shelf in the eastern Weddell Sea. *Source: J Gutt, from the ANTXXI/2 cruise on board of the RV Polarstern.*

Chemical ecology in Antarctic benthic ecosystems

Marine organisms produce a wide variety of molecules, often unique and critical for their survival in terms of feeding, reproduction, and/or protection (Amsler *et al.*, 2001; Puglisi *et al.*, 2014). These **natural products** (NPs) may affect species distribution, feeding patterns, community structure, and biodiversity (McClintock & Baker, 2001). Marine NPs, mostly secondary metabolites, often regulate the species' biology without participating directly in their primary metabolism (*i.e.*, growth, development, and reproduction; see Torssel, 1983). Although descriptive chemical studies on novel NPs from marine invertebrates are constantly growing in number, the ecological functions of NPs have received less attention (see reviews Lebar *et al.*, 2007; Avila *et al.*, 2008; McClintock *et al.*, 2010; Núñez-Pons & Avila, 2015). Among the ecological functions of these compounds, **anti-predatory** properties have raised more interest. In particular, several studies in McMurdo Sound (Ross Sea), the western Antarctic Peninsula (see McClintock & Baker, 1997; Amsler *et al.*, 2001, 2014; Avila *et al.*, 2008; McClintock *et al.*, 2010), and the eastern Weddell Sea and Bouvet Island (*e.g.*, Davies-Coleman, 2006; Taboada *et al.*, 2013; Figuerola *et al.*, 2013; Núñez-Pons & Avila, 2014; Moles *et al.*, 2015b) indicate that anti-predatory chemical defences are widespread among Antarctic species. The concentration and body allocation of NPs among and within individuals may vary with life history, season, and ecological interactions (López-Legentil *et al.*, 2005; Loh & Pawlik, 2014). According to the **Optimal Defence Theory** (ODT), NPs should be allocated effectively in most vulnerable or valuable structures, thus compensating the energetic requirements for growth, reproduction, and defence (Rhoades & Gates, 1976). Since the keystone predators in Antarctica are asteroids, which generally consume the surface of prey with their eversible cardiac stomach (Hyman, 1955), they may have driven the evolution of differential allocation of defences to the most exposed tissues in potential prey organisms (*e.g.*, Furrow *et al.*, 2003; Fairhead *et al.*, 2005; Peters *et al.*, 2009). Additionally, high densities of opportunistic crustacean predators, such as amphipods, might also graze upon sessile organisms (Dayton *et al.*, 1974), and accordingly, sessile taxa have developed NPs to protect themselves (Núñez-Pons *et al.*, 2012a; Figuerola *et al.*, 2013). Sessile taxa display strong repellence activities, with ascidians, cnidarians, and sponges usually being the best chemically protected. Defensive NPs are often of nonpolar/lipophilic nature (Taboada *et al.*, 2013; Moles *et al.*, 2015b), such as phlorotannins and terpene alcohols in algae, alkaloids and terpenoids in poriferans and tunicates, dithiocarbamates in hydrozoans or terpenoids in sea slugs (Lindquist *et al.*, 1992; Cronin *et al.*, 1995; Davies-Coleman, 2006; Toth *et al.*, 2007; Cutignano *et al.*, 2012; Núñez-Pons *et al.*, 2012b). Antarctic colonial ascidians belonging to the genera *Aplidium* and *Synoicum* present meroterpenoids and indole alkaloids as effective anti-predatory devices against sea stars and amphipods (Núñez-Pons *et al.*, 2010, 2012b). Antarctic soft corals of the genus *Alcyonium* yield illudalane sesquiterpenes and wax esters also deterring sympatric predators (Núñez-Pons & Avila, 2015). Antarctic demosponges are greatly defended against predation (Peters *et al.*, 2009), but even Antarctic glass sponges (Hexactinellida), such as *Rossella*, possess keto-steroids, and *Anoxycalyx* yielded a

taurine-like organic acid that displayed repellence against sympatric predators (Núñez-Pons *et al.*, 2012b; Núñez-Pons & Avila, 2014b). Overall, NPs, by mediating trophic interactions between prey and their potential predators, play an important role in structuring Antarctic benthic ecosystems.

Chemical ecology in sea slugs (Mollusca: Gastropoda: Heterobranchia)

Sea slugs occupy many different ecological niches and display a wide array of trophic relationships with organisms from many different phyla. Marine sea slugs are gastropod molluscs traditionally classified as **opisthobranchs** (see Figure 3), although they are currently included in the monophyletic Heterobranchia (including pulmonates). Marine heterobranchs are excellent models to understand evolution driven by sympatric predators through the study of their chemical defences and the glandular structures involved, since they possess a wide array of defensive strategies (Wägele & Klussmann-Kolb, 2005; Wägele *et al.*, 2006; Wilson *et al.*, 2013). Nearly all heterobranch taxa contain shelled and naked representatives, besides nudibranchs. Recent phylogenies therefore suggest that shell loss happened several times during the evolution of heterobranchs (Medina *et al.*, 2011; Zapata *et al.*, 2014; Wägele *et al.*, 2014). From an evolutionary perspective, the loss of the shell represents an advantage in terms of energy saving, which would otherwise be used for shell production and transportation, as well as other respiratory and excretory advantages. However, it simultaneously entails the investment in alternative defence strategies to survive in front of putative predators. In fact, the loss of the shell in sea slugs promoted a panoply of defensive strategies, including the use of chemicals (Avila, 1995; Cimino & Ghiselin, 2009; Putz *et al.*, 2010). Among the key innovations behind the evolutionary success of sea slugs are the abilities to steal functional structures (*i.e.*, kleptoplasty, kleptocnides) or NPs (*i.e.*, kleptochemistry) from other organisms. Sacoglossan heterobranchs, such as *Elysia viridis* (Figure 3), steal chloroplasts from algae to obtain energy and camouflage (*i.e.*, kleptoplasty; Händeler *et al.*, 2009), while most aeolideans steal nematocysts from cnidarians to use as protective devices (*i.e.*, kleptocnides; Putz *et al.*, 2010). **Kleptochemistry**, instead, is the incorporation of NPs from the diet, which may then be used for their own defence (see Avila, 1995; Cimino & Ghiselin, 2009). Bioactive metabolites derived from the diet may be transferred and accumulated in exposed, vulnerable areas, such as the mantle, foot, and gills (see Figure 3); within mucus or ink secretions; in specialized glands; and also occasionally in eggs, embryos, and larval stages (*e.g.*, Avila, 1995; Wägele *et al.*, 2006; Cimino & Ghiselin, 2009). For instance, species of the nudibranch *Felimare* from the Mediterranean gather furanoterpenoids from their sponge preys *Dysidea* spp. and locate them along the exposed mantle rim to defend against fish and crustacean predators (Avila *et al.*, 1991a; Fontana *et al.*, 1994). Some species are able to **biotransform** the dietary metabolites to make them less toxic for the slug itself, or more noxious and deterrent towards predators (Avila, 1995, 2006; Cimino & Ghiselin, 2009). This is the case of *F. orsinii* (Vérany, 1846),

which obtains the sesterterpenoid scalaradial from the sponge *Cacospongia mollior* Schmidt, 1862 and transforms it to deoxoscalarin by deoxygenation (Cimino *et al.*, 1993). Finally, some sea slugs may completely ***de novo* biosynthesise** some chemicals from simple precursors (Cimino & Ghiselin, 1999; Cimino *et al.*, 2001). For example, the nudibranch *Dendrodoris limbata* (Cuvier, 1804) and *D. grandiflora* (Rapp, 1827) build up drimane sesquiterpenoids and accumulate them in the mantle and egg masses for defence against fish predators (Avila *et al.*, 1991b). Overall, heterobranch molluscs possess a wide range of bioactive compounds protecting them against potential predators, and thereby enhancing their ecological performance.

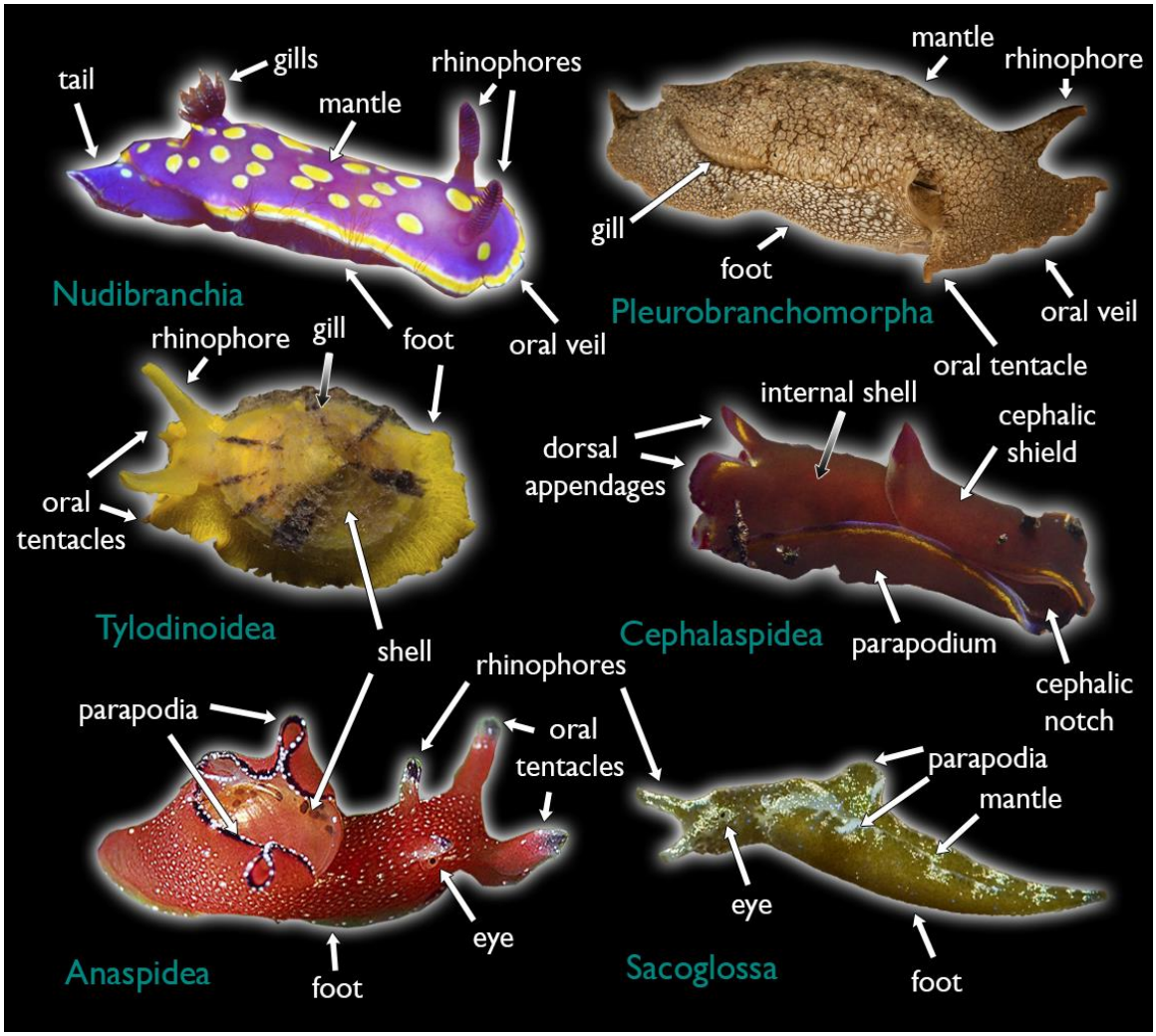


Figure 3. External morphological features of the main marine heterobranch clades. *Source: Mediterranean pictures gathered from the GROC website (http://www.opistobranquis.org/).*

Antarctic chemical ecology in sea slugs

Despite the large number of chemical studies on molluscs from temperate and tropical areas, little is known about secondary metabolism in Antarctic nudibranchs (Davies-Coleman, 2006; Avila *et al.*, 2008). Only four species of Antarctic sea slugs have been chemically analysed to date, all of them containing defensive NPs in the mantle, used

against sympatric predators (McClintock & Baker, 1997b; Avila *et al.*, 2000, 2008; Iken *et al.*, 2002; Davies-Coleman, 2006). Pteroenone, a polypropionate-derived NP from the pelagic pteropod *Clione antarctica* Smith, 1902 displayed feeding repellence against fish predators (McClintock & Janssen, 1990; Yoshida *et al.*, 1995). *De novo* biosynthesis of bioactive terpene metabolites has been hypothesized for two anthobranch nudibranchs: *Bathydoris hodgsoni* Eliot, 1907 and *Doris kerguelenensis* (Bergh, 1884)(Avila *et al.*, 2000; Iken *et al.*, 2002). Hodgsonal, a sesquiterpene isolated exclusively from the notum and papillae of *B. hodgsoni* (Iken *et al.*, 1998), showed repellence against *O. validus* (Avila *et al.*, 2000). *Doris kerguelenensis* was proved to possess a variety of diterpene diacylglycerols in the notum (Gavagnin *et al.*, 1995; 1999a, b; 2003a, b; Diyabalanage *et al.*, 2006; Maschek *et al.*, 2012), some of them displaying anti-predatory activity against *O. validus* (Iken *et al.*, 2002). These metabolites are synthesised through diverse metabolic routes with a remarkable variability among individuals (Cutignano *et al.*, 2011). This, in combination with molecular phylogenetic analyses led Wilson *et al.* (2013) to suggest cryptic speciation driven by predation in this species complex. Finally, the dendronotid nudibranch *Tritoniella belli* Eliot, 1907 is the only Antarctic nudibranch investigated so far that obtains its defensive NP from its food, the stoloniferan soft coral *Clavularia frankliniana* Roule, 1902. This is a chimyl alcohol which also displays repellent activity against *O. validus* (McClintock *et al.*, 1994).

Among nudibranchs, the family Charcotiidae possesses four Antarctic – mostly circum-Antarctic – endemic species, one of the genus *Charcotia* and three of *Pseudotritonia*, and one species endemic from South Africa of the genus *Leminda* (Wägele, 1991). Within this family, only *L. millecra* Griffiths, 1985 was chemically analysed and four bioactive sesquiterpenes were described (Pika & Faulkner, 1994). In this sense, elucidation of the chemical structure of the NPs in the widely distributed Antarctic shallow-water nudibranch ***Charcotia granulosa*** Vayssière, 1906 (Barnes & Bullough, 1996; Arnaud *et al.*, 2001; Barnes & Brockington, 2003; Shields *et al.*, 2009) has never been assessed before (**Chapter 1**). This species is currently assigned to **Cladobranchia**, to whom it shares a ramified digestive gland (Wägele *et al.*, 1995; Wägele & Willan, 2000; Pola & Gosliner, 2010). Cladobranchia are not well investigated yet regarding their chemical ecology. Only a very few species from the genera *Melibe* and *Doto* are known to synthesise NPs themselves (Putz *et al.*, 2010; 2011). Regarding the location of the anti-predatory NPs, special **glandular structures** on the external and most vulnerable parts of the slug normally gather them (Avila & Paul, 1997; Wägele *et al.*, 2006; Carbone *et al.*, 2013). These structures can be epidermal and subepithelial glands, or complex glandular structures (see Wägele *et al.*, 2006). Complex glandular cells, such as mantle dermal formations (MDFs) or similar structures, produce and/or accumulate chemical defences (Avila & Durfort, 1996). These can be found in nudibranchs, cephalaspideans, and sacoglossans. Nonetheless, knowledge on the origin, location, and function of the NPs in *C. granulosa* still is unexplored (**Chapter 2**).

During our research on *C. granulosa*, we also found an unreported copepod crawling in the notum of the nudibranch. Unlike heterobranchs from temperate and tropical waters, Antarctic sea slugs had never been reported to present **symbiotic relationships**, neither ecto- nor endosymbionts. **Copepods** have been highly successful in forming associations with other marine organisms, among which molluscs seem to be one of the most preferred hosts. According to Ho (1997), a total of 246 copepod species have been described in association with 458 species of molluscs. These symbionts belong to five orders: Harpacticoida, Misophrioida, Cyclopoida, Siphonostomatoida, and Poecilostomatoida, the last of which includes about 73% of the known copepod associates of Mollusca. Indeed, poecilostomatoid species of the family **Anthessiidae** are mostly associated with molluscs (Boxshall & Halsey, 2004a,b), while some are found associated with algae, plankton, crustaceans, and teleost fish (Ho, 1997; Conradi *et al.*, 2012). Although more than 50 species of Anthessiidae have been recorded worldwide, none of them is known from the Southern Ocean. In this thesis we found it interesting to report a new species of *Anthessius* as the first record from Antarctic waters and the first **ectosymbiotic** association with a nudibranch, *i.e.*, *C. granulosa* (**Chapter 3**). Additionally, several **endoparasites** were also documented for a new species of Cephalaspidea (**Chapter 5**), therefore increasing the current knowledge on macro-symbiotic relationships in Heterobranchia.

Although adult nudibranchs studied in the field present secondary metabolites, their specific ontogenetic origin, in species with *de novo* biosynthesis, is not assessed. As mentioned above, both *B. hodgsoni* and *D. kerguelenensis* possess bioactive molecules that protect the adults from sympatric predators and which are likely *de novo* biosynthesised by the slugs (Avila *et al.*, 2000; Iken *et al.*, 2002; Cutignano *et al.*, 2011). Both species are circumpolar, eurybathic, and present a broad dietary spectrum, *B. hodgsoni* is a generalist omnivorous predator (Avila *et al.*, 2000), while *D. kerguelenenses* feeds on a wide variety of demosponges and hexactinellids (reviewed in McDonald & Nybakken, 1997). The cold Antarctic waters favour a slow growth rate, longevity, and a delayed maturity in Antarctic benthic fauna (Pearse *et al.*, 1991; Clarke, 2003; Peck *et al.*, 2007). Low temperatures and/or differences in seasonal availability of organic matter favour protected intracapsular development as a common strategy in Antarctic species, to protect early stages of their life cycle (Wray & Raff, 1991; Peck *et al.*, 2006). Intracapsular or direct developing molluscs usually produce few, large eggs (Thompson, 1967; Todd & Doyle, 1981; Hain & Arnaud, 1992), this seems to be also the case of *B. hodgsoni* and, to a less extent, *D. kerguelenensis* (Wägele, 1989b, 1996). However, little is known about their ontogenetic development and chemical protection of embryonic stages, and further insight is gained in **Chapter 4**.

Systematics and taxonomy of heterobranchs

Gastropoda, the clade of molluscs that include snails and slugs, is extremely diverse with respect to species number, morphology, habitat, and many other attributes. They radiated in marine, freshwater, and terrestrial systems, and thus display extensive body plan disparity. Gastropods are characterized by having a single shell and an operculum, at least in the larval stage, and by undergoing torsion during development. Classically, gastropod molluscs were divided into three subclasses: Prosobranchia, **Opisthobranchia**, and Pulmonata, on the basis of the position and type of their respiratory organs. Molecular phylogenies recovered prosobranchs polyphyletic and opisthobranchs and pulmonates paraphyletic (Ponder & Lindberg, 1997; Zapata *et al.*, 2014). A plethora of studies dealt with opisthobranch phylogeny concluding that, although this is still a matter of great controversy, Opisthobranchia together with Pulmonata and some "prosobranchs" are very closely related. Haszprunar (1985) recovered the taxon **Heterobranchia** (coined by Gray, 1840), which includes the synapomorphies: hyperstrophy, pallial kidney, hypobranchial gland in anterior position, absence of odontophoral cartilages, hermaphroditism, loss of parasperm, spiral-shaped sperm, coarse fibres, and intra-axonemal dense granules. This phylogenetic hypothesis has been recurrently tested and it has now reached a consensus, although internal relationships among clades within the tree remain yet unsolved (see review in Wägele *et al.*, 2014). The phylogeny given in Figure 4 shows the relationships among the main heterobranch subclades. Therefore, nowadays the "Opisthobranchia" concept is of historical and emotional value "only".

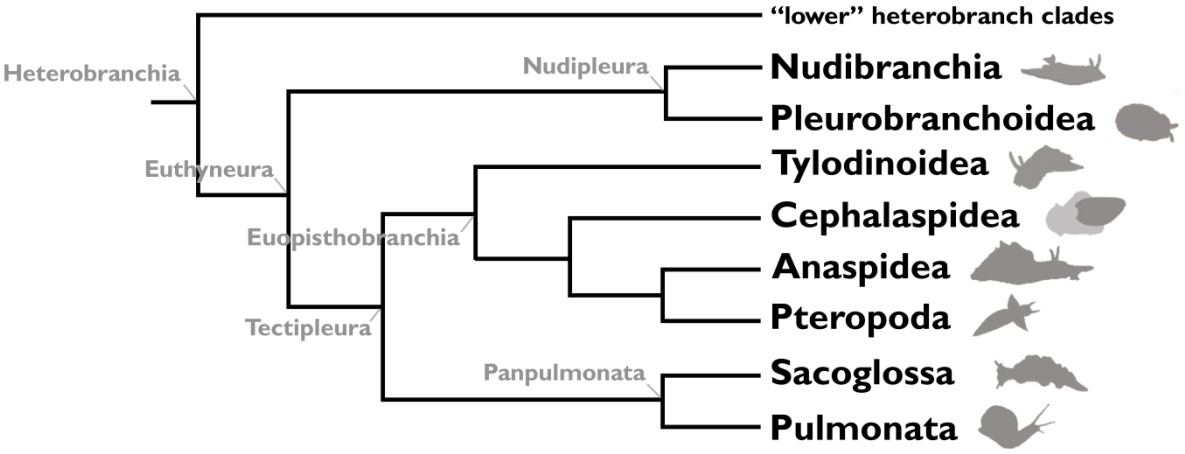


Figure 4. Phylogenetic tree consensus of the Heterobranch main subclades.
Source: modified after Wägele et al. *(2014) and Zapata* et al. *(2014).*

Heterobranchia is divided into some "lower clades" and **Euthyneura**, the latter characterized by **detorsion** of the nervous system (= euthyneury) and by having rhinophores innervated by N3 (rhinophoral nerve). Euthyneura comprises **Nudipleura** (*sensu* Wägele & Willan, 2000), and **Tectipleura** (*sensu* Schrödl *et al.*, 2011), which is a monaulic taxon. Nudipleura consists of side-gilled

Pleurobranchoidea and **Nudibranchia**, the latter are the sea slugs in a strict sense. The characters defining Nudipleura are the possession of a blood gland, androdiaulic reproductive system, and loss of the osphradium (Wägele & Willan, 2000). The main synapomorphy of Tectipleura, instead, is the monauly (possession of a single flow-through for autosperm, allosperm, and ovules) and are divided into **Panpulmonata**, including **Sacoglossa**, and **Pulmonata**, and **Euopisthobranchia**, which present a cuticularized oesophagus (Schrödl *et al.*, 2011; Wägele *et al.*, 2014). The latter clade includes several well-known opisthobranch groups: the side-gilled **Tylodinoidea**, the bubble-shelled **Cephalaspidea**, the sea hares **Anaspidea**, and the pelagic **Pteropoda**.

Antarctic heterobranchs

Although Antarctic and Subantarctic heterobranch diversity has been surveyed in several campaigns during the XIX and XX centuries (Box I), large areas of knowledge on this group remain still underexplored. Only a few heterobranch taxa are present in the SO (Box II), whereas charismatic taxa such as the solar-powered sacoglossans and sea hares are not represented. The latter taxa are shallow-water specialized herbivores from subpolar, temperate, and tropical waters (*i.e.*, stenothermics; Carefoot, 1987; Jensen, 2007). They might have never been able to cope with the harsh environmental conditions of the SO, especially since primary production (algae) is seasonally limited (Barnes & Clarke, 1995). Alternatively, they could have been extinguished during glacial maxima (see above) and/or were unable to cross the PF. There are approximately 80 species of heterobranchs described in the SO hitherto, among which Nudibranchia (~35) and Cephalaspidea (~25) are the most speciose orders (De Broyer *et al.*, 2016). Although less diverse, "lower heterobranchs", pteropods, and pleurobranchomorphs are also found in Antarctica, as well as one Subantarctic species of *Siphonaria* (Pulmonata). In this sense, SO heterobranch's diversity is not as high in terms of species and higher taxa as in other oceans. Nonetheless, several families and genera are only found in SO waters, being sometimes crucial for the phylogenetic comprehension of the evolution of heterobranch lineages. Wägele *et al.* (2008) and Martynov & Schrödl (2009) noticed how basal members of some major Nudipleura linages have an Antarctic origin. For instance, the nudibranchs *Bathydoris* Bergh, 1884 and *Prodoris* Baranetz & Minichev, 1995 are deep-sea genera basal to Anthobranchia (Valdés, 2002); Charcotiidae, with species such as *C. granulosa*, is sister group to the Aeolidoidea (Wägele *et al.*, 1995); *Notaeolidia* Eliot, 1905 is the most basal group of Aeolidoidea (Wägele, 1990); *Tritoniella* Eliot, 1907 is considered basal among Tritoniidae (Wägele, 1989); among others. Even the pleurobranchs *Bathyberthella antarctica* Willan & Bertsch, 1987 and *Tomthompsonia antarctica* (Thiele, 1912) represent the basal offshoot of the Pleurobranchidae, leading Göbbeler and Klussmann-Kolb (2010) to hypothesize an Antarctic origin of the Pleurobranchoidea. Despite the low diversity of SO heterobranchs in comparison with other oceans, all

the evidence strikes into the importance of studying Antarctic systematics and taxonomy of this key group. Moreover, recent advances in phylogenetic analysis are showing a high prevalence of cryptic speciation in apparent circumpolar species such as *D. kerguelenensis*, to which more than 36 different linages had been discovered to date (Wilson *et al.*, 2009, 2013; Wilson's pers. comm.). Summarizing, Antarctic heterobranchs appear revealing in systematics, but their diversity is far from being explored yet. In **Chapters 5**, **6**, and **7** we aim to contribute to the knowledge of Antarctic heterobranch diversity and systematics.

BOX 1

Antarctic expeditions and researchers who contributed to the knowledge of the SO heterobranch fauna during the XIX and XX centuries

- d'Orbigny's explorations in South America (1835–1846)
- Watson (1886) *Challenger* (1873–1876)
- Bergh (1898) L. Plate's expedition to South America
- Pelseneer (1903) Belgian Antarctic Expedition (1897–1899)
- Eliot (1907a) expedition to the Falkland Islands
- Strebel (1908) and Odhner (1926) Swedish South Polar Expedition
- Thiele (1912) German South Polar Expedition (1901–1903)
- Eliot (1905, 1907b) Scottish National Antarctic Expedition (1901–1904)
- Vayssière (1906, 1917) French Antarctic Expeditions (1903–1905 and 1908–1910)
- Eales (1923) and Odhner (1934) British Antarctic *Terra Nova* Expedition (1910–1913)
- Hedley (1916) Australasian Antarctic Expedition (1911–1914)
- Odhner (1924) Mortensen's Pacific Expedition
- Odhner (1944) Norwegian Antarctic Expeditions (1927, 1928 et seq.)
- Powell (1951) *Discovery* Investigations (1925–1939)
- Powell (1955, 1957, 1958, 1960, 1965) British-Australian-New Zealand Antarctic Research Expedition (1929-1931) with Sub-Antarctic islands
- Er. Marcus (1959) Lund University Chile Expedition (1957)
- Vicente & Arnaud (1974) 12th (1961–1963) and 15th (1964–1965) French Antarctic Expeditions
- Minichev (1972) Davis Sea collection

While the origin of nudibranchs and pleurobranchomorphs has been suggested to be Antarctic (Wägele *et al.*, 2008; Göbbeler & Klussmann-Kolb, 2010), little is known about the origin of the **Cephalaspidea**. This taxon is distributed worldwide (OBIS, 2016), usually restricted from shallow to deep interstitial muddy bottoms, but some species live in association with seagrasses, algae or sessile invertebrates (Gosliner *et al.*, 2008). The original diagnostic character of Cephalaspidea is the presence of a cephalic shield. This, together with sessile eyes and posterior tentacular folds, are characteristic features related mostly to their burrowing habits, other than true synapomorphies (Mikkelsen, 2002). The diagnostic characters of the Cephalaspidea *sensu stricto* (without Runcinacea and Acteonoidea) (Mikkelsen, 1996; Malaquias *et al.*, 2009) are the presence of three hardened oesophageal gizzard plates, flexed ciliated strips in the mantle cavity, a prepharyngeal nerve ring (*i.e.*, located anterior to the pharynx), and the genital ganglion located on the visceral nerve loop

(Mikkelsen, 1996). Later, Mikkelsen (2002) recognized only the two first characters as valid autapomorphies, rejecting the other two. Among the cephalaspidean families, **Diaphanidae** Odhner, 1914 (Amphisphyridae Gray, 1857) has been for a long time considered a basal family within Cephalaspidea, because they exhibit plesiomorphic morphological features (Jensen, 1996). Diaphanidae was primarily defined on negative characters: absence of parapodia, jaws, and gizzard plates (Eliot, 1906; Odhner, 1914; Thiele, 1931) and their apparent resemblances were interpreted as homoplastic adaptations to epifaunal habits and suctorial feeding. Consequently, the family became a wastebasket taxon, where several genera were included. In fact, several families have been designed subsequently to include most genera of Diaphanidae *sensu lato.* However, the relationships of the Antarctic monotypic genus ***Newnesia*** Smith, 1902, which in former times was also included in the Diaphanidae, remain so far untested (**Chapter 5**). This genus is currently restricted to Antarctic and Subantarctic circumpolar waters at depths ranging from 16 to 655 m (Aldea & Troncoso, 2008).

Several worldwide distributed heterobranch families exhibit Antarctic representatives, although these are usually little diversified in the SO. For instance, the nudibranch family **Dotidae** Gray, 1853, has a single representative, ***Doto antarctica*** Eliot, 1907 described from Antarctica hitherto, based on a single specimen from McMurdo Sound (Victoria Land). However, although accurate details of the **external anatomy** and radula of *D. antarctica* were reported (Eliot, 1907a; Odhner, 1934), no internal description of the digestive and reproductive system was provided (**Chapter 6**). Since then, several specimens have been collected from nearly all around the SO (Thiele, 1912; Odhner, 1934; Powell, 1960; Lovell & Trego, 2003; Schiaparelli *et al.*, 2006), indicating a putative circum-Antarctic distribution. Additional undetermined species of *Doto* have been recorded at Bouvet Island (Arntz *et al.*, 2005) and Ross Sea (Schiaparelli *et al.*, 2006; Ghiglione *et al.*, 2013). Nonetheless, no taxonomic description has been provided for these specimens. Although very diverse, with 87 species recognised to date covering a cosmopolitan distribution (WoRMS, 2016), the genus *Doto* is understudied in the SO, and potentially new species are awaiting to be discovered (**Chapter 6**). Although some heterobranch families are worldwide distributed, some other are restricted to the SO or even they present a disjunct distribution between poles. A disjunct distribution of sister taxa covering the northern and southern hemispheres is a phenomenon known as **bipolarity** (Stepanjants *et al.*, 2006). Bipolar distributions can occur either at the species, genus or higher taxonomic levels (Allcock & Griffiths, 2015). In molluscs, approximately 30 % of living Antarctic bivalve and gastropod families are bipolar, including heterobranch genera such as *Philine* Ascanius, 1772 and the diaphanid *Toledonia* Dall, 1902 (Rudman, 1972; Warén, 1989; Dell, 1990; Crame, 1993). The wide fossil record of molluscs suggests at least three paleontological periods in which bipolar events occurred: Late Jurassic (~150 Mya), Paleogene-Neogene (~23 Mya), and Neogene-Pleistocene (~2.6 Mya; Crame, 1993). Current disjunct distributions might be the result of **transequatorial** dispersal during glacial maxima cooling or, alternatively, a prior cosmopolitan species isolated vicariantly in high latitudes during interglacial periods (Allcock & Griffiths, 2015).

Vicariant cases imply that species once placed in the tropics might have sheltered in deep waters during interglacial periods, a phenomenon called **equatorial submergence** (Stepanjants *et al.*, 2006). This is applicable for *Philine* for example, which is distributed in deep waters of all world oceans (OBIS, 2016). The nudibranch family **Akiodoridídae** Millen & Martynov, 2005 is a further example of bipolar distribution. Akiodoridídae is considered to be a basal family within **Onchidoridoidea**, presently related to Goniodoridídae based on the reproductive system (Hallas & Gosliner, 2015). Among the Akiodoridídae genera: *Akiodoris* Bergh, 1879 is confined to the N Pacific, *Armodoris* Minichev, 1972 is from the SO, and ***Doridunculus*** Sars, 1878 is from the N Pacific and N Atlantic, each with two described species; while *Echinocorambe* Valdés & Bouchet, 1998 inhabits the Norwegian Sea and *Prodoridunculus* Thiele, 1912 is from the Davies Sea, and both are monotypic. Therefore, all Akiodoridídae genera are either restricted to northern or southern hemispheres (**Chapter 7**).

Overall, knowledge on the biodiversity of Antarctic benthic communities is essential to identify biological responses to predicted environmental changes in Antarctica. In this sense, we contribute here to the current knowledge of Antarctic heterobranch diversity by describing three new species (**Chapters 6**, **7**, and **8**). Description of new heterobranch species requires dissection, radula preparation, and internal anatomy description, thus, usually at least one specimen is almost completely destroyed during the process. Since holotype specimens should remain intact for their deposit in a museum after description, we performed 3D reconstruction analysis, by using micro-CT techniques, to describe a *Doto* (**Chapter 6**) and *Doridunculus* (**Chapter 7**) species. Thereby, unique type material from regions difficult to survey is investigated in a non-destructive way. Several heterobranchs have been previously described using 3D reconstructive techniques, including interstitial acochlideans (Rückert *et al.*, 2008; Jörger *et al.*, 2008; Brenzinger *et al.*, 2013a), cephalaspideans (Brenzinger *et al.*, 2013b), and nudibranchs (Martynov *et al.*, 2011). However, these 3D reconstructions were performed by taking digital photographs of histological slices; therefore the specimens were completely damaged after it. Here, micro-tomographic scanning of intact specimens and posterior 3D reconstruction was performed in both **Chapters 6** and **7**, thus we assessed the potential of micro-CT for non-invasive description of unique type material.

BOX II
Heterobranchs described in the Southern Ocean to date

"LOWER HETEROBRANCHS"
Acteonidae
Acteon antarcticus Thiele, 1912
Neactaeonina edentula (Watson, 1883)
Neactaeonina fragilis Thiele, 1912
Mathildidae
Turritellopsis gratissima Thiele, 1912
Turritellopsis latior Thiele, 1912
Omalogyridae
Omalogyra atomus (Philippi, 1841)
Orbitestellidae
Microdiscula subcanaliculata (E. A. Smith, 1875)
Microdiscula vanhoeffeni Thiele, 1912
Pyramidellidae
Streptocionella pluralis Dell, 1990
Rissoellidae
Rissoella notabilis (Thiele, 1912)
Rissoella powelli Ponder, 1983

CEPHALASPIDEA
Cylichnidae
Cylichna cumberlandiana (Strebel, 1908)
Cylichna gelida (E. A. Smith, 1907)
Cylichna georgiana (Strebel, 1908)
Toledonia elata Thiele, 1912
Toledonia globosa Hedley, 1916
Toledonia limnaeaeformis (E. A. Smith, 1879)
Toledonia major (Hedley, 1911)
Toledonia palmeri Dell, 1990
Toledonia parelata Dell, 1990
Toledonia punctata Thiele, 1912
Toledonia striata Thiele, 1912
Diaphanidae
Diaphana anderssoni (Strebel, 1908)
Diaphana inflata (Strebel, 1908)
Diaphana paessleri (Strebel, 1905)
Diaphana pfefferi (Strebel, 1908)
Newnesiidae
Newnesia antarctica E. A. Smith, 1902
Philinidae
Philine antarctica E. A. Smith, 1902
Philine apertissima E. A. Smith, 1902
Philine kerguelensis Thiele, 1925
Philinorbidae
Antarctophiline alata (Thiele, 1912)
Antarctophiline amoena (Thiele, 1925)
Antarctophiline gibba (Strebel, 1908)
Scaphandridae
Kaitoa scaphandroides Powell, 1951

PTEROPODA
Cliidae
Clio piatkowskii van der Spoel, Schalk & Bleeker, 1992
Clio pyramidata Linnaeus, 1767
Clionidae
Clione limacina (Phipps, 1774)
Limacinidae
Limacina helicina (Phipps, 1774)
Limacina rangii (d'Orbigny, 1834)
Limacina retroversa (Fleming, 1823)
Thielea helicoides (Jeffreys, 1877)
Peraclidae
Peracle reticulata (d'Orbigny, 1834)
Pneumodermatidae
Spongiobranchaea australis d'Orbigny, 1836

NUDIBRANCHIA
Aegiridae
Aegires albus Thiele, 1912
Akiodoridiidae
Armodoris antarctica Minichev, 1972
Armodoris anudeorum Valdés, Moran & Woods, 2011
Prodoridunculus gaussianus Thiele, 1912
Bathydoridiidae
Bathydoris hodgsoni Eliot, 1907
Prodoris clavigera (Thiele, 1912)
Cadlinidae
Cadlina affinis Odhner, 1934
Cadlina georgiensis Schrödl, 2000
Cadlina kerguelensis Thiele, 1912
Cadlina magellanica Odhner, 1926
Charcotiidae
Charcotia granulosa Vayssière, 1906
Pseudotritonia antarctica (Odhner, 1934)
Pseudotritonia gracilidens Odhner, 1944
Pseudotritonia quadrangularis Thiele, 1912
Doridiidae
Doris kerguelenensis (Bergh, 1884)
Dotidae
Doto antarctica Eliot, 1907
Eubranchidae
Eubranchus glacialis (Thiele, 1912)
Eubranchus adarensis Odhner, 1934
Galvinella antarctica Eliot, 1907
Notaeolidiidae
Notaeolidia gigas Eliot, 1905
Notaeolidia schmekelae Wägele, 1990
Notaeolidia depressa Eliot, 1907
Tergipedidae
Cuthona crinita Minichev, 1972
Cuthona elioti (Eliot, 1907)
Cuthona georgiana (Pfeffer in Martens & Pfeffer, 1886)
Cuthona giarannae Valdés, Moran & Woods, 2012
Cuthona modesta (Eliot, 1907)
Guyvalvoria francaisi Vayssière, 1906
Guyvalvoria paradoxa (Eliot, 1907)
Tergipes antarcticus Pelseneer, 1903
Tritoniidae
Tritonia challengeriana Bergh, 1884
Tritonia dantarti Ballesteros & Avila, 2006
Tritonia vorax (Odhner, 1926)
Tritoniella belli Eliot, 1907

PLEUROBRANCHOIDEA
Pleurobranchidae
Bathyberthella antarctica Willan & Bertsch, 1987
Bathyberthella orcadensis (García, García-Gómez, Troncoso & Cervera, 1994)
Bathyberthella tomasi (García, Troncoso, Cervera & García-Gómez, 1996)
Tomthompsonia antarctica (Thiele, 1912)

PULMONATA
Siphonariidae
Siphonaria lateralis Gould, 1846

OBJECTIVES

The present PhD thesis covers several topics of the ecology, taxonomy, and systematics of selected Antarctic heterobranchs. The main objective is **to study the ecological and diversity patterns of some Antarctic marine sea slugs** by applying multidisciplinary methodologies.

According to the general subjects treated here, the work can be divided into two main divisions. **Section I**, ecology, including three chapters on chemical ecology, ectosymbiosis, and development, includes the description of a new natural product of the nudibranch *Charcotia granulosa* (**Chapter 1**), the analysis of its origin, function, and anatomical location (**Chapter 2**), and the description of a new species of copepod ectosymbiont of this nudibranch (**Chapter 3**). Moreover, we studied the development of two large anthobranchs (*B. hodgsoni* and *D. kerguelenensis*) and evaluated the ontogenetic origin of their compounds (**Chapter 4**). **Section II**, includes the chapters on taxonomy and systematics, including three articles on Cephalaspidea and Nudibranchia. Here we describe a new species and family of Cephalaspidean and shed light into the origin of Cephalaspidea (**Chapter 5**), describe two new species of nudibranchs of the genera *Doto* (**Chapter 6**) and *Doridunculus* (**Chapter 7**) and provide a comprehensible discussion about their phylogenetic relationships.

The specific objectives for each chapter are summarized below.

Section I

- Chapter 1: **Granuloside, a unique linear homosesterterpene from the Antarctic nudibranch *Charcotia granulosa*.** Our aim is to (1) investigate the nature of the NPs of the specimens of *C. granulosa* collected by scuba diving in shallow-waters of Deception Island; (2) chemically elucidate the structure of the secondary metabolites of *C. granulosa* by using spectroscopic techniques; and (3) shed light into the origin of granuloside and chemical aspects.

- Chapter 2: **Distribution of granuloside in the Antarctic nudibranch *Charcotia granulosa* (Gastropoda: Heterobranchia: Charcotiidae).** Our main objectives are four: (1) to localize granuloside in the animal tissues; (2) to chemically analyse the egg masses of *C. granulosa* and its prey *Beania erecta*, to shed light into the possible origin of granuloside; (3) to describe histologically and ultrastructurally the notum and egg masses of the nudibranch, in order to identify putative storage areas for the compounds; and (4) to test the feeding repellence of *C. granulosa* through *in situ* bioassays with the sea star *O. validus*.

- Chapter 3: ***Anthessius antarcticus* n. sp. (Copepoda: Poecilostomatoida: Anthessidae) from Antarctic waters living in association with *Charcotia granulosa* (Mollusca: Nudibranchia: Charcotiidae).** Our scope is to (1) describe

the ectosymbiont found in the slug, a new species of Anthesiidae copepod living in association of *C. granulosa*; and (2) to discuss its systematic relationships among congeners from all over the world.

- Chapter 4: **The slugs that laid giant egg masses: Embryonic development in two Antarctic anthobranchs (Gastropoda: Nudibranchia)**. We aim to (1) evaluate the developmental stages of the Antarctic intracapsular developers' *B. hodgsoni* and *D. kerguelenensis* by rearing their egg masses for several months and subsequently investigate them with histological methods; (2) unravel the defensive strategies in early stages of these nudibranchs by analysing the presence/absence of their NPs in various ontogenetic stages of *D. kerguelenensis*; and (3) gain further information on the origin of the compounds of *D. kerguelenensis* by analysing their occurrence in four of the preyed sponges collected together at same sites as the slugs.

Section II

- Chapter 5: **An Antarctic opisthobranch clade is sister to all other Cephalaspidea (Gastropoda: Heterobranchia).** We aim to (1) describe a new *Newnesia* species from Antarctic deep waters; (2) provide a formal taxonomical description by using morphological and molecular characters; (3) compare the morphology of the new species to the rest of genera of Diaphanoidea *s. l.*; and (4) provide a phylogenetic hypothesis for the position of the species within Cephalaspidea, potentially evaluating their ancestral features in a phylogenetic context.

- Chapter 6: **The end of the cold loneliness: 3D comparison between *Doto antarctica* and a new sympatric species of *Doto* (Heterobranchia: Nudibranchia).** In this chapter our scope is to (1) explore the anatomy and the egg mass characteristics of *D. antarctica*, by both histological and tomographic techniques; (2) assess the potential of micro-CT for non-invasive description of unique type material; (3) describe a new species based on a single specimen, *D. carinova* n. sp., collected in the Weddell Sea, by 3D reconstruction of micro-CT images; (4) sequence *D. antarctica* from the Weddell Sea and compare it to specimens from the Ross Sea; and (5) disentangle the phylogenetic conundrum of *Doto* species, by providing an evolutionary scenario of the changes in *Doto* anatomy for all the species where molecular data are available to date.

- Chapter 7: **Bipolarity in sea slugs: On the description of *Doridunculus punkus* n. sp. (Nudibranchia, Onchidoridoidea) from Antarctica.** We aim to (1) describe a single specimen of *Doridunculus punkus* n. sp., collected in the eastern Weddell Sea, by using micro-CT techniques; (2) provide a comparative anatomical description between *Doridunculus* and the rest of Akiodoridídae genera; and (3) explain the bipolar distribution of this enigmatic family.

SUPERVISOR'S REPORT

Conxita Avila, PhD, Director of the PhD thesis entitled "**Antarctic heterobranch molluscs: diving into their challenging ecology, taxonomy, and systematics**", certifies that the thesis presented here is the result of the work carried out by Juan Moles Sánchez under my guidance and supervision. The contribution of the PhD candidate to each one of the manuscripts included in the thesis is detailed below.

Chapter 1. Granuloside, a unique linear homosesterterpene from the Antarctic nudibranch *Charcotia granulosa*
Cutignano A*, **Moles J***, Avila C, Fontana A
Journal of Natural Products 78:1761–1764 (2015)
Impact Factor (2014): 3.798
*Equal contribution

JM: sample collection, identification, chemical extraction, chemical purification, manuscript writing.

Chapter 2. Distribution of granuloside in the Antarctic nudibranch *Charcotia granulosa* (Gastropoda: Heterobranchia: Charcotiidae)
Moles J, Wägele H, Cutignano A, Fontana A, Avila C
Marine Biology 163:54 (2016)
Impact Factor (2014): 2.391

JM: sample collection, identification, histological sections, TEM preparations, chemical analyses, results interpretation, manuscript writing.

Chapter 3. *Anthessius antarcticus* n. sp. (Copepoda: Poecilostomatoida: Anthessiidae) from Antarctic waters living in association with *Charcotia granulosa* (Mollusca: Nudibranchia: Charcotiidae)
Moles J, Avila C, Kim I-H
Journal of Crustacean Biology 35: 97–104 (2015)
Impact Factor (2014): 1.081

JM: sample collection, SEM preparations, interpretation of part of the results, manuscript writing.

Chapter 4. The sea slugs that laid giant egg masses: Embryonic development in two Antarctic anthobranchs (Mollusca: Gastropoda: Nudibranchia)
Moles J, Wägele H, Cutignano A, Fontana A, Ballesteros M, Avila C
(*In prep*)

JM: histological sections, chemical analyses, interpretation of part of the results, as well as part of the manuscript writing.

Chapter 5. An Antarctic opisthobranch clade is sister to all other Cephalaspidea (Gastropoda: Heterobranchia)
Moles J, Wägele H, Schrödl M, Avila C
Zoologica Scripta (*In press*)
Impact Factor (2014): 3.224

JM: sample identification, dissection, histological sections, DNA extraction, phylogenetic analyses, results interpretation, manuscript writing.

Chapter 6. The end of the cold loneliness: 3D reconstruction of *Doto antarctica* (Heterobranchia: Nudibranchia) and description of the sympatric *D. carinova* n. sp.
Moles J, Wägele H, Ballesteros M, Pujals Á, Uhl G, Avila C
PLoS ONE (*In press*)
Impact Factor (2014): 2.717

JM: sample identification, histological sections, microCT reconstruction, DNA extraction, phylogenetic analyses, results interpretation, manuscript writing.

Chapter 7. Bipolarity in sea slugs: On the description of *Doridunculus punkus* n. sp. (Nudibranchia, Onchidoridoidea) from Antarctica
Moles J, Wägele H, Uhl G, Avila C
Organisms Diversity & Evolution (*Submitted*)

JM: sample identification, microCT reconstruction, results interpretation, manuscript writing.

From all the co-authors of the different chapters, AP has not been awarded a PhD degree. I hereafter guarantee that none of the information contained in the chapter co-authored by him will be used to elaborate any other part of someone else's PhD thesis.

For all the above, I consider that the work developed by the PhD candidate grants him the right to defend his thesis in front of a scientific committee.

Barcelona, June 2nd, 2016.

Dr. **Conxita Avila**

Other papers from the author related to this Doctoral Thesis

1. Figuerola B, Núñez-Pons L, **Moles J**, Avila C (2013) Feeding repellence in Antarctic bryozoans. *Naturwissenschaften* 100:1069−1081

2. Moles J, Torrent A, Alcaraz MJ, Ruhí R, Avila C (2014) Anti-inflammatory activity in selected Antarctic benthic organisms. *Frontiers in Marine Science* 1:24

3. Moles J, Figuerola B, Campanyà-Llovet N, Monleón-Getino T, Taboada S, Avila C (2015) Distribution patterns in Antarctic and Subantarctic echinoderms. *Polar Biology* 38:799−813

4. Moles J, Núñez-Pons L, Taboada S, Figuerola B, Cristobo J, Avila C (2015) Anti-predatory chemical defences in Antarctic benthic fauna. *Marine Biology* 162:1813−1821

5. Nuzzo G, Cutignano A, **Moles J**, Avila C, Fontana A (2016) Exiguapyrone and exiguaone, new polypropionates from the Mediterranean cephalaspidean mollusc *Haminoea exigua*. *Tetrahedron Letters* 57:71−74

6. Avila C, Núñez-Pons L, **Moles J** (*in press*) From the tropics to the poles: Chemical defense strategies in sea slugs (Mollusca: Heterobranchia). In Puglisi-Weening M, Becerro M, Paul V (eds) *Chemical ecology: The ecological impacts of marine natural products.* Taylor & Francis, CRC Press

Section I

Ecological interactions in sea slugs

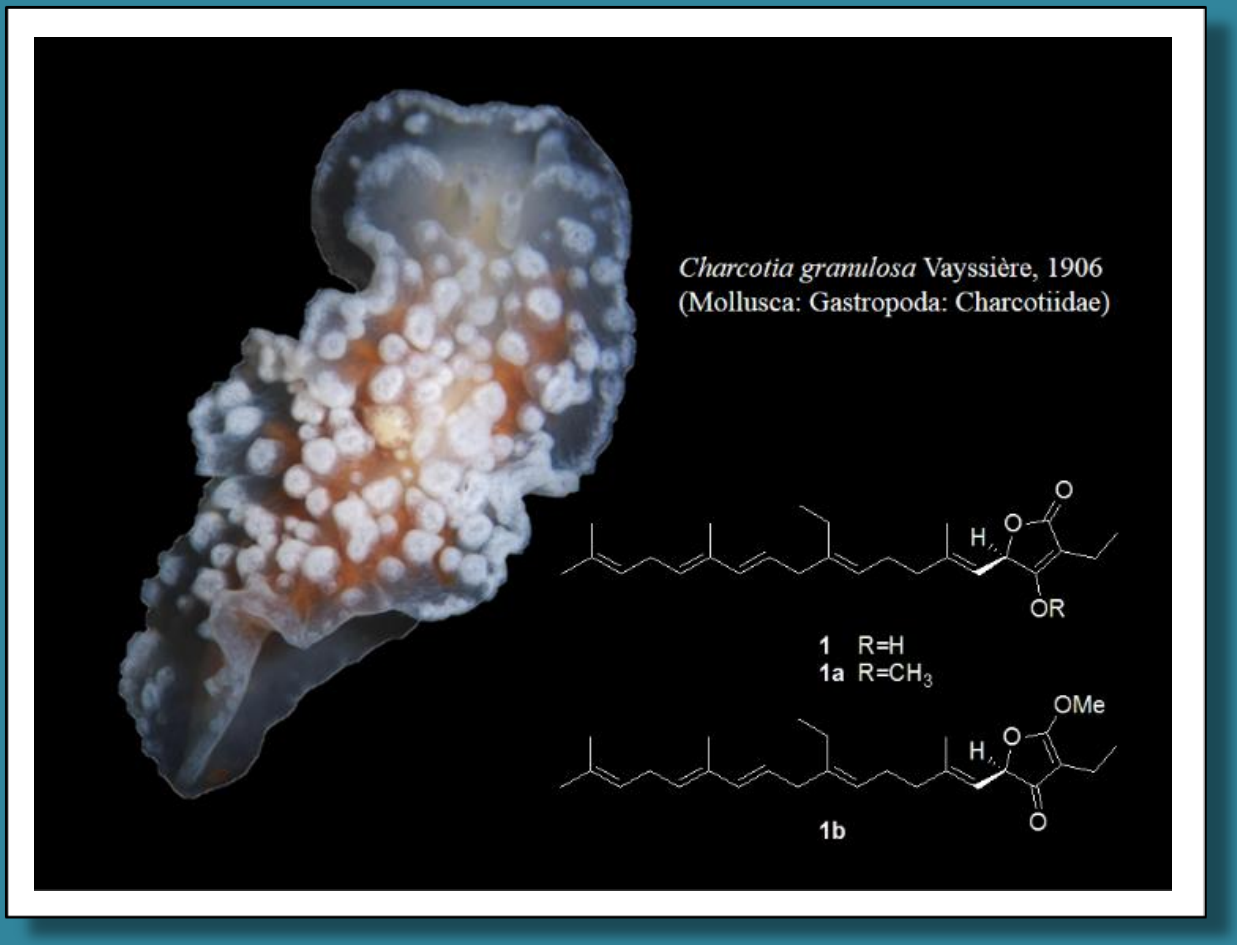
“Have you gone mad?”

“I’m afraid so, you are entirely bonkers. But I’ll tell you a secret. All the best people are.”

Lewis Carroll, Alice’s Adventures in Wonderland

Chapter 1

Granuloside, a unique linear homosesterterpene from the Antarctic nudibranch *Charcotia granulosa*



Cutignano A, **Moles J**, Avila C, Fontana A (2015) Granuloside, A Unique Linear Homosesterterpene from the Antarctic Nudibranch *Charcotia granulosa*. *Journal of Natural Products* 78:1761−1764

Chapter 1. **Granuloside, a unique linear homosesterterpene from the Antarctic nudibranch *Charcotia granulosa***

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**Equal contribution*

ABSTRACT

A new homosesterterpene with a unique linear skeleton, named granuloside (1), has been fully characterized from the Antarctic nudibranch *Charcotia granulosa* Vayssière, 1906 (Mollusca: Gastropoda). The planar structure of 1 was determined by extensive spectroscopic techniques on the methyl derivatives (1a and 1b), and the R absolute configuration at C-4 is suggested by comparison of experimental and calculated ECD spectra of 1b. Granuloside (1) is the first linear homosesterterpene skeleton ever reported and, despite the low molecular complexity, its chemical structure poses many questions about its biogenesis and origin in the nudibranch.

Key words: sea slug, natural product, terpene, Charcotiidae

Capítulo 1. **Granuloside, un homosesterterpene linear único aislado del nudibranquio antártico *Charcotia granulosa***

RESUMEN

Un nuevo homosesterterpeno con un esqueleto linear único, al que hemos llamado granuloside (1), del nudibranquio antártico *Charcotia granulosa* Vayssière, 1906 (Mollusca: Gastropoda), ha sido completamente caracterizado. La estructura planar de 1 fue determinada mediante técnicas espectroscópicas extensivas sobre los derivados metilados (1a y 1b), y se propone la configuración R absoluta en C-4 de 1b debido a la comparación de los espectros ECD experimentales y a los calculados. El granuloside (1) es el primer homosesterterpeno con esqueleto linear reportado hasta ahora y, pese a su baja complejidad molecular, su estructura química origina numerosas preguntas sobre su biogénesis y su origen en el nudibranquio.

Palabras clave: babosa marina, producto natural, terpenos, Charcotiidae

INTRODUCTION

Antarctic benthic ecosystems are characterized by low temperatures, pronounced seasonality, and low food supplies (Barnes & Clarke, 1995). The environmental stability has led to the conclusion that the Antarctic benthic community is structured to a great extent by biological factors such as predation and competition (Dayton *et al.*, 1974). Accordingly, effective defense mechanisms come to be crucial for the survival of the species. In particular, shell loss in nudibranch molluscs led to the development of protective strategies to deter predators, including chemical defense (Avila *et al.*, 2008). Despite the large number of chemical studies on molluscs from temperate and tropical areas, little is known about secondary metabolism in Antarctic nudibranchs (Avila *et al.*, 2008). As part of our worldwide exploration of chemistry and ecology of marine invertebrates, we studied the Antarctic nudibranch *Charcotia granulosa* Vayssière, 1906 (Mollusca: Gastropoda: Charcotiidae) which, despite its wide geographic distribution (Barnes & Bullough, 1996; Arnaud *et al.*, 2001; Barnes & Brockington, 2003; Shields, 2009) has not been analyzed to date.

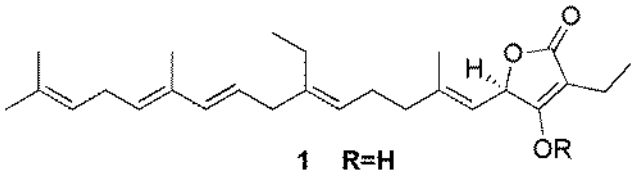
Frozen individuals (n = 61) of *C. granulosa* were extracted by gentle sonication of the outer tissues in acetone (3 × 15 mL). The animals were then successively ground with a mortar and pestle and sonicated in acetone to obtain the whole body extract. This material was concentrated under reduced pressure and the resulting aqueous residue partitioned against diethyl ether. Chromatographic analysis of the diethyl ether-soluble fractions showed the presence of an UV-absorbing component in the outer part of the animal only. Silica gel chromatography using 70% diethyl ether in petroleum ether gave product **1**, which was analyzed by mass spectrometry and NMR spectroscopy.

RESULTS AND DISCUSSION

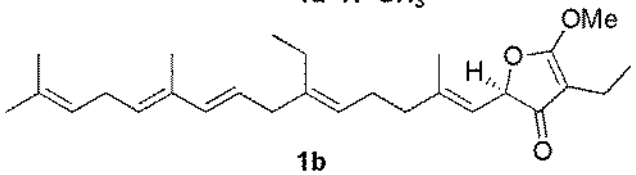
High resolution mass data HRESIMS gave a single $[M + Na]^+$ ion at m/z 421.2711 accounting for the molecular formula $C_{26}H_{38}O_3$. The ^1H NMR spectrum ($CDCl_3$, 600 MHz) revealed the typical terpene fingerprinting with four methyl singlets and aliphatic methine/methylene multiplets in the region 0.5−3.0 ppm but also a few deshielded signals that integrated for less than one proton indicating the presence of an equilibrium between two or more chemical species (Table 1). Any attempt to resolve this mixture by chromatographic techniques failed. However, methylation of this fraction with diazomethane gave two distinct products, which were successfully separated by HPLC as the isobaric methyl derivatives **1a** and **1b**.

Compound **1a** showed a sodium adduct ion $[M + Na]^+$ at *m/z* 435.2868 consistent with a molecular formula $C_{27}H_{40}O_3$. Accordingly, compared to the natural compound, the ^1H NMR spectrum ($CDCl_3$, 600 MHz) of **1a** showed an additional methoxy singlet resonating at δ 3.96 (OCH_3 58.8 ppm). The ^1H NMR spectrum

displayed also six methyl (*δ* 1.82, 1.75, 1.69, 1.64, 1.12, 0.95) and six methylene (*δ* 2.81, 2.75, 2.33, 2.15, 2.11, 2.02) resonances, together with six olefinic (*δ* 6.04, 5.50, 5.33, 5.11, 5.06, 5.00) and one oxymethine (*δ* 5.37) signals.



1 R=H
1a R=CH_3



1b

In addition to the above 19 protonated carbons, the ^{13}C NMR data indicated seven nonprotonated sp^2 carbons, including two oxygenated carbons at 172.8 and 173.4 ppm. Seven out of the eight formal degrees of unsaturation required by the molecular formula were fulfilled by six double bonds and one carbonyl group, thus suggesting the presence of a cyclic system. This latter substructure was identified as an *α,β*-unsaturated, trisubstituted *α*-ethyl-*β*-methoxy-*γ*-alkyl-*γ*-butenolide ring on the basis of the correlations of the methoxy group and the oxymethine proton (*δ* 5.37) with the oxygenated carbon signal at 172.8 ppm (C-3). Furthermore, HMBC cross peaks of signals at *δ* 2.33 (CH2-20) and *δ* 1.12 (CH3-21) with carbons at 104.1 (C-2), 173.4 (C-1), and 172.8 (C-3) ppm indicated the presence of an ethyl residue at C-2 of the ring moiety. The coupling of the olefinic proton at *δ* 5.00 with the oxymethine at *δ* 5.37 secured the linkage of the trisubstituted double bond C-5/C-6 to the butenolide ring at C-4 (73.7 ppm). The remaining part of the molecule was an acyclic terpenoidic structure containing three spin systems (H-7/H-9, H-11/H-13, and H-15/H-17) that were connected by heteronuclear two-dimensional experiments (Table 1). Thus, long-range correlations were observed between the allylic methyl group at *δ* 1.82 (C-22, 16.8 ppm) and the methylene protons at *δ* 2.11 (C-7, 39.8 ppm), whereas two bis-allylic methylenes at *δ* 2.75 (C-11, 40.0 ppm) and 2.81 (C-16, 27.1 ppm) connected the central diene system C-12/C-15 with the trisubstituted double bonds C-9/C-10 and C-17/C-18, the latter of which bore the two geminal methyl singlets at *δ* 1.69 (C-19, 25.6 ppm) and *δ* 1.64 (C-26 17.6 ppm) of the chain end. HMBC data located the remaining two vinyl methyl groups at *δ* 1.82 (C-22, 16.8 ppm) and *δ* 1.75 (C-25, 12.5 ppm) on the quaternary carbons C-6 (145.5 ppm) and C-14 (132.6 ppm), respectively. As depicted in **1a**, the resulting structure contained an ethyl branch (*δ* 2.02, 23.1 ppm of CH_2-23; *δ* 0.95, 12.4 ppm of CH_3-24) in place of the usual methyl group at C- 10 and accounted for a new homosesterterpene skeleton for the natural compound, here named granuloside (**1**) (Table 1). The double bonds exhibited all *trans* configurations as deduced by the H-12/H-13 coupling constant (*J* = 15.6 Hz), the chemical shifts of the vinyl methyl groups (below 20 ppm), and of the allylic methylene carbons (above 30 ppm; Stothers, 1972).

Table 1. ^{1}H and ^{13}C NMR Data for Granuloside (**I**) and Its Methyl Derivatives **Ia** and **Ib** (600 MHz).

	I		**Ia**				**Ib**	
	$CDCl_3$		$CDCl_3$		C_6D_6		C_6D_6	
position	δ_C, type	δ_H, *J* (Hz)	δ_C, type	δ_H, *J* (Hz)	δ_C, type	δ_H, *J* (Hz)	δ_C, type	δ_H, *J* (Hz)
1	172.4, C [172.9][a]		173.4, C		173.3, C		179.8, C	
2	104.0, C [47.0]	[2.95/2.97]	104.1, C		105.0, C		94.1, C	
3	171.0, C [207.9]		172.8, C		172.3, C		196.0, C	
4	73.9, CH [81.3/81.9]	5.36[b] [5.29, d (8.0)/5.42, d (8.3)]	73.7, CH	5.37, d (9.0)	73.6, CH	4.93, s[b]	83.4, CH	5.03, C (8.6)
5	117.2, CH [114.8]	5.02, d (9.0) [5.07/5.11]	118.1, CH	5.00, dd (9.0, 1.1)	119.8, CH	4.93, s[b]	117.5, CH	5.20, m
6	147.0, C		145.5, C		144.7, C		144.9, C	
7	39.5, CH_2	2.11, m	39.8, CH_2	2.11, m	40.3, CH_2	1.87, t (7.7)	40.0, CH_2	1.98, t (7.3)
8	25.1, CH_2	2.15, m	25.5, CH_2	2.15, m	26.4, CH_2	2.01, q (7.6)	25.7, CH_2	2.10, dt (7.3, 7.1)
9	123.2, CH	5.05, m	123.6, CH	5.06, t (6.8)	124.3, CH	5.10, t (7.0)	124.1, CH	5.18[b], t (7.1)
10	141.0, C		140.6, C		141.0, C		141.2, C	
11	39.7, CH_2	2.76, d, 6.1	40.0, CH_2	2.75, d (7.0)	40.9, CH_2	2.79, d (7.1)	40.7, CH_2	2.80, d (7.6)
12	125.3, CH	5.51, dt (15.5, 6.9)	125.3, CH	5.50, dt (15.6, 7.0)	125.7, CH	5.61, dt (15.6, 7.1)	125.6, CH	5.64, dt (15.4, 7.1)
13	135.7, CH	6.05, d (15.5)	135.7, CH	6.04, d (15.6)	136.9, CH	6.20, d (15.6)	136.3, CH	6.21, d (15.4)
14	129.6, C		132.6, C		133.5, C		133.5, C	
15	129.0, C	5.35[b], m	129.4, CH	5.33, t (7.1)	130.0, CH	5.49, t (7.2)	129.2, CH	5.48, t (7.5)
16	26.8, CH_2	2.81, t (7.0)	27.1, CH_2	2.81, t (7.1)	27.8, CH_2	2.83, t (7.2)	27.3, CH_2	2.81, t (7.5)
17	122.1, CH	5.11, brt (7.0)	122.4, CH	5.11, m	123.3, CH	5.20, brt (7.0)	123.2, CH	5.18[b], m
18	131.6, C		130.6, C		131.8, C		131.6, C	
19	25.3, CH_3	1.70, s	25.6, CH_3	1.69, s	26.0, CH_3	1.63, s	25.7, CH_3	1.52, s
20	14.5, CH_2 [19.5]	2.22, q (7.6) [1.97, q (7.4)]	15.9, CH_2	2.33, q (7.5)	16.8, CH_2	2.28, q (7.5)	13.4, CH_2	2.32, q (7.5)
21	11.4, CH_3 [10.6]	1.12, t (7.6) [1.02, t (7.4)/ 1.05, t (7.4)]	13.8, CH_3	1.12, t (7.5)	14.4, CH_3	1.10, t (7.5)	13.0, CH_3	1.20, t (7.5)
22	16.8, CH_3	1.83, s [1.82]	16.8, CH_3	1.82, d (1.1)	17.1, CH_3	1.44, s	16.9, CH_3	1.65, s
23	22.8, CH_2	2.00, m	23.1, CH_2	2.02, q (7.5)	24.0, CH_2	1.98, q (7.5)	23.4, CH_2	2.00, q (7.5)
24	12.7, CH_3	0.96, t (7.5) [0.95, t (7.5)]	12.4, CH_3	0.95, t (7.5)	13.6, CH_3	0.92, t (7.5)	12.9, CH_3	0.92, t (7.5)
25	11.8, CH_3	1.75, s	12.5, CH_3	1.75, s	12.9, CH_3	1.77, s	12.3, CH_3	1.77, s
26	17.3, CH_3	1.64, s	17.6, CH_3	1.64, s	18.0, CH_3	1.53, s	175, CH_3	1.52, s
OMe			58.8, CH_3	3.96, s	57.1, CH_3	3.13, s	54.2, CH_3	3.23, s

[a]Values in brackets [ ] refer to diketo form II. See Figure 1 and text for details. [b]Overlapping signals within the same column.

With the assignment of **1a**, the multiple series of signals in the natural product spectrum of **1** was attributed to a keto-enolic equilibrium of the tetronic acid derivative. In effect, a comparison of the NMR data (C_6D_6, 600 MHz) of 1a and 1b indicated that the main differences were ascribable to the part of the molecule surrounding the five-membered ring (Table 1). In particular, the *α,β*-unsaturated ester function of **1a** was replaced by an *α,β*-unsaturated keto group in **1b**, as indicated by a carbonyl group resonating at 196.0 ppm bearing an α-ethyl group C-20 (*δ* 2.32)/C-21 (*δ* 1.20) and a *β*-methoxy group.

Full NMR assignment confirmed the whole molecular skeleton of **1b** (Table 1). A closer inspection of two- dimensional spectra of the natural compound **1** indicated that the predominant species at equilibrium were the enol-lactone (I) and the 1,3-dicarbonyl forms (II) (Figure 1). This latter species apparently occurred as a mixture of epimers at C-2, as inferred by two sets of signals correlating the H-4 oxymethine protons at *δ* 5.29/5.42 (81.3/81.9 ppm) with a keto group at 207.9 ppm (C-3), which in turn was connected to a bis-allylic methine at *δ* 2.95/2.97 (C-3, 47.0 ppm) and a methylene signal centered at *δ* 1.97.

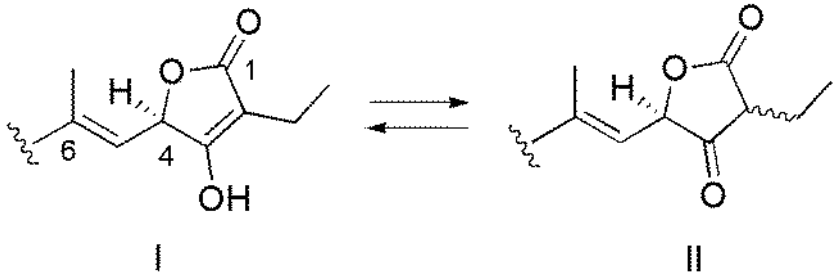


Figure 1. Mixture of tautomers observed for granuloside (**1**) in CDCl3 solution.

The absolute configuration of the stereogenic carbon C-4 was proposed by a chiroptical approach (Nugroho & Morita, 2014). Compound **1a** turned out to be a very labile molecule and degraded during the analytical work up. Hence, we concentrated our analysis on isomer **1b**. The circular dichroism spectrum in MeOH of this product showed two maximum values at 281 nm (negative band) and 258 nm (positive band) as depicted in Figure 2. For the ECD calculation, a conformational analysis was carried out by using the MMFF94 molecular mechanics force field method to obtain the most stable conformer for both enantiomers, which in turn was used for geometry optimization by density functional theory at the B3LYP/6-31G(d) level. A computed ECD spectrum in MeOH with B3LYP/aug-cc-pVDZ level was finally performed. The calculated ECD spectrum for the *R*-enantiomer was in good agreement with the experimental spectrum of **1b** (Figure 2), thus suggesting the *R*-configuration at C-4 of the natural granuloside (**1**).

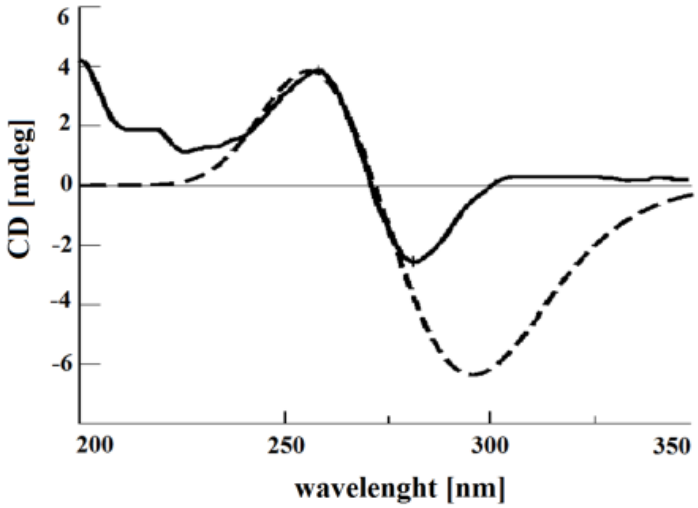


Figure 2. Experimental (solid line) and calculated (dashed line) ECD spectra of compound **1b**.

Sesterterpenes are a group of secondary metabolites not common in nature but typically found in a few genera of higher plants, fungi, insects, and marine invertebrates such as sponges and nudibranchs. Marine sesterterpenes can exhibit a linear or a cyclic carbon skeleton often combined with an *α,β*-unsaturated-*γ*-hydroxy-lactone ring and display important biological properties such as antibacterial, anti-inflammatory, and cytotoxic activities (Ebada *et al.*, 2010; Li *et al.*, 2013; Wang *et al.*, 2013). Marine homosesterterpenes are very rare, and the only examples described so far are restricted to the cyclic derivatives of the homoscalarane family from sponges of the family Thorectidae and from Doridoidea nudibranchs that are specialized to prey on sponges (Nakagawa *et al.*, 1987; Alvi & Crews, 1992; Bergquist *et al.*, 1999; Fontana *et al.*, 2000). The here reported granuloside (**1**) is the first example of a linear homosesterterpene ever described in nature and represents the first report of nonscalarane homosesterterpenes from marine opisthobranchs. Beyond the apparent absence of complexity, this product evokes intriguing biosynthetic questions, as to the origin of the additional methyl group and the very unusual closure of the butenolide ring after oxidation of the methyl branch of the terminal unit. The origin of homoterpenes in nature is largely unknown. In higher plants, homoterpenes are major volatile components and originate from higher homologues by an oxidative cleavage of the terminal isoprene unit (Tholl *et al.*, 2011). By contrast, ethyl-branched farnesoic acids that act as juvenile pheromones in insects are derived from a 3-hydroxy-3-ethylglutaryl-CoA intermediate arising from two units of acetate and one of propionate (Brindle *et al.*, 1988). Recently, a methyltransferase has been shown to carry out methylation of the terpene part of telocidin in *Streptomyces* (Awakawa *et al.*, 2014), and postcyclization methylation has been suggested in the biosynthesis of the sacculatane derivatives isolated from a marine sponge of the genus *Psammoclema* (Rudi *et al.*, 1995). According to these studies and in analogy with alkylation of sterol side chains in marine sponges (Djerassi & Silva, 1991; Giner, 1993), the biogenesis of granuloside (**1**) may proceed by methylation of the geranylfarnesyl skeleton. P450-mediated oxidation of a

putative α-farnesene intermediate likely generates the polyoxygenated scaffold, prompting lactone formation.

The structural novelty of **1** and the absence of previous chemical studies on the genus *Charcotia* indicated that further investigations to establish the function of this natural product are warranted. To this aim, feeding experiments and ecological tests are planned in the future to address the biosynthetic origin and the potential defensive role of **1**.

MATERIAL AND METHODS

General Experimental Procedures

Optical rotations were measured on a Jasco P2000 digital polarimeter. UV spectra were acquired on a Jasco V-650 spectrophotometer. ECD spectra were acquired on a Jasco J-815 polarimeter. IR spectra were measured on a Jasco FT-IR 4100 spectrometer. NMR spectra were recorded on a Bruker Avance DRX 600 equipped with a cryoprobe operating at 600 MHz for proton. Chemical shifts values are reported in ppm and referenced to internal signals of residual protons ($CDCl_3$, ^1H δ 7.26, ^{13}C 77.0 ppm; C_6D_6, ^1H δ 7.15, ^{13}C 128.0 ppm). High resolution mass spectra were acquired on a Q-Exactive hybrid quadrupole-orbitrap mass spectrometer (Thermo Scientific); HPLC analyses were performed on a Jasco system (PU-2089 Plus Quaternary Gradient Pump equipped with a Jasco MD-2018 Plus Photodiode Array Detector).

Biological Material

Samples of *Charcotia granulosa* were collected by scuba diving at depths ranging from 5 to 15 m depth near Deception and Livingston Islands (South Shetland Islands) during the ACTIQUIM-3 (December 2011−February 2012) and ACTIQUIM-4 (December 2012−February 2013) cruises. Samples were immediately frozen at −20 °C for chemical investigations.

Extraction of Biological Material

Sixty-one individuals of *C. granulosa* were soaked in acetone and extracted (3 × 15 mL) in an ultrasonic bath (~1 min) to obtain an outer (*i.e.*, mantle and foot) extract. The animals were successively ground in a mortar with a pestle, and the organic material was exhaustively extracted again with acetone (3 × 15 mL), affording an inner extract (digestive gland). Both extracts were concentrated under vacuum, and the resulting aqueous suspensions were partitioned with diethyl ether (3 × 20 mL). TLC comparative analyses of the lipid extracts were carried out in light petroleum/diethyl ether (8:2, 1:1, and 2:8) and $CHCl_3$/MeOH (9:1). Purification of the nudibranch extract obtained from external tissues was performed on a silica gel column using a petroleum

ether/diethyl ether gradient and afforded 2.5 mg of granuloside (**1**), which was absent in the inner organs.

*Granuloside (**1**).* Colorless oil; $[\alpha]_D$ −6.6 (*c* 0.04, MeOH); IR (film KBr) ν_{max} 1745 cm^{-1}; ^1H and ^{13}C data, Table 1; HRESIMS *m/z* 421.2711 $[M + Na]^+$ (calcd for $C_{26}H_{38}O_3Na$, *m/z* 421.2713).

*Methylation of Granuloside (**1**) and HPLC Purification of Methylated Products (**1a** and **1b**)*

Granuloside (**1**) (2 mg) was methylated (1 h, rt) with an excess of ethereal diazomethane (1 mL) freshly prepared from Diazald. The reaction product gave two UV−visible spots by TLC analysis in light petroleum/diethyl ether (1:1) with R_f 0.5 and 0.6. Compounds **1a** and **1b** (0.4 mg each) were separated by HPLC on a silica column (Kromasil Silica-Phenomenex, 5 µm, 100A, 250 mm × 4.6 mm) by isocratic elution with *n*-hexane/2-propanol 99:1 (flow 1 mL/min) monitoring UV absorption at 230 nm.

*Compound **1a**.* Colorless oil; $[\alpha]_D$ −3.24 (*c* 0.025, MeOH); UV (MeOH) λ_{max} (log ε) 238 (4.37) nm; ^1H and ^{13}C data, Table 1; HRESIMS *m/z* 435.2868 $[M + Na]^+$ (calcd for $C_{27}H_{40}O_3Na$, m/z 435.2870).

*Compound **1b**.* Colorless oil; $[\alpha]_D$ +2.11 (*c* 0.025, MeOH); UV (MeOH) λ_{max} (log ε) 240 (4.06), 265 (3.78) nm; IR (film KBr) ν_{max} 1644 cm^{-1}; ^1H and ^{13}C data, Table 1; HRESIMS *m/z* 435.2865 $[M + Na]^+$ (calcd for $C_{27}H_{40}O_3Na$, *m/z* 435.2870).

Computational Analysis

DFT geometric optimizations and TD DFT excitation energies calculations were performed with the Gaussian 09 (revision D.01) package by using the B3LYP functional and a generic basis set 6-31G(d) for geometry optimization and aug- cc-pVDZ for TD analyses.

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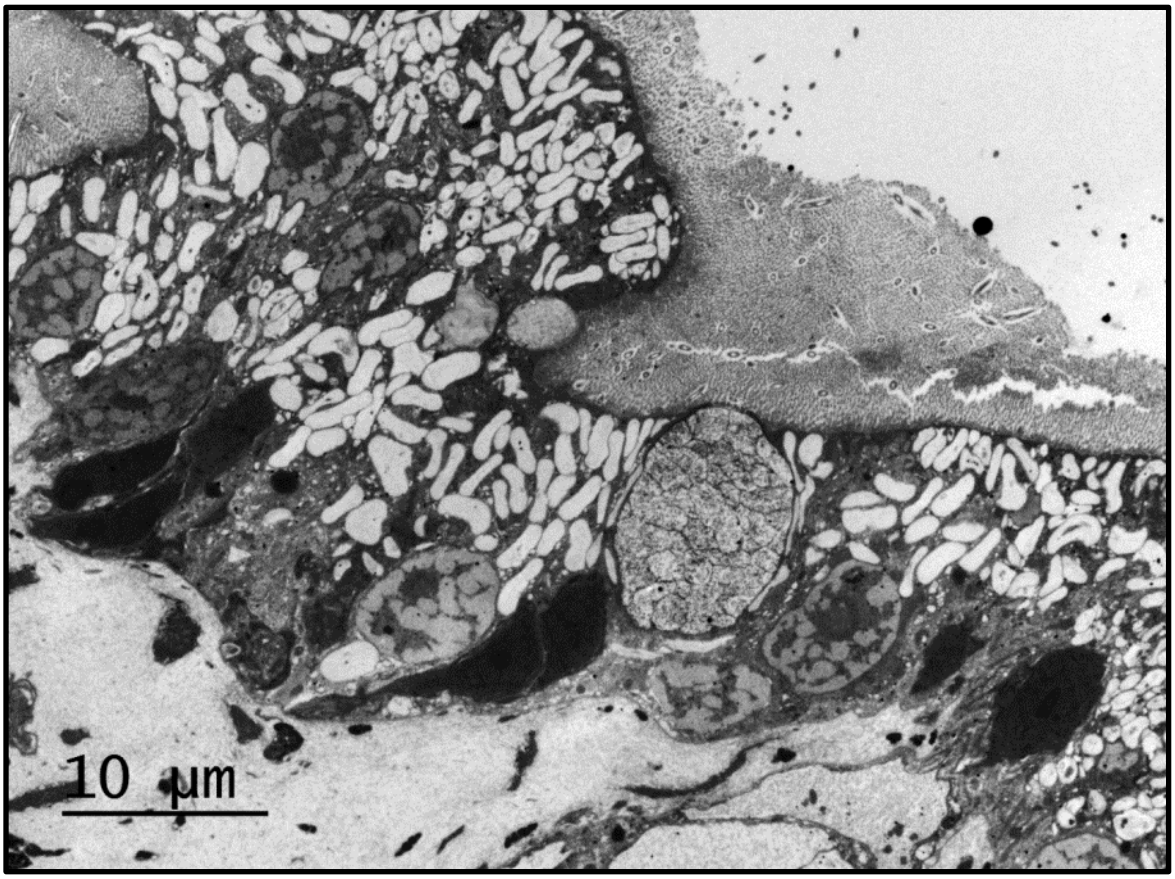
Chapter 2

Distribution of granuloside in the Antarctic nudibranch *Charcotia granulosa* (Gastropoda: Heterobranchia: Charcotiidae)



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Chapter 2. Distribution of granuloside in the Antarctic nudibranch *Charcotia granulosa* (Gastropoda: Heterobranchia: Charcotiidae)

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ABSTRACT

The loss of the shell in nudibranch gastropods has been related to the acquisition of chemical defensive strategies during evolution, such as the use of natural products to deter predation. In the present study we investigated the origin, location, and putative role of granuloside (**1**), a homosesterterpene lactone recently isolated from the Antarctic nudibranch *Charcotia granulosa* Vayssière, 1906. Several adults, egg masses, and its bryozoan prey, *Beania erecta* Waters, 1904, were chemically analyzed by chromatographic and spectroscopic techniques. Light- (LM) and transmission electron microscopy (TEM) of the mantle revealed complex glandular structures, which might be associated with the storage of defensive compounds in analogy to mantle dermal formations (MDFs) described in other nudibranchs. Although preliminary *in situ* repellence bioassays with live specimens of the nudibranch showed avoidance against the Antarctic generalist sea star predator *Odontaster validus*, the specific role of the terpene granuloside requires further investigation. The egg masses do not present granuloside and the glandular structures are absent in the trochophore larvae. Our results suggest that *C. granulosa* synthesizes granuloside *de novo* in early stages of its ontogeny, instead of obtaining it from the prey. Considering the wide geographic area inhabited by this slug, this may be advantageous, because natural products produced by the slug will not be affected by fluctuant food availability. Overall, the Antarctic sea slug *C. granulosa* seems to possess defensive strategies that are similar, in terms of production and storage, to nudibranchs from other regions of the world. This species is one of the few cladobranchs investigated so far that present *de novo* biosynthesis of a defensive compound.

Key words: feeding repellence; marine natural products; mantle dermal formations; Nudibranchia; sea slugs

Capítulo 2. Distribución del granuloside en el nudibranquio antártico *Charcotia granulosa* (Gastropoda: Heterobranchia: Charcotiidae)

RESUMEN

A lo largo de la evolución, la pérdida de la concha en los nudibranquios (gasterópodos) ha ido relacionada con la adquisición de estrategias químicas defensivas, tales como el uso de productos naturales para disuadir a los depredadores. En este estudio, investigamos el origen, localización y el posible papel del granuloside (**1**), un homosesterterpeno lactona, aislado recientemente del nudibranquio antártico *Charcotia granulosa* Vayssière, 1906. Varios ejemplares adultos, sus puestas, y su presa, el briozoo *Beania erecta* Waters, 1904, fueron analizados químicamente mediante técnicas cromatográficas y espectroscópicas. Los análisis de microscopía óptica y de transmisión (TEM) del manto revelaron la presencia de estructuras glandulares complejas, que podrían estar asociadas al almacenamiento de compuestos defensivos, en analogía a las formaciones dérmicas del manto (MDFs) descritas para otros nudibranquios. Pese a que los bioensayos preliminares de repelencia *in situ* con especímenes vivos demuestran una clara protección frente a la estrella de mar generalista antártica *Odontaster validus*, para establecer la función específica del terpeno granuloside se requieren más estudios detallados. Las puestas no presentan granuloside y las estructuras glandulares no se observan en la larva trocófora. Nuestros resultados sugieren que *C. granulosa* sintetiza granuloside *de novo* en los estadios tempranos de su ontogenia, en lugar de obtenerlo de su presa. Considerando la amplia distribución geográfica habitada por esta babosa, este hecho debe ser ventajoso para la especie, ya que los productos naturales no se verán afectados por una disponibilidad fluctuante de alimento. El nudibranquio antártico *C. granulosa* parece poseer estrategias defensivas que son similares, en términos de producción y almacenamiento, a nudibranquios similares de otras regiones del mundo. Sin embargo, ésta es una de las pocas especies de cladobranquio investigados hasta la fecha que sintetiza *de novo* un compuesto defensivo.

Palabras clave: repelencia alimentaria; productos naturales marinos; formaciones dérmicas del manto; Nudibranchia; babosas marinas

INTRODUCTION

Marine sea slugs are gastropod molluscs traditionally classified as opisthobranchs, although these are currently included in the monophyletic Heterobranchia (including pulmonates). Heterobranch sea slugs are excellent models to understand evolution driven by predation through the study of chemical defenses and the glandular structures involved (Wägele & Klussmann-Kolb, 2005; Wägele *et al*., 2006; Wilson *et al*., 2013). Nearly all heterobranch taxa contain shelled and naked representatives, besides nudibranchs. Recent phylogenies therefore suggest that shell loss has been acquired several times during the evolution of heterobranchs (Wägele *et al*., 2014; Zapata *et al*., 2014). The loss of the shell in sea slugs promoted a panoply of defensive strategies, including the use of chemicals (Avila, 1995; Cimino & Ghiselin, 2009; Putz *et al*., 2010). Bioactive metabolites can be either sequestered from the diet (cleptochemicals) or synthesized *de novo* (*e.g*., Avila, 1995; Gavagnin *et al*., 2001; Cimino *et al*., 2004; Putz *et al*., 2011). It has been widely shown that metabolites present in the notum (=mantle) of nudibranchs, but not in the digestive tract, are usually involved in chemical defense (Cimino & Ghiselin, 2009). Defensive natural products are frequently localized in special glandular structures on the external and most vulnerable parts of the slug (*e.g*., notum, rhinophores, gills, cerata), displaying anti-predatory activities (Avila & Paul, 1997; Wägele *et al*., 2006; Carbone *et al*., 2013). These structures can be epidermal and subepithelial glands, or complex glandular structures (see Wägele *et al*., 2006). Epithelial cells are the ultimate responsible of the mucus cover secreted by the slugs. Complex glandular cells, such as mantle dermal formations (MDFs) or similar structures, produce and/or accumulate chemical defenses. These can be found in nudibranchs, cephalaspideans, and sacoglossans. Cladobranch nudibranchs (*i.e*., with ramified digestive gland) possess terminal sacs for the excretion of digestive products. A special modification of these into cnidosacs is found in some aeolids, where nematocysts from corals are stored and extruded for defense. The strategic allocation compensates the energetic requirements invested for growth, reproduction, and defense, following the postulates of the optimal defense theory (ODT; Rhoades & Gates, 1976; *et al*., 2000; Iken *et al*., 2002).

Antarctic benthic invertebrates are generally preyed upon by sea stars, which are the dominant predators in shallow waters (Dayton *et al*., 1974). In order to test for chemical defenses, thus, *in situ* chemical ecology experiments have been commonly performed using the generalist-feeder and ubiquitous sea star *Odontaster validus* (*e.g*., McClintock *et al*., 1994; Avila *et al*., 2000; Iken *et al*., 2002). However, only four species of Antarctic sea slugs have been chemically analyzed to date, all of them containing defensive natural products in the mantle, used against sympatric predators (McClintock & Baker, 1997a; Avila *et al*., 2000, 2008; Iken *et al*., 2002; Davies-Coleman, 2006). Pteroenone, a polypropionate-derived natural product from the pelagic pteropod *Clione antarctica* displayed feeding repellence against fish predators (McClintock & Janssen, 1990; Yoshida *et al*., 1995). *De novo* biosynthesis of bioactive terpene metabolites has been hypothesized for two anthobranch nudibranchs: *Bathydoris*

hodgsoni and *Doris kerguelenensis*. Hodgsonal, a sesquiterpene isolated exclusively from the notum and papillae of *B. hodgsoni* (Iken *et al*., 1998), showed repellence against *O. validus* (Avila *et al*., 2000). *Doris kerguelenensis* was proven to possess a variety of diterpene diacylglycerols in the notum (Gavagnin *et al*., 1995, 1999a,b; 2003a,b; Diyabalanage *et al*., 2010), some of them displaying anti-predatory activity against *O. validus* (Iken *et al*., 2002). These metabolites are synthesized through diverse metabolic routes with a remarkable variability among individuals (Cutignano *et al*., 2011). This, in combination with molecular phylogenetic analyses led Wilson *et al*. (2013) to suggest cryptic speciation driven by predation in this species complex. Finally, the dendronotid *Tritoniella belli* is the only Antarctic nudibranch investigated so far that obtains its defensive natural product from its food, the stoloniferan soft coral *Clavularia frankliniana*. This is a chimyl alcohol which also displays repellent activity against *O. validus* (McClintock *et al*., 1994).

Recently, we described a novel homosesterterpene lactone, granuloside (**1**), from the notum of the Antarctic nudibranch *Charcotia granulosa* Vayssière, 1906 (Cutignano *et al*., 2015). This species is currently assigned to Cladobranchia by having a ramified digestive gland (Wägele *et al*., 1995a; Wägele & Willan, 2000; Pola & Gosliner, 2010). Cladobranchia are not well investigated yet regarding their chemical ecology. Only a few species from the genera *Melibe* and *Doto* are known to synthesize natural products themselves (see review by Putz *et al*., 2010, 2011). The family Charcotiidae possesses four Antarctic endemic species – mostly circum-Antarctic – of the genera *Charcotia*, *Pseudotritonia*, and *Telarma*, and one species, endemic from South Africa, of the genus *Leminda* (Wägele, 1991a). Within this family, only the African monotypic *Leminda millecra* Griffiths, 1985 was chemically analyzed (Pika & Faulkner, 1994) and four bioactive sesquiterpenes were described. These compounds are chemically related to metabolites of the octocoral upon which the nudibranch feeds. However, the presence of different octocoral spicules in the digestive tract of *L. millecra* suggested that its diet includes a variety of prey species. This added to the evidence that the nudibranch sequesters its defensive metabolites from different octocoral species (McPhail *et al*., 2001). In contrast, *Pseudotritonia* and *Charcotia* appear to be specialist bryozoan feeders (Barnes & Bullough, 1996). Actually, *C. granulosa*'s diet is species specific to one locally abundant bryozoan, *Beania erecta* Waters, 1904 (Barnes & Bullough, 1996). *Charcotia granulosa* was first described from a single specimen of Wandel Island in the western Antarctic Peninsula (Vayssière, 1906). More recently Wägele *et al*. (1995a) redescribed this species from Signy Island (South Orkney Islands, Scotia Sea) including its internal anatomy.

In the present study we investigated the chemical ecology of *C. granulosa*. We aimed to: 1) localize granuloside (**1**) in the animal tissues; 2) to chemically analyze the egg masses of *C. granulosa* and its prey *B. erecta,* to shed light into the possible origin of granuloside; 3) to describe histologically and ultrastructurally the notum and egg masses of the nudibranch; and 4) to test the feeding repellence of *C. granulosa* through *in situ* bioassays with the sea star *O. validus*.

MATERIAL AND METHODS

Collection methods

Samples were collected by scuba diving at depths between 5 to 15 m from Deception (62°59.33'S, 60°33.45'W) and Livingston (62°42.7'W, 60°23'W) Islands (South Shetland Islands), during ACTIQUIM-3 (December 2011–February 2012) and ACTIQUIM-4 cruises (December 2012–February 2013) by J. Moles and C. Avila. Additionally, one specimen from Cape Legoupil (63°19.53'S, 57°56.95'W) in the Antarctic Peninsula was collected by J. Moles in the latter campaign. *Charcotia granulosa* specimens were usually found near the bryozoan *B. erecta*, which covered rocks and other substrates from where it was collected. Egg masses of the nudibranch were observed in February and only a few of them were collected. Samples for chemical investigations were frozen at –20 °C until further analysis. One adult and one egg mass (Fig. 1) were preserved for both histological and cytological analyses (see below).

Histological and ultrastructural analyses

Samples for light microscopy (LM) were preserved in 4% formaldehyde/ sea water, subsequently dehydrated in ethanol and embedded in HEMA (Kulzer; see Wägele, 1997). Serial sections (2.5 µm) were stained with Toluidine blue, which specifically stains acidic mucopolysaccharides red to violet, and neutral mucopolysaccharides and nucleic acids, as well as proteins, in various shades of blue. Additionally, histological slides obtained in the same way as described above of *C. granulosa*, *Pseudotritonia gracilidens* Odhner, 1944, and *Telarma antarctica* Odhner, 1934 available from one of the authors (HW) were analyzed for comparison and are discussed herein.

Transmission electron microscopy (TEM) was used to describe the ultrastructure of epithelial glands. Fixation of an adult and an egg mass was performed in 2.5% glutaraldehyde in 0.2 M Milloning's phosphate buffer (MPB) and 1.4 M sodium chloride for 1 h. Samples were then rinsed with MPB for 40 min, post-fixed in 2% osmium tetroxide in MPB, dehydrated in a graded acetone series and embedded in Spurr's resin. Ultrathin sections obtained with an Ultracut Reichert-Jung ultramicrotome were mounted on gold grids and stained with 2% uranyl acetate for 30 min, followed by lead citrate for 10 min (Reynolds, 1963). Observations were conducted with a JEOL 1010 transmission electron microscope operating at 80 kV and fitted with a Gatan module for acquisition of digital images at the CCiT (UB).

Chemical analysis

As previously reported, 61 frozen individuals of *C. granulosa* were extracted with acetone (3×10 mL) by gentle ultrasound effect (Cutignano *et al*., 2015). The extracted specimens were later grounded with a mortar and pestle and extracted again by the same procedure. Considering that anatomical dissection of frozen animals is not suitable for this species, the extraction procedure allowed a rough approach to the

compounds present in the external and the internal tissues. Two egg masses of the nudibranch from Deception and Livingston Islands were also extracted with acetone. Additionally, methanol extraction of several colonies of the nudibranch's prey, the bryozoan *B. erecta*, was performed. Tiny colonies of this bryozoan were found covering different substrates; they were combined and analyzed together. Organic fractions were evaporated *in vacuo*, and the resulting aqueous suspension was partitioned with diethyl ether (3×50 mL) and *n*-butanol (2×50 mL). The raw ether extracts were evaluated by SiO_2-TLC (thin layer chromatography) with petroleum ether/diethyl ether (1:1) and then revealed with sulfate reagent. The organic extracts of egg masses, *C. granulosa* and *B. erecta* were purified on a silica column using an increasing gradient of petroleum ether/diethyl ether and chloroform/methanol and compared for chemical content. Fractions were analyzed by TLC and ^{1}H-NMR spectroscopy at Servizio NMR at Istituto di Chimica Biomolecolare (ICB).

Feeding repellence assays

Twenty specimens of *Odontaster validus*, ranging 6–10.5 cm in total diameter and collected in proximity of the target nudibranchs, were randomly used in the feeding repellence tests during the ACTIQUIM-4 cruise. Sea stars were distributed in large tanks with current sea water pumped directly from Foster's bay into the laboratory at the Spanish Antarctic Base "Gabriel de Castilla" (Deception Island). After five days of starvation, ten sea stars were placed individually in small tanks (250 mL) and one living individual of *C. granulosa* was placed under each sea star's mouth. A parallel set of sea stars, with shrimp cubes offered instead, was performed as control (see Avila *et al*., 2000). Consumption was evaluated after 24 h of the experiment. Statistics were calculated by contrasting the difference in ingestion rates between the living nudibranchs referred to the simultaneous control by applying the Fisher's exact test (Sokal and Rohlf, 1995).

RESULTS

Glandular structures

All investigated live animals exhibited a rather transparent epidermis, with the ramified brownish digestive gland shining through (Fig. 1A). The notum epithelium is formed by a unicellular layer of multivacuolated cells (specialized vacuolated epithelium), interspersed with two types of mucus glandular cells (Fig. 2A; 3A,B). These multivacuolated cells were prismatic in shape, had a basal nucleus, and presented microvilli all over the apical part. Cilia were seldom observed between the microvilli and might actually belong to another cell type. The mid to apical part contained abundant elongate to "sausage-like" shaped vacuoles with an electron-lucent substance. Sometimes a less electron-lucent central material (spindle) was present. Mucus gland

cells with dark violet stained contents (acid mucopolysaccharides) had granules in different degrees of condensation (Fig. 2A,F), usually being highly electron-dense (Fig. 3C,D). Secretion granules were sometimes homogeneously fused when exocytosis occurred (Fig. 2A). These mucus cells were occasionally extending subepidermally, but were strictly related to the epidermis. Additionally, cup-like macrovacuolated cells presenting a basal nucleus surrounding a huge vacuole were also present. This vacuole stained light blue (neutral mucopolysaccharides) and had a fibrillar appearance under TEM (Fig. 3B).

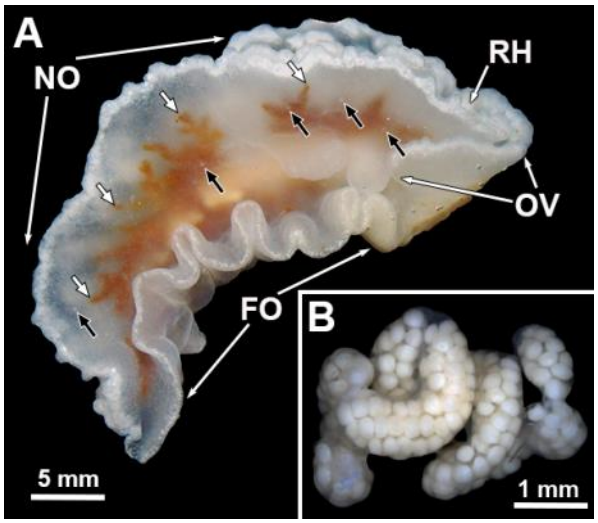


Figure 1. *In vivo* photographs of *Charcotia granulosa* collected at Whalers Bay (62°59.33'S, 60°33.45'W; Deception Island). **A** – Right lateral view of an adult; white arrows show the end of the digestive gland ramifications, black arrows show MDFs-like structures by transparency; **B** – Egg mass. *FO* foot; *NO* notum; *OV* oral veil; *RH* rhinophores

Special glandular structures with unusual characteristics were observed within the epidermis, but also extending far into the notum tissues. These glands resemble the MDFs described from doridoidean species and are therefore called "MDF-like" structures herein (*sensu* Wägele et al. 2006). A total of 65 and 71 MDF-like structures were found, mainly in the notum, in the two specimens investigated here. They were abundant in the dorsal papillae and notal edge. Additionally, they were also present in the oral veil and at the base of the rhinophores (see black arrows in Fig. 1A). MDF-like structures analyzed measured 100.2 ± 14.33 µm (mean ± sd) and were spherical in shape (Fig. 2B, D). They were composed of surrounding epithelial tissue with cells containing a highly active nucleus (Fig. 2C–D). The surrounding epithelium presented cells full of cisternae of rough endoplasmic reticulum (RER) and vacuoles in formation (Fig. 3E,F). These cells contained a substance(s) that stained homogenously light blue (LM) or exhibited a granulose appearance under TEM. The substance(s) was stored in large vacuoles with variable electron-dense properties (Fig. 3F). Vacuoles were observed to fuse occasionally, forming larger droplets. Lipid droplets were also present within the vacuoles (Fig. 3F). Some of the MDF-like structures were observed to open to the exterior, through a channel composed of epidermal cells, often with high density of mucus glandular cells (Fig. 2B,E). The content was still surrounded by a membrane when transported outside (Fig. 2B,E). The exudation channel was not observed in all MDF-like structures found.

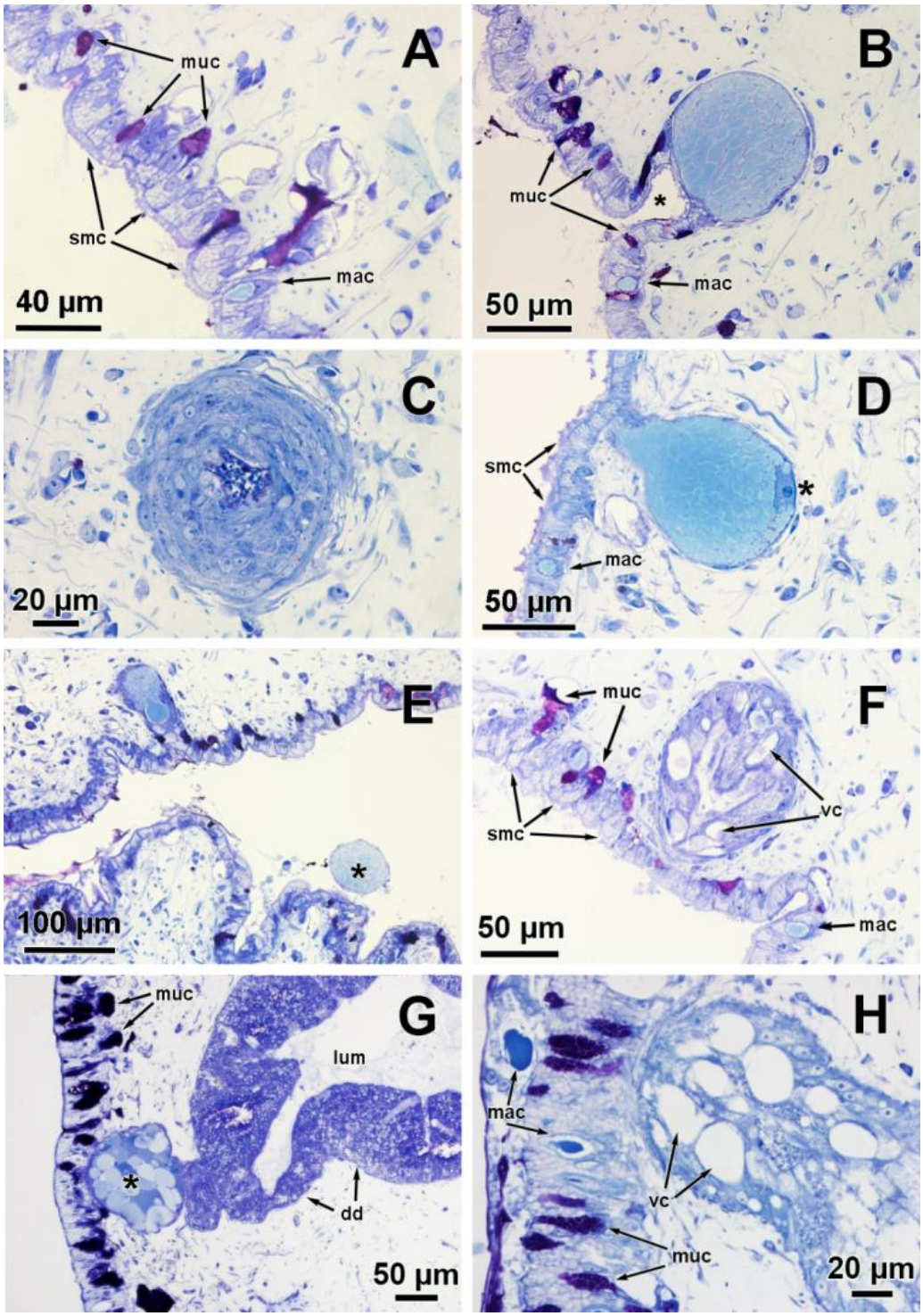


Figure 2. Histological sections of *Charcotia granulosa* at light microscopy (A–F), *Pseudotritonia gracilidens* (G), and *Telarma antarctica* (H). **A** – Notal epithelium. **B** – MDF-like structure connected to the outside through a channel (asterisk). **C** – Possible formation of a MDF-like structure, where different secretory cells surround an internal vacuolated matrix. **D** – MDF-like structure protruding its content to the outside, showing one of its surrounding cells with a large nucleus (asterisk). **E** – Two MDF-like structures, one being released (asterisk). **F** – Terminal sac of *C. granulosa* near the epidermis showing vacuolated cells. **G** – Terminal sac of *P. gracilidens* presenting bluish-staining vacuoles (asterisk). **H** – Detail of epithelium and terminal sac of *T. antarctica*. *dd* digestive diverticulum; *lum* lumen; *mac* macrovacuolated cell; *muc* glandular mucus cell; *smc* specialized multivacuolated cell; *vc* vacuolated cells

Similarly to other Cladobranchia, the digestive gland in the family Charcotiidae is ramified (see white arrows in Fig. 1A) with terminal sacs in the tip of some of its diverticula. Terminal sacs lie subepithelially and consist of greatly enlarged cells containing very large non-staining vacuoles in *C. granulosa* (Fig. 2F) and *T. antarctica* (Fig. 2H), while *P. gracilidens* presents big cells with big bluish vacuoles (Fig. 2G). Further analysis of some histological preparations of *P. gracilidens* and *T. antarctica* revealed similar epithelial cells to those described above for *C. granulosa*, but no MDF-like structures were found there.

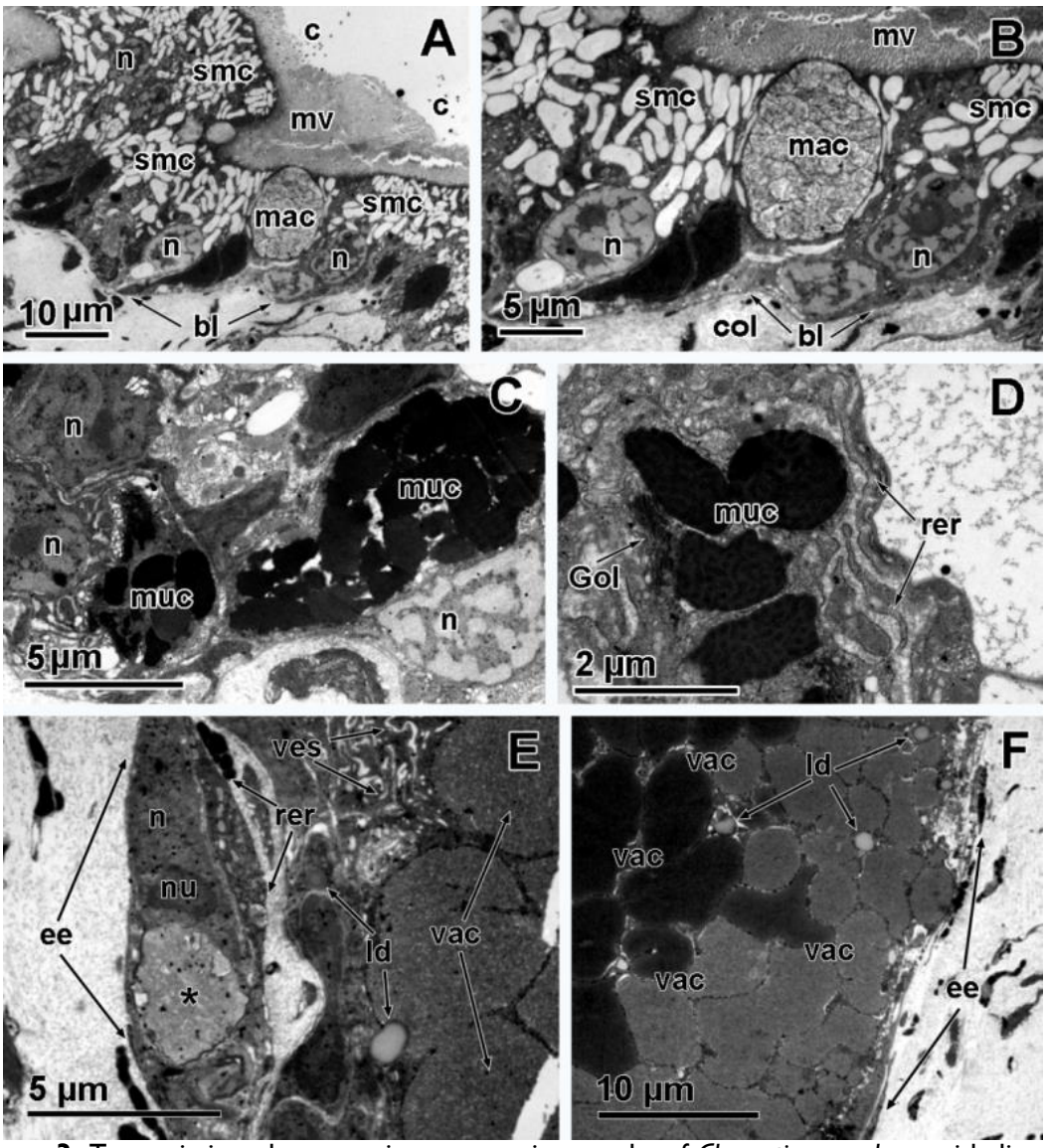


Figure 3. Transmission electron microscopy micrographs of *Charcotia granulosa* epithelium. **A** – General view of the epithelium. **B** – Close view of multivacuolated, mucus glandular, and macrovacuolated cells. **C** – Detail of glandular mucus cells. **D** – Internal mucus granules being produced. **E** – External epithelium of the MDF-like structure, showing a vacuole in formation (asterisk). **F** – Detail of vacuoles from a MDF-like structure, more or less electron-dense. *bl* basal lamina; *c* cilia; *col* collagen; *ee* external epithelium; *Gol* Golgi apparatus; *ld* lipid droplets; *mac* macrovacuolated cell; *mv* microvilli; *muc* glandular mucus cell; *n* nucleus; *rer* rough endoplasmic reticulum; *smc* specialized multivacuolated cell; *vac* vacuoles; *ves* vesicles

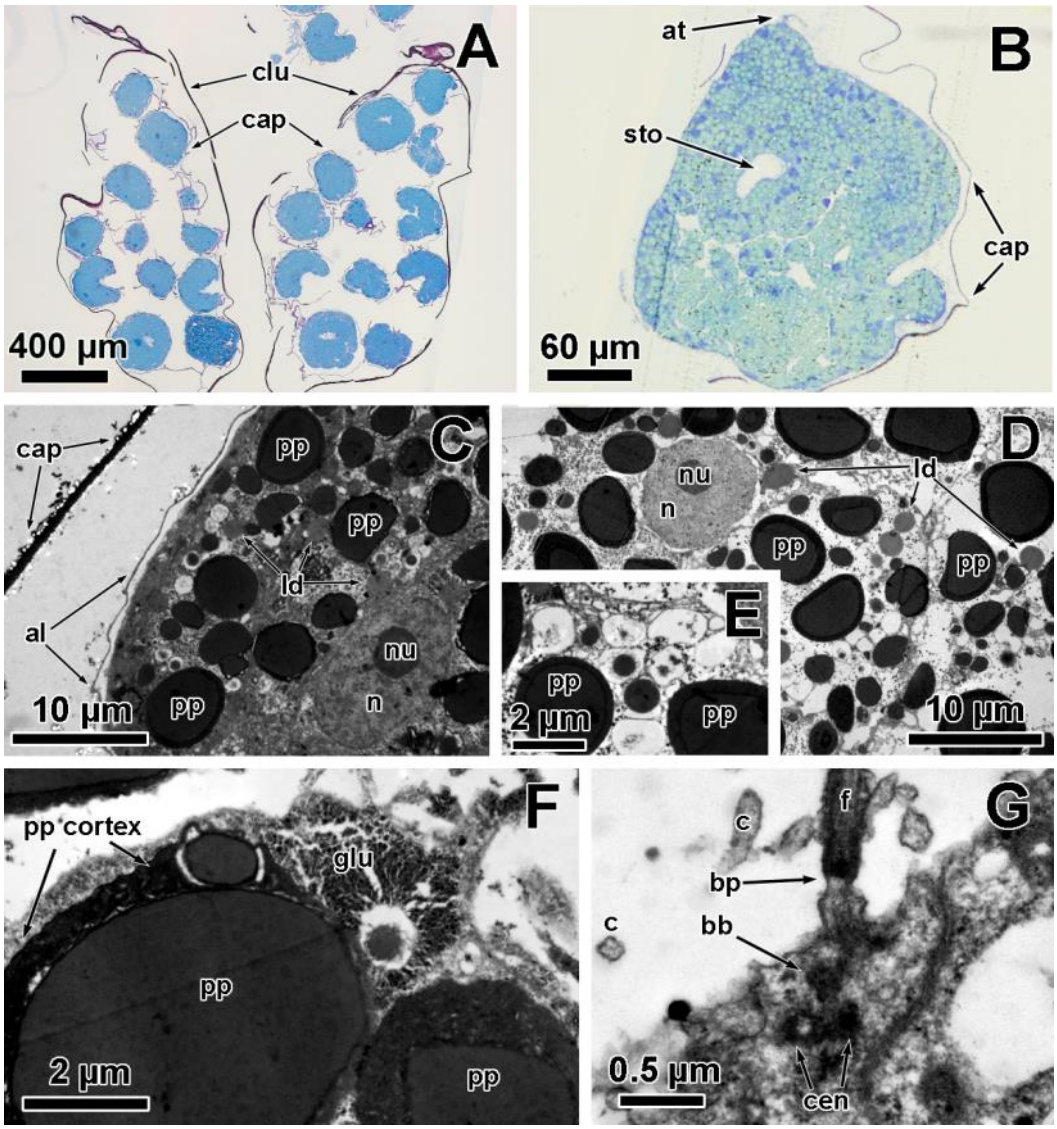


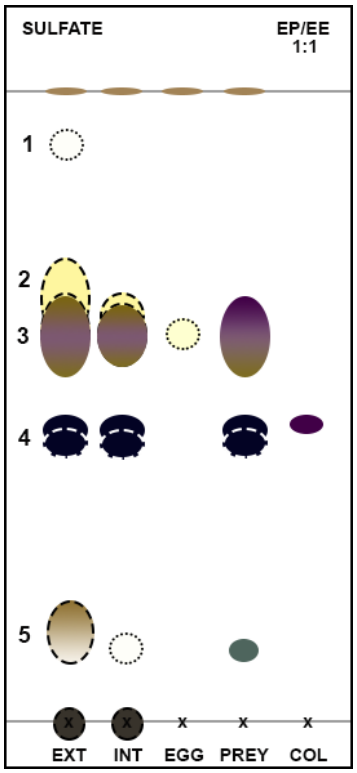
Figure 4. Light- and transmission electron microcopy micrographs of *Charcotia granulosa*'s egg masses. **A** – Cross section of egg string showing embryos' capsule and outer clutch. **B** – Trochophore larva. **C** – Detail of albumen and capsule layers of trochophore. **D** – Blastomere with proteid yolk platelets and lipid droplets. **E** – Close up of proteinaceous platelets in different degrees of digestion. **F** – Detail of glucogen clusters inside blastomere. **G** – Flagellum insertion in the apical tuft of the trochophore. *al* albumen layer; *at* apical tuft; *bb* basal body; *bp* basal plate; *c* cilia; *cap* capsule; *cen* centriole; *clu* a coil of the clutch; *f* flagellum; *glu* glucogen; *ld* lipid droplets; *nu* nucleolus; *n* nucleus; *pp* proteid platelet; *sto* stomodeum

Egg masses of *C. granulosa* were laid during February 2012 and 2013, attached to rocks near the prey, the bryozoan *Beania erecta*. They were cylindrical, capsule-filled strings, attached repeatedly along the outer mucous cover, thus conferring an irregularly-arranged coiled appearance (Fig. 1B). Eggs measured 304.77 ± 17.18 µm in diameter and were surrounded by a thin membranous layer, probably albumen (Fig. 4A–C). The eggs and the albumen layer were surrounded by a compact mucoid layer, thus forming a capsule. The capsules were surrounded additionally by an outer, thin and translucent mucus layer. Both egg masses prepared for LM and TEM were in an early stage of development, *i.e.*, trochophore larva (Fig. 4B). Several blastomeres with

big nuclei were found containing abundant proteid yolk platelets, with some probably being digested (Fig. 4D,E). Mature platelets had a distinct layered cortex from a less electron-dense homogeneous central core, and some of them were aggregated (Fig. 4F). Several lipid droplets, smaller and sparser than the proteid platelets were observed. Glucogen at different degrees of aggregation was observed widespread in the cytoplasm (Fig. 4F). Some metaphase nuclei were seen in LM. Blastomeres from the apical tuft of the larva had several flagella. Each flagellum had a centriole containing two kinetochores, a basal body, and a distinct basal plate anchored to the blastomere (Fig. 4G).

Origin and role of granuloside

Ether extracts of the "outer" and "inner" tissues of *C. granulosa*'s adult specimens, egg masses, and the prey *B. erecta* evaluated by TLC showed differences in their chemical pattern (see Fig. 5 for a schematic representation). The extract of the external part of *C. granulosa* contained granuloside (**1**), as previously reported (Cutignano *et al*., 2015). The terpene **1** was absent in the "inner" organs of the animal. Using the same procedures (chromatographic purification of the ether extracts and NMR characterization of the obtained fractions), neither eggs, nor the bryozoan prey revealed the presence of granuloside or any precursor of the terpene skeleton.



With regards to the feeding assays, there was no consumption of live individuals of the nudibranch *C. granulosa* by the sea star *O. validus*, whereas all shrimp food cubes were eaten in the parallel control (Fisher's exact test: p-value=0.000).

Figure 5. Schematic diagram of the SiO_2-TLC comparing ether extracts of *Charcotia granulosa*: external part (*EXT*), internal part (*INT*), egg mass (*EGG*), and its prey, the bryozoan *Beania erecta*, using colesterol (*COL*) as a reference. Dashed lines indicate UV-visible products. Petroleum ether/diethyl ether (1:1) was used as eluent and sulfate reagent to reveal organic spots. Main spots are as follows: 1 – UV-visible uncharacterized terpene. 2 – Fatty acids. 3 – Sterols. 4 – $\Delta^{5,7}$ sterols (UV-visible). 5 – Granuloside (brown, UV-visible)

DISCUSSION

Charcotia granulosa has been recorded in Adelaide, Livingston, Signy, and Wandel Islands, as well as in the Ross Sea (Vayssière, 1906; Wägele *et al*., 1995a; Arnaud *et al*., 2001; Barnes & Brockington, 2003; Shields, 2009). Our specimens were collected at Deception and Livingston Islands, and at the northern part of the Antarctic Peninsula.

Thus, now its geographic distribution covers the South Orkney Islands, South Shetland Islands, and Western Antarctic Peninsula until the Ross Sea. This may indicate a circum-Antarctic distribution, related to that of its prey, the bryozoan *Beania erecta* (OBIS, 2014). The present study shows that *C. granulosa* is protected against the sea star *Odontaster validus*, a predator commonly found sympatrically in shallow-water Antarctic benthic communities (Dayton *et al*., 1974; Moles *et al*., 2015a).

Vacuolated cells were found throughout the epithelium of the notum and foot of *C. granulosa,* resembling the so-called "specialized vacuolated epithelium" found generally in nudibranchs (see Wägele, 1998; Wägele & Willan, 2000). Vacuolated cells were suggested to play a role in the uptake of soluble substances, especially in the digestive system, which is only found in cladobranchs (Schmekel, 1982). Recent investigations showed that these cells possess an internal spindle of chitinous nature, which may act reducing damage from cnidarian nematocyst attacks (Martin *et al*., 2007a). Thus, the possession of the special vacuolated epithelium in the digestive tract presumably is related to the sequestration of nematocysts in members of the Cladobranchia. As seen for other nudibranchs that do not feed on cnidarians (Wägele, 1998; Martin *et al*., 2007b), this specialized epithelium is only developed in the most external and vulnerable parts exposed to nematocyst aggressions, but not in the digestive tract. This could be the case of *C. granulosa* since it feeds on bryozoans (Barnes & Bullough, 1996; authors pers. obs.). Two typical nudibranch cell types (macrovacuolated and mucus glandular cells) in the epidermis probably secret acid and neutral mucus, and are thus responsible for the slime secreted by *C. granulosa*. A structural protection in the form of masses of intracellular grains in vacuolated epithelial cells, together with mucous secretions, may be a first physical protection in *C. granulosa* against parasites, microbes, and even cnidarian attacks (Avila & Durfort, 1996; Wägele *et al*., 2006; Martin *et al*., 2007b). However, a specialist ectosymbiont copepod has recently been discovered living on the notum of *C. granulosa* (Moles *et al*., 2015b).

The term "MDF-like" structures, *sensu* Wägele *et al*. (2006), is used here for the glandular structures described in *C. granulosa*, because they had an opening to the outside, lacked the muscular clot and the surrounding muscular layer typical of the MDF, and because of their diameter (approximately 100 µm). MDF and MDF-like structures have been proven to store natural products for defensive purposes (*e.g*., García-Gómez *et al*., 1990; Avila *et al*., 1991; Avila, 1995; Avila & Paul, 1997). They are widely distributed in Chromodorididae, storing defensive natural products accumulated from their sponge diet (*e.g*., Avila *et al*., 1991; Fontana *et al*., 1994). However, Wägele *et al*. (2006) found these structures also in other nudibranchs, such as Doridinae and Triophinae, and even in some cephalaspideans and sacoglossans. This adds more evidence to the current hypothesis of Wägele *et al*. (2006), suggesting that complex glandular structures (*i.e*., MDF and MDF-like) may have constraints concerning structure – and therefore function – since they are found widespread in completely unrelated heterobranch taxa. The number and location of MDF-like

structures in the most vulnerable parts of *C. granulosa* (*i.e.*, rhinophores, notal edges) suggests a defensive role against predators (following the postulates of the ODT; Rhoades & Gates, 1976).

Contrastingly, the charcotiids *Pseudotritonia gracilidens* and *Telarma antarctica* did not present the complex glandular MDF-like structures of *C. granulosa*, although scarce material was available. Thus, we cannot completely discard its presence; in fact the three species studied had the same type of epithelial and subepithelial singular glandular cells. However, there were clear differences concerning the terminal sacs typical of the Charcotiidae. Terminal sacs are saccular structures placed at the terminations of the diverticula of the digestive gland in some charcotiid, arminid, embletonid, and aeolid cladobranchs. The presence of terminal sacs represents an apomorphic state within Cladobranchia, and is considered homologous to the Aeolidoidea cnidosacs (Wägele & Willan, 2000). These authors suggested an excretory function of the terminal sacs, since they directly connect the lumen of the digestive gland to the epidermis, and they present huge vacuolar cells. In fact, dendronotacean species, such as *Hancockia*, present several small cnidosacs in each cerata which open to the exterior to expulse nematocysts (Martin *et al*., 2009). In our study, *Telarma* and *Charcotia* specimens presented similar terminal sacs, as mentioned above, thus we suggest an excretory function for them. *Pseudotritonia gracilidens*, instead, presented huge vacuoles staining bluish. These vacuoles resemble the MDF-like structures of *C. granulosa*, but they are structurally different, and its origin is endodermal (not ectodermal like the MDF-like structures), therefore they are not attached to the epidermis. Again, functional constraints related to the need to exude substances might have led to the similar morphology, although their developmental origin is different.

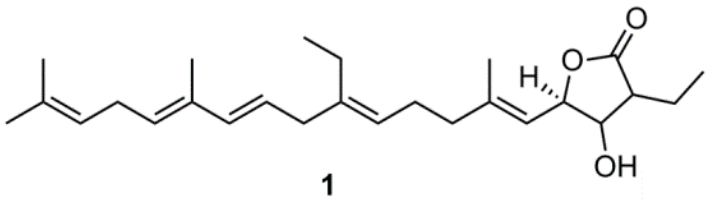


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Figure 6. Structure of granuloside (**1**)

Granuloside (**1**) was isolated from the lipophilic extract of the external part (*i.e.*, notum and foot) of the nudibranch *C. granulosa*. Since the compound was absent in the gut contents and the digestive gland, we suggest that the putative defensive homosesterterpene is not of dietary origin. Accordingly, neither terpene **1** nor any other related molecule were present in its specialist prey, the bryozoan *B. erecta* collected together with the nudibranch. In several Antarctic bryozoans, anti-predatory strategies have been shown to vary from physical to chemical protection (Figuerola *et al*., 2013). In *B. erecta*, probably the huge and abundant "bird's beak" avicularia provide adequate mechanical protection (Hayward, 1995). From our data, granuloside (**1**) was unequally distributed between skin and inner organs of the nudibranch, and was absent in its common prey, therefore *de novo* biosynthesis is suggested. Moreover, the

presence of numerous RER cisternae, active nuclei, and vesicles in the surrounding epithelial tissue of the MDF-like structures provides evidence of an active synthesis. Further biosynthetic experiments with isotopic labelled precursors are needed to confirm both the *de novo* biosynthesis and the metabolic terpenoidic route. Although the origin of some particular secondary metabolites in some molluscs has been associated to bacterial symbionts (Davis *et al*., 2013; Lin *et al*., 2013), in our study, LM and TEM did not reveal the presence of associated bacteria in the tissue structures under study. Thus production of granuloside by symbiont partnership seems not to be supported. Finally, further bioassays with the isolated compound should determine if granuloside is the ultimate responsible for the chemical repellence in *C. granulosa* against *O. validus*. However, the chemical lability of the molecule will make the ecological assays difficult.

Some previous studies demonstrated that egg masses and embryos of some invertebrates were chemically protected (McClintock & Baker, 1997b; Benkendorff *et al*., 2001). Here, the egg masses analyzed did not present granuloside or any related terpene, neither the specialized structures supposed to store the chemicals. The high number of proteinaceous platelets in the trochophore larva suggests a provision of food for postembryonic development, after the veliger stage (Morrill, 1964). The rather thin mucus layers of the egg masses, which probably are degraded much faster than those of, for example, the sympatrically occurring *D. kerguelenensis* (Wägele, 1989, 1996), also indicate a rather short developmental time within the egg clutches. Thus, after a relatively short intracapsular period of time, *C. granulosa* juveniles probably hatch anatomically complete, except for the reproductive system. It seems that the egg clutches of *C. granulosa* provide enough physical protection at this stage, and subsequent juvenile stages of the nudibranch may further develop MDF-like structures and produce granuloside, as described for MDFs in chromodoridid nudibranchs (Wägele *et al*., 2006).

The present study on the Antarctic nudibranch *C. granulosa* is a multidisciplinary approach to chemical ecology with microscopical, ultrastructural, ecological, and chemical methods. *Charcotia granulosa* from Antarctica offers evidence of synthesizing and delivering of natural products as a defensive strategy against the sea star *O. validus*. We suggest a non-dietary origin of the homosesterterpene granuloside in this charcotiid species, which is likely *de novo* biosynthesized in early juvenile stages. Our findings together with literature data indicate that, additional to Dotidae and Tethydidae, Charcotiidae is the third cladobranch family where members seem to rely on *de novo* biosynthesis of natural compounds (Putz *et al*., 2010, 2011). *De novo* biosynthesis allows the species to be independent from diet for obtaining their defensive compounds. In addition, we provide the first description of *C. granulosa* spawn, showing that egg masses do not contain granuloside. A physical protection of the clutch together with a fast development is assumed to be the strategy to protect early intracapsular development, reducing the exposition time to predators.

Although single glandular cells are commonly found and widespread in the family, no evidence of MDF-like structures has been found so far in members of the genera *Pseudotritonia* and *Telarma*. Further histological analyses as well as chemical studies are needed to unravel the relationships and life strategies among congeners of Charcotiidae; also, further biosynthetic studies with stable-labelled precursors could provide indication about the origin of the linear homosesterterpene **1** in the nudibranch *C. granulosa*.

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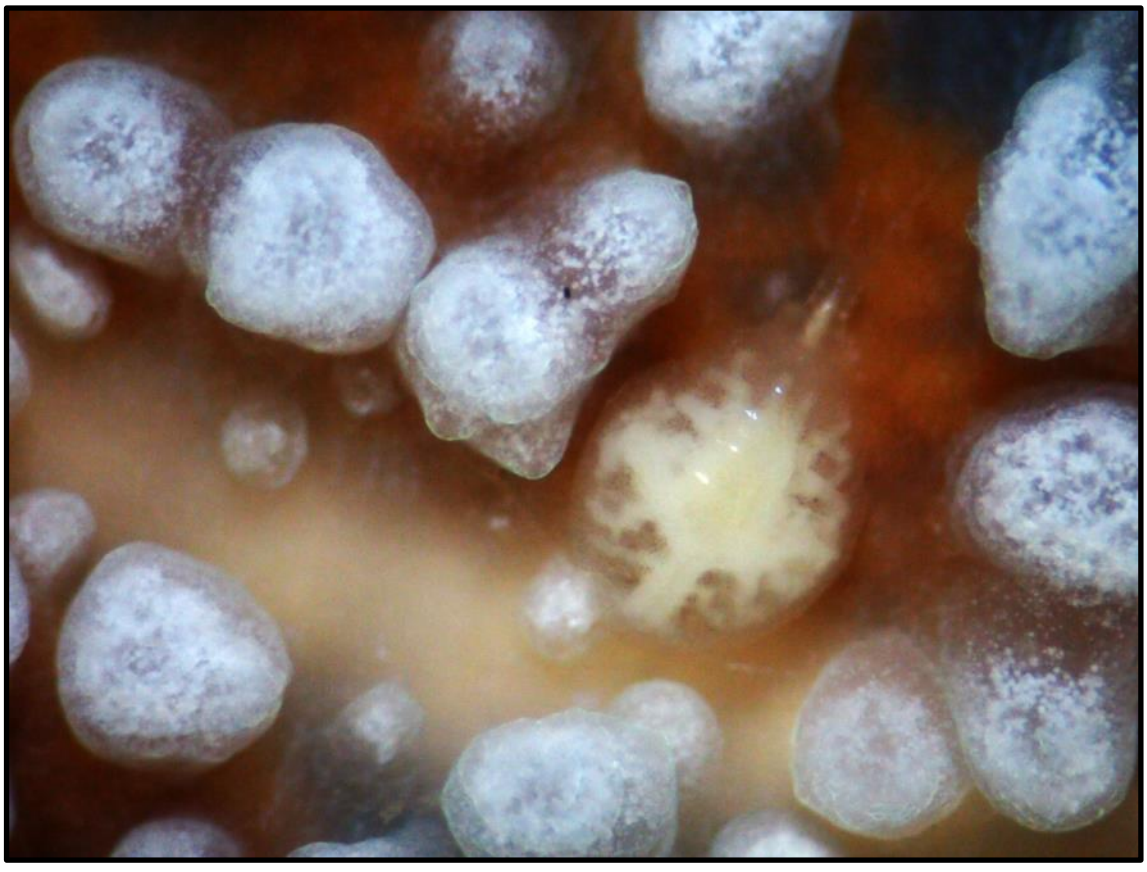
Chapter 3

Anthessius antarcticus n. sp. (Copepoda: Poecilostomatoida: Anthessiidae) from Antarctic waters living in association with *Charcotia granulosa* (Mollusca: Nudibranchia: Charcotiidae)



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Chapter 3. *Anthessius antarcticus* n. sp. (Copepoda: Poecilostomatoida: Anthessiidae) from Antarctic waters living in association with *Charcotia granulosa* (Mollusca: Nudibranchia: Charcotiidae)

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ABSTRACT

A new species of the genus *Anthessius* Della Valle, 1880 is described under the name *A. antarcticus*. It is ectosymbiont of the nudibranch *Charcotia granulosa* Vayssière, 1906 from the South Shetland Islands in the Southern Ocean. The female of the new species is distinguished from its congeners by the following combination of diagnostic morphological characters: 1) antenna with two terminal claws; 2) mandible with a seta between distal and outer lashes; 3) third exopodal segment of leg 4 with four spines and five setae (formula: III, I, 5); and 4) caudal ramus 2.40 times as long as wide. Its relationship with its congeners and other anthessiid genera are discussed. This is the first species of the genus found to be related to a nudibranch, and remarkably, it is also the only record of Anthessiidae from Antarctica.

Key words: *Anthessius antarcticus* n. sp.; ectosymbiosis; sea slug; Deception Island; marine benthos

Capítulo 3. *Anthessius antarcticus* n. sp. (Copepoda: Poecilostomatoida: Anthessiidae) de aguas antárticas hallado en asociación con *Charcotia granulosa* (Mollusca: Nudibranchia: Charcotiidae)

RESUMEN

Se describe una especie nueva del género *Anthessius* Della Valle, 1880 bajo el nombre de *A. antarcticus*. Ésta especie es ectosimbionte del nudibranquio *Charcotia granulosa* Vayssière, 1906 procedente de las islas Shetland del Sur en el océano austral. La hembra de la nueva especie se diferencia de sus congéneres por la siguiente combinación de caracteres diagnósticos: 1) antena con dos uñas terminales; 2) mandíbula con una seta entre los látigos distal y exterior; 3) tercer segmento del exopodito de la 4ª pata con cuatro espinas y cinco setas (fórmula: III, I, 5); y 4) ramo caudal 2,4 veces más largo que ancho. Se discute su relación con sus congéneres y otros géneros de la família Anthesiidae. Ésta es la primera especie del género hallada en un nudibranquio, y notablemente, también es el único registro hasta la fecha de esta familia en la Antártida.

Palabras clave: *Anthessius antarcticus* n. sp.; ectosimbiosis; babosa marina; isla Decepción; bentos

INTRODUCTION

Copepods have been highly successful in forming associations with other marine organisms, among which molluscs seem to be one of the most preferred partners. According to Ho (1997), a total of 246 copepod species have been described in association with 458 species of mollusc. These symbionts belong to five orders: Harpacticoida, Misophrioida, Cyclopoida, Siphonostomatoida, and Poecilostomatoida, the last of which includes about 73% of the known copepod associates of Mollusca. Indeed, poecilostomatoid species of the family Anthessiidae are mostly associated with molluscs (Boxshall & Halsey, 2004a, b), while some are found associated with algae, plankton, crustaceans, and teleost fish (Ho, 1997; Conradi *et al*., 2012). Copepods of the family Anthessiidae are currently classified into five genera: *Anthessius* Della Valle, 1880, *Katanthessius* Stock, 1960, *Neanthessius* Izawa, 1976, *Panaietis* Stebbing, 1900, and *Rhinomolgus* Sars, 1918 (Humes, 1986; Boxshall & Halsey, 2004a, b). *Anthessius* is the most specious genus in the family, with species generally associated with marine bivalves and gastropods, some of which are of commercial importance (Uyeno & Nagasawa, 2012). There are 44 nominal species of *Anthessius* described to date, all of them inhabiting temperate and warm waters (Conradi *et al*., 2012; Uyeno & Nagasawa, 2012; Walter & Boxshall, 2014). Among them only 11 species are associated with opisthobranchs, generally from the orders Anaspidea and Pleurobranchomorpha, but they have never been found in association with nudibranchs (Illg, 1960; Stock *et al*., 1963; Humes & Ho, 1965). Although more than 50 species of Anthessiidae have been recorded worldwide, none of them is known from the Southern Ocean. The present study reports a new species of *Anthessius* as the first record from Antarctic waters and the first association with a nudibranch, *Charcotia granulosa* Vayssière (Charcotiidae).

MATERIAL AND METHODS

A total of 64 specimens of the nudibranch *Charcotia granulosa* were collected by SCUBA-diving in Port Foster, Deception Island (South Shetland Islands, Antarctica) during the ACTIQUIM-4 cruise in February, 2013. One specimen of the copepod *Anthessius antarcticus* n. sp. was found in ectosymbiosis on *C. granulosa* collected in the area of Whalers Bay (62°59.33'S; 60°33.45'W), 14 m water depth. *Charcotia granulosa* specimens were collected from shallow rocky bottoms where its prey, the bryozoan *Beania erecta*, was abundant, covering the substrate and other sessile animals. The benthic ecosystem was dominated by demosponges (*Mycale* (*Oxymycale*) *acerata*, *Dendrilla antarctica*), soft corals (*Alcyonium haddoni*), solitary ascidians (*Cnemidocarpa verrucosa*), and wandering fauna (mainly echinoderms: *Odontaster validus*, *Ophionotus victoriae*, *Sterechinus neumayeri*). Other nudibranch species were collected in the area: 18 specimens of *Doris kerguelenensis* (Bergh, 1884) and four of *Cuthona crinita* Minichev, 1972, but no *Anthessius* spp. or other ectosymbiotic copepods were found.

Prior to preservation in 96% ethanol, the holotype was photographed alive with a camera (Invenio 5S 5MPixel CMOS) adapted to a stereomicroscope (Zeiss Stemi 2000-C) (Fig. 1). The animal was transferred to the Department of Animal Biology at the University of Barcelona for further morphological analysis. The organism in 96% ethanol was dehydrated in a graded series of alcohol, dried to the critical point, mounted, carbon-coated, and imaged using a Hitachi H-4100FE scanning electron microscope (SEM) (University of Barcelona) (Fig. 2). Live and SEM photographs were edited using Adobe Photoshop CS6, making the background black and enhancing contrast. Following SEM micrography, the animal was restored for anatomical analysis. Carbon-coating was partly removed by treating the sample with HCN gas for three days, following the method of Leslie and Mitchell (2007). Subsequently, the dried specimen was soaked in 0.5% Na_3PO_4 for 10 min in order to return the specimen as close as possible to its original condition. The specimen was immersed in lactic acid before dissection and afterwards observed using the reverse slide method of Humes and Gooding (1964). All illustrations were drawn with the aid of a drawing tube mounted on an Olympus BH-2 microscope. In the armature formula of appendages, spines are indicated by Roman numerals and setae by Arabic numerals.

SYSTEMATICS

Order Poecilostomatoida Burmeister, 1835
Anthessiidae Humes, 1986
***Anthessius* Della Valle, 1880**
Anthessius antarcticus n. sp.
(Figures 1–5)

Types.—One ♀ (holotype) collected on the body surface (notum) of the nudibranch *Charcotia granulosa* Vayssière, 1906 (Charcotiidae) from Deception Island, South Shetland Islands, Antarctica, 05 February 2013, collected by C. Avila and J. Moles. The holotype (dissected and mounted on a glass slide) has been deposited in the National Institute of Biological Resources (NIBR), Incheon, Korea (Catalog number NIBRIV0000293978).

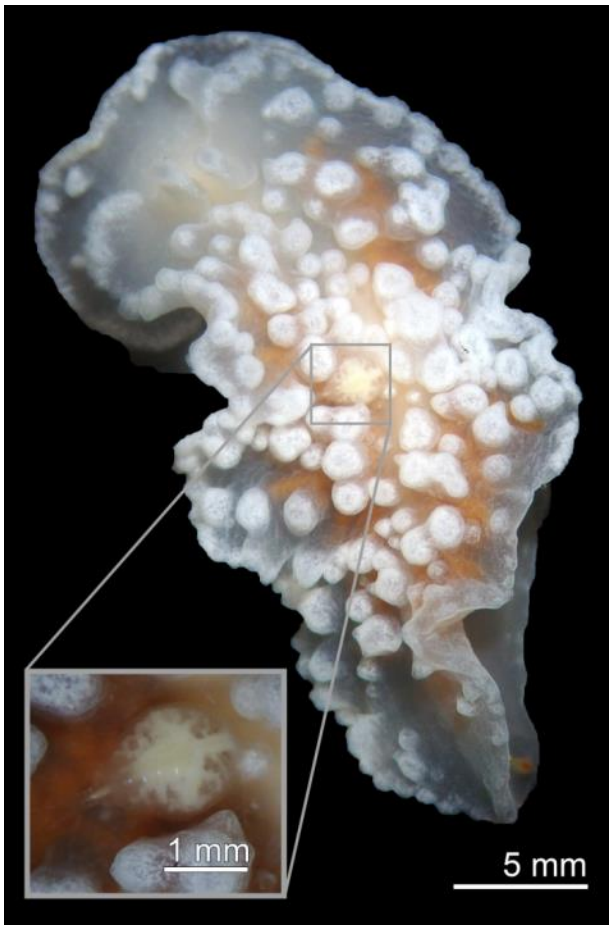

Figure 1. The nudibranch *Charcotia granulosa* with a close up of the copepod *Anthessius antarcticus* n. sp. on the nudibranch's notum (alive). Picture taken at the "Gabriel de Castilla" Spanish Antarctic Base.

Female.— Body (Figs. 2A, 3A) dorsoventrally flattened and 2.55 mm long, not including caudal setae. Prosome oval, 1.62 mm long along midline, representing about 64% of body length; greatest width 1.34 mm; length:width ratio = 1.21:1. Dorsal suture line distinct between cephalosome and first pedigerous somite. Posterolateral corners of all prosomal somites rounded. Third pedigerous somite longer than other pedigerous somites. Urosome (Fig. 3B) 5-segmented. First urosomite (fifth pedigerous somite) 458 µm wide, much wider than genital double-somite, with tapering lateral margins. Genital double-somite and abdominal somites with finely pectinate posteroventral margins (Fig. 2B). Genital double-somite 246 × 385 µm, 1.57 times wider than long and consisting of strongly expanded anterior two-thirds and narrower posterior one-third (225 µm wide across this region), with narrow horizontal sclerotized band on dorsal anterior region and faint transverse line on dorsal surface at two-thirds of somite length; genital apertures large and located dorsolaterally. Three free abdominal somites 80 × 209, 80 × 191, and 188 × 218 µm in length and width, respectively. Anal somite longer than two preceding somites combined, smooth without spinules or denticles on ventral surface, with distinct posteromedial notch and large anal area; anal operculum not prominent. Caudal rami slightly divergent and separated widely from each other; each ramus (Fig. 3C) tapering, 192 × 80 µm (length:width ratio = 2.40:1), probably with 6 setae, and with minute spinules at ventro-distal margin near bases of setae III and IV (Fig. 2C); two mid-terminal setae (setae IV and V) much longer than ramus; other setae shorter than ramus; insertion of outer lateral seta (seta II) about 45% of ramus length.

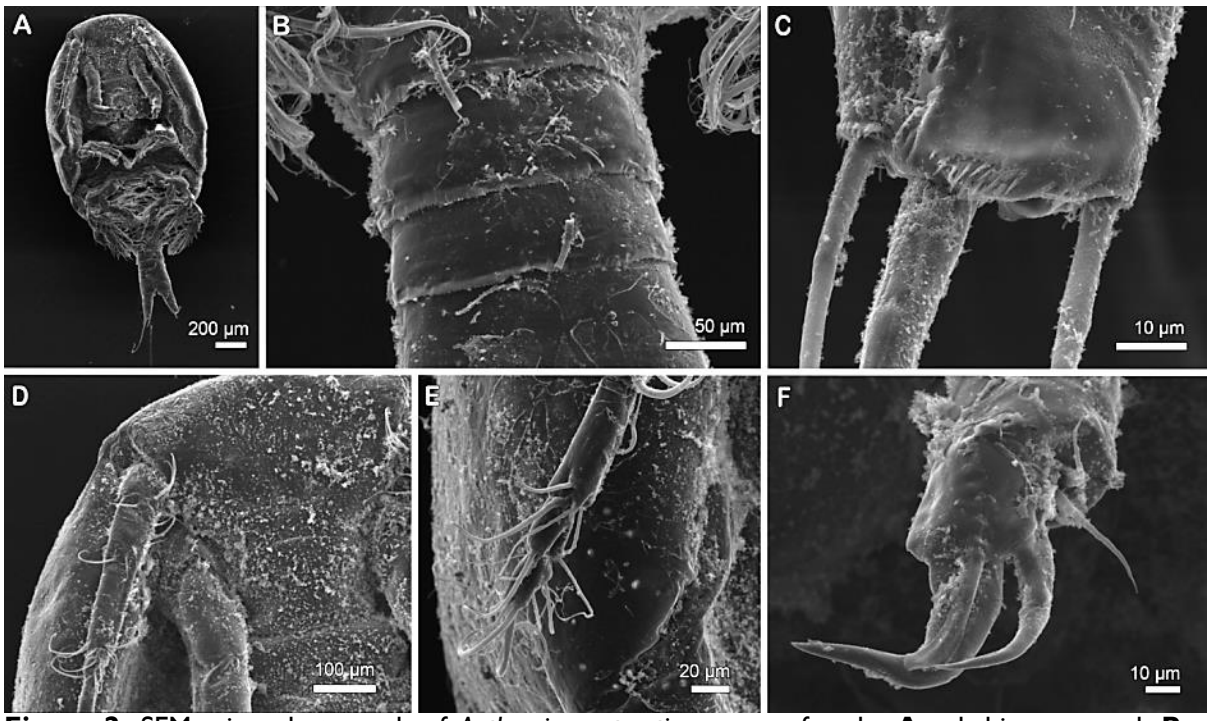

Figure 2. SEM microphotograph of *Anthessius antarcticus* n. sp., female. **A –** habitus, ventral; **B –** abdomen, ventral; **C –** distal part of caudal ramus, ventral, showing setae III, IV, and VI; **D –** antero-lateral part of cephalothorax, ventral; **E –** distal part of antennules; **F** – distal part of antenna.

Rostral area broad, but rostrum absent (Figs. 2A, 3D). Antennule (Figs. 2D, E, 3E) 735 μm long and 7-segmented; second segment longest, 288 μm long (39% of length of antennule); fourth segment second-longest, 146 μm long (20% of length of antennule); two terminal segments markedly short; first segment armed with 4 setae; armature formula of second to terminal segments (observed from SEM microphotographs) 15, 6, 3, 4 + aesthetasc, 2 + aesthetasc, and 7 + aesthetasc; all observed setae naked. Antenna (Fig. 3F) 3-segmented, consisting of basis and 2-segmented endopod; basis about 160 × 110 μm in length and width, with 1 distal seta; proximal endopodal segment 183 × 108 μm, with 1 small subdistal seta; distal endopodal segment 192 × 96 μm (length:width ratio 2.23:1), distinctly narrower than two proximal segments, and armed with 3 setae on medial margin, 3 setae on outer subdistal region, and 2 unequal claws and 2 setae distally (Fig. 2F); medial claw 104 μm long, broad and strong; outer claw 82 μm long, much narrower than medial one; all setae on antenna small.

Labrum (Figs. 3G, 4A) with divergent, tapering posterior lobes and shallow posteromedial incision, also with a pair of hyaline rims, each bearing row of fine spinules on medial region of incision. Mandible (Fig. 3H) with large distal and outer lashes; convex medial side with 2 dentiform elements, proximal one bifid and distal one quadrifid; distal lash serrate along proximal two-thirds of convex medial margin, but smooth along outer margin; outer lash slightly shorter than distal lash and also serrate along proximal region of medial margin; 1 slender but conspicuous seta present between distal and outer lashes (this seta about half as long as outer lash and spinulose on medial margin). Paragnath (Fig. 3I) as sub-globular lobe bearing long spinules along medial margin. Maxillule (Fig. 4B) lamella-like, bearing 6 setae (or setiform elements) on distal margin, one of them much larger than others. Maxilla (Fig. 4C) 2-segmented; proximal segment very broad and unarmed; distal segment terminating in stiff spiniform process and armed with small proximal seta (seta III) with swollen basal portion, broad anterior seta (seta II), 4 large spines on subdistal region of convex outer margin, and 3 denticles (including minute proximal one) on subdistal part of concave medial margin. Small pore present near insertion of anterior seta. Maxilliped (Fig. 4D) indistinctly 3-segmented; first segment unarmed; suture between first and second segments unclear, represented only by fine wrinkles; second segment with one rudimentary seta (minute knob) subdistally on medial margin; boundary between second and third segments represented by lateral constriction; third segment tapering, apically, with small seta and small, blunt setiform process, and with flap-like expansion along outer side.

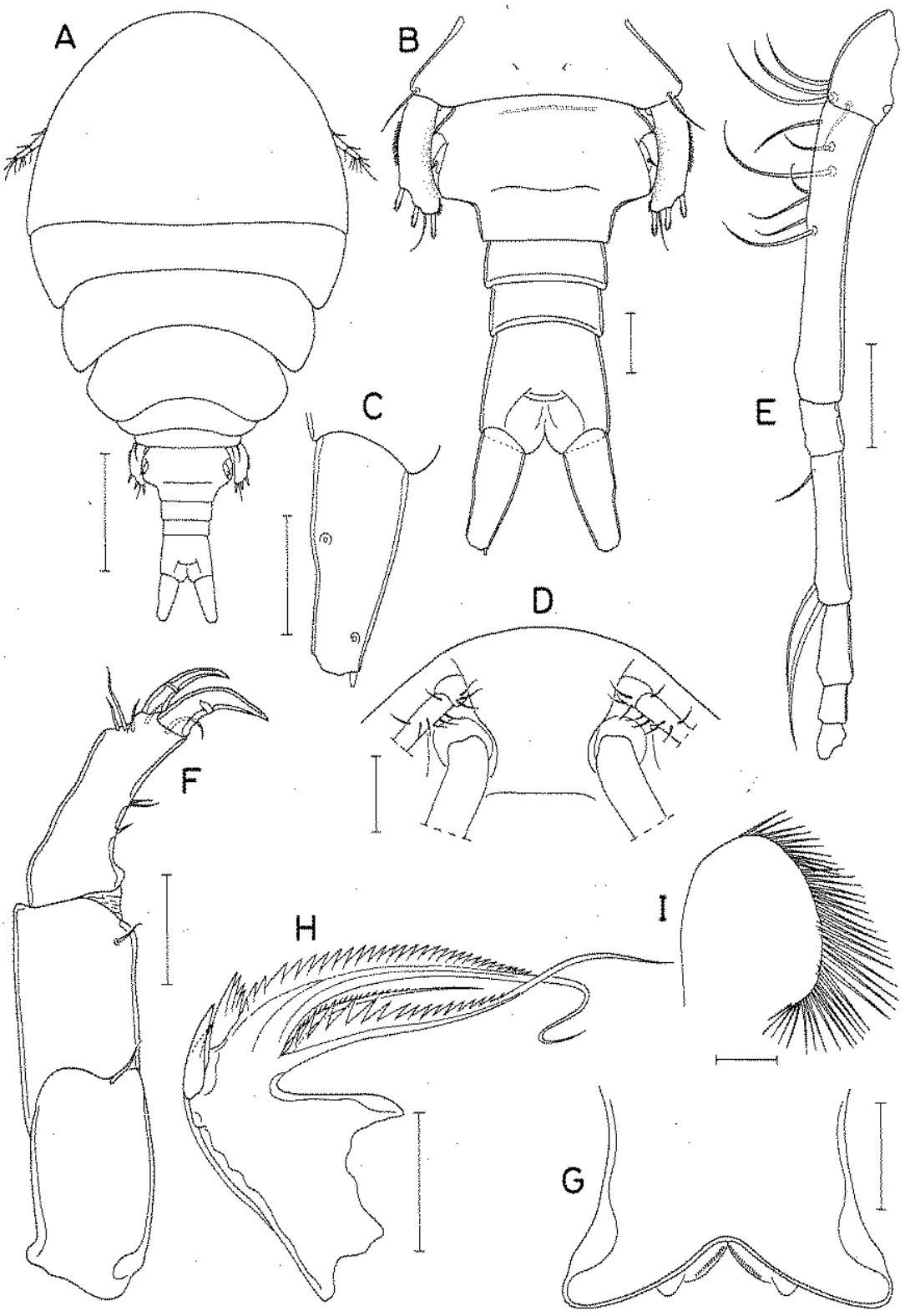

Figure 3. *Anthessius antarcticus* n. sp., female. **A –** habitus, dorsal; **B –** urosome, dorsal; **C –** left caudal ramus, dorsal (setae omitted); **D –** rostral area, ventral; **E –** antennules (most of setae omitted); **F –** antenna; **G –** labrum; **H –** mandible; **I –** paragnath. Scale bars: A, 0.5 mm; B, C, E, F, 0.1 mm; D, 0.2 mm; G, H, 0.05 mm; I, 0.02 mm.

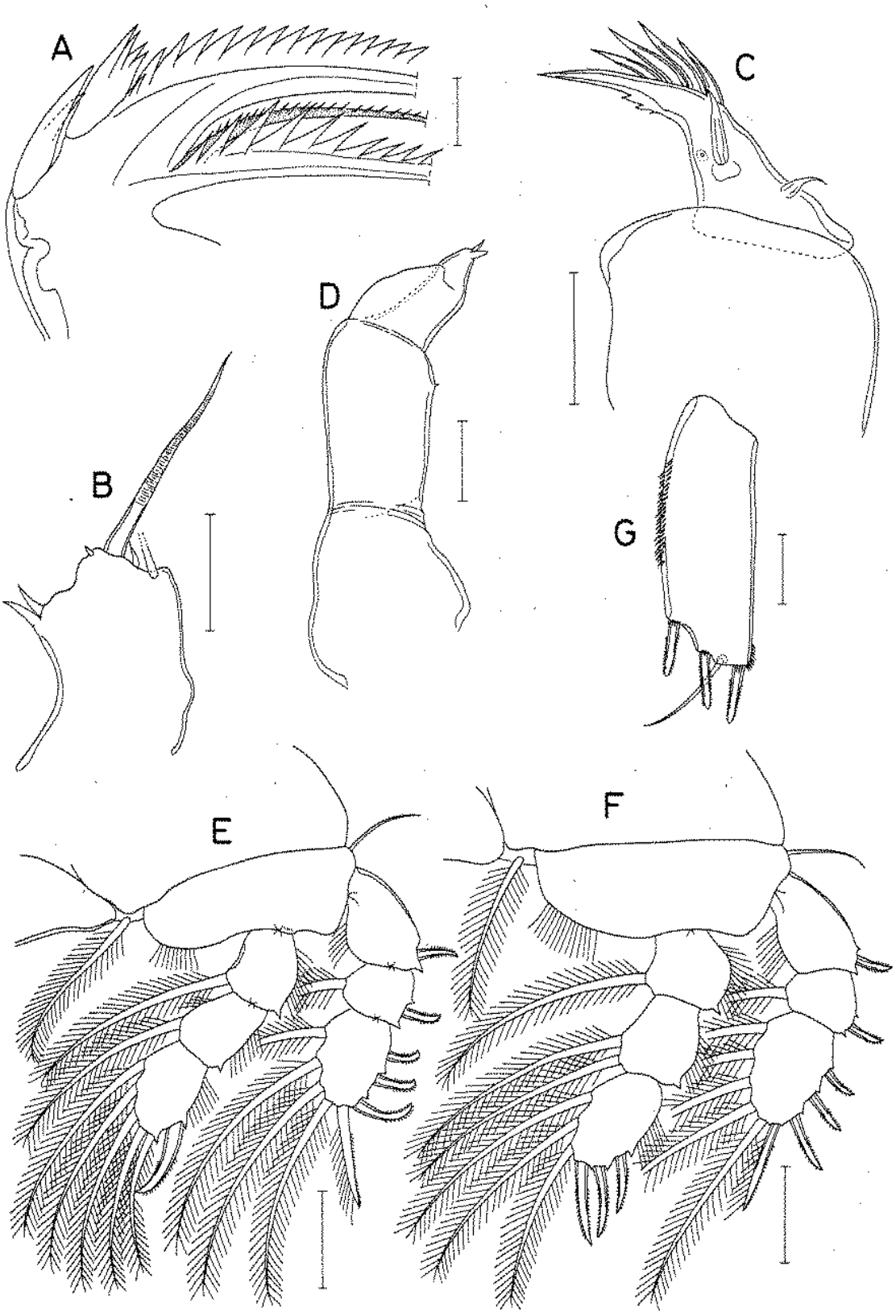

Figure 4. *Anthessius antarcticus* n. sp., female. **A –** proximal region of mandibular lashes; **B –** maxillule; **C –** maxilla; **D –** maxilliped; **E –** leg 1; **F –** leg 2; **G –** exopod of leg 5. Scale bars: A, 0.02 mm; B-D, G, 0.05 mm; E, F, 0.1 mm.

Legs 1–4 (Figs. 4E, F, 5A, B) with 3-segmented rami; outer seta on basis naked; all setae on coxa and rami pinnate; outer margin of endopodal segments with row of setules; outer distal corners of first and second segments of the endopods with pointed process; outer margin of exopodal segments and third endopodal segments with pointed processes near base of outer spines; spines on endopods and outer spines on exopods slender and spinulose; medio-distal margin of basis with setules. Leg 3 similar to leg 2, except for bearing 4 spines and 2 setae on third endopodal segment. Armature formula of legs 1–4 presented in Table 1.

Table 1. Spine and setal formula for swimming legs 1 to 4 of *Anthessius antarcticus* n. sp.

	Coxa	Basis	Exopod	Endopod
Leg 1	0-1	1-0	I-0; I-1; III, I, 4	0-1; 0-1; I, 2, 3
Leg 2	0-1	1-0	I-0; I-1; III, I, 5	0-1; 0-2; II, I, 3
Leg 3	0-1	1-0	I-0; I-1; III, I, 5	0-1; 0-2; III, I, 2
Leg 4	0-1	1-0	I-0; I-1; III, I, 5	0-1; 0-2; III, I, 1

Leg 5 consisting of one dorsolateral seta on fifth pedigerous somite and free exopod. Exopod (Fig. 4G) 195 × 69 µm (length:width ratio 2.83:1); outer margin slightly convex, with distal tuft of minute spinules near middle region; medial margin straight, with small distal tuft of minute spinules; distal margin armed with 3 rod-shaped, spinulose spines of similar length (40 µm) and shape, and 1 naked seta; row of spinules present at bases of spines. Leg 6 represented by thick seta and spiniform element on genital operculum (Fig. 5C).

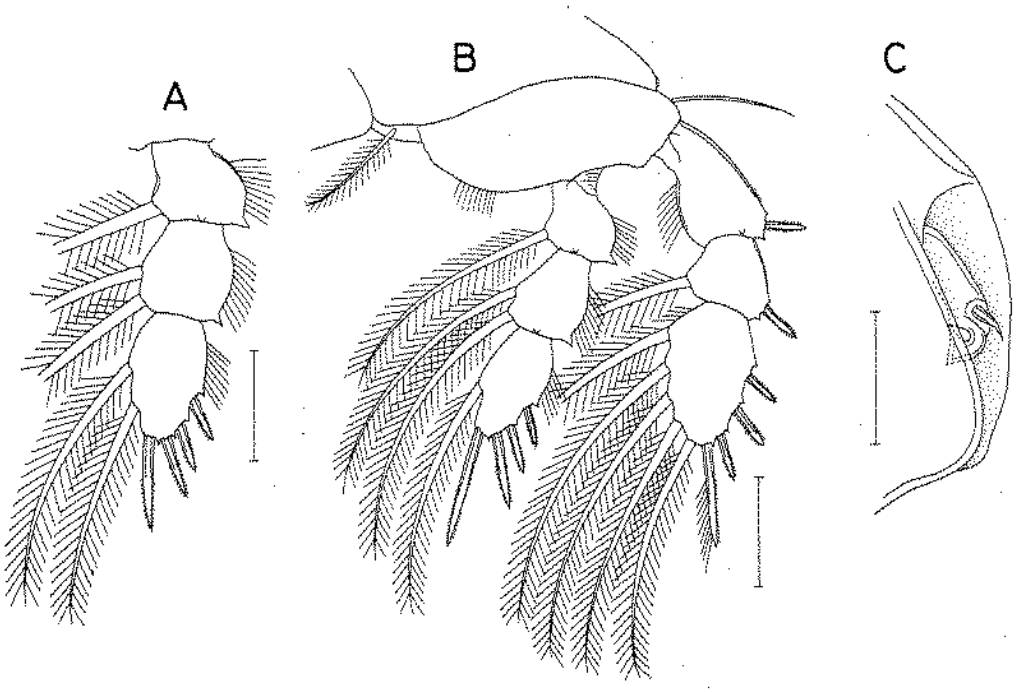

Figure 5. *Anthessius antarcticus* n. sp., female. **A –** endopod of leg 3; **B –** leg 4; **C –** right genital aperture, dorsal. Scale bars: A, B, 0.1 mm; C, 0.05 mm.

The color in life in transmitted light is translucent to white; the digestive system is whitish to creamy.

Male.—Unknown. Etymology.**—**The specific name is derived from the geographic area, Antarctica, from which the type specimen was collected. This is the first species of the genus, and the first in the family Anthessiidae, described from there.

DISCUSSION

Anthessius antarcticus is clearly distinguished from its 44 congeners by the following combination of diagnostic features: 1) antenna with two terminal claws; 2) mandible with a seta between distal and outer lashes; 3) third exopodal segment of leg 4 with four spines and five setae (formula: III, I, 5); and 4) caudal ramus 2.40 times as long as wide. Among these characters, the first and second in particular seem to be phylogenetically valuable. A cladistic analysis of *Anthessius* done by Ho (1997) showed that the number of terminal claws on the antenna is important in the taxonomy of the genus. Species of this genus usually have three or four terminal claws on the antenna (Humes, 1986). Exceptions to this are represented by only two species: there are no claws in *A. brevifurca* Sewell, 1949, recovered from weed washings in the Maldive Islands (Sewell, 1949), and two claws in *A. pinnae* Humes 1959, is associated with a bivalve in Madagascar (Humes, 1959). *Anthessius antarcticus* n. sp. is similar to *A. pinnae* in having two terminal claws on the antenna, but *A. pinnae* is clearly distinguished by other features: it has a rostrum (absent in *A. antarcticus*), the caudal ramus is about 3.4 times as long as wide (versus 2.4 times), the third exopodal segment of leg 4 bears three spines (versus four), and the mandible has no element between the distal and outer lashes (versus a seta) (Humes, 1959).

The second significant morphological feature of the new species, the presence of a prominent seta between the distal and outer lashes of the mandible seems to be unique within the genus *Anthessius*. In most species of this genus this element is absent as in *A. pinnae* Humes, 1958 and *A. nosybensis* Kim, 2009 (Humes, 1959; Kim, 2009) or it appears as a hyaline tapering foliaceous lamella as in *A. nortoni* Illg, 1960 and *A. pinctadae* Humes, 1973 (Illg, 1960; Humes, 1973), or bifurcate as in *A. brevicauda* (Leigh-Sharpe, 1934) (see Humes, 1973), and *A. saecularis* Stock, 1964 (Stock, 1964). Although in four species, *viz*., *A. arcuatus* López-González, Conradi, Naranjo and García-Gómez, 1992, *A. concinnus* (A. Scott, 1909), *A. obtusispina* Ho, 1983, and *A. ovalipes* Stock, Humes and Gooding, 1963 have an elongate linguiform extension of the mandible at this site, none of these four species or other congeners is known to have a true seta between the distal and outer lashes of the mandible, as observed in *A. antarcticus*.

Within the Anthessiidae, however, such a seta is found between the distal and outer lashes in species of *Katanthessius* Stock, 1960. This genus consists of two known species: *K. delamarei* Stock, 1960 from the Mediterranean and *K. stocki* Humes, 1997 from California, both found in association with nudibranch gastropods (Stock, 1960; Humes, 1997). It is assumed that this element between the distal and outer lashes is one of the five ancestral gnathobase elements of the mandible known in primitive poecilostomatoid families, such as the Oncaeidae and Corycaeidae of Huys & Boxshall (1991: 342), along with the two tooth-like elements on the convex side, and the distal and outer lashes. Therefore, the retention of the elongate setiform condition of the element in *Katanthessius* and *A. antarcticus* may be interpreted as a primitive condition of the mandible within the Anthessiidae. In addition to having a similar form of the mandible and the same group of hosts (Nudibranchia), they also share a similar form of antenna, tipped with two claws. Nonetheless, it seems premature at present to treat them as congeneric; *Katanthessius* is currently differentiated from other anthessiid genera by the reduction of the segmentation and/or setation of the posterior swimming legs (Humes, 1986; Boxshall & Halsey, 2004a, b).

Table 2. *Anthessius* species associated with opisthobranch molluscs.

Species of *Anthessius*	**Host species**	**Order**	**References**
A. antarcticus n. sp.	*Charcotia granulosa* Vayssière, 1906	Nudibranchia	Present study
A. arcuatus López-González, Conradi, Naranjo and García-Gómez, 1992	*Berthella stellata* (Risso, 1826)	Pleurobranchomorpha	López-González *et al.* (1992)
A. dolabellae Humes and Ho, 1965	*Dolabella auricularia* (Lightfoot, 1786)	Anaspidea	Humes & Ho (1965)
A. hawaiiensis (C.B. Wilson, 1921)	*Pleurobranchus* Cuvier, 1804	Pleurobranchomorpha	Wilson (1935); Illg (1960)
A. lighti Illg, 1960	*Aplysia californica* J. G. Cooper, 1863	Anaspidea	Illg (1960)
A. navanacis (C.B. Wilson, 1935)	*Navanax inermis* (J. G. Cooper, 1862)	Cephalaspidea	Wilson (1935); Illg (1960)
A. obtusispina Ho, 1983	*Pleurobranchaea californica* MacFarland, 1966	Pleurobranchomorpha	Ho (1983)
A. ovalipes Stock, Humes and Gooding, 1963	*Pleurobranchus areolatus* Mörch, 1863	Pleurobranchomorpha	Stock *et al.* (1963)
A. pleurobrancheae Della Valle, 1880	*Pleurobranchaea meckeli* (Blainville, 1825)	Pleurobranchomorpha	Della Valle (1880)
A. proximus Stock, Humes and Gooding, 1963	*Dolabrifera dolabrifera* (Rang, 1828), *Petalifera petalifera* (Rang, 1828)	Anaspidea	Stock *et al.* (1963)
A. stylocheili Humes and Ho, 1965	*Stylocheilus longicauda* (Quoy and Gaimard, 1825)	Anaspidea	Humes & Ho (1965)
A. varidens Stock, Humes and Gooding, 1963	*Aplysia dactylomela* Rang, 1828, *Bursatella leachii* Blainville, 1817	Anaspidea	Stock *et al.* (1963)

Among the 44 nominal species of the genus *Anthessius* described to date, only 11 are ectosymbionts of opisthobranch molluscs; concretely, five from Anaspidea, five from Pleurobranchomorpha, and one in Cephalaspidea (Table 2). *Anthessius antarcticus* n. sp. is only known to inhabit the notum of a nudibranch, *Charcotia granulosa*. As this association occurred at a low incidence (one ectosymbiosis out of 64 potential hosts) in the locality of Deception Island, it is not possible to suggest specificity of the copepod to a single host species (monoxenous development), even if it was not found on the other nudibranchs collected, *Doris kerguelenensis* and *Cuthona crinita*. It seems reasonable to suggest that many species of *Anthessius* probably remain to be discovered, since this is the first species described to date from Antarctica.

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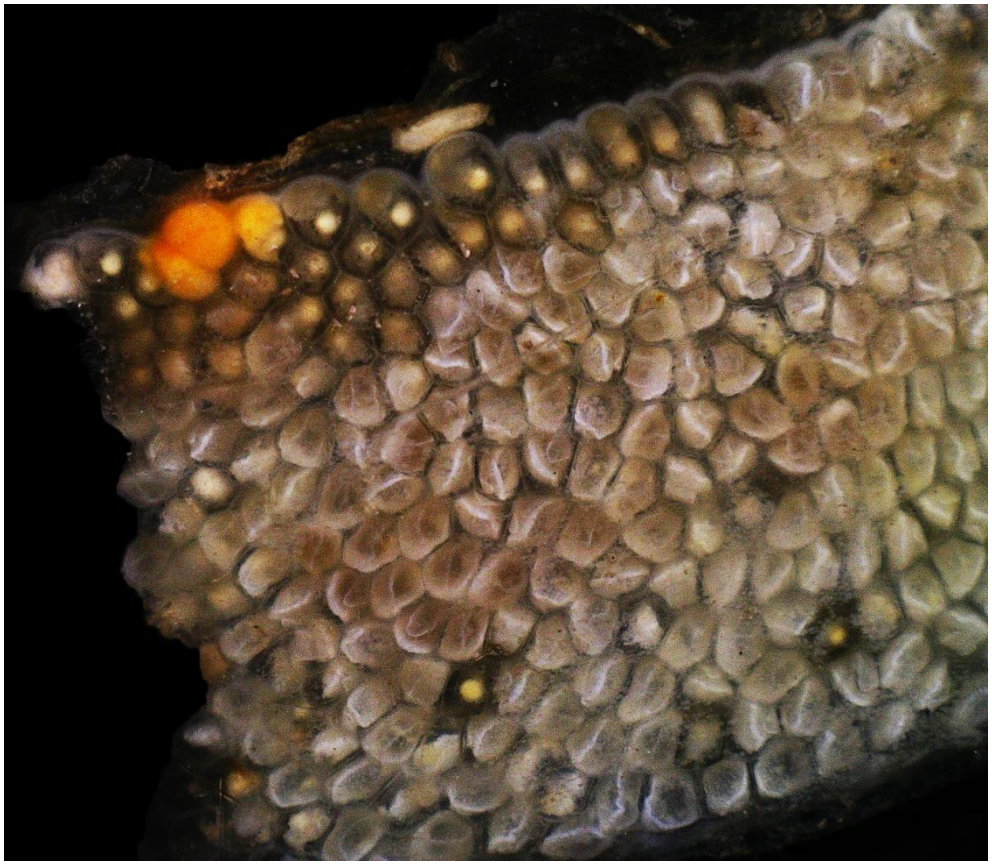
Chapter 4

The sea slugs that laid giant egg masses: Embryonic development in two Antarctic anthobranchs (Mollusca: Gastropoda: Nudibranchia)



Moles J, Wägele H, Cutignano A, Fontana A, Ballesteros M, Avila C (*In prep*) The sea slugs that laid giant egg masses: Embryonic development in two Antarctic anthobranchs (Mollusca: Gastropoda: Nudibranchia)

Chapter 4. The sea slugs that laid giant egg masses: Embryonic development in two Antarctic anthobranchs (Mollusca: Gastropoda: Nudibranchia)

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ABSTRACT

Doris kerguelenensis and *Bathydoris hodgsoni* are two of the largest Antarctic nudibranchs, both protected by chemical defences against potential sympatric predators. They are very common, circumpolar species with a broad bathymetric distribution, although *Bathydoris* is restricted to deep waters in the high Antarctic zone. Both species exhibit similar reproductive strategies, but differ in egg capsule size and number. Egg masses and juveniles of these species were collected in the eastern Weddell Sea in different ontogenetic stages. New data about egg mass characteristics and ontogeny of both *B. hodgsoni* and *D. kerguelenensis* is presented herein, applying histological methods in reared or directly caught embryos and juveniles. We propose a continuous reproduction throughout the year and an estimated embryonic period of 13 months for *D. kerguelenensis*. *Bathydoris hodgsoni* possesses the largest egg capsules and embryos ever found in molluscs, and we estimate a long embryonic period of up to 10 years. The high yolk content observed and the ingestion of the capsule by embryos might be vital strategies for ensuring these long embryonic periods in both species. Thick egg capsules might also act as a physical defense strategy for embryos, while hatched and 'vulnerable' juveniles might rely on chemical defence, as adults do.

Keywords: *Bathydoris hodgsoni*; *Doris kerguelenensis*; ontogeny; chemical defence; natural products; terpenoids; Weddell Sea

Capítulo 4. Las babosas de mar que ponen huevos gigantes: desarrollo embrionario de dos antobranquios antárticos (Mollusca: Gastropoda: Nudibranchia)

RESUMEN

Doris kerguelenensis y *Bathydoris hodgsoni* son dos de los nudibranquios antárticos de mayor tamaño, ambos protegidos por defensas químicas contra posibles depredadores simpátricos. Son especies circumpolares muy comunes, con una amplia distribución batimétrica, aunque *Bathydoris* se limita a aguas profundas en altas latitudes del océano austral. Ambas especies presentan estrategias reproductivas similares, pero difieren en número y tamaño de la cápsula del huevo. Las puestas y juveniles de estas especies fueron recolectads al este del mar de Weddell en diferentes etapas ontogenéticas. En este estudio, presentamos nuevos datos sobre las características de la puesta y la ontogenia de ambas especies, *B. hodgsoni* y *D. kerguelenensis*, aplicando métodos histológicos para el estudio de los embriones y los juveniles cultivados o directamente capturados. Se sugiere una reproducción continua durante todo el año y un período embrionario estimado de 13 meses para *D. kerguelenensis*. *Bathydoris hodgsoni* posee las mayores cápsulas ovígeras y embriones que se han encontrado en moluscos, y estimamos una duración del período embrionario de hasta 10 años. El alto contenido en vitelo observado y la ingestión de la cápsula por los embriones podrían ser estrategias vitales para asegurar estos períodos embrionarios tan largos en ambas especies. El grosor de las cápsulas de los huevos también puede actuar como una estrategia física defensiva para los embriones, mientras que los juveniles eclosionados y "más vulnerables" están defendidos químicamente, como lo están los adultos.

Palabras clave: *Bathydoris hodgsoni*; *Doris kerguelenensis*; ontogenia; defensa química; productos naturales; terpenoides; mar de Weddell

INTRODUCTION

The isolation of the Antarctic continent and the formation of the Antarctic Circumpolar Current allowed benthic species to co-evolve in habitats characterized by low and relatively stable temperatures (Clarke, 1992; Dayton *et al.*, 1994; Clarke *et al.*, 2004). In general, cold water conditions a slow growth rate, longevity, and a delayed age of maturity in Antarctic benthic fauna (Pearse *et al.*, 1991; Clarke, 2003; Peck *et al.*, 2007). Low temperatures and/or differences in seasonal availability of organic matter favour protected intracapsular development as a common strategy among Antarctic species to protect early stages of their life cycles (Wray & Raff, 1991; Peck *et al.*, 2006). Intracapsular development in invertebrates is a slow process, and in molluscs it takes even longer than, for example, in barnacles, echinoids, and teleost fishes (Palmer, 1994; Peck *et al.*, 2007). Intracapsular or direct developing molluscs usually produce few, large eggs (Thompson, 1967; Todd & Doyle, 1981; Hain & Arnaud, 1992; Peck *et al.*, 2007). There is a positive correlation between egg size and time to hatch, and an inverse correlation between these and the number of eggs (Thompson, 1967; Ros, 1981). Accordingly, juveniles of the Subantarctic cephalaspidean *Philine gibba* (Cephalaspidea) hatched after 120 days and measured 500 µm (Seager, 1979). However, *Philine* spp. from warmer waters present shorter embryonic periods and smaller eggs and juveniles (Schaefer, 1996). Usually, in high-latitudinal Antarctic heterobranchs development takes longer. For instance, late veliger larvae of the Antarctic *Philine alata* hatched after 180 days (Hain & Arnaud, 1992), and 1.6 mm juveniles of *Bathyberthella antarctica* (Pleurobranchomorpha) hatched after 100 days (Wägele, 1996).

Among Antarctic nudibranchs, *Bathydoris hodgsoni* is one of the largest anthobranchs worldwide, with an eurybathic (*i.e.*, 152–2,757 m depth) and circumpolar Antarctic distribution (Valdés, 2002). Only three egg masses of *B. hodgsoni* had been found to date. They contain 2 to 4 oval, elongated, flat, big-sized egg capsules each, arranged in a line (Wägele, 1996). The whole and largest egg mass was up to 100 x 62 x 14 mm (length:width:height), and embryos were 15 mm long after the egg mass was kept for 460 days in the aquarium. Wägele (1996) suggested this species to have an embryonic period of at least 2.5 years. Similarly, *Doris kerguelenensis* is a very common, circumpolar species with a broad bathymetric distribution, ranging from 1 to 1,550 m depth (Iken *et al.*, 2002; Wilson *et al.*, 2013). It possesses spiral, flat, yellowish egg masses (Gibson *et al.*, 1970; Wägele, 1989a). The few egg masses analysed up to now measured 70–80 x 12–18 mm (length:width), and contained about 1,280–2,380 egg capsules. Developed embryos of 2–4 mm were described in the capsules of a single spawn, leading Gibson *et al.* (1970) to suggest *D. kerguelenensis* is a direct developer. However, eggs of 500 µm and veliger larvae of 900 µm were observed inside 1.2–1.9 mm egg capsules (Wägele, 1989b, 1996; Hain & Arnaud, 1992), suggesting an intracapsular indirect development. Hain (1992) reported an embryonic period in *D.*

kerguelenensis of 21 months (1.75 years). After that period, hatched juveniles measured 2 mm and survived for 37 weeks without feeding in aquarium.

Both anthobranchs present a broad dietary spectrum. *Bathydoris hodgsoni* is a generalist omnivorous predator feeding on a wide variety of invertebrates, including sponges, cnidarians, bryozoans, polychaetes, molluscs, crustaceans, and echinoderms (Avila *et al.*, 2000). *Doris kerguelenensis*, instead, is reported to feed on a wide variety of demosponges, including the genera *Calyx*, *Dendrilla, Halichondria*, *Haliclona*, *Homaxinella*, *Hymeniacidon*, *Isodictya*, *Lissodendoryx*, *Microxina*, *Polymastia*, *Sphaerotylus*, and *Tetilla*; as well as the hexactinellid genera *Anoxycalyx* and *Rossella* (reviewed in McDonald and Nybakken, 1997).

Nudibranchs usually possess bioactive molecules to ensure their survival against potential predators (Avila, 1995; Cimino *et al.*, 2001). These natural products (NPs) may be *de novo* biosynthesized by the slug or derived from its diet (Cimino & Ghiselin, 2009). *De novo* biosynthesis of terpenoid NPs has been hypothesized for both *B. hodgsoni* and *D. kerguelenensis*, and these were proved to protect the adults from sympatric predators (Avila *et al.*, 2000; Iken *et al.*, 2002; Cutignano *et al.*, 2011). Hodgsonal, a sesquiterpene isolated exclusively from the notum and dorsal papillae of *B. hodgsoni* (Iken *et al.*, 1998), showed repellence against the sympatric sea star predator *Odontaster validus* (Avila *et al.*, 2000). *Doris kerguelenensis* was proven to possess a wide variety of terpene acylglycerols in the notum (Gavagnin *et al.*, 1995, 1999a,b, 2003a,b; Diyabalanage *et al.*, 2006; Cutignano *et al.*, 2011; Maschek *et al.*, 2012), some of them proven to display anti-predatory activity against *O. validus* (Iken *et al.*, 2002). The metabolites of *D. kerguelenensis* are synthesized through diverse metabolic routes with a remarkable variability among individuals of even the same population (Cutignano *et al.*, 2011). Notwithstanding chemical studies on adults of both species have been performed, whether the egg masses or the embryos of these two species are chemically protected has never been studied before.

In this study, we aim to (1) evaluate the developmental stages of the Antarctic intracapsular developers' *B. hodgsoni* and *D. kerguelenensis*, and provide new information about their egg mass characteristics, embryos, and juveniles, subsequently investigating them with histological methods; and (2) unravel the defensive strategies in early stages of these nudibranchs by rearing the egg masses for several months and analysing the presence/absence of their NPs at different ontogenetic stages of *D. kerguelenensis*; further information on the origin of the compounds is gained by analysing the occurrence of NPs in four of the preyed sponges.

MATERIAL AND METHODS

Sample collection and rearing

Adults, juveniles, and egg masses of *B. hodgsoni* and *D. kerguelenensis* were collected in the eastern Weddell Sea and King George Island by using Agassiz and bottom trawls during the ANT XV/3 (1998) and ANT XXI/2 (2003–2004) cruises on board of the R/V Polarstern. They were preserved in 4% formalin/seawater and subsequently transferred into 70% EtOH. During the latter campaign four egg masses of *D. kerguelenensis* were maintained alive in aquaria and afterwards reared in the lab until 2005 (see Table 1). The egg masses were kept separated in sea water tanks at -2°C, and water was changed every 2–3 days. Additional samples of *D. kerguelenensis* were collected by scuba diving in Livingston Island (South Shetland Islands), during the ACTIQUIM-3 campaign on board of the BIO Las Palmas (2012). Moreover, four sponges, where we found *D. kerguelenensis* feeding on, were collected: the hexactinellid *Rossella* cf. *fibulata* during ANTXXI/2 at 295 m depth, and the demosponges *Haliclona* sp., *Dendrilla antarctica*, and *Mycale (Oxymycale) acerata* during the ACTIQUIM-3 cruise at 15 m depth. Samples selected for chemical analysis were preserved at –20 °C after collection, while those for histological analysis were preserved in 10% formaldehyde/sea water.

Histological analysis

Samples of all developmental stages were dehydrated in a series of alcohol and subsequently embedded in HEMA (Kulzer's method, see Wägele, 1997). Serial sections (2.5 µm thick) were stained with Toluidine blue, which specifically stains acid mucopolysaccharides red to violet, and neutral mucopolysaccharides and nucleic acids, as well as proteins in various blue shades.

Estimation of embryonic periods

In order to estimate the developmental time of the two species, we applied the equation proposed by Thompson and Jarman (1986) for heterobranchs, which considers egg capsule size and water temperature:

$$P = (2.78 \cdot 10^{-8}) \cdot D^{0.775} \cdot e^{4687/T}$$

where P is the embryonic period in days, D the egg capsule diameter in micrometres, and T the absolute temperature in Kelvins (273.5 K or 0 °C).

Table 1. Samples of *D. kerguelenensis* collected during the Antarctic cruises ANTXV/3, ANTXXI/2, and ACTIQUIM-3 in the Weddell Sea and South Shetland Islands (King George and Livingston Islands) and analysed in this study. Austasen, Kapp Norvegia, North Halley, and Vestkapp refer to the eastern Weddell Sea. Some early juveniles were artificially hatched with a scalpel.

Sample	*N*	Location	Latitude (S)	Longitude (W)	Depth (m)	Gear[a]	Collected	Fixed	Fixed in	Analysis
Egg mass	1	Austasen	70° 57.00'	10° 33.02'	333	BT	24/12/2003	09/09/2005	10 % Formaline	morphology/ histology
Egg mass	1	Austasen	70° 57.00'	10° 33.02'	333	BT	24/12/2003	08/06/2005	Frozen	chemistry
Egg mass	1	Austasen	70° 52.75'	10° 51.24'	295	BT	27/12/2003	11/01/2004	10 % Formaline	morphology/ histology
Egg mass	1	Austasen	70° 52.75'	10° 51.24'	295	BT	27/12/2003	09/09/2005	Frozen	chemistry
Egg mass	1	Austasen	71° 07.15'	11° 26.23'	228	AT	29/12/2003	09/09/2005	10 % Formaline	morphology/ histology
Egg mass	1	Austasen	71° 07.15'	11° 26.23'	228	AT	29/12/2003	21/12/2005	Frozen	chemistry
Egg mass	1	Austasen	70° 57.00'	10° 33.02'	333	BT	24/12/2003	24/12/2003	10 % Formaline	morphology/ histology
Egg mass	1	Austasen	70° 57.11'	10° 33.32'	337	BT	16/12/2003	11/01/2004	10 % Formaline	morphology/ histology
Egg mass	1	Austasen	70° 56.67'	10° 32.05'	302	BT	13/12/2003	11/03/2004	70% EtOH	morphology
Egg mass	2	Livingston Island	62° 39.9'	60° 36.2'	0-15	SD	02/09/2012	02/09/2012	Frozen	chemistry
Early, artificially-hatched juvenile from egg mass	1	Austasen	70° 56.67'	10° 32.05'	302	BT	06/01/2004	11/01/2004	10 % Formaline	morphology/ histology
Early, artificially-hatched juvenile from egg mass	1	Austasen	70° 56.67'	10° 32.05'	302	BT	13/12/2003	20/12/2003	70% EtOH	morphology
Early, naturally-hatched juvenile from egg mass	2	Austasen	70° 56.67'	10° 32.05'	302	BT	01/01/2004	11/01/2004	10 % Formaline	morphology/ histology
Early, naturally-hatched juvenile from egg mass	2	Austasen	70° 56.67'	10° 32.05'	302	BT	13/12/2003	20/12/2003	10 % Formaline	morphology/ histology
Early, naturally-hatched juvenile from egg mass	1	Austasen	70° 56.67'	10° 32.05'	302	BT	13/12/2003	20/12/2003	70% EtOH	morphology

Juvenile found in the field	1	Vestkapp	72° 54.52'	19° 47.74'	694	RD	03/01/2004	03/01/2004	10 % Formaline	morphology/ histology
Juvenile found in the field	1	Austasen	70° 51.8'	10° 26.5'	266	AT	30/01/1998	30/01/1998	Frozen	chemistry
Juvenile found in the field	1	Austasen	70° 52.3'	10° 29.0'	246	AT	31/01/1998	31/01/1998	Frozen	chemistry
Juvenile found in the field	1	Austasen	70° 54.0'	10° 28.2'	232	AT	31/01/1998	31/01/1998	Frozen	chemistry
Juvenile found in the field	2	North Halley	74° 40.3'	27° 6.0'	567	BT	11/02/1998	11/02/1998	Frozen	chemistry
Juvenile found in the field	1	North Halley	75° 26.9'	26° 48.3'	225	BT	12/02/1998	12/02/1998	Frozen	chemistry
Juvenile found in the field	1	Kapp Norvegia	71° 18.0'	12° 15.0'	184	AT	27/02/1998	27/02/1998	Frozen	chemistry
Juvenile found in the field	1	King George Island	62° 20.3'	58° 35.6'	601	AT	17/03/1998	17/03/1998	Frozen	chemistry
Adult	1	Kapp Norvegia	71° 40.3'	12° 43.5'	244	AT	15/02/1998	15/02/1998	Frozen	chemistry
Adult	1	North Halley	75° 04.9'	27° 25.1'	411	BT	02/12/1998	02/12/1998	Frozen	chemistry
Adults	2	Austasen	70° 46.8'	10° 21.5'	309	BT	02/01/1988	14/03/1988	Frozen	chemistry
Adult	1	North Halley	73° 34.3'	22° 00.9'	519	BT	02/05/1998	02/05/1998	Frozen	chemistry
Adults	2	Austasen	70° 50.5'	10° 41.8'	307	BT	19/02/1998	19/02/1998	Frozen	chemistry
Adult	1	Vestkapp	72° 49.8'	19° 26.4'	473	AT	25/02/1988	25/02/1988	Frozen	chemistry
Adult	1	Kapp Norvegia	71° 14.0'	12° 27.9'	247	AT	16/02/1998	16/02/1998	Frozen	chemistry
Adult	1	Vestkapp	72° 50.5'	19° 24.2'	439	BT	02/03/1998	02/03/1998	Frozen	chemistry
Adult	1	Vestkapp	72° 50.5'	19° 28.0'	463	BT	02/03/1998	02/03/1998	Frozen	chemistry
Adults	2	North Halley	74° 40.3'	27° 06.0'	567	BT	02/11/1998	02/11/1998	Frozen	chemistry
Adult	1	Kapp Norvegia	71° 17.0'	12° 36.2'	414	AT	16/02/1998	16/02/1998	Frozen	chemistry
Adult	1	Austasen	70° 50.5'	10° 41.8'	231	BT	19/02/1998	19/02/1998	Frozen	chemistry
Small (2.4 mm) and large (149 and 160 mm) adults	3	Austasen	70° 52.75'	10° 51.24'	295	BT	27/12/2003	27/12/2003	Frozen	chemistry
Adult	1	North Halley	73° 36.5'	22° 23.8'	748	BT	02/07/1998	02/07/1998	Frozen	chemistry
Small adults (26 and 28 mm)	2	Livingston Island	62° 41.8'	60° 19.7'	0-15	SD	13/02/2012	13/02/2012	Frozen	chemistry
Adults	15	Livingston Island	62° 39.9'	60° 36.2'	0-15	SD	02/09/2012	02/09/2012	Frozen	chemistry

[a] *AT* Agassiz trawl, *BT* bottom trawl, *SD* scuba diving

Chemical analyses

Egg masses as well as early developmental stage samples (including eggs, embryos, and juveniles) of *D. kerguelenensis* were extracted individually, while adults were dissected into mantle and viscera. Samples were soaked in acetone and extracted in an ultrasonic bath (~1 min), successively ground in a mortar with a pestle (thrice). Extracts were concentrated under *vacuum*, and the resulting aqueous suspensions were partitioned with diethyl ether (thrice). TLC comparative analyses of the lipid extracts were carried out in light petroleum/diethyl ether (1:1). Purification of the extract was performed on a silica gel column using a petroleum ether/diethyl ether gradient. We followed the procedures of Cutignano *et al.* (2011) for isolation and characterization of the compounds. All ethereal extracts and purified fractions were analysed by LC-APCI/MS and/or NMR spectroscopy.

The four diet sponges collected were also separately grounded in a mortar with pestle and extracted (thrice) with methanol after ultrasonic bath (~5 min). The organic fraction of the extracts was evaporated *in vacuo*, and the resulting aqueous suspension was partitioned into diethyl ether (thrice). Ether extracts were analysed by TLC with petroleum ether/diethyl ether (8:2, 1:1, 2:8), and then revealed by UV and cerium sulfate. The ether extracts were purified on a silica column using an eluent gradient of light petroleum ether (LP)/diethyl ether (EE) (100% LP 9:1, 8:2, 7:3, 1:1, 2:8, 100% EE). Fractions were analysed by ^{1}H-NMR and LC-MS.

All NMR spectra were acquired in $CDCl_3$ (shifts are referenced to residual proton signal at δ 7.26) on a Bruker DRX-600 operating at 600 MHz, using an inverse TCI CryoProbe fitted with a gradient along the Z-axis. LC-MS analyses were carried out under isocratic conditions with *n*-hexane/2-propanol 97:3 for monoacyl- and 99.8:0.2 for diacyl-glycerides by a silica gel column (Phenomenex, Kromasil Si 5µm, 100A, 250x4.6mm, flow 1ml/min) on Alliance HPLC system (Waters) coupled with a QTof*micro* (Waters) equipped with an APCI probe operating in positive ionization mode.

RESULTS

Bathydoris hodgsoni *egg masses and embryos*

(Figures 1, 2)

Material examined: Four egg masses collected from the eastern Weddell Sea during ANT XXI/2. Two with a single egg capsule each collected at 274 m depth, 11/12/2003 (PS65/121), and two with two egg capsules each at 302 m depth, 13/12/2003 (PS65/148), and 337 m depth, 16/12/2003 (PS65/175).

Egg mass (Fig. 1A): Maximum size of 124 x 68 x 14 mm (length:width:height). One or two egg capsules, elongated, flat, large, yellowish, slightly iridescent; measuring 48.8 ± 3.6 x 44.6 ± 0.55 x 12.6 ± 0.9 mm (mean±sd; length:width:height). Egg clutch thick, membranous, semi-transparent. A single egg capsule containing cream-coloured, crescent-shaped body within basal part, shining through capsule wall. One late juvenile observed inside each capsule.

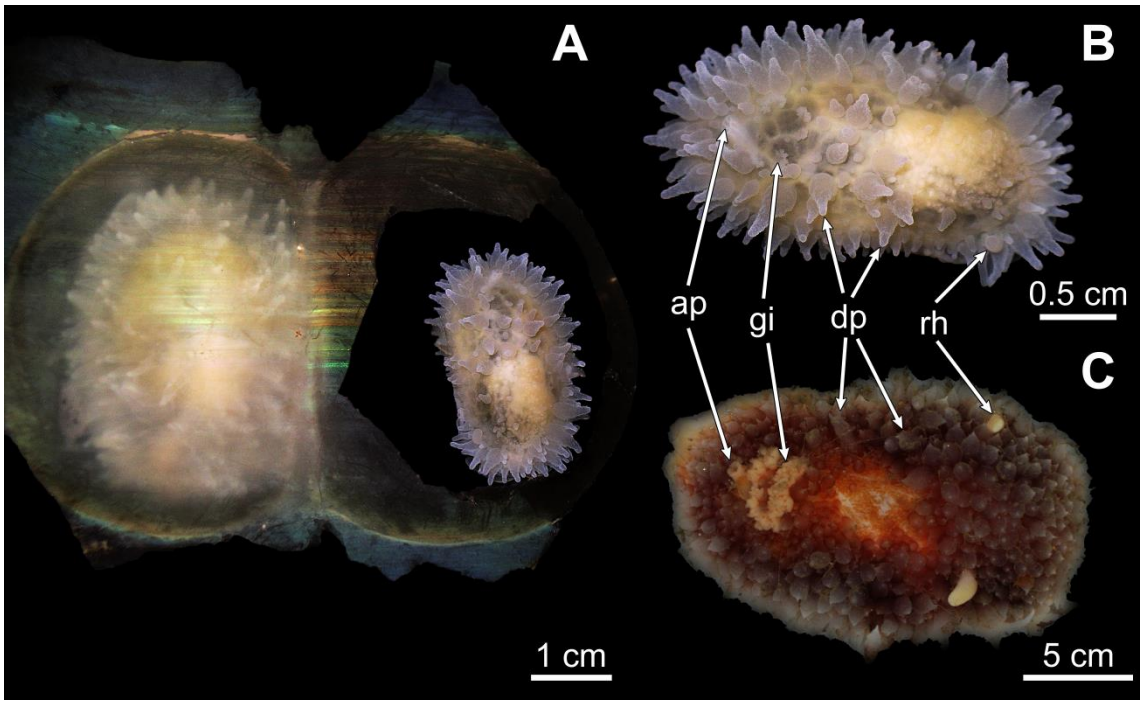

Figure 1. Developmental stages of *Bathydoris hodgsoni*. **A** – Egg mass with two egg capsules containing developed embryos; one capsule was artificially opened. **B** – Detailed view of a 29 mm long developed embryo. **C** –Adult. *ap* anal papilla; *dp* dorsal papillae; *gi* gills; *rh* rhinophores.

External morphology (Fig. 1B): Late juveniles found inside egg capsule measured up to 29 x 18 mm (length:width); white-cream coloured; notum thin, transparent; internal organs brownish. Rhinophores developed. Six tiny gills surrounding anal papilla dorsally in semicircle. Velar tentacles present. Papillae rugose, white-transparent, conical, variable in size; covering dorsal notal surface and margins; some easily released upon manipulation. Foot developed, whitish. Notum and foot with reticulated pattern seen by transparency due to yolk content and tissue involved (see below).

General anatomical and histological considerations: Epidermis consisting of specialized vacuolated cells, mucus cells containing granules of acid mucopolysaccharides interspersed (Fig. 2A); better developed in anterior and

posterior body regions. Dorsal papillae containing cells with large non-staining vacuoles; some completely filled with yolk; containing muscles at base (possible autotomy function) and in longitudinal direction, allowing contraction. Connective tissue and muscles in anterior part of body less developed than posterior part. Visceral cavity filled with yolk, containing many cells, representing embryonic connective tissue cells. Oral tentacles filled with yolk. Rhinophores filled with connective tissue and muscles; large cells, presenting large non-staining vacuole at base. Notum wall composed of few muscle fibres and interspersed connective cells, but mainly filled with yolk; containing large dorsal cells filled with numerous, tiny, blue-staining granules and a very large nucleus (Fig. 2B), sometimes leading outside, probably representing excretory cells. Connective tissue and muscles present around kidney and heart; much better developed in right and posterior body regions. Foot gland follicles developed.

Digestive system: Digestive system generally well developed, completely filled with lipid-rich yolk, homogenously staining dark-blue. Oral tube extremely short, labial disc lying in mouth region. Oral glands few, not developed. Jaws present. Radula present, at least with several rows and several teeth per row. Pharynx containing few, distinct muscles. Oesophagus developed, highly folded, epithelium covered by thin cuticle; some cells disintegrating, probably due to inadequate preservation. Stomach wide, folded, with columnar, ciliated cells (Fig. 2C). Digestive gland incompletely developed, better developed in posterior part; large, composed of large follicles, forming compact mass (Fig. 2D); cells filled with huge vacuoles staining homogenously dark-blue, similarly to yolk (Fig. 2E). Intestine forming a loop. Anal papilla lying dorsally, internally folded, cells containing long cilia.

Genital system: Undeveloped.

Nervous system: Cerebral, pleural, and pedal ganglia present. Velar and rhinophoral nerves present, large neuronal nuclei containing heterochromatin surrounding central axis. Statocysts developed. Nerves within dorsal papillae present.

Circulatory and excretory systems (Fig. 2F–H): Pericardium with muscular ventricle and thin, non-muscular auricle developed. Blood gland composed of numerous glandular follicular glands (as described in Wägele, 1989a), also occurring outside of pericardium, very close to auricle (Fig. 2G, H); differing from large excretory cells lying subepithelial (Fig. 2B). Kidney developed, forming saccular structure, lying on top of digestive gland; forming folds intermingled with digestive gland; cells small, containing distinct nucleus and non-staining vacuole. Syrinx internally highly folded, with long cilia (Fig. 2F).

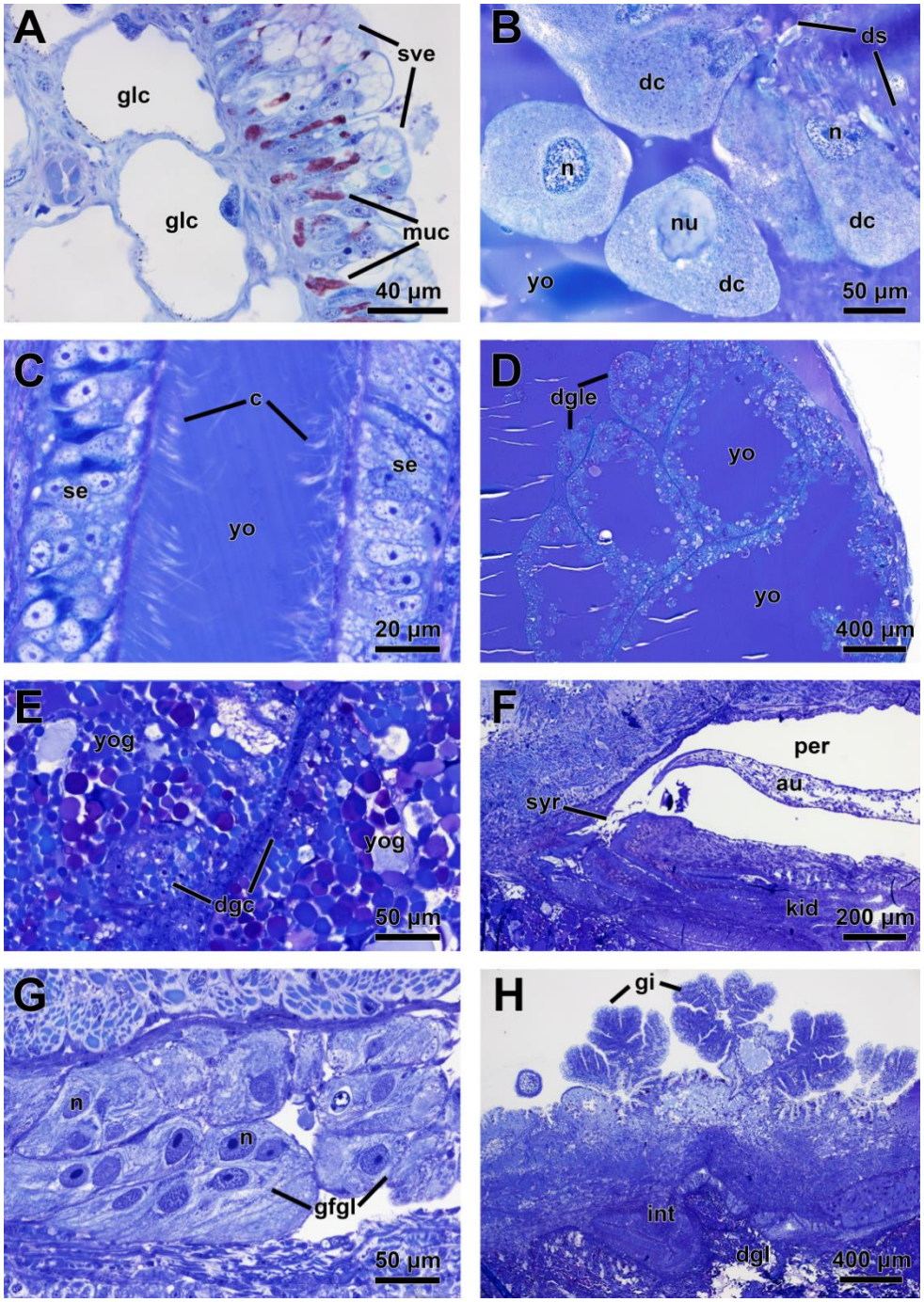

Figure 2. Histological sections of *Bathydoris hodgsoni* juveniles. **A** – Detail of epithelium of dorsal papillae with vacuolized epidermal cells and large, non-staining, subepithelial gland cells. **B** – Large dorsal cells lying in dorsal notal tissue within a large yolk mass. **C** – Detail of stomach containing yolk. **D** – Digestive gland with yolk in the lumen of the digestive gland tubes. **E** – Detail of digestive gland epithelium. **F** – Cross section through heart region; syrinx connecting excretory with circulatory systems. **G** – Follicles of glandular cells lying in auricle and close to it. **H** – Transverse section of posterior body region, showing the intestine close to the dorsal gills. *au* auricle; *c* cillia; *dc* dorsal cell; *dgc* digestive gland cells; *dgle* digestive gland epithelium; *dgl* digestive gland; *ds* dorsal septum; *gfgl* glandular follicular glands; *gi* gills; *glc* glandular cells; *int* intestine; *kid* kidney; *muc* mucus glandular cells; *n* nucleus; *nu* nucleolus; *per* pericardium; *se* stomach epithelium; *sve* specialized vacuolated epithelium; *syr* syrinx; *yo* yolk; *yog* yolk granules.

D. kerguelenensis *egg masses, embryos, and juveniles*

(Figures 3–5)

Material examined: see Table 1. Juveniles were classified into (1) early, artificially-hatched, with a scalpel at the lab; (2) early, naturally-hatched; and (3) late, found in the sea, larger and more developed.

Egg masses (Fig. 3A–C): Ribbon-like, surrounded by transparent membrane, disposed in semi-close circle; yellowish; measuring 115 ± 64 x 27 ± 6 x 3.5 ± 0.7 mm (mean±sd; length:width:height); containing 1,500–2,400 egg capsules (25–28 egg/cm^2). Egg capsules measuring 1,740 ± 684 x 1,222 ± 395 µm (mean±sd; length:width), capsule thickness 219 ± 54 µm; yellowish; squared- or rhomboid-shaped. Egg capsule containing spherical holes (Fig. 4A) increasing in number and size throughout larval development; thinner when juveniles hatch. Two to eight cell and morula stages seen externally inside egg capsules (Fig. 3B). Veliger larvae not visible through egg capsule.

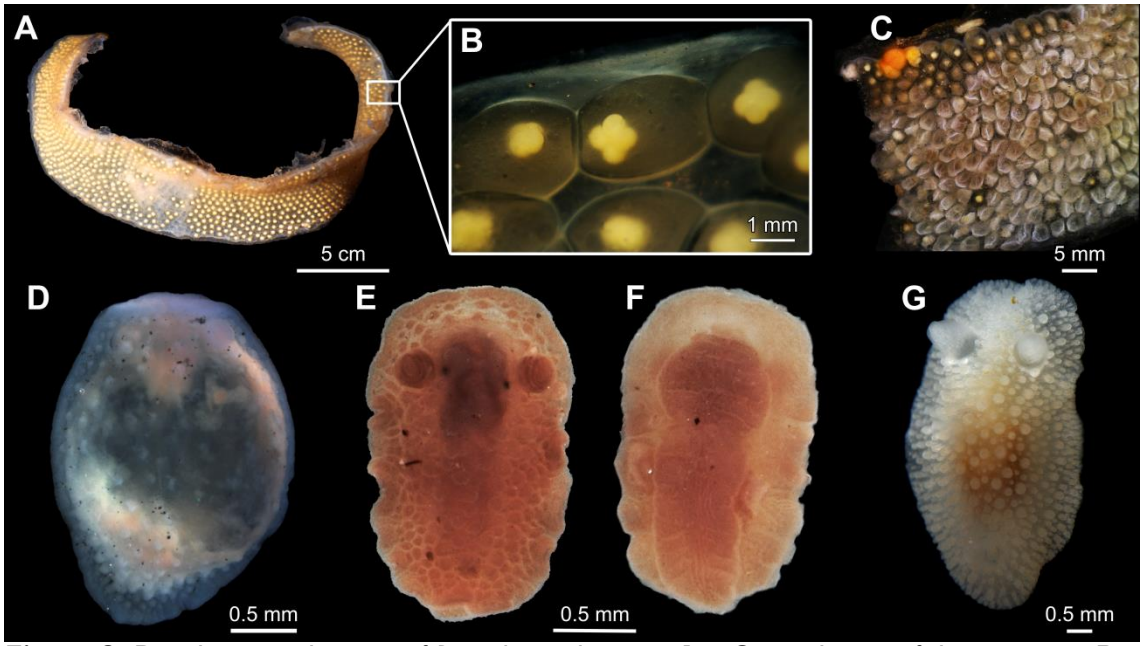

Figure 3. Developmental stages of *Doris kerguelenensis*. **A** – General view of the egg mass. **B** – Close-up of the egg mass edge showing embryos at 4-cells stage. **C** – Detail of the egg mass with early juveniles probably ready to hatch inside egg capsules. **D** – Early, artificially-hatched juvenile (2.5 mm long) with chubby appearance, note absence of anus on the dorsal side. **E** – Early, naturally-hatched juveniles (3 mm) with dorsal anus. **F** – Late, well-developed juvenile (5 mm long) found inside the hexactinellid sponge *Rossella* cf. *fibulata*, gills present.

Larval development (Fig. 3, 4): Histological sections showed veliger larvae with a distinct velum, shell, statocysts, and foot (Fig. 4A); in close touch with capsule elements (Fig. 4B), but only when veliger is fully formed. External, live investigations showed

development slightly heterogeneous along egg mass; juveniles observed inside capsule (Fig. 3C).

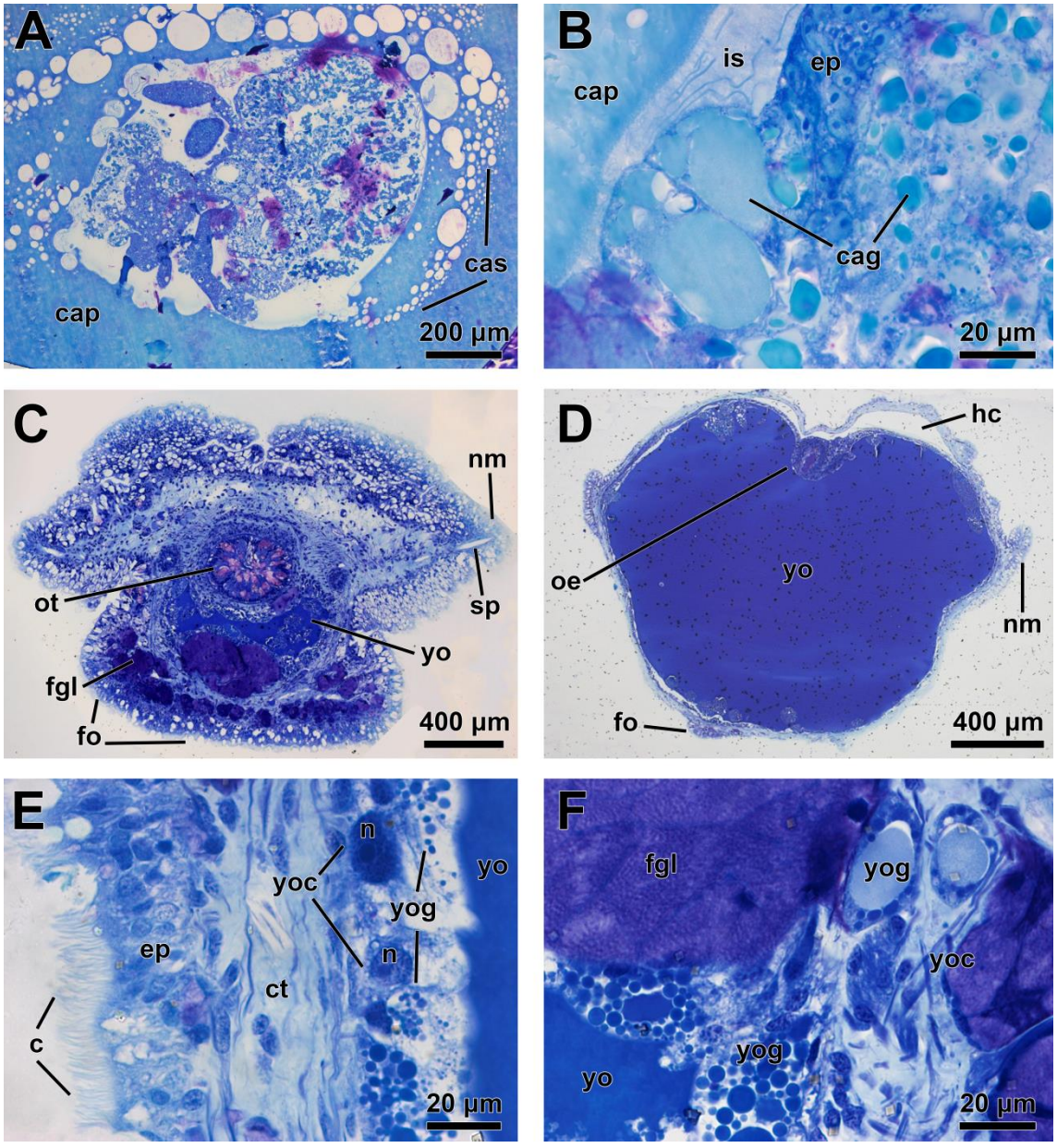

Figure 4. Histological sections of *Doris kerguelenensis* the veliger larva, and an early, artificially-hatched juvenile. **A** – Veliger larva inside egg capsule, note large spherical holes in capsule and the close contact of embryonic tissue with the walls within the holes. **B** – Detail of the veliger, note capsule digestion and granule uptake by the larva. **C** – Transverse section of the anterior region of an early juvenile. **D** – Transverse section of the middle region of the same early juvenile. **E** – Detail of yolk cells uptake close to external epithelium. **F** – Detail of yolk cells uptake close to foot gland. *c* cillia; *cag* capsule granules; *cap* capsule; *cas* capsule spheres; *ct* connective tissue; *ep* epithelium; *fgl* foot gland; *fo* foot; *hc* hemolymphatic cavity; *is* interstitial space; *n* nucleus; *nm* notal margin; *oe* oesophagus; *ot* oral tube; *sp* spicule; *yo* yolk; *yoc* yolk cells; *yog* yolk granules.

External morphology of juveniles (Fig. 3D–G): Early, hatching juveniles (36 individuals analysed) measuring 2.91 ± 0.33 x 1.93 ± 0.28 mm (length:width) on average, some reaching 4.7 mm in length; moving actively inside egg capsule, crawling actively when hatched. Notum covered by small, conical tubercles, increasing in size while cultivated in aquarium; subepithelial spicules few, interspersed. Rhinophores present, laminae scarce, less distinguished than in late juveniles (Fig. 5A, B). Eyes visible through transparent notum. Oral tentacles present. Foot developed. Gills not developed in these stages, only in late juveniles already hatched in the field (5 mm; Fig. 3G), and collected inside the sponge *Rossella* cf. *fibulata*. Early, artificially-hatched juveniles chubby due to lipid content (Fig. 3D).

General anatomical and histological considerations: Organs and tissues better developed in anterior and posterior body parts, but not so much in the middle area (Fig. 4C, D). Specialized vacuolated epithelium present. Glandular cells containing huge vacuoles, with non-staining or light-blue contents; ubiquitous in epithelium, densely concentrated in notal rim and dorsal tubercles. Mucus glandular cells containing acid mucopolysaccharides scarcely interspersed in epithelium. Connective tissue cells, muscle fibres, and spicules in connective tissue of notum wall present; spicules more abundant in late juveniles. Hemolymphatic cavity distinct, dorsal of digestive system. Foot glands developed in anterior and posterior body regions of early, artificially-hatched juveniles; homogenously spread along foot in early, naturally-hatched and late juveniles. Yolk homogeneously dark-purple; placed mainly ventrally in between digestive tract and foot; very voluminous, especially in middle region of body (giving rounded appearance to early, artificially-hatched juveniles; Fig. 3D, 4D); periphery of yolk mass with cells containing yolk droplets, probably being transported to other tissues (Fig. 4E, F); anterior and posterior body regions containing more yolk droplets; completely lacking in late juveniles.

Digestive system: Oral tube developed (Fig. 4C); oral glands rather scarce in early juveniles. Labial disc present. Jaws not completely developed. Pharynx with cuticle lining. Odontophore developed, muscle fibres present. Radular teeth increasing in number and length throughout ontogeny (Fig. 5E, F). Salivary glands paired, increasing in side throughout development. Oesophagus present (Fig. 4D). Stomach not clearly delimited; undistinguished from whole yolk mass. Digestive gland not distinguishable in early juveniles; developed in late juveniles (Fig. 5G, H). Intestine developed; running backwards. Anus lying posteroventrally between posterior notum and foot tail in early, artificially-hatched juveniles (Fig. 3D); lying dorsally in early, naturally-hatched and late juveniles (Fig. 3E, F).

Reproductive system: Not developed in any of the juveniles analysed.

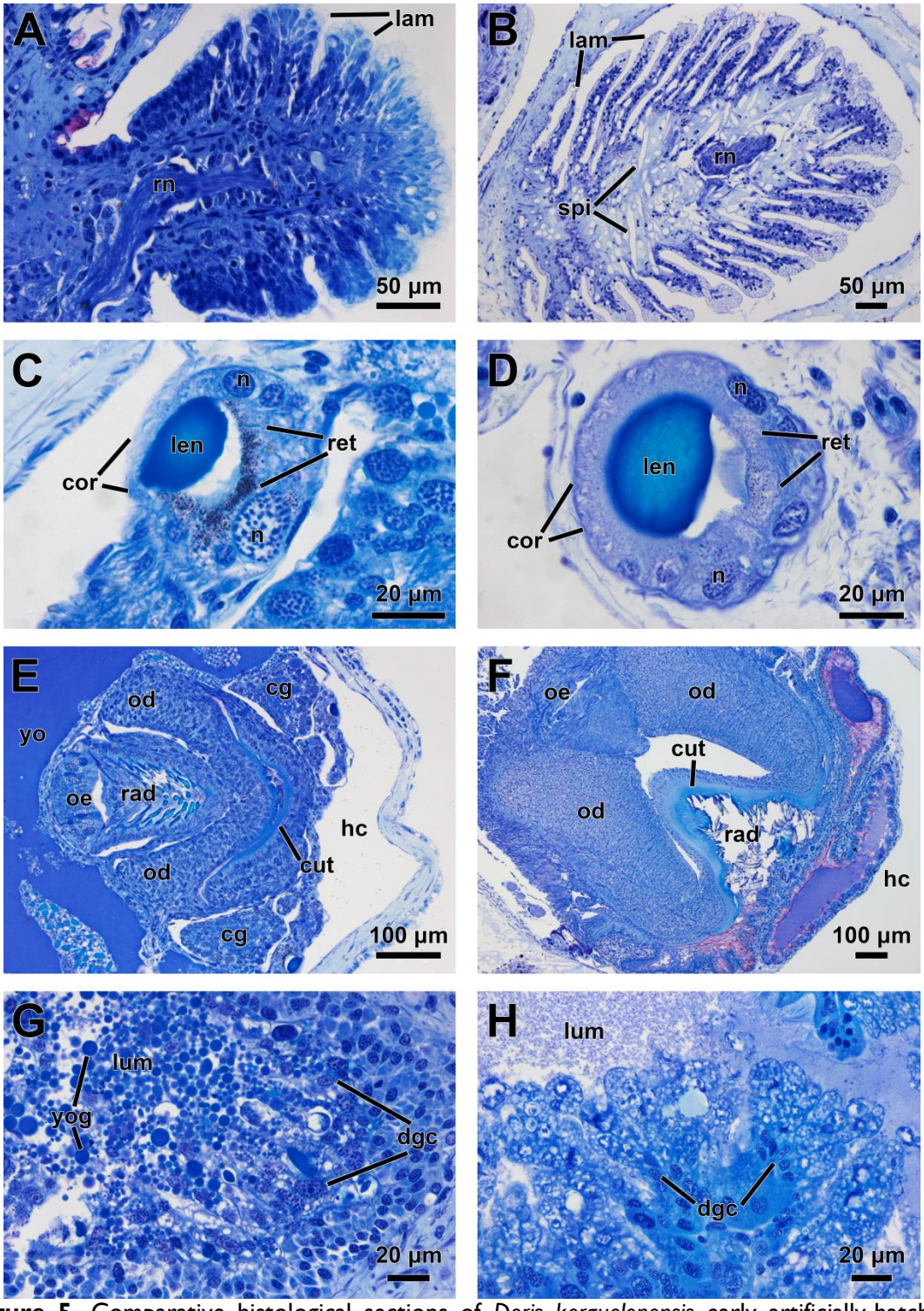

Figure 5. Comparative histological sections of *Doris kerguelenensis* early artificially-hatched juveniles (2.5 mm; A, C, E, G), and late, hatched in the sea (5 mm; B, D, F, H). **A, B** – Rhinophore, note the smaller number of lamellae in the smaller animal. **C, D** – Eye, note the less developed lens and cornea. **E, F** – Pharynx. **G, H** – Detail of digestive gland with many yolk granules still present in the smaller animal and none in the larger. *cg* cerebral ganglion; *cor* cornea; *cut* cuticle; *dgc* digestive gland cells; *hc* hemolymphatic cavity; *lam* laminae; *len* lens; *lum* lumen; *n* nucleus; *nm* notal margin; *od* odontophore; *oe* oesophagus; *rad* radula; *ret* retina; *rn* rhinophoral nerve; *spi* spicule; *yo* yolk; *yog* yolk granules.

Nervous system: Cerebral ganglia present. Rhinophoral nerve in central axis less structured in early juveniles than in late juveniles. Eyes containing lens and retina, cornea not well developed in early juveniles (Fig. 5C, D). Pleural and pedal ganglia present, as well as statocysts in between. Cortex clearly differentiated from neuropile in the ganglia; cortical neurons with large nucleus containing heterochromatin in early juveniles. Ganglia larger and cortex better differentiated from neuropile in late juveniles.

Circulatory and excretory systems: Pericardium present. Auricle and ventricle undifferentiated in early juveniles; clearly differentiated in late juveniles. Kidney and syrinx present. Nephroduct close to anal papilla; ventrally in early juveniles, dorsally in late juveniles. Gills absent in early juveniles; present in late juveniles (Fig. 3D–F).

Chemical analyses of D. kerguelenensis

The extracts of all sampled developmental stages of *D. kerguelenensis* were analysed by TLC, LC-MS, and NMR. Extracts of whole egg ribbons immediately collected from deep waters of the eastern Weddell Sea (two) and shallow waters from Livingston Island (two) had no trace of either monoacyl- or diacyl-glycerides. Intracapsular ontogenetic stages of the three egg masses reared were extracted individually and compared by LC-MS with known monoacyl- and diacyl-glycerides LC-MS data, showing no trace of the NPs. Eight juveniles (< 10 mm) collected in the sea (eastern Weddell Sea) presented terpene monoacyl- and diacyl-glyceride derivatives detected by TLC and LC-MS ($M\text{-}H_20+H^+$ at *m/z* 361 and 403, respectively). All adults evaluated presented NPs, exclusively present in mantle tissues: those from the Weddell Sea presented monoacyl- and diacyl- terpene glycerides showing a labda-8-en-15-oyl skeleton (Cutignano *et al.*, 2011), while specimens from Livingston Island contained palmadorin C (Diyabalanage *et al.*, 2010). Finally, none of the four diet sponges analysed showed the typical glycerides isolated from *D. kerguelenensis*, or their precursors.

DISCUSSION

Both *B. hodgsoni* and *D. kerguelenensis* possess similar reproductive strategies, but differ considerably in egg capsule size and number. Egg capsules of *B. hodgsoni* are few (1 to 4), and larger (52.4 x 45.2 x 13.5; length:width:height), than in any other mollusc taxon (Table 2), perhaps representing the largest eggs of any marine invertebrate. Contrary, *D. kerguelenensis* possesses ribbon-like egg masses containing thousands of egg capsules (Gibson *et al.*, 1970; Wägele, 1989a). We presented evidence of intracapsular development in the two anthobranchs studied (Type 1; Hain & Arnaud, 1992), with crawling juveniles of 29 and 3 mm in length after hatching for *B. hodgsoni* and *D. kerguelenensis*, respectively. Extraembryonic yolk uptake occurs either at larval or

juvenile stages, thus allowing the growth of such large embryos. Consequently, adults of *B. hodgsoni* reach up to 200 mm in length and 472 g in mass (see Fig. 1C; Avila *et al.* 2000). However, an obligate correlation of egg capsule and adult seems unlikely since larger heterobranch species than *B. hodgsoni*, such as *Aplysia* spp. (up to several kg in mass) present very small egg capsules (< 150 µm; Ros, 1981). In addition, *D. kerguelenensis* reaches up to 160 mm length with a mass of 172.5 g (Iken *et al.*, 2002), while its egg capsules are far smaller than those of *B. hodgsoni*.

Egg masses of both species collected in two austral seasons (late spring and summer) were in different developmental stages, starting from an early morula up to juveniles of a few millimetres. Therefore, our data support the hypothesis of continuous reproduction throughout the year, as described for deep-sea and other Antarctic benthic invertebrates (Picken, 1979, Tyler *et al.*, 1982, Pearse *et al.*, 1991). According to our estimations, the two anthobranchs studied here seem to have the longest embryonic period known for molluscs. We estimated an embryonic period of 3,577 days (9.8 years) for *B. hodgsoni*, and 390 days (13 months) for *D. kerguelenensis*, using the formula reported above (Thompson & Jarman, 1986). These estimates are longer than the time previously suggested for *B. hodgsoni* (2.5 years; Wägele, 1996), and shorter than that described for *D. kerguelenensis* (21 months; Hain, 1989). If the estimates for *B. hodgsoni* are correct, this species would have the longest lifetime of any heterobranch mollusc. The benefit for such long embryonic periods is unknown. In fact, long developmental times may be a consequence of slow metabolism in the cold, highly stable environments of the Southern Ocean. The equation to calculate this, however, was not formulated for such large egg capsules (Thompson & Jarman, 1986), and therefore these estimations should be considered with caution. Our data support the assumption, though, that *B. hodgsoni*, as other Antarctic invertebrates (Pearse *et al.*, 1991), are distinguished by a high longevity, only comparable to *Nautilus* molluscs (up to 20 years; Saunders, 1984).

Long intracapsular development requires large amounts of yolk, and even their own capsule may serve as an additional nutrition, as suggested for both species (Wägele, 1989a, 1996). The capsule is the result of packing the usually viscous albumen into a compact layer, therefore providing more stability for the egg capsule (Klussmann-Kolb & Wägele, 2001). Here, our histological observations on *D. kerguelenensis* show the uptake of capsule elements, thus this species consumes the capsule during the veliger and posterior embryonic stages. In this way, juveniles of *D. kerguelenensis* have more food available and can hatch more easily from the egg capsules, when the capsule is thinner. In the median part of *D. kerguelenensis* early juvenile, along the longitudinal axis, a large reservoir of yolk seems to be present, containing peripheral connective cells that uptake and transport yolk granules. Yolk is completely digested in later juveniles found in the field, where the digestive gland is fully developed, and the reproductive system begins to mature. In the two species

studied, some organs seem to develop first, which are lying more in the anterior or posterior parts of the body. These are the central nervous system and the anterior and posterior parts of the digestive, excretory, and circulatory systems. We observed a delayed development at least in the rhinophores, eyes, radula and digestive glands (Fig. 5). In the case of *D. kerguelenensis,* the anus is subventral in earlier postlarval stages, and later migrates to the dorsum, as sugested for cryptobranch anthobranchs (Martynov, 2011). However, this still has to be shown for the genus *Bathydoris*.

Thorson's rule states that there is a trend toward increased egg size (with more yolk available for nutrition) and non-planktonic development along gradients of increasing latitude and water depth (Thorson, 1936). Nevertheless, there are many exceptions described (Pearse *et al.*, 1991; Palmer, 1994; Levin & Bridges, 1995; Clarke, 2008), including some nudibranchs (Clark & Goetzfried, 1978; Ros, 1981; Moles *et al.*, 2016). Factors such as food availability or energy budgets may have a strong influence on reproductive strategies. However, these factors might not be a limitation to both anthobranchs studied herein, since they possess a broad dietary spectrum, and most of the food items have a lifetime of many years (McDonald & Nybakken, 1996; Avila *et al.*, 2000; Iken *et al.*, 2002). Thus, limited dispersal of embryos would be enough for ensuring the survival of the species studied here, which live in a very stable environment with high predictability and continuity of food conditions. Since long developmental times for embryos of both species thrives the exposure to predators (Pearse *et al.*, 1991; Wägele, 1996), embryos of both species might rely on physical defence of such thick egg capsules (2 mm in *B. hodgsoni* and 0.3 mm in *D. kerguelenensis*; Wägele, 1989b, 1996). Here, we found that the four diet sponges of *D. kerguelenensis* evaluated lacked NPs, as well as all egg masses and the embryos within the eggs. However, the small juveniles collected from the sponge already showed distinct traces of diacylglycerols. Unfortunately, we do not have any information about *Bathydoris* juveniles after hatching, but individuals of *B. hodgsoni* of 9.5 cm long already contain defensive NPs (ref). The hodgsonal identified in these individuals is found in similar concentrations as in larger adults (0.08% dry weight in the mantle), and is suggested to have a biosynthetic origin (Avila *et al.*, 2000). All these facts provide good evidence that hatched juveniles of both species rely on biosynthesized NPs as a chemical anti-predatory strategy, similarly to other Antarctic nudibranchs (Moles *et al.*, 2016). Overall, we suggest that both anthobranchs compensate the low numbers of juveniles produced by reducing the mortality during both embryonic and adult stages. This is achieved by a physical defence strategy (very thick egg capsule) in the intracapsular development and by a chemical defence strategy, as soon as the animals hatch. In conclusion, the complementarity of developmental, defensive, and trophic strategies becomes essential in the harsh environmental conditions of the Southern Ocean, and this might partly explain the evolutionary success of both ubiquitous and abundant sea slug species. More studies are needed in other species to establish whether this is a general trend of Antarctic heterobranch molluscs.

Table 2. Largest sizes reported for molluscan egg capsules.

Species	Group	Length (mm)	Distribution	Reference
Terrestial				
Archachatina sp.	Heterobranchia: Eupulmonata	23	Africa	Abbott (1989)
Megalobulimus popelairianus	Heterobranchia: Eupulmonata	51	South America	Standen (1917)
Marine				
Nautilus belauensis	Cephalopoda: Nautilida	20	Pacific Ocean	Arnold & Carlson (1986)
Graneledone boreopacifica	Cephalopoda: Octopoda	39.6	North Pacific	Voight & Drazen (2004)
Megaleledone setebos	Cephalopoda: Octopoda	41.5	Antarctica	Allcock *et al.* (2003)
Bathydoris clavigera	Heterobranchia: Nudibranchia	13	Antarctica	Wägele (1989b)
Bathydoris hodgsoni	Heterobranchia: Nudibranchia	52	Antarctica	This study

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Section II

Beyond taxonomy:

Towards the past and present of Heterobranchia

“The Opisthobranch Gastropoda are to the Mollusca what the orchids are to the angiosperms, or the butterflies to the arthropods”

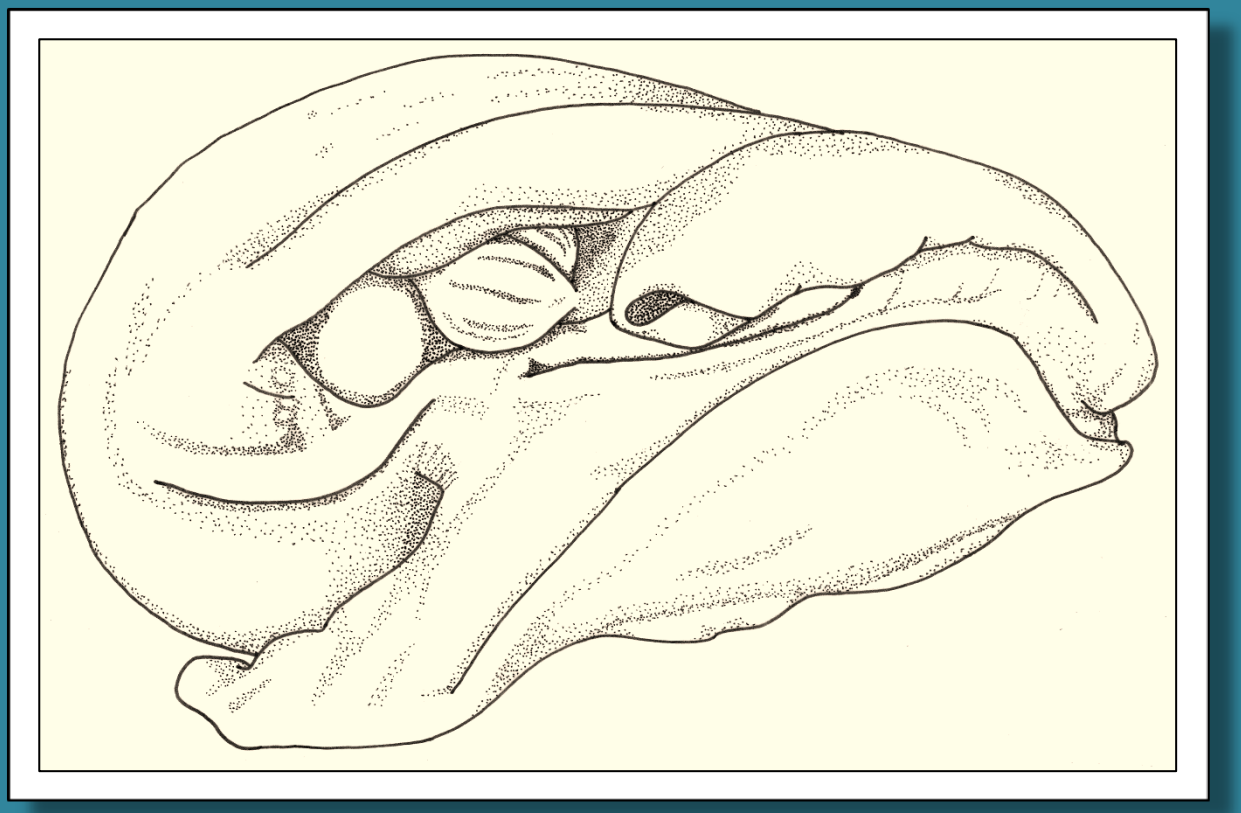
T. E. Thompson

“…besides the Antarctic ones, these are colorless.”

J. Moles

Chapter 5

An Antarctic opisthobranch clade is sister to all other Cephalaspidea (Gastropoda: Heterobranchia)



Moles J, Wägele H, Schrödl M, Avila C (*In press*) An Antarctic opisthobranch clade is sister to all other Cephalaspidea (Gastropoda: Heterobranchia). *Zoologica Scripta*

Chapter 5. A new Antarctic heterobranch clade is sister to all other Cephalaspidea (Mollusca: Gastropoda)

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ABSTRACT

For a long time Diaphanidae has been considered a basal family within Cephalaspidea, based on the presence of plesiomorphic morphological features within this taxon. Traditionally the family contained the genera *Bogasonia*, *Colobocephalus*, *Colpodaspis*, *Diaphana*, *Newnesia*, *Toledonia*, and *Woodbridgea.* Some phylogenetic analyses of several of these genera support the basal position of Diaphanidae within Cephalaspidea *sensu stricto.* However, the family is presently confirmed to be a polyphyletic taxon in which only the genus *Diaphana* is included. Several genera previously embraced within the family, such as the monotypic *Newnesia*, have never been previously analyzed in molecular studies. Here we provide an extensive morphological, anatomical, and histological description of a new species of *Newnesia* from Antarctic deep waters (967–1227 m depth) in the Drake Passage. We also discuss the similarities to the traditional Diaphanidae genera to try to shed light into this phylogenetic conundrum. We sequenced cytochrome *c* oxidase subunit I, 16S rRNA, 28S rRNA, and histone H3 markers of *Newnesia antarctica* and *Newnesia joani* n. sp. We analyzed a comprehensive dataset of sequenced genera to evaluate the placement of both *Newnesia* species within the cephalaspidean families. Maximum likelihood and Bayesian phylograms support the monophyly of *N. joani* n. sp. and suggest cryptic speciation in *N. antarctica* specimens. *Newnesia* is recovered as the most basal offshoot of Cephalaspidea, suggesting the establishment of a new family restricted to Antarctic waters, named Newnesiidae n. fam., to hold both species. The possible Antarctic origin of Cephalaspidea is discussed.

Key words: Diaphanidae, Antarctica, Taxonomy, Newnesiidae, *Newnesia*

Capítulo 5. Un nuevo clado antártico de heterobranquios es hermano de todos los cefalaspídeos (Mollusca: Gastropoda)

RESUMEN

Durante mucho tiempo la familia Diaphanidae se ha considerado basal dentro de los Cephalaspidea, debido a la presencia de características morfológicas plesiomórficas dentro de este taxón. Tradicionalmente la familia contenía los géneros *Bogasonia*, *Colobocephalus*, *Colpodaspis*, *Diaphana*, *Newnesia*, *Toledonia* y *Woodbridgea*. Algunos análisis filogenéticos de varios de estos géneros apoyan la posición basal de Diaphanidae dentro de Cephalaspidea *sensu stricto*. Sin embargo, la familia se confirma actualmente como polifilética, y actualmente sólo incluye el género *Diaphana*. Varios géneros considerados previamente dentro de la familia, como el monotípico *Newnesia*, nunca han sido analizados previamente en estudios moleculares. En este estudio proporcionamos una extensa descripción morfológica, anatómica e histológica de una nueva especie de *Newnesia* de aguas profundas de la Antártida (967 a 1.227 m de profundidad) del Paso del Drake. También se discuten las similitudes con los géneros de Diaphanidae tradicionales para tratar de aportar información sobre su filogenia. Hemos secuenciado los marcadores moleculares citocromo *c* oxidasa subunidad I, 16S ARNr, 28S ARNr, y los de la histona H3 de *Newnesia antarctica* y *Newnesia joani* n. sp. Éstos se analizaron juntamente con varios géneros previamente secuenciados, para evaluar el estatus de ambas especies de *Newnesia* dentro de las diferentes familias de cefalaspídeos. Los filogramas de máxima verosimilitud y bayesianos apoyan la monofilia de *N. Joani* n. sp. y sugieren la existencia de especiación críptica en *N. antarctica*. *Newnesia* aparece como la rama más basal de Cephalaspidea, lo que sugiere que es necesario crear una nueva familia, restringida a las aguas antárticas, que hemos llamado Newnesiidae n. fam., para incluir ambas especies. Se propone asimismo un posible origen antártico para los Cephalaspidea.

Palabras clave: Diaphanidae, Antártida, Taxonomía, Newnesiidae, *Newnesia*

INTRODUCTION

Heterobranch sea slugs and snails are traditionally grouped into the paraphyletic group "Opisthobranchia" (*e.g.*, Wägele *et al.*, 2014). Among them, monophyletic Cephalaspidea is a taxon distributed worldwide (OBIS, 2016), usually restricted from shallow to deep interstitial muddy bottoms, but some species live in association with seagrasses, algae or sessile invertebrates (Gosliner *et al.*, 2008). The original diagnostic character of Cephalaspidea is the presence of a cephalic shield. This, together with sessile eyes and posterior tentacular folds, are characteristic features related mostly to their burrowing habits, other than true synapomorphies (Mikkelsen, 2002). The diagnostic characters of the Cephalaspidea *sensu stricto* (without Runcinacea and Acteonoidea; Mikkelsen, 1996; Malaquias *et al.*, 2009) are the presence of three hardened oesophageal gizzard plates, flexed ciliated strips in the mantle cavity, a prepharyngeal nerve ring (*i.e.*, located anterior to the pharynx), and the genital ganglion located on the visceral nerve loop (Mikkelsen, 1996). Later, Mikkelsen (2002) recognized only the two first characters as valid autopomorphies, rejecting the other two.

Diaphanidae Odhner, 1914 (Amphisphyridae Gray,1857) has been for a long time considered a basal family within Cephalaspidea, because they exhibit plesiomorphic morphological features (Jensen, 1996). For instance, they present a fully formed shell, cephalic tentacles, and, although having an armed oesophagus, they lack distinct gizzard plates (Schiøtte, 1998). The family was first erected to embrace the genera *Diaphana* Brown, 1827, *Toledonia* Dall, 1902 (described under the name *Ptisanula* Odhner, 1913), and provisionally *Newnesia* Smith, 1902 (Odhner, 1914). Diaphanidae was primarily defined on negative characters: absence of parapodia, jaws, and gizzard plates (Eliot, 1906; Odhner, 1914; Thiele, 1931). Its members also present rudimentary oral tentacles, a narrow radula, and an external sperm groove. Jensen (1996) stated that these were autopomorphies or symplesiomorphies, rather than synapomorphic characters. Therefore, the apparent resemblances were interpreted as homoplastic adaptations to epifaunal habits and suctorial feeding. Consequently, the family became a wastebasket taxon, where several genera have been included since then (see below). Phylogenetic analyses of some of its genera supported the basal position of the family Diaphanidae within Cephalaspidea *s. s.*, although only *Diaphana* retrieved basal, while the other diaphanids included in these studies appeared polyphyletic (Thollesson, 1999; Malaquias *et al.*, 2009; Jörger *et al.*, 2010; Oskars *et al.*, 2015).

The genera *Bogasonia* Warén, 1989 was later described based on dried specimens, and its resemblances to *Toledonia* (*i.e.*, volute shell and three-seriate radula) lead Warén (1989) to suggest the new subfamily Toledoniinae. This separation was corroborated by recent molecular analyses, which, however, suggested to place *Toledonia* (and subsequently *Bogasonia*) into the Cylichnidae (Oskars *et al.*, 2015). The subfamily Diaphaniinae Odhner, 1914, thus, included *Diaphana*, *Newnesia*, and *Woodbridgea* Berry, 1953. The latter was described only from a unique shell and was

never found again (Berry, 1953). The genera *Colpodaspis* M. Sars, 1870, with two nominal species, and the monotypic *Colobocephalus* M. Sars, 1870 were included into Diaphanidae based on shell characters (Garstang, 1894; Odhner, 1939). Lately, a more accurate description of live specimens of these three species (Brown, 1979; Ohnheiser & Malaquias, 2014), together with phylogenetic analyses, placed both genera in the new family Colpodaspididae Oskars, Bouchet & Malaquias, 2015, far away from Diaphanidae *s. s.* (Oskars *et al.*, 2015). Moreover, the genus *Rhinodiaphana* was also considered to be a diaphanid, but it has been recently transferred to Philinidae (Ohnheiser & Malaquias, 2013). Additionally, the controversial family Notodiaphanidae Thiele, 1931, previously considered parent of Diaphanoidea, is considered *incertae sedis* within the Cephalaspidea (Ortea *et al.*, 2013; Oskars *et al.*, 2015). Therefore, several families have been designed subsequently to include most genera of Diaphanidae *sensu lato*. However, the relationships of the Antarctic genus *Newnesia* and the elusive *Woodbridgea*, which in former times were also included in the Diaphanidae, remain so far untested.

The monospecific genus *Newnesia* was first described by Smith (1902) based on four specimens of *N. antarctica* collected in Cape Adare (Ross Sea). The description included shell and radula features. Later, Eliot (1906) re-described the same specimens and gave a short description of the internal soft organs. Strebel (1908) described a new genus and species named *Anderssonia sphinx* from Paulet Island (north of the Antarctic Peninsula), later synonymized with *N. antarctica* by Odhner (1926). Jensen (1996) gave an accurate and comparative description of the internal anatomy of *N. antarctica*. This species is currently restricted to Antarctic and Subantarctic circumpolar waters at depths ranging from 16 to 655 m (Aldea & Troncoso, 2008).

In this study we aim (1) to describe a new *Newnesia* species from Antarctic deep waters by using morphological and molecular characters; (2) to compare the morphology of the new species to the rest of the Diaphanoidea *s. l.* genera; (3) to provide a phylogenetic hypothesis for the position of the genus *Newnesia* within Cephalaspidea; and (4) to evaluate the ancestral features of this genus in a phylogenetic context.

MATERIAL AND METHODS

Sample collection

Samples of *Newnesia joani* n. sp. were collected with Agassiz trawl in muddy bottoms at the Drake Passage, north of King George Island (Antarctica), during the Antarctic cruise ANT XV/3 of the R/V Polarstern (Gutt & Arntz, 1999). All specimens were collected in a single dredge operation (48/336) on 19th of March 1998, at a 967–1227 m depth range from 61°27.6'S, 58°4.1'W to 61°26.5'S, 58°7.4'W (Fig. 1). Twenty-seven specimens were collected; 8 were preserved in 70 % ethanol for anatomical and histological analyses, the rest were frozen and two of these were transferred to absolute ethanol for genetic extraction. Specimens of *N. antarctica* were collected

during different campaigns. During ANT XXI/2, December the 24th, 2003 (PS65/259-1), *N. antarctica* (1) was collected from the Austasen Bank in the eastern Weddell Sea (70° 57' S, 10° 33.02' W) with a bottom trawl, at 333 m depth. During Andeep I, ANT XIX, January the 30th, 2002 (PS61/046-7), *N. antarctica* (2; voucher n° ZSMMoll20021145) was collected from north of the South Scotia Ridge (start 60°39.19'S, 53°56.85'W; end 60°38.06'S, 53°57.51'W) at 2889–2893 m depth with an epibenthic sledge.

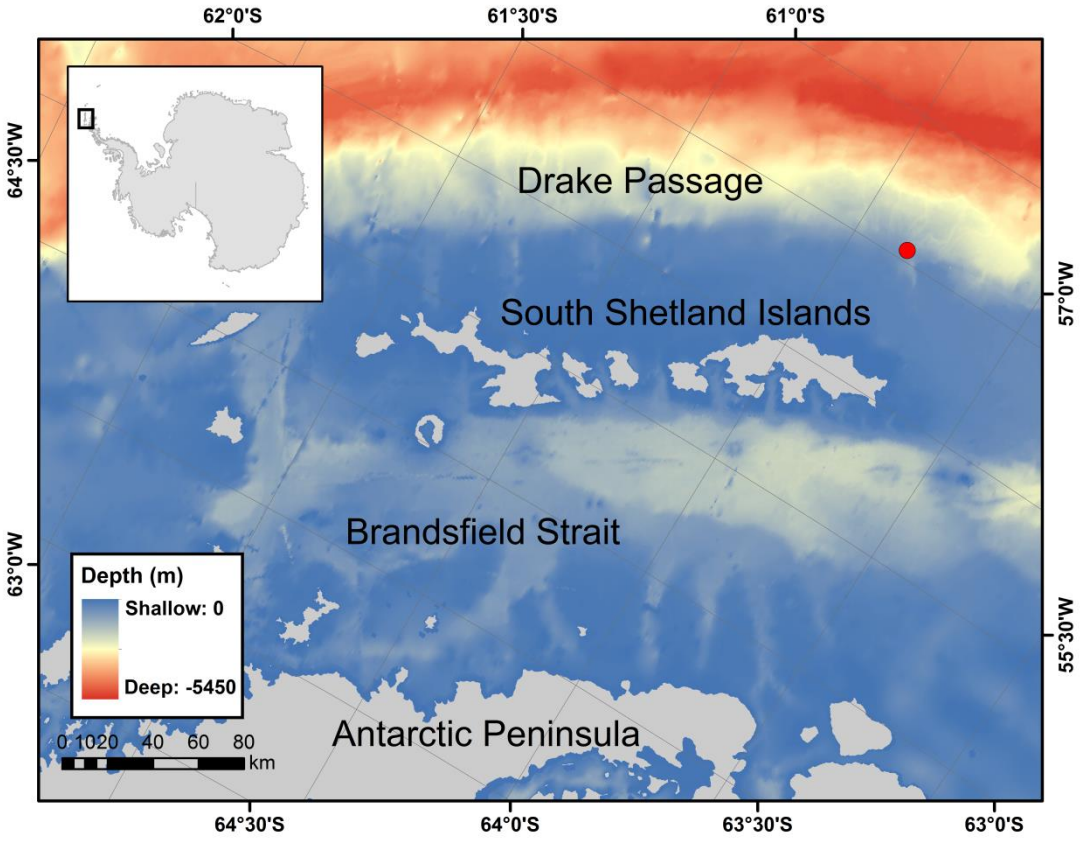

Figure 1. Map of the South Shetland Islands and surrounding waters showing the position of the station AGT 48/336 (red dot), where *Newnesia joani* n. sp. was collected.

Additionally to the four sequenced *Newnesia* specimens, sequences of 38 cephalaspidean species and 13 outgroup taxa were obtained from GenBank (see Supplementary Table 1). Taxon sampling was designed to cover representatives of all available sequenced cephalaspidean families. Outgroups consisting of 13 species representing seven Heterobranchia clades of similar ranking to that of Cephalaspidea (Jörger *et al.*, 2010) were included in the analyses (*i.e.*, Acochlidia, Acteonoidea, Anaspidea, Nudibranchia, Runcinacea, Sacoglossa, and Umbraculida). The trees were rooted with the nudibranch species *Aldisa smaragdina* a sister lineage to the Tectipleura (Euopisthobranchia + Panpulmonata) molluscs (Zapata *et al.*, 2014). In total this study includes 154 sequences.

Morphological analysis

Three specimens of *N. joani* n. sp. were dissected under a stereomicroscope for anatomical analysis. Both buccal masses and shells were immersed in potassium hydroxide for up to three hours to dissolve the organic tissues, and then rinsed with distilled water. Shells and radulae were mounted on metallic stubs with bioadhesive carbon sticky tabs and coated with carbon for scanning electron microscopy (SEM). One individual was dehydrated in an ethanol series and embedded in HEMA for histological analysis (Kulzer method; see Wägele, 1997). Serial sections (2.5 µm thick) were stained with Toluidine blue, which specifically stains acid mucopolysaccharides red to violet, and neutral mucopolysaccharides and nucleic acids, as well as proteins in various blue shades.

DNA amplification

Total genomic DNA was extracted from small pieces of foot tissue for most samples, using DNeasy Tissue Kit (Qiagen, Valencia, CA, USA). Molecular markers included three fragments of the mitochondrial genes cytochrome *c* oxidase subunit I (COI), 16S rRNA and 28S rRNA, and the nuclear gene histone H3. A fragment of *ca.* 720bp of the mitochondrial protein-encoding gene COI was amplified using the primers LCO1490 and HCO2198 (Folmer *et al.*, 1994). A fragment of *ca.* 465bp of the 16S rRNA gene was amplified using the primer pair 16Sar-L and 16Sbr-H (Palumbi *et al.*, 2002). A fragment of *ca.* 746 bp of the 28S gene was amplified using the primer pairs LSU5-F (Littlewood *et al.*, 2000) and LSU1600-R (Williams *et al.*, 2003). A fragment of *ca.* 318 bp of the protein-encoding gene histone H3 was amplified using the primer pair H3AD5'3' and H3BD5'3' (Colgan *et al.*, 1998). PCR amplifications were carried out in a 24 µL-reaction volume including 18.25 µL Sigma dH_2O, 2.5 µL CoraLLoad buffer, 1.25 µL MgCl, 0.5 µL dNTP, 0.5 µL of each primer, 0.5 µL Taq, and 0.5µL of genomic DNA. Polymerase chain reaction (PCR) program for COI and 16S rRNA involve an initial denaturing step (95 °C for 15 min) followed by 25 cycles of denaturation (94 °C for 45 s), annealing (40–55°C for 1:30min), and extension (72 °C for 1:30 min), with a final extension step at 72 °C for 10 min. For 28S rRNA and histone H3, the PCR started with an initial denaturation step at 95°C for 3 min followed by 35 cycles including denaturation at 94 °C for 45 s, annealing at 50–52 °C for 45 s, and extension at 72 °C for 2 min, with a final extension step at 72°C for 10 min. Amplified products were purified using microCLEAN (Microzone Ltd., Sussex, UK) and sequenced at the UB Scientific and Technological Centers (CCiT-UB) on an ABI 3730XL DNA Analyzer (Applied Biosystems).

Phylogenetic analysis

Chromatograms were visualized and sequences were assembled in Geneious Pro 8.1.5 (Drummond *et al.*, 2010). These were compared against the GenBank nucleotide database with the BLAST algorithm (Altschul *et al.*, 1997) to check for contamination. Alignments were trimmed to a position at which more than 50% of the sequences had

nucleotides and missing positions at the ends were coded as missing data. All new sequences have been deposited in GenBank (see Supplementary Table 1 for accession numbers). We used GBlocks 0.91b on the final trimmed alignment for identifying and excluding blocks of ambiguous data in single, non-codifying gene alignments (16S and 28S) with relaxed settings (Talavera & Castresana, 2007).

Bayesian inference (BI) was performed on the concatenated alignment of the four genes, using MrBayes ver. 3.2.5 (Ronquist *et al.*, 2011) with a GTR model of sequence evolution (Tavaré, 1986), corrections for a discrete gamma distribution, and a proportion of invariant sites (GTR + Γ + I; Yang, 1996) specified for each gene partition, as selected in jModelTest ver. 2.1.7 (Posada, 2008) under the Akaike Information Criterion (Posada & Buckley, 2004). Two runs, each with three hot chains and one cold chain, were conducted in MrBayes for 20 million generations, sampling every 2,000th generation, using random starting trees. The analysis was performed twice, and 25% of the runs were discarded as burn-in after checking for stationarity with Tracer v.1.6. (Rambaut *et al.*, 2014). The remaining trees were combined to find the maximum *a posteriori* probability estimate of phylogeny.

Maximum likelihood (ML) analyses were conducted using RAxML ver. 8.1.2 (Stamatakis, 2014). For the maximum likelihood searches, a GTR model of sequence evolution with corrections for a discrete gamma distribution (GTR + Γ; Yang, 1996) was specified for each data partition, and 500 independent searches were conducted. Nodal support was estimated via the rapid bootstrap algorithm (1,000 replicates) using the GTR-CAT model (Stamatakis *et al.*, 2008). Bootstrap resampling frequencies were thereafter mapped onto the optimal tree from the independent searches. Additionally, we assessed saturation by constructing a tree without the third codon position of the protein coding genes COI and H3, and, as there were no differences, we used the alignment with the third position.

COI uncorrected *p*-distances were calculated using MEGA 7 for all species of the dataset which had more than one congener (Table 1).

Table 1. Matrix for COI uncorrected *p*-distances ± standard deviation for the genera with several species included in the phylogenetic analyses.

	N. joani n. sp. (1)	*N. joani* n. sp. (2)	*N. antarctica* (1)	*N. antarctica* (2)	*B. striata*	*B. ampulla*	*P. babai*	*P. indisticta*	*Philinorbis* sp. A	*Philinorbis* sp. B	*L. quadrata*	*L. ventricosa*	*Colinatys* sp. A (1)	*Colinatys* sp. A (2)	*D. globosa*	*Diaphana* sp. EED
Newnesia joani n. sp. (2)	0 ± 0															
Newnesia antarctica (1)	12.9 ± 1.5	12.9 ± 1.5														
Newnesia antarctica (2)	9.2 ± 1.2	9.2 ± 1.2	11.4 ± 1.4													
Bulla striata	21.6 ± 1.8	21.6 ± 1.8	22.5 ± 1.8	22.3 ± 1.8												
Bulla ampulla	22.1 ± 1.8	22.1 ± 1.8	24.0 ± 1.9	23.3 ± 1.8	17.4 ± 1.7											
Philine babai	17.8 ± 1.7	17.8 ± 1.7	19.9 ± 1.7	20.1 ± 1.7	20.6 ± 1.8	19.9 ± 1.7										
Philine indisticta	20.6 ± 1.7	20.6 ± 1.7	21.2 ± 1.7	19.5 ± 1.6	21.6 ± 1.8	19.5 ± 1.7	15.6 ± 1.5									
Philinorbis sp. A	21.6 ± 1.8	21.6 ± 1.8	22.0 ± 1.8	19.9 ± 1.7	23.6 ± 1.9	23.1 ± 1.9	18.4 ± 1.7	19.1 ± 1.7								
Philinorbis sp. B	21.0 ± 1.8	21.0 ± 1.8	22.5 ± 1.9	20.6 ± 1.8	23.1 ± 1.9	22.7 ± 1.9	18.4 ± 1.8	16.9 ± 1.6	6.6 ± 1							
Laona quadrata	19.3 ± 1.7	19.3 ± 1.7	19.1 ± 1.7	19.7 ± 1.7	22.3 ± 1.9	22.0 ± 1.8	15.4 ± 1.6	20.5 ± 1.7	18.6 ± 1.7	19.3 ± 1.7						
Laona ventricosa	19.5 ± 1.7	19.5 ± 1.7	23.1 ± 1.8	21.0 ± 1.7	21.0 ± 1.7	22.0 ± 1.7	19.3 ± 1.8	20.3 ± 1.7	18.6 ± 1.7	19.9 ± 1.8	18.9 ± 1.6					
Colinatys sp. A (1)	20.6 ± 1.8	20.6 ± 1.8	19.7 ± 1.8	21.6 ± 1.8	19.3 ± 1.8	20.3 ± 1.8	18.2 ± 1.7	18.0 ± 1.7	19.3 ± 1.8	19.5 ± 1.9	20.3 ± 1.8	20.8 ± 1.8				
Colinatys sp. A (2)	20.6 ± 1.8	20.6 ± 1.8	19.7 ± 1.8	21.6 ± 1.8	19.3 ± 1.8	20.3 ± 1.8	18.2 ± 1.7	18.0 ± 1.7	19.3 ± 1.8	19.5 ± 1.9	20.3 ± 1.8	20.8 ± 1.8	0 ± 0			
Diaphana globosa	17.4 ± 1.6	17.4 ± 1.6	19.1 ± 1.7	17.1 ± 1.6	24.0 ± 1.8	23.8 ± 1.8	19.9 ± 1.7	19.1 ± 1.6	21.2 ± 1.8	22.9 ± 1.8	19.9 ± 1.7	21.0 ± 1.7	21.0 ± 1.7	21.0 ± 1.7		
Diaphana sp. EED	18.0 ± 1.7	18.0 ± 1.7	19.7 ± 1.7	17.6 ± 1.6	23.5 ± 1.8	22.9 ± 1.8	20.3 ± 1.8	18.8 ± 1.6	21.4 ± 1.8	22.9 ± 1.8	20.3 ± 1.7	21.4 ± 1.7	21.6 ± 1.8	21.6 ± 1.8	1.7 ± 0.6	
Diaphana minuta	17.6 ± 1.6	17.6 ± 1.6	18.4 ± 1.7	18.0 ± 1.6	23.1 ± 1.7	22.5 ± 1.8	18.8 ± 1.7	19.1 ± 1.6	19.5 ± 1.7	20.3 ± 1.7	18.2 ± 1.6	20.8 ± 1.7	18.6 ± 1.6	18.6 ± 1.6	13.5 ± 1.5	13.9 ± 1.5

RESULTS

Systematic description

Gastropoda Cuvier, 1795

Cephalaspidea Fischer, 1883

Newnesiidae Moles, Wägele, Schrödl & Avila n. fam.

http://zoobank.org/NomenclaturalActs/6650E66C-F4F1-4606-929E-8821C2372FF1

Diagnosis: Shell external or internal, globose, thin; apical area flattened, with large aperture. Radular formula: 0.1.0 or 1.1.1 (see Fig. 2). Sharp unicuspidated rachidian teeth with denticles along borders. Broad cephalic shield, posterolateral cephalic lobes present. Tentacular processes absent. Jaws and gizzard plates absent. Cuticularized and spinous stomach. External sperm groove present, running laterally on right side of body from gonopore to penial pore. Parapodia absent. Two gills lying in roof and floor of mantle cavity, respectively. Two repugnatorial glands present: one placed on left antero-lateral side, and one on right postero-lateral side right after mantle cavity (infrapallial lobe).

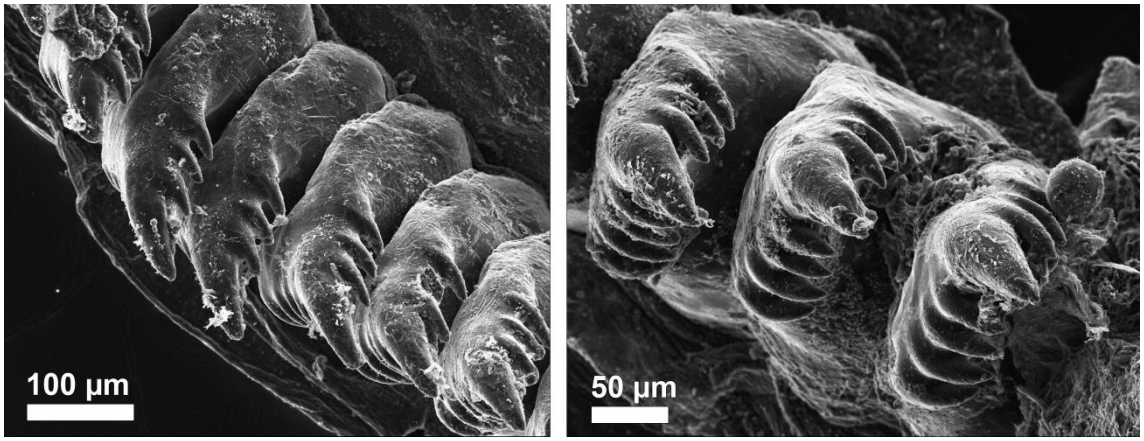

Figure 2. Scanning electron microscopy (SEM) micrographs of the rachidian teeth of *Newnesia antarctica* (1) from the Weddell Sea. Uneven number of denticles observed.

Geographical distribution: from 16 to 1227 m depth, endemic to Antarctic and Subantarctic waters.

Type genus: *Newnesia* Smith, 1902; **Type species**: *Newnesia antarctica* Smith, 1902; by monotypy; Ross Sea.

***Newnesia joani* n. sp.**

(Figures 3–7)

http://zoobank.org/NomenclaturalActs/0B175ACB-D90D-4203-8FDE-ED11218A2CFF

Holotype (Fig. 4a–d): 15.7 mm, preserved in 70 % ethanol. Deposited in SNSB Zoologische Staatssammlung München (Catalog number ZSM Moll 20150456).

Paratypes: (1) 21 mm, dissected; (2) 19 mm, dissected; (3) 18 mm, dissected; (4) 10.7 mm, sectioned; (5) 10.4 mm, preserved in 70 % ethanol; (6) 8.5 mm, preserved in 70 % ethanol. Dissected and un-dissected specimens are deposited at SNSB Zoologische Staatssammlung München (Catalog number ZSM Moll 20150456). The sectioned individual, as well as radula and shell SEM preparations, are deposited at the University of Barcelona. Paratype (1) is deposited at the CRBA (Centre de Recursos de Biodiversitat Animal, http://www.ub.edu/crba/english/index.htm) under the Catalog number CRBA2024.

Shell (Fig. 3a, b): Maximum height 16.5 mm; maximum width 12 mm. Internal, thin, white; concave, slightly globose in shape, composed of 2.5 whorls, presenting wide aperture strongly oblique to shell axis. Shell covering whole viscera. Protoconch not protruding. Apical area flat, apex barely acute. Surface ornamentation consisting of faint parallel spiral lines with some thin transverse lines producing a reticulate pattern, sometimes thinner lines alternating with wider ones. Umbilicus absent. Lip present, thin, not ornamented, parietal callus absent. Periostracum external, thin, translucent, yellowish, and elastic.

Radula (Fig. 3c–e): Radular formula 19–21 x 1.1.1. Three-seriated, composed by large denticulate teeth with large, hollow, partly overlapping bases. Rachidian teeth with a central sharp cusp, one small denticle at each side positioned in an angle of 45° to central cusp. 5–6 further denticles along rachidian border, each one having sharp cusp curved towards inner edge; these gradually decreasing in size towards base. Lateral teeth thin, lamellate, with strongly convex anterior margin; placed with their basal edges in longitudinal direction, having concave outer surface.

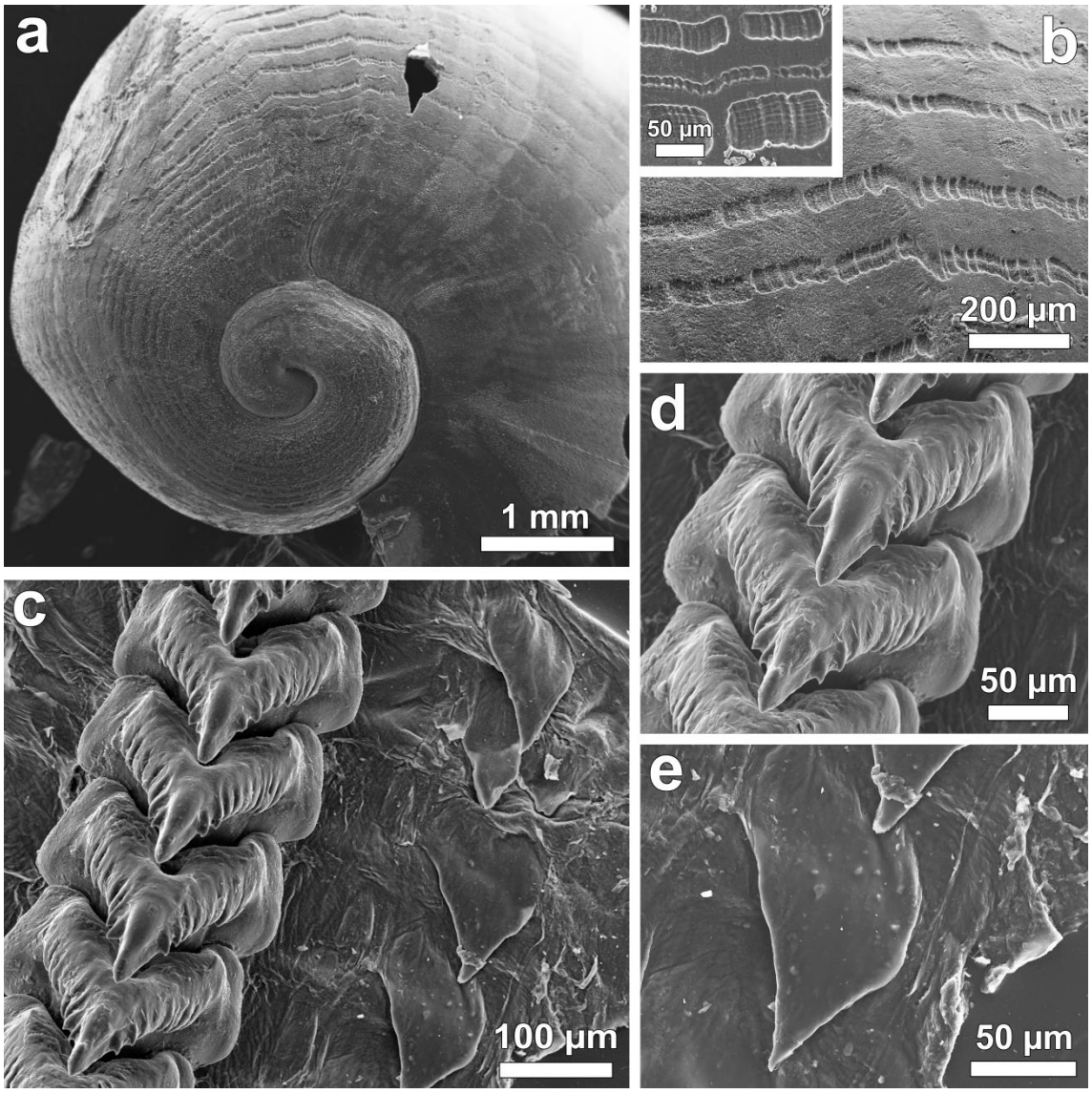

Figure 3. Scanning electron microscopy (SEM) micrographs of *Newnesia joani* n. sp. **a** – shell, apical area. **b** – shell microsculpture, close up showing distinct ornamentation in the same shell. **c** – general view of the radula. **d** – detail of the rachidian tooth. **e** – detail of the lateral tooth.

External morphology (Fig. 4): Live specimens beige to light brown in color, beige and whitish when fixed. A picture of the live animal can be seen in Rauschert & Arntz (2015; plate 41, page 48). Body oval shaped, margin only interrupted by two posterior cephalic lobes, when looking from dorsal view. Cephalic shield broad, thickened, trapezoidal; mouth opening lying ventrally; eyes shining through transparent notal tissue, located in mid-anterior lateral edges; head with two large, folded, postero-lateral orientated velar lobes displaying ciliated grooves; penial opening placed in the right anterior notch under cephalic lobe. Foot broad, not overpassing body perimeter; propodium squared and slightly lobulated, metapodium oval. Pedal gland opening in the middle foot, visible as an ovate furrow. Conical funnel in frontal left side of notum, lying above left cephalic lobe. Mantle cavity placed on right side and partially covered by shell; inside with prominent, plicate, primary gill; anus opening posteriorly on right

side of body close to edge of mantle cavity in small anal papilla (Fig. 5a). Kidney forming a dorsal bulge in mantle cavity, which is partly covered by plicated accessory gill. Accessory gill smaller than primary, which is placed directly underneath.

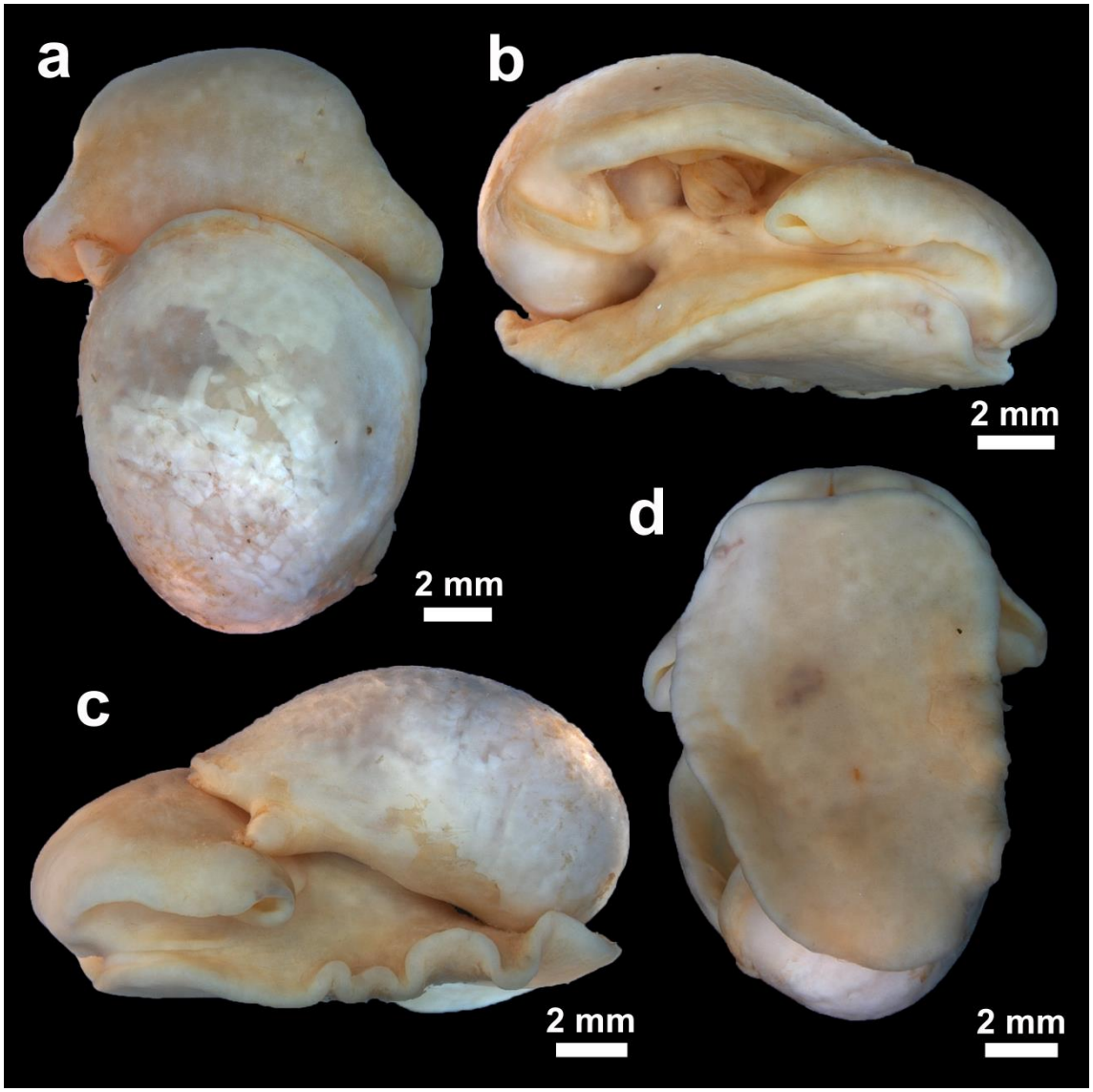

Figure 4. External view of *Newnesia joani* n. sp. preserved holotype. **a**– dorsal view. **b** – right lateral view. **c** – left lateral view. **d** – ventral view.

Digestive system (Fig. 5b): Mouth lying above horizontal furrow between propodium and anterior cephalic shield. Oral glands subepidermal, follicular, containing acid and neutral mucopolysaccharides, opening directly at each side into oral tube, without distinct tube (Fig. 7c). Anterior pharynx elongated and lined with thin cuticle, later on covered with knobbed or spiniform cuticular structures lying on thicker cuticle layer. Posterior pharynx containing odontophore, lined with smooth cuticle. Few denticulate processes, only observed next to radula. Posterior pharynx surrounded by thick muscle layers, whereas anterior pharynx exhibiting fewer muscles. No jaws detected.

Salivary glands open into pharynx through thin multiciliated paired ducts. Salivary glands sausage shaped with narrow section, lying close to oesophagus and stomach while extending until medium body length. Oesophagus running to left side, widening posteriorly, and entering stomach on left side; anterior region with thin cuticle, "T" shaped in cross section, and presenting multiple folds. Epithelium composed of large macrovacuolated cells with bluish, fibrillar content and columnar cells, all having basal nucleus (Fig. 7d); these were not seen in rest of digestive tract epithelium; thin cuticle lining oesophagus. Stomach lying in mid-left section of the animal, anteriorly presenting interior cuticle with knobs, and larger spines posteriorly (Fig. 6e). Gizzard plates absent. Digestive gland occupying most of visceral whorl; composed of numerous diverticula, connected by continuous and expansive lumen. Digestive gland epithelium composed of at least three cell types (Fig. 6f): (1) digestive cells containing spherical, pinkish food vacuoles; (2) microtubule-containing cells with large vacuoles of fibrillar content; and (3) secretory cells containing reddish vacuoles, see (Kress *et al.*, 1994). Intestine originating from stomach, on left side, and running dorsally towards right side, just in front of digestive gland. Rectum cells multiciliated, containing acidic mucopolysaccharides.

Juvenile specimens had similar digestive system arrangement compared to adults, but also presenting two large digestive gland diverticula. First one, reaching far into mid-ventral cephalic region and connecting posteriorly to digestive tract. Second one, extending anteriorly in visceral mass and under shell, right behind anterior repugnatorial gland into mid-right section, occupying almost entirely transversal section of animal. Both diverticula composed by four cell types: (1) columnar multiciliated cells close to reduced lumen, (2) globular cells with large nucleus, (3) cells aggregated into follicles, and (4) bluish granulated cells (Fig. 7e). Diverticula surrounded by transversal and longitudinal muscular fibers; longitudinal muscular fibers found at both sides of ventral digestive gland diverticulum.

Reproductive system (Fig. 5c): Monaulic. Gonad (ovotestis) large, slightly lobulated, granular; intermingling with digestive gland, reaching into body whorls; connecting directly to nidamental glands by tiny duct. Albumen gland elongated, lobulated, connecting separately to other two parts of nidamental glands (capsule and membrane glands). Capsule gland plicated, convoluted, connecting to membrane gland of similar arrangement, but with different texture. Nidamental glands directly enter vagina. Receptaculum seminis wide, globose, entering proximally vagina by short duct. Bursa copulatrix thin, saccular, opening distally into vagina by long duct. Gonopore situated mid-laterally under primary gill, connecting to distinct, external sperm groove (Fig. 6c), leading into opening of highly muscular penial sheath, under right cephalic lobe. Penis unarmed, retractile, connecting directly into single, tubular prostate gland.

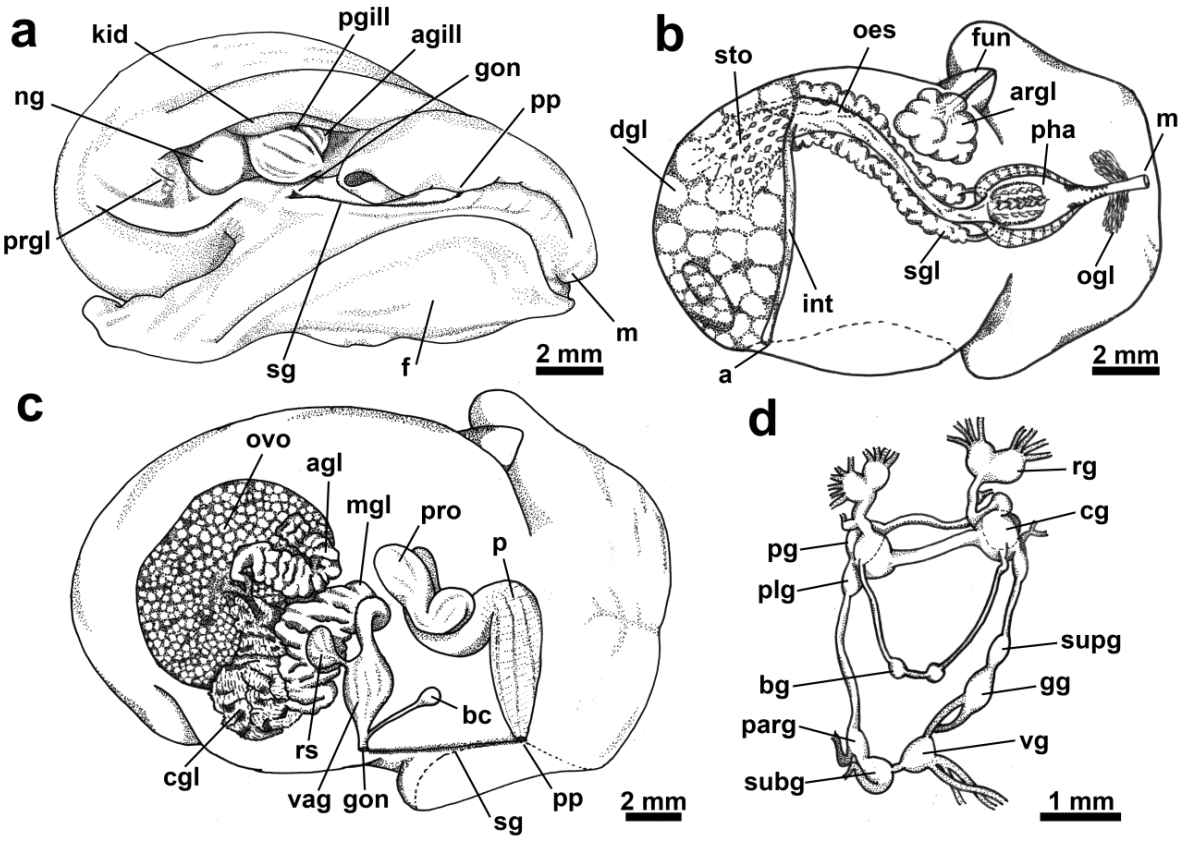

Figure 5. Schematic representation of *Newnesia joani* n. sp. **a** – external view of the right side of the body showing mantle cavity organs. *agill* accessory gill; *f* foot; *gon* gonopore; *kid* kidney; *m* mouth; *ng* nidamental glands; *pgill* primary gill; *pp* penial pore; *prgl* posterior repugnatory gland; *sg* sperm groove. **b** – dorsal schematic view of the digestive system. *a* anus; *argl* anterior repugnatorial gland; *dgl* digestive gland; *fun* funnel; *int* intestine; *m* mouth; *oes* oesophagus; *ogl* oral glands; *pha* pharynx; *sgl* salivary gland; *sto* stomach. **c** – dorsal schematic view of the reproductive system. *agl* albumen gland; *bc* bursa copulatrix; *cgl* capsule gland; *gon* gonopore; *mgl* membrane gland; *ovo* ovotestis; *p* penis; *pp* penial pore; *pro* prostate; *rs* receptaculum seminis; *sg* sperm groove; *vag* vagina. **d** – nervous system showing the prepharyngeal and visceral nerve loops. *bg* buccal ganglion; *cg* cerebral ganglion; *gg* genital ganglion; *parg* parietal ganglion; *pg* pedal ganglion; *plg* pleural ganglion; *subg* subintestinal ganglion; *supg* supraintestinal ganglion; *rg* rhinophoreal ganglion; *vg* visceral ganglion.

Nervous system (Fig. 5d): Composed of prepharyngeal nerve ring connected to visceral ring loop, reaching far back along digestive system. Two cerebral ganglia situated above prepharyngeal region, connected by distinct long commissure. Optical nerves short, leading to small optical ganglion. Distal optical nerve long, up to four times longer than diameter of eye. Eyes with lens, vitreous humor, and retina. Rhinophoral ganglion bilobed, sending nerves forward anteriorly and laterally; one nerve running to small ganglion from where posterior cephalic lobes are innervated. Sensory neuronal cells organized into highly innervated follicles (Fig. 7b), thus chemosensory function is assumed. Each follicle with cortical layer of arranged neuronal cells; each of these with dendrites leading into center of follicle, far into the epidermis; tip of dendrite with several cilia lying outside. These cells are organized into cephalic sensory organ, called lip organ, in the anterior part of cephalic lobe, and

Hancock's organ in posterior part. Two small buccal ganglia located below pharynx at the base of salivary ducts and near oesophagus, separated by small commissure and connected by connectives to cerebral ganglia.

Pedal ganglia placed below pharynx and connected to cerebral and pleural ganglia by one relatively long connective nerve. Statocyst with several ovate otogonia, close to pedal ganglia. Right pleural ganglion connected to supraintestinal ganglion, this in turn connected to small genital ganglion, while left pleural ganglion only connected to smaller distinct parietal ganglion, connected in turn to subintestinal ganglion; this and supraintestinal ganglion connect to visceral (=abdominal) ganglion.

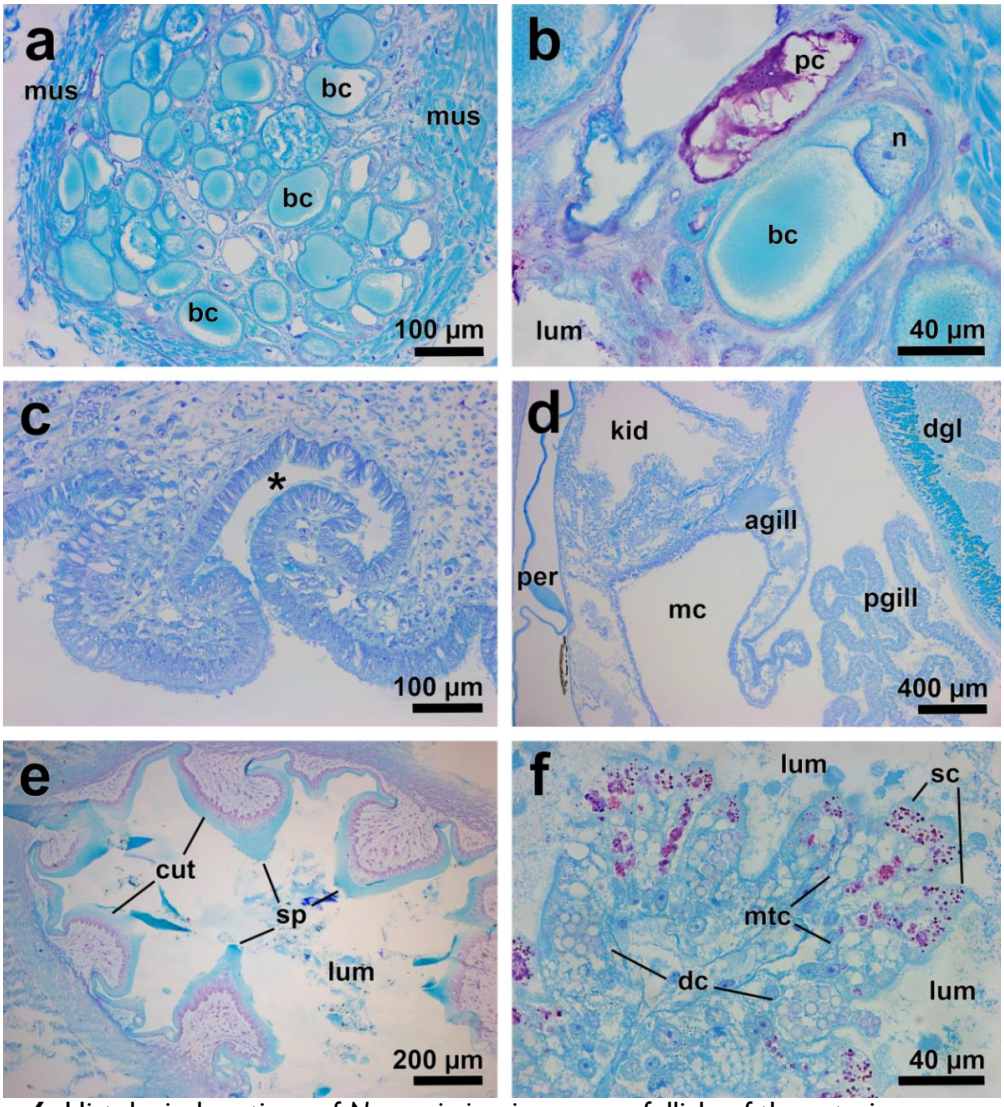

Figure 6. Histological sections of *Newnesia joani* n. sp. **a** – follicle of the anterior repugnatorial gland composed by blue staining cells (*bc*) and surrounded by muscular fibers (*mus*). **b** – detail of the lumen (*lum*), purple (*pc*) and blue macrovacuolar (*bc*) glandular cells and its nucleus (*n*), of the anterior repugnatorial organ. **c** – cross section of the sperm groove showing sperm groove (asterisk). **d** – detail of the mantle cavity roof (*mc*) where kidney (*kid*), accessory (*agill*) and primary *(pgill*) gills are found; digestive gland (*dgl*) and shell periostracum (*per*) are also seen. **e** – cross section of the stomach lumen (*lum*) delimitated by spines (*sp*) with a thick cuticle (*cut*). **f** – detail of the digestive gland cells (*dc*), microtubule-containing cells (*mc*), and secretory cells (*sc*); all surrounding the digestive gland lumen (*lum*).

Circulatory, excretory, and respiratory systems: Pericardial complex (composed of one auricle and ventricle within pericardium) aligning transversely across longitudinal axis of body, lying in mid-anterior region under shell. Kidney large, saccular, occupying anterior right part of visceral mass, lying under shell in mantle cavity roof, attached to right side of pericardium (Fig. 6d), as well as to accessory gill, which has thin lamellae. Primary gill larger than accessory gill.

Glandular organs: Huge bluish glandular cells – probably containing neutral mucopolysaccharides – placed at cephalic and propodium edges (Fig. 7a), missing in notch between two cephalic lobes. Smaller glandular cells widespread in epidermis, commonly staining blue in cephalic region and purple-reddish ventrally in foot. Funnel located anterior to left part of visceral mass, connecting to follicular organ through duct paved with columnar multiciliated cells. This organ consisting in up to twenty follicles of 593.9 µm ± 112 µm (mean ± sd) surrounded by layer of 83.6 µm ± 34.6 µm of muscles (Fig. 6a). Each follicle containing two types of glandular cells, all leading into common lumen (Fig. 6b); first type stained purple, containing acid mucopolysaccharides; second one with macrovacuole occupying the entire cytoplasm, staining light blue; maximum size of these cells 90.83 µm ± 12.8 µm. Both cell types also leading into lumen of funnel. Similar follicles also placed at posterior right side between shell and protuberated notum rim; each one leading individually to outside with distinct duct. There is no thick muscle lining in this posterior lying organ, only some muscle fibers. Pedal gland opening ventrally in middle part of foot; pyriform, composed only by follicles of glandular cells containing acid mucopolysaccharides.

Ecology: Twenty-seven animals of different sizes, including juveniles and reproductive adults, were found in muddy bottoms dominated by asteroids, ophiuroids, polychaetes, echiurids, and the dendronotid nudibranch, *Tritoniella belli* Eliot, 1907. Usually, the digestive tract was mainly empty; however in some animals the stomach and intestine contained sand particles, sclerotized structures, and spicules of, probably, soft corals. Occasionally, cellular structures of unidentified origin were found. Broad and thick cephalic shield together with habitat suggests burrowing habits. Moreover, a sectioned specimen presented six different endoparasites (*i.e.*, copepods and/or nematodes) in the cephalic lobes and foot (Fig. 7f).

Etymology: *Newnesia joani* n. sp. is named after Joan Giménez, a cetacean biologist and esteemed colleague, in recognition of his support and friendship.

Type locality: Between 967–1227 m depth in the Drake Passage, north of King George Island (South Shetland Islands, Antarctica).

Remarks: *N. joani* n. sp. is mainly characterized by the presence of: (1) internal and globose shell; (2) three-seriate radula with sharp unicuspid rachidian tooth and lamellate laterals; (3) broad cephalic shield and posterolateral tentacular lobes; (4) left anterolateral repugnatorial gland (with a distinct funnel) and right posterolateral

repugnatorial gland; (5) presence of distinct parietal ganglion. Uncorrected COI *p*-distances between both specimens of *N. joani* n. sp. was zero, and 12.9 ± 1.5%, and 9.2 ± 1.2%, respectively between *N. joani* n. sp. and the two specimens of *N. antarctica.*

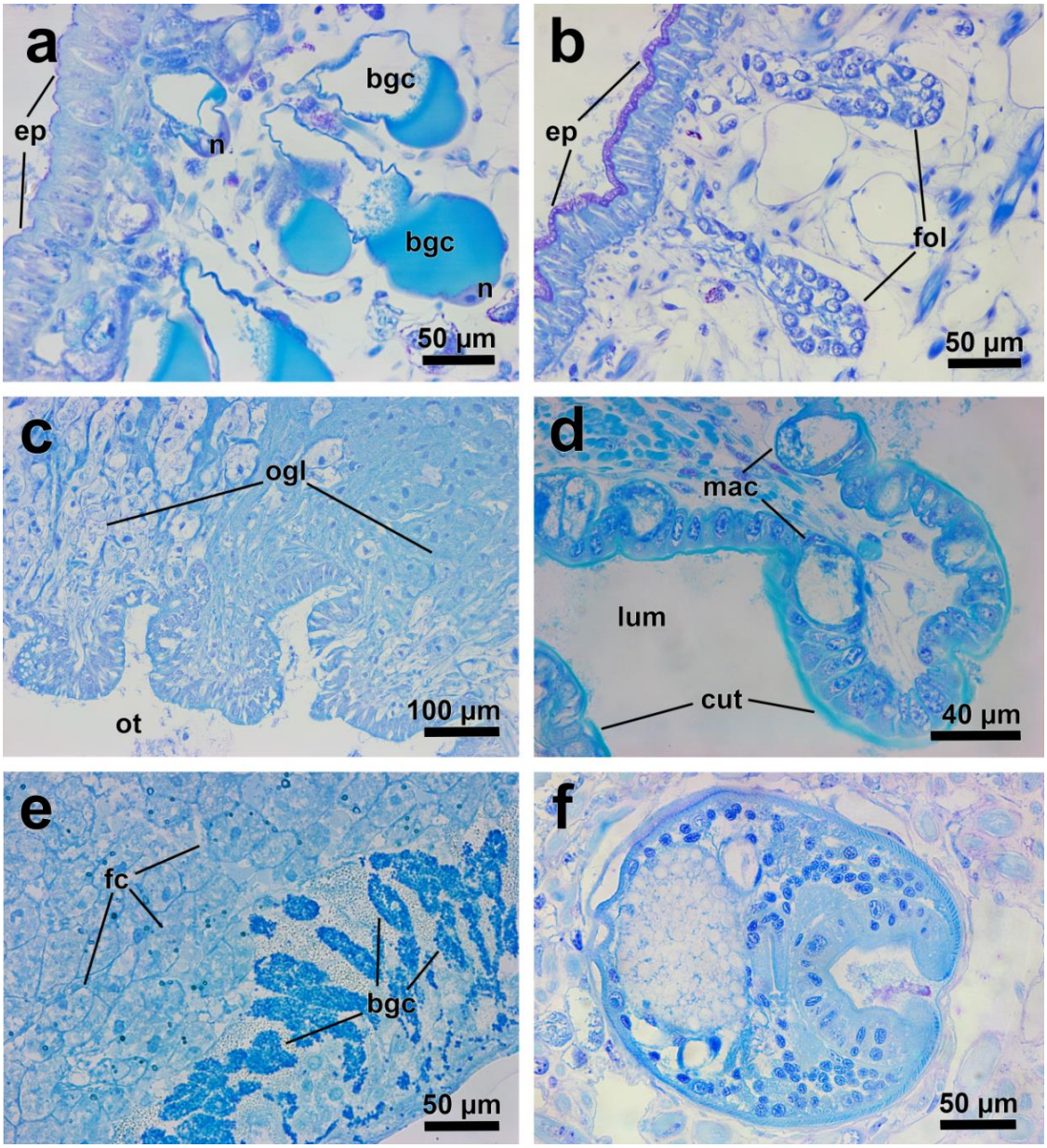

Figure 7. Histological sections of *Newnesia joani* n. sp. **a** – cephalic epithelium (*ep*) containing huge blue glandular cells (*bgc*) and nuclei (*n*). **b** – sensory neuronal cells organized into follicles (*fol*) near the cephalic lobe's epithelium (*ep*). **c** – oral glands (*ogl*) found near the oral tube (*ot*). **d** – oesophagus epithelium folded and composed of large macrovacuolated cells with bluish, fibrillar content (*mac*) and columnar cells (*cc*); these are lined by a thin cuticle (*cut*) in contact to the lumen (*lum*) of the digestive tract. **e** – detail of the digestive reservoir glands of juveniles showing follicular cells (*fc*) and bluish granulated cells (*bgc*). **f** – cross section of a parasite found in the right cephalic lobe.

Phylogenetic analyses

The total dataset contained 40 cephalaspidean species, corresponding to all families sequenced hitherto, and 13 outgroup taxa. The concatenated alignment consisted of 2,203 characters, of which COI had 614 characters, 16S 352 characters, 28S 928 characters, and H3 had 309 characters. ML and BI analysis recovered a tree with strong support for monophyletic Newnesiidae n. fam. (PP=1; BS=100), composed by both *N. antarctica* and *N. joani* n. sp., which was in turn the earliest branching Cephalaspidea *s. s.* group (Fig. 8). In general, the topology of the phylogenetic tree is in accordance to previous studies including the same taxa (Oskars *et al.*, 2015).

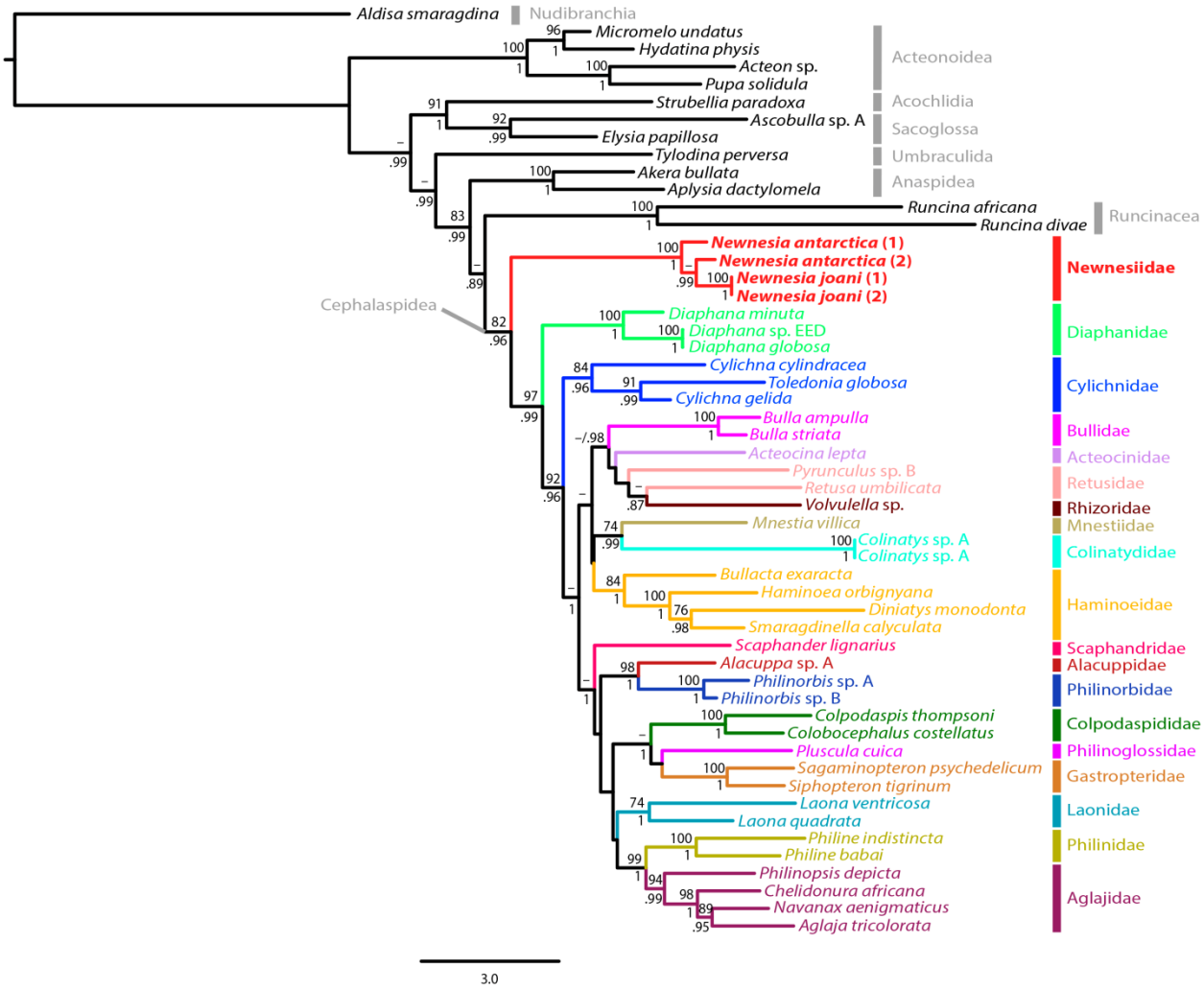

Figure 8. Phylogenetic tree of the Cephalaspidea based on the combined COI, 16S, 28S, and H3 genes using maximum-likelihood (ML) and Bayesian inference (BI). Numbers on nodes indicate bootstrap support values (ML) and posterior probability values (BI). Cephalaspidean families are marked in colors corresponding to families at the right side, while outgroup clades are in grey.

DISCUSSION

A new species of cephalaspidean mollusc from deep waters in the Drake Passage (Antarctica; 967–1227 m) is described here under the name *N. joani* n. sp. *Newnesia joani* n. sp. was found to be related to *N. antarctica* using both morphological and molecular analyses (Smith, 1902; Odhner, 1926; Jensen, 1996), although specific morphological traits of the new species clearly separate both species. The genus *Newnesia* forms a distinct lineage at the base of the Cephalaspidea (PP=1, BS=100), and we thus consider it to represent a discrete family named Newnesiidae n. fam. separated from Diaphanidae. Molecular markers show a clear differentiation between *N. joani* n. sp. and the two specimens of *N. antarctica.* Moreover, COI *p*-distances of 9.2–12.9 % between both *Newnesia* species indicate cryptic speciation of *N. antarctica* specimens. In fact, both *N. antarctica* specimens analyzed here were collected at very distant locations (eastern Weddell Sea and Scotia Ridge). Similarly, cryptic speciation has been shown in other heterobranchs of Antarctic circumpolar waters (Wilson *et al.*, 2009, 2013). However, a thorough taxon sampling of *N. antarctica* from additional locations is needed to corroborate this hypothesis.

Here, Diaphanidae *s. l.* is recovered polyphyletic and we found further support on the families described recently by Oskars *et al.* (2015). However, we included an additionally basal lineage to Cephalaspidea *s. s.*, the new family Newnesiidae. The basal position based on molecular analyses is also reflected by the presence of such a broad array of plesiomorphic morphological features not found again within other cephalaspidean groups: *e.g.*, the presence of a well-developed cephalic shield, the absence of anterior tentacular processes and gizzard plates. The simple cuticle lining in the oesophagus and stomach of *Newnesia* (as well as in *Toledonia*) may constitute a precursor of the complex gizzard plates of some cephalaspidean groups. Further plesiomorphic features in euthyneuran heterobranchs are the lateral position of the mantle cavity with the gonopore opening posteriorly, the prepharyngeal position of the nerve ring, as well as the long visceral nerve loop (Wägele *et al.*, 2014). Moreover, in *Newnesia* as well as in *Diaphana*, the cerebral and pleural ganglia are still separated by a distinct commissure (Huber, 1993). However, only *N. joani* n. sp. has a pentaganglionate visceral loop with a distinct parietal ganglion. The pentaganglionate condition has been proposed as a synapomorphy of Euthyneura (=Pentaganglionata, Haszprunar, 1985), only present in 'basal' taxa of all major Heterobranchia *s. l.* clades (Brenzinger *et al.*, 2013a). Eliot (1906) described a second gill-like organ that he considered to be an osphradium in *N. antarctica*, but histological sections herein demonstrated that this is a true gill, which together with the primary gill typically form a plicatidium (Morton, 1972). This is similar to those of other heterobranchs with burrowing habits, such as *Akera* or *Acteon* (Fretter & Graham, 1954; Morton, 1972).

The new family Newnesiidae is characterized by an unusual big trapezoidal cephalic shield with folded posterior cephalic lobes. Cephalic lobes might act as chemosensory organs since neuronal follicles were ubiquitously seen. The presence of two follicular and multicellular repugnatorial glands is another defining characteristic of

the family. These repugnatorial glands might represent modified Blochmann's glands, a gland type that is seen in other heterobranch species too (Brenzinger *et al.*, 2013b). These glandular organs are surrounded by musculature helping to release the contents outside, probably in a similar way as in the mantle dermal formations (MDFs) of doridoideans (Avila & Durfort, 1996), some cladobranchs (Moles *et al.*, 2016), and other heterobranchs (Wägele *et al.*, 2006). This mechanism seems to be improved in the frontal gland of *N. joani* n. sp. since it is connected through a funnel to the exterior. However, its follicular arrangement and the presence of distinct secreting ducts lead to conclude these are not MDFs, in contrast to previous interpretations (Wägele *et al.*, 2006), but a distinct glandular organ only found in the family Newnesiidae to date.

Newnesiidae n. fam. presents some shared morphological characters to the genera originally assigned to the family Diaphanidae (see Table 2). The new family bears a globose shell similar to that of *Colpodaspis*, *Colobocephalus*, and some species of the genus *Diaphana*, although it is internal only in the Colpodaspididae (Ohnheiser & Malaquias, 2014) and in *N. joani* n. sp. The radula, however, differs considerably: the genera *Colpodaspis*, *Colobocephalus*, and *Diaphana* present long hooked laterals and lack a rachidian in both colpodaspidids, while it is bilobed in *Diaphana* (Brown, 1979; Schiøtte, 1998). Lateral teeth are very thin and likely vestigial in *N. joani* n. sp., while *N. antarctica* lacks them. Dissection of *N. antarctica* (1) from the Weddell Sea revealed the typical radular formula of 25 x 0.1.0, although having 4 denticles along its right border and 3 denticles in the left border of the rachidian teeth. This has never been reported before for *N. antarctica* and it was not observed in the specimens of *N. joani* n. sp. The radula of *Newnesia* resembles that of *Toledonia* and *Bogasonia*, since they also present a unicuspid rachidian and sometimes thin lamellate laterals (Warén, 1989), as for *N. joani* n. sp. However, the uniseriate radula with unicuspid teeth (together with a muscular and voluminous pharynx) may be an adaptation to suctorial feeding rather than a homology (Jensen, 1996). Both *Toledonia* and *Bogasonia* present a shell with an elongated spire (Marcus, 1976; Warén, 1989), and therefore morphologically differ from *Newnesia.* In fact, morphological evidence lead Odhner (1926) and Warén (1989) to propose several subfamilies within Diaphanidae *s. l.*, some of which have been supported as distinct families in recent molecular phylogenies (Oskars *et al.*, 2015). Therefore, the apparent similarities clustering the primal Diaphanidae *s. l.* genera may be interpreted as homoplastic adaptations to epifaunal habits and suctorial feeding (Jensen, 1996). For instance, the pedal gland is present in different species with epifaunal habits, thus it might be either a plesiomorphy or a homoplasy of *Toledonia*, *Colpodaspis*, and *Newnesia* (Jensen, 1996). Further studies should ascertain these questions in the future.

There are approximately 80 species of heterobranchs described in Antarctica, being Cephalaspidea (~25) one of the most speciose groups (De Broyer *et al.*, 2016). Interestingly, several families and genera are found only in Antarctic waters, and they are crucial for the phylogenetic comprehension of the evolution of heterobranch lineages. In fact, basal members of some major Nudipleura (Nudibranchia +

Pleurobranchomorpha) linages are exclusively Antarctic. This, together with molecular clock analyses, suggested a possible Antarctic origin of nudibranchs and pleurobranchomorphs (Wägele *et al.*, 2008; Martynov & Schrödl, 2009; Göbbeler & Klussmann-Kolb, 2010). Giving the data presented here, we also propose an Antarctic origin for the Cephalaspidea. Likewise to Nudipleura species, cephalaspideans may have dispersed through deep-sea waters thanks to the Antarctic Bottom Water (Stepanjants *et al.*, 2006). Migration through deep waters to the Atlantic and Pacific Ocean Basins might have occurred during glacial maxima, similarly to what happens in other benthic phyla, such as cnidarians, crustaceans, and echinoderms, among others (Vinogradova, 1997; Stepanjants *et al.*, 2006). This is also supported by the occurrence of other basal lineages such as *Diaphana* and *Toledonia* in Antarctic and deep-water areas (Marcus, 1976; Schiøtte, 1998). Further molecular clock analyses should shed light on the geographical origin of Cephalaspidea.

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Table 2. Comparative table of diagnostic characters of the former Diaphanidae genera compared to the Newnesiidae n. fam.

	***Newnesia joani* n. sp.**	***Newnesia antarctica***	***Diaphana***	***Toledonia***	***Bogasonia***	***Woodbridgea***	***Colpodaspis***	***Colobocephalus***
Shell	internal	external	external	external	external	external	internal	internal
Shape	globose	globose	globose-elongate	elongate	elongate	globose	globose	globose
Radula	1.1.1	0.1.0	0-1.1.1.1.1-0	0-1.0-1.1.0-1.1-0	1.1.1	?	1.0.1	1.0.1
Rachidian	unicuspid	unicuspid	bilobed	unicusped	unicusped	?	absent	absent
1st lateral	lamellate	absent	hook shaped	absent	lamellate	?	hook shaped	hook shaped
Tentacular processes	absent	absent	present	present	present	?	present	present
Prostate	undivided	absent?	divided or undivided	undivided	?	?	undivided	undivided
Family	Newnesiidae n. fam.	Newnesiidae n. fam.	Diaphanidae	Cylichnidae	Cylichnidae?	?	Colpodaspididae	Colpodaspididae
Reference	Present study	Jensen (1996)	Schiøtte (1998)	Marcus (1976)	Warén (1989)	Berry (1953)	Brown (1979)	Ohnheiser & Malaquias (2014)

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Supplementary Table 1. Data of the species included in the phylogenetic analyses and information considered in this study. Voucher accession numbers [SNSB Zoologische Staatssammlung München] are given along the text for the species sequenced herein, and GenBank accession numbers for all the genes included in the analyses, being the sequences generated for this study in **bold** letters.

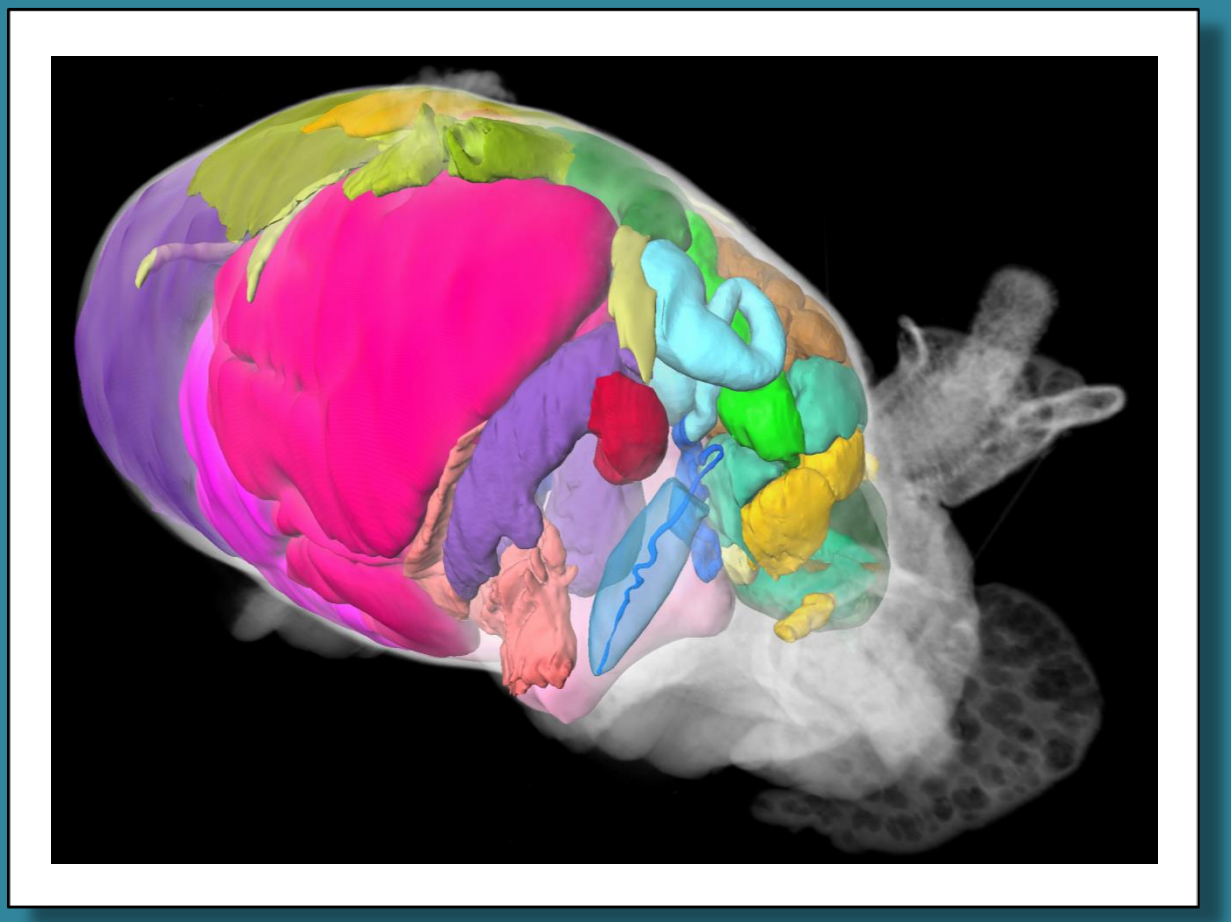
Higher taxa	Family	Species	COI	16S	28S	H3
Cephalaspidea	Acteocinidae Dall, 1913	*Acteocina lepta* Woodring, 1928	KF992197	KJ022827	KJ023022	KJ022891
	Aglajidae Pilsbry, 1895	*Aglaja tricolorata* Reiner, 1807	AM421902	AM421854	AM421950	–
		Chelidonura africana Pruvot-Fol, 1953	DQ974654	KJ022777	DQ927216	KJ022928
		Navanax aenigmaticus (Bergh, 1893)	JN402059	JN402144	–	JN402117
		Philinopsis depicta (Renier, 1807)	AM421892	AM421831	AM421954	–
	Bullidae Gray, 1827	*Bulla ampulla* Linnaeus, 1758	DQ986524	DQ986584	DQ986647	KJ022885
		Bulla striata Bruguière, 1792	DQ986565	DQ986630	DQ986692	KJ022886
	Colpodaspididae Oskars, Bouchet & Malaquias, 2015	*Colobocephalus costellatus* Sars, 1870	KJ023013	KJ022873	KF992207	KJ02286
		Colpodaspis thompsoni Brown, 1979	KF992158	KJ022774	DQ927222	KJ022947
	Colinatydidae Oskars, Bouchet & Malaquias, 2015	*Colinatys* sp. A	DQ974665	KJ022776	DQ927223	KJ022946
		Colinatys sp. A	DQ974666	KJ022783	DQ927224	KJ022939
	Cylichnidae Adams & Adams, 1854	*Cylichna cylindracea* (Pennant, 1777)	KF992159	K022779	KJ23057	KJ022943
		Cylichna gelida (Smith, 1907)	–	EF489326	EF489374	–
		Toledonia globosa Hedley, 1916	EF489395	EF489327	EF489375	–
	Diaphanidae Odhner, 1914	*Diaphana globosa* (Lovén, 1846)	KF992162	KJ022791	KJ23056	KJ022930
		Diaphana minuta Brown, 1827	KF643345	AJ223404	–	–
		Diaphana sp. EED	EF489394	EF489325	EF489373	–

Gastropteridae Swainson, 1840	*Sagaminopteron psychedelicum* Carlson & Hoff, 1974	DQ974667	KJ022787	DQ927225	KJ022934
	Siphopteron tigrinum Gosliner, 1989	DQ974668	KJ022788	DQ927226	KJ022933
Haminoeidae Pilsbry, 1895	*Bullacta exarata* (Philippi, 1849)	GQ332576	KJ022800	HM100714	KJ022920
	Diniatys monodonta (Adams, 1850)	KF992178	KJ022809	KJ023040	KJ022912
	Haminoea orbignyana (Férussac, 1822)	KF615813	KJ022794	KF615776	KJ022927
	Smaragdinella calyculata (Broderip & Sowerby I, 1829)	KF992185	KJ022815	KJ023034	KJ022905
Laonidae Pruvot-Fol, 1954	*Laona quadrata* (Wood, 1839)	JX944809	KJ022793	KJ023010	KJ022952
	Laona ventricosa (Jeffreys, 1865)	JX944803	KJ022831	KJ023008	KJ022978
Mnestiidae Oskars, Bouchet & Malaquias, 2015	*Mnestia villica* (Gould, 1859)	KF992161	KJ022789	DQ927236	KJ022931
Newnesiidae Moles, Wägele, Schrödl & Avila, 2016	*Newnesia joani* n. sp. Moles, Wägele, Schrödl & Avila, 2016 (1)	**KX238906**	**KU939089**	–	**KX238902**
	Newnesia joani n. sp. Moles, Wägele, Schrödl & Avila, 2016 (2)	**KX238907**	**KU939090**	**KU939091**	**KX238903**
	Newnesia antarctica Smith, 1902 (1)	**KX238908**	**KU939085**	**KU939087**	**KX238904**
	Newnesia antarctica Smith, 1902 (2)	**KX238909**	**KU939086**	**KU939088**	**KX238905**
Philinidae Gray, 1850	*Philine babai* Valdés, 2008	KF877702	KJ022854	KJ022989	KJ022968
	Philine indistincta Ohnheiser & Malaquias, 2013	JX944798	KJ022832	–	KJ022950
Philinoglossidae Hertling, 1932	*Pluscula cuica* Marcus, 1953	KF992203	KJ022837	KJ023016	KJ022881
Philinorbidae Oskars, Bouchet & Malaquias, 2015	*Philinorbis* sp. A	KF877715	KJ022869	KJ022999	KJ022960
	Philinorbis sp. B	KF877716	KJ022853	KJ022990	KJ022979

	Retusidae Thiele, 1925	*Pyrunculus* sp. B	DQ974678	KJ022773	DQ927237	KJ022948
		Retusa umbilicata (Montagu, 1803)	KF992163	KJ022792	KJ023055	KJ022929
	Rhizoridae Dell, 1952	*Volvulella* sp.	DQ974684	KJ022785	DQ927244	KJ022937
	Scaphandridae Sars, 1878	*Sabatia* sp. A	KF992204	KJ022863	KJ023015	KJ022876
		Scaphander lignarius (Linnaeus, 1758)	KC351563	KC351526	KC351545	KJ094553
Acteonidea	Acteonidae d'Orbigny, 1843	*Acteon* sp.	DQ974648	KJ022782	DQ927213	KJ022940
		Pupa solidula (Linnaeus, 1758)	DQ238006	EF489319	AY427481	EF133483
	Aplustridae Gray, 1847	*Hydatina physis* (Linnaeus, 1758)	DQ986572	DQ986637	DQ986699	–
		Micromelo undatus (Bruguière, 1792)	DQ974653	KJ022778	DQ927214	KJ022944
Acochlidia	Acochlidiidae Küthe, 1935	*Strubellia paradoxa* (Strubell, 1892)	HQ168457	HQ168419	HQ168445	–
Anaspidea	Akeridae Mazzarelli, 1891	*Akera bullata* Müller, 1776	KF992164	KJ022795	KJ023054	KJ022926
	Aplysiidae Lamarck, 1809	*Aplysia dactylomela* Rang, 1828	KF992168	KJ022798	KJ023050	KJ022921
Nudibranchia	Cadlinidae Bergh, 1891	*Aldisa smaragdina* Ortea, Pérez & Llera, 1982	KF992175	KJ022806	KJ023043	KJ022914
Runcinacea	Runcinidae Adams & Adams, 1854	*Runcina africana* Pruvot-Fol, 1953	DQ974680	KJ022780	DQ927240	KJ022942
		Runcina divae (Marcus & Marcus, 1963)	KF992195	KJ022825	KJ023024	KJ022893
Sacoglossa	Plakobranchidae Gray, 1840	*Elysia papillosa* Verrill, 1901	HQ616844	HQ616815	–	HQ616869
	Volvatellidae Pilsbry, 1895	*Ascobulla* sp. A	DQ974683	KJ022781	DQ927243	KJ022883
Umbraculida	Tylodinidae Gray, 1847	*Tylodina perversa* (Gmelin, 1791)	KF992172	KJ022803	KJ023046	KJ022917

Chapter 6

The end of the cold loneliness: 3D comparison between *Doto antarctica* and a new sympatric species of *Doto* (Heterobranchia: Nudibranchia)



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Chapter 6. The end of the cold loneliness: 3D comparison between *Doto antarctica* and a new sympatric species of *Doto* (Heterobranchia: Nudibranchia)

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ABSTRACT

Although several studies are devoted to determining the diversity of Antarctic heterobranch sea slugs, new species are still being discovered. Among nudibranchs, *Doto antarctica* Eliot, 1907 is the single species of this genus described from Antarctica hitherto, the type locality being the Ross Sea. *Doto antarctica* was described mainly using external features. During our Antarctic research on marine benthic invertebrates, we found *D. antarctica* in the Weddell Sea and Bouvet Island, suggesting a circumpolar distribution. Species affiliation is herein supported by molecular analyses using cytochrome *c* oxidase subunit I, 16S rRNA, and histone H3 markers. We redescribe *D. antarctica* using histology, micro-computed tomography (micro-CT), and 3D-reconstruction of the internal organs. Moreover, we describe a new, sympatric species, namely *D. carinova* Moles, Avila & Wägele n. sp., and provide an anatomical comparison between the two Antarctic *Doto* species. Egg masses in both species are also described here for the first time. We demonstrate that micro-CT is a useful tool for non-destructive anatomical description of valuable specimens. Furthermore, our high resolution micro-CT data reveal that the central nervous system of both *Doto* species possesses numerous accessory giant cells, suggested to be neurons herein. In addition, the phylogenetic tree of all *Doto* species sequenced to date suggests a scenario for the evolution of the reproductive system in this genus: bursa copulatrix seems to have been reduced and the acquisition of a distal connection of the oviduct to the nidamental glands is a synapomorphy of the Antarctic *Doto* species. Overall, the combination of thorough morphological and anatomical description and molecular analyses provides a comprehensive means to characterize and delineate species, thus suggesting evolutionary scenarios.

Keywords: Antarctica, Cladobranchia, Dotidae, Weddell Sea, micro-CT, giant cells, nervous system, reproductive system

Capítulo 6. El final de la fría soledad: comparación 3D entre *Doto antarctica* y una nueva especie simpátrica de *Doto* (Heterobranchia: Nudibranchia)

RESUMEN

A pesar de que diversos estudios han abordado la diversidad de babosas marinas (Heterobranchia) antárticas, se descubren aún regularmente nuevas especies. Entre los nudibranquios, *Doto antarctica* Eliot 1907 es la única especie de este género descrita en la Antártida hasta el momento, siendo la localidad tipo el Mar de Ross. *Doto antarctica* fue descrita utilizando principalmente carácteres de morfología externa. En el transcurso de nuestra investigación antártica en invertebrados bentónicos marinos, encontramos especímenes de *D. antarctica* en el Mar de Weddell y en la isla de Bouvet, lo que sugiere una distribución circumpolar. La afiliación a esta especie está apoyada por análisis moleculares, utilizando los marcadores citocromo *c* oxidasa subunidad I, 16S rRNA, y la histona H3. En este estudio, redescribimos *D. antarctica* usando técnicas histológicas, tomográficas micro-computarizadas (micro-CT) y de reconstrucción en 3D de los órganos internos. Por otra parte, se describe una nueva especie simpátrica, denominada *D. carinova* Moles, Ávila y Wägele n. sp., y proporcionamos una comparación anatómica entre las dos especies antárticas de *Doto*. También describimos las puestas de ambas especies por primera vez. Este estudio demuestra que la técnica de micro-CT es una herramienta muy útil para la descripción anatómica, no destructiva, de especímenes valiosos. Además, nuestros datos de micro-CT de alta resolución revelan que el sistema nervioso central de ambas especies de *Doto* posee numerosas células gigantes accesorias, que sugerimos que pueden ser neuronas. Además, el árbol filogenético de todas las especies secuenciadas hasta la fecha del género *Doto* sugiere un interesante escenario para la evolución del sistema reproductivo en este género: por un lado la bursa copulatrix parece haberse reducido, y por otro, la adquisición de una conexión distal del oviducto a las glándulas nidamentales es una sinapomorfía de las especies antárticas de *Doto*. En general, la combinación de una descripción morfológica y anatómica completa, y el análisis molecular, proporcionan un método integral para caracterizar y delimitar las especies, lo que sugiere escenarios evolutivos de gran interés.

Palabras clave: Antártida, Cladobranchia, Dotidae, micro-CT, células gigantes, sistema nervioso, sistema reproductivo

INTRODUCTION

Heterobranch sea slugs are worldwide-distributed molluscs with new species being discovered regularly from tropical and temperate areas, while Polar Regions are less explored (Clarke & Johnston, 2003). Interestingly, basal members of some major Nudipleura and Pleurobranchomorpha linages have an Antarctic origin (Wägele *et al.*, 2008; Martynov & Schrödl, 2009; Göbbeler & Klussmann-Kolb, 2010). Among nudibranchs, the family Dotidae Gray, 1853 is presently considered to be a monophyletic taxon within Cladobranchia (Willan *et al.*, 1984). However, the relationships between Dotidae and the other cladobranch families remain undefined (Pola & Gosliner, 2010), although RNAseq analyses of Goodheart *et al.* (2015) indicate a closer relationship to Dendronotida. In fact, Dotidae was traditionally placed into Dendronotida (formerly Dendronotoidea) (Bouchet & Rocroi, 2005) based on the presence of rhinophoral sheaths, into which the rhinophores can be retracted (Odhner, 1936). A cuticle lining of the stomach and tentacular expansions of the oral veil were lately advocated as additional autapomorphies of Dendronotida (Wägele & Willan, 2000). Since these traits are not present in Dotidae, their systematic position within Dendronotida is questionable.

Dotidae comprises four genera: *Doto* Oken, 1815, *Caecinella* Bergh, 1870, *Miesea* Marcus, 1961, and the recently described *Kabeiro* Shipman & Gosliner, 2015. However, the taxonomic status of the monotypic *Caecinella* and *Miesea* is often considered doubtful (Thiele, 1931; Odhner, 1936). *Doto* differs from *Miesea* in having rhinophoral sheaths (Marcus, 1961), and from *Kabeiro* by the shape and arrangement of the cerata, the pericardium size, and the absence of a penial gland (Shipman & Gosliner, 2015). *Doto*, with 87 species recognised to date (WoRMS, 2015) shows a cosmopolitan distribution, and the species are usually defined only on the basis of external characters, *i.e.*, colouration, number and shape of cerata, and shape of the rhinophoral sheath (Ortea & Urgorri, 1978). Lemche (1976) took a wider approach including not only body colour pattern, but also food preference and shape of the egg mass. However, information on anatomical characters of the digestive, reproductive, circulatory, nervous, or excretory systems for most *Doto* species remains poorly known.

Only *Doto antarctica* Eliot, 1907 has been described from Antarctica to date, based on a single specimen from McMurdo Sound (Victoria Land; Eliot, 1907). A brightly-yellow coloured species of *Doto* was recorded from the Davies Sea (eastern Antarctica) and roughly described by Thiele (1912). However, the material was insufficient to properly describe the species. Later, Odhner (1934) added details of the external anatomy and radula of *D. antarctica* from Cape Adare (Victoria Land) but did not provide internal description of the digestive and reproductive systems. Since then, *D. antarctica* has been found in King George Island (South Shetland Islands) at 160 m (Lovell & Trego, 2003) and in the Victoria Land (Ross Sea) at 80–500 m depth (Powell, 1960; Schiaparelli *et al.*, 2006).

Additional undetermined species of *Doto* have been recorded in Bouvet Island (Arntz *et al.*, 2005) and the Ross Sea (Schiaparelli *et al.*, 2006; Ghiglione *et al.*, 2013).

Here, we use histological and tomographic techniques to explore the organ systems and the egg masses of *D. antarctica.* In addition, we newly describe a single specimen of *Doto carinova* n. sp., collected in the Weddell Sea, by 3D reconstruction of micro-CT images. Thereby, we assessed the potential of micro-CT for non-invasive description of singleton type material (Rückert *et al.*, 2008; Jörger *et al.*, 2008)). Moreover, we sequenced *D. antarctica* from the Weddell Sea and compare it to specimens from the Ross Sea. The aims of this study are thus threefold: (1) to improve species delimitation of the two Antarctic *Doto* species; (2) to provide a basis for anatomical comparison of the species of *Doto*; and (3) to disentangle the phylogenetic conundrum of *Doto* species. We also provide an evolutionary scenario of the changes in *Doto* anatomy for all the species for which molecular data are available.

MATERIAL AND METHODS

Sample collection

Nudibranch samples were collected in the eastern Weddell Sea (Antarctica) during the ANT XV/3 cruise (1998) (Gutt & Arntz, 1999), and during the ANT XXI/2 cruise (2003–2004) of the R/V Polarstern (Alfred Wegener Institute, Bremerhaven, Germany). Several specimens of *D. antarctica* and egg masses were collected at depths ranging from 65 to 433 m at several stations (see Table 1). For *D. carinova* Moles, Avila & Wägele n. sp., only one specimen and its egg masses were collected from station code PS65/276-1. Samples were photographed alive, anaesthetised with 10% magnesium chloride for 1h, and then transferred to 70% ethanol for morphological analysis. One specimen of *D. antarctica* was preserved in 10% formaldehyde/sea water for histology (PS65/166-1), and two specimens were frozen and then transferred to 100% ethanol for sequencing (48/033). Additional *Doto* species were also collected from the Mediterranean Sea and sequenced to increase the number of taxa from the ones already available in GenBank (see Supplementary Table 1). Antarctic samples were collected with the permission of the Spanish Polar Committee (CPE; www.idi.mineco.gob.es/portal/site/MICINN). Mediterranean samples were collected under the permission issued by the Catalan Government (www.gencat.cat/darp).

Table 1. Sampling stations where *D. antarctica* and *D. carinova* Moles, Avila & Wägele n. sp. were collected. Kapp Norvegia is situated in the eastern Weddell Sea.

Specimens (nº)	Cruise	Date	Area	Station code	Operation	Latitude (S)	Longitude (W)	Depth (m)
D. antarctica (12) + egg masses (8)	ANTXV/3	29/01/98	North of Kapp Norvegia	48/033	TV grab	71° 7.3' S	11° 28.3' W	65
D. antarctica (1)	ANTXV/3	29/01/98	North of Kapp Norvegia	48/037	Dredge	71° 6.7' S	11° 28' W	146
D. antarctica (1)	ANTXV/3	01/02/98	North of Kapp Norvegia	48/071	Bottom trawl	71° 50.5' S	10° 32.8' W	231
Egg masses *D. antarctica* (3)	ANTXV/3	02/02/98	North of Kapp Norvegia	48/077	Agassiz trawl	71° 8.6' S	12° 26.6' W	433
D. antarctica (1)	ANTXV/3	16/02/98	Kapp Norvegia	48/198	Dredge	71° 17' S	12° 36.6' W	416
D. antarctica (1)	ANTXV/3	18/02/98	Kapp Norvegia	48/214	Dredge	71° 7.2' S	11° 28.8' W	110
Egg masses *D. antarctica* (6)	ANTXV/3	27/02/98	Kapp Norvegia	48/277	Agassiz trawl	71° 18.2' S	12° 16.4' W	177
D. antarctica (1)	ANTXXI/2	25/11/03	Bouvet Island	PS65/029-1	Agassiz trawl	54° 31.59' S	3° 13.05' E	377
D. antarctica (1)	ANTXXI/2	15/12/03	Eastern Weddell Sea	PS65/166-1	Bottom trawl	70° 56.83' S	10° 32.61' W	338
D. antarctica (1)	ANTXXI/2	29/12/03	Eastern Weddell Sea	PS65/280-1	Agassiz trawl	71° 7.15' S	11° 26.23' W	228
Doto carinova n. sp. (1) + egg masses (4)	ANTXXI/2	28/12/03	Eastern Weddell Sea	PS65/276-1	Agassiz trawl	71° 6.44' S	11° 27.76' W	277

Morphological analysis

The buccal mass of *D. antarctica* (48/033) was immersed in potassium hydroxide for up to three hours to dissolve the organic tissues and then rinsed with distilled water. The radula was mounted on metallic stubs with bioadhesive carbon sticky tabs and coated with carbon for scanning electron microscopy (SEM). One specimen of *D. antarctica* (PS65/166-1), an egg mass (48/033), and the egg mass of *D. carinova* n. sp. (PS65/276-1) were dehydrated in an ethanol series and embedded in HEMA for histological analysis (Kulzer's method; see Wägele, 1997). Serial sections (2.5 µm thick) were stained with Toluidine blue, which specifically stains acid mucopolysaccharides red to violet, and neutral mucopolysaccharides and nucleic acids, as well as proteins in various shades of blue.

For micro-CT analysis, *D. antarctica* (PS65/280-1) and *D. carinova* n. sp. (PS65/276-1) specimens (Fig. 1) were dehydrated in a graded series of ethanol, contrasted with 1% iodine metal (I_2) dissolved in 100% ethanol (I2E) for 24 h, and transferred to 100% ethanol. Subsequently, each specimen was mounted on a pipette tip containing 100% ethanol, with the specimen arrested, later mounted on a pin with superglue. The microscopic X-ray tomography scan was performed with an XRadia Micro XCT-200 (Carl Zeiss X-ray Microscopy Inc.) using the 4x object lens unit, at 40 kV and 200 µA, with a pixel size of 5.77 and 4.98 µm for *D. antarctica* and *D. carinova* n. sp., respectively. Tomography projections were then reconstructed using the software provided by XRadia. For image segmentation the software platform Amira® 5.4. (FEI, Visualization Science Group) was used. Images from micro-CT scans were compared with histological sections (2.5 µm thick) for reciprocal illumination. A graphical 3D PDF reconstruction of both species was performed using Deep Exploration. 3D PDFs can be opened in Adobe Acrobat Reader and activated by clicking on it (see Supporting Information).

DNA amplification

We sequenced two specimens of *D. antarctica* (Station code 48/033; see Table 1), but we were not able to sequence *D. carinova* n. sp. due to inadequate chemical fixation for molecular analyses. Total genomic DNA was extracted from small pieces of foot tissue using DNeasy Tissue Kit (Qiagen, Valencia, CA, USA). Molecular markers included two fragments of the mitochondrial genes cytochrome *c* oxidase subunit I (COI) and 16S rRNA, and the nuclear gene histone H3. A fragment of about 593 bp of the mitochondrial protein-encoding gene COI was amplified using the primers LCO1490 and HCO2198 (Folmer *et al.*, 1994). A fragment of about 383 bp of the 16S rRNA gene was amplified using the primer pair 16Sar-L and 16Sbr-H (Palumbi *et al.*, 2002). A fragment of about 310 bp of the protein-encoding gene histone H3 was amplified using the primer pair H3AD5'3' and H3BD5'3' (Colgan *et al.*, 1998). PCR amplifications were carried out in a 10 µL-reaction volume including 5.1 µL of Sigma dH_2O, 3.3 µL REDExtract-N-Amp™ PCR ReadyMix (Sigma Aldrich, St. Louis, MO, USA), 0.3 µL of

each primer, and 1 µL of genomic DNA. Polymerase chain reaction (PCR) programs for COI and 16S rRNA involve an initial denaturing step (94 °C for 5 min) followed 40 cycles of denaturation (94 °C for 30 s), annealing (44–50 °C for 30 s), and extension (72 °C for 30 s), with a final extension step at 72 °C for 5 min. For histone H3, the initial denaturation step was conducted at 94 °C for 3 min followed by 35 cycles including denaturation at 94 °C for 35 s, annealing at 50 °C for 1 min, and extension at 72 °C for 15 s, with a final extension step at 72 °C for 2 min. Amplified products were purified using microCLEAN (Microzone Ltd., Sussex, UK) and sequenced at the UB Scientific and Technological Centers (CCiT-UB) on an ABI 3730XL DNA Analyzer (Applied Biosystems).

Phylogenetic analysis

Chromatograms were visualized and sequences were assembled in Geneious Pro 8.1.5 (Drummond *et al.*, 2010). These were compared against the GenBank nucleotide database with the BLAST algorithm (Altschul *et al.*, 1997) to check for contamination. Alignments were trimmed to a position at which more than 50% of the sequences had nucleotides and missing positions at the ends were coded as missing data. All new sequences have been deposited in GenBank (see Supplementary Table 1 for accession numbers). We used GBlocks 0.91b on the final trimmed alignment for identifying and excluding blocks of ambiguous data in single, non-codifying gene alignments (16S) with relaxed settings (Talavera & Castresana, 2007).

Bayesian inference (BI) and maximum likelihood (ML) analyses were conducted on the concatenated alignment of the three genes. BI analyses were performed using MrBayes ver. 3.2.5 (Ronquist *et al.*, 2011) with a unique GTR model of sequence evolution (Tavaré, 1986) with corrections for a discrete gamma distribution and a proportion of invariant sites (GTR + Γ + I; Yangm 1996) specified for each partition, as selected in jModelTest ver. 2.1.7 (Posada, 2008) under the Akaike Information Criterion (Posada & Buckley, 2004). Two runs, each with three hot chains and one cold chain, were conducted in MrBayes for 20 million generations, sampling every 2000th generation, using random starting trees. The analysis was performed twice, and 25% of the runs were discarded as burn-in after checking for stationarity with Tracer v.1.6 (Rambaut *et al.*, 2014). The remaining trees were combined to find the maximum *a posteriori* probability estimate of phylogeny.

ML analyses were conducted using RAxML ver. 8.1.2 (Stamatakis, 2014). For the maximum likelihood searches, a unique GTR model of sequence evolution with corrections for a discrete gamma distribution (GTR + Γ; Yang, 1996) was specified for each data partition, and 500 independent searches were conducted. Nodal support was estimated via the rapid bootstrap algorithm (1000 replicates) using the GTR-CAT model (Stamatakis *et al.*, 2008). Bootstrap resampling frequencies were thereafter mapped onto the optimal tree from the independent searches.

RESULTS

Systematics

Cladobranchia Willan & Morton, 1984

Dotidae Gray, 1853

Doto Oken, 1815

Type species: *Doris coronata* Gmelin, 1791

***Doto antarctica* Eliot, 1907**

(Figures 1–4) (See 3D PDF of the reconstructed anatomy of the anterior region of the specimen in Supplementary Material 1)

Material examined. See Table 1. Deposited in SNSB Zoologische Staatssammlung München (Catalog number ZSM Moll 2016115).

Distribution. Ross and Weddell Seas, King George Island (South Shetland Islands), and Bouvet Island.

External morphology (Fig. 1A, C, E). Body short and bulged dorsally mostly due to reproductive system; young and adult specimens measured 4–9 x 2–5 x 2–3.5 mm (length:width:height). Body and cerata pale brown, intensified in cerata; containing bright white spots (corresponding to huge glandular cells) on tip of rhinophores, edge of rhinophoral sheaths, and each tubercle on cerata. Velum broad and rounded. Tentacular processes absent. Rhinophores transparent, thin, smooth, blunt; rhinophoral sheath 1/3 of rhinophore size, elongated in frontal side (giving resemblance of "calla lily" inflorescence). Six cerata pairs, short, rounded, 2–3 mm high, progressively smaller towards tail, connected to body by narrow connection; 4–5 circlets containing up to 12 short tubercles (largest circlet) close to each other, apex of cerata of same shape. Pseudobranchs absent. Cerata in live specimens easily autotomized upon manipulation, discharging white content of glandular cells. Anal papilla large, placed dorsally in mid-right position. Genital apertures below and in between 1st and 2nd cerata on right side. Foot narrow, linear, rounded anteriorly, tapering posteriorly to short, blunt tail.

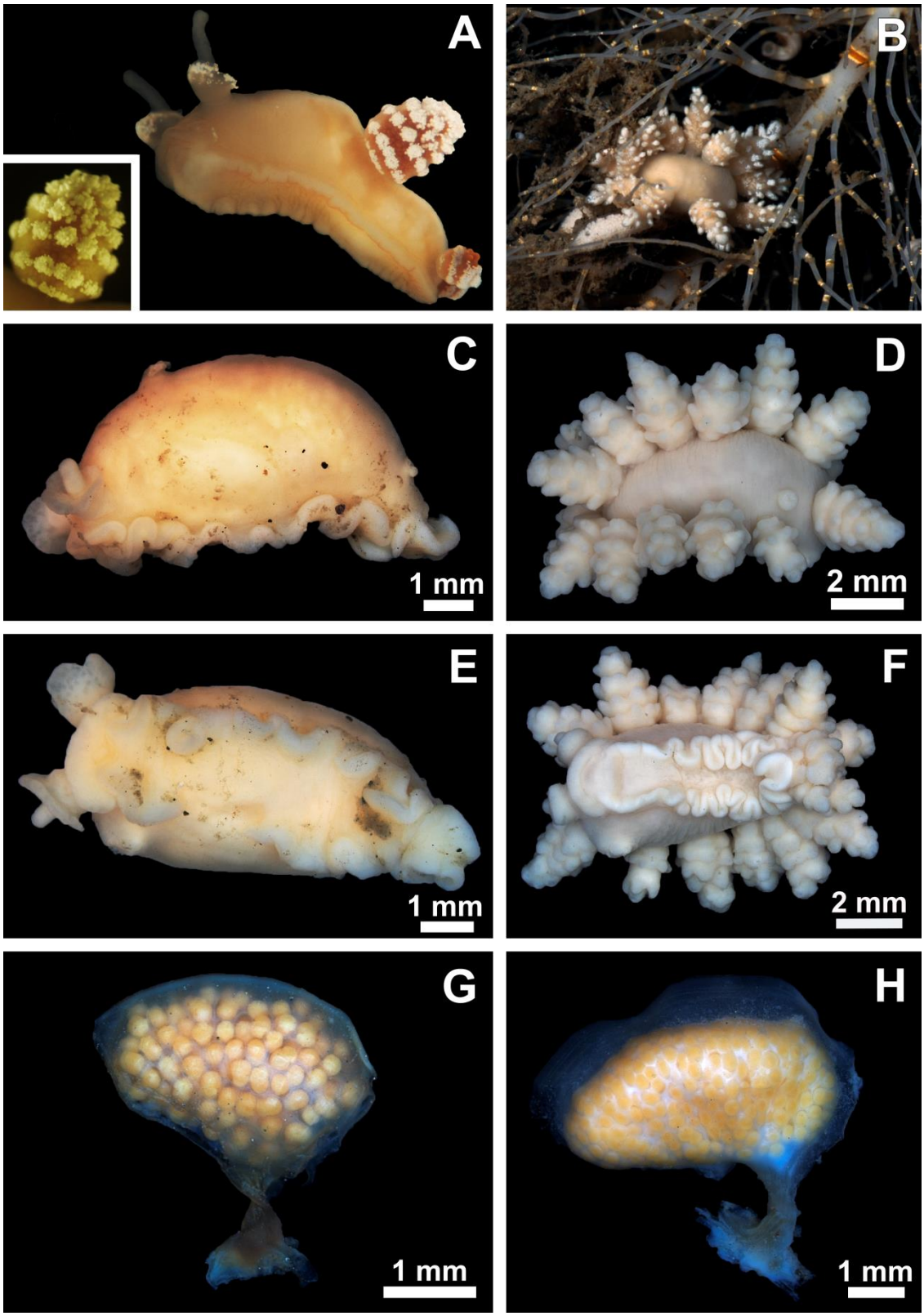

Figure 1. Photographs of *D. antarctica* (left column: A, C, E, G) and *D. carinova* Moles, Avila & Wägele n. sp. (right column: B, D, F, H); specimens subjected to micro-CT reconstruction. **A** Live animal, where most of the cerata were lost; close up of the cerata. **B** Live picture right after collection, showing the *D. carinova* n. sp. spawning on top of the gorgonian *Primnoisis antarctica* (Isididae). **C–D** Lateral and dorsal view of the preserved animals. **E–F** Ventral view of the preserved animals. **G–H** Lateral view of the preserved egg masses.

Digestive system (Fig. 2A, C). Mouth opening ventrally between oral veil and foot; oral tube relatively short and surrounded by follicular, blue-staining, oral glands (Fig. 3C). Jaws thin, membranous, without any appreciable ornamentation. Pharynx bulbous, inner lining presenting thin cuticle; posteriorly projected downwards due to odontophore and upwards (suctorial pump). Radular formula 72 x 0.1.0; rachidian arched, pointed tip; bearing five denticles along border, unequal among and within teeth (see Fig. 4). Paired, saccular salivary glands leading dorso-posteriorly to pharynx by short duct; attached to posterior part of cerebropleural ganglia; composed by huge granular cells containing high amount of secretory vacuoles (see Fig. 3D). Oesophagus opening at upper end of pharynx; widening, connecting to stomach right before location of anal papilla. Stomach widening, becoming flattened; from there, several digestive glandular diverticula reaching into cerata; from anterior part of stomach, two digestive gland ducts reaching into left and right cerata; from posterior part of stomach, one large digestive gland duct opening, composed of several diverticula reaching posterior cerata. Posterior branch and diverticula covered by gonad. Digestive gland containing typical digestive epithelium only present in cerata, here forming rather diffuse, racemose tissue (not depicted in Fig. 2). Intestine short, thick, leading through densely ciliated anal papilla to outside.

Reproductive system (Fig. 2E,G). Diaulic. Ovotestis occupying whole posterior body region, reaching dorsally far until mid-longitudinal section. Ampulla convoluted; gonoducts connecting to small, globose, bean-shaped ampulla diverticulum placed in proximal part, ending blind; lying dorsally between gonad, mucus and membrane glands; proximally to diverticulum, elongated ampulla narrowing to distal gonoduct, branching into vas deferens and oviduct. Proximal vas deferens widening into prostate, the latter forming a loop in dorso-anterior region of animal, becoming thinner; composed by elongate cells, containing basal nucleus, cytoplasm filled of small, blue-staining granules (Fig. 3E). Distal vas deferens after prostatic part decreasing in diameter, connecting to penis. Penis unarmed, conical, relatively short; placed in ovoid penial sheath, which can be sometimes seen through penial pore in preserved specimens. Male and female genital openings in antero-lateral right position.

Oviduct starting with a sphincter, widening, entering distally the vaginal duct, which widens directly into receptaculum seminis (flow through system). This connection separated by a distinct sphincter. At same area oviduct entering nidamental glands.

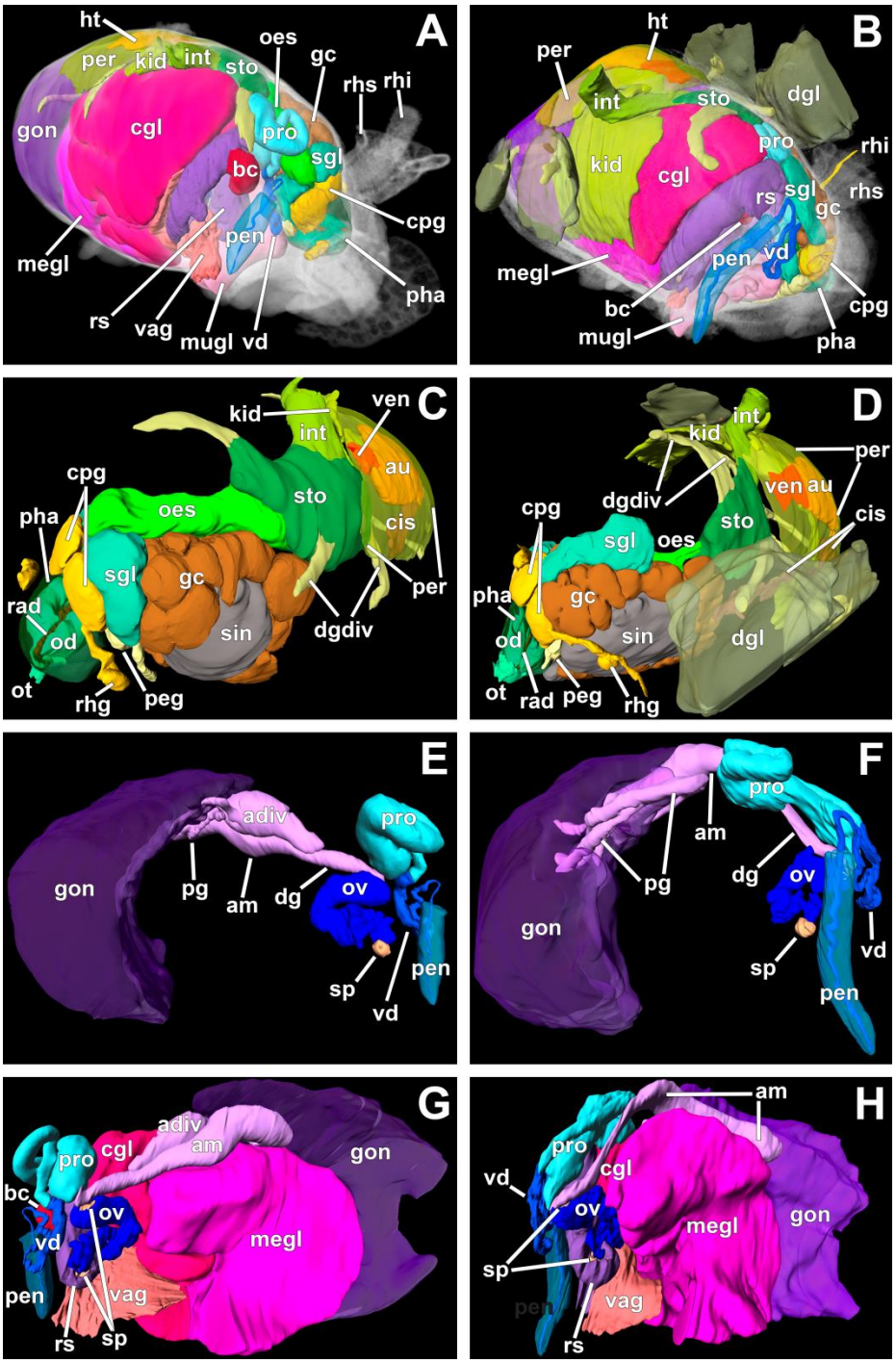

Figure 2. Micro-CT reconstructions of the internal organs of *D. antarctica* (left column) and *D. carinova* Moles, Avila & Wägele n. sp. (right column). **A–B** Right antero-lateral view of all reconstructed organs. **C–D** Left antero-lateral view of the circulatory, digestive, excretory, and nervous systems. **E–F** Right lateral view of the male reproductive system. **G–H** Left lateral view of the reproductive system (mucus gland is not depicted here since it covers the whole view). *am* ampulla; *adiv* ampulla diverticulum; *au* auricle; *bc* bursa copulatrix; *cis* circulatory sinuses; *cgl* capsule gland; *cpg* cerebropleural ganglion; *dg* distal gonoduct; *dgdiv* digestive gland diverticula; *dgl* digestive gland (only depicted in *D. carinova* n. sp.); *gc* giant cells; *gon* gonad; *ht* heart; *int* intestine; *kid* kidney; *megl* membrane gland; *mugl* mucus gland; *oes* oesophagus; *od* odontophore; *ot* oral tube; *ov* oviduct; *peg* pedal ganglion; *pen* penis; *per* pericardium; *pg* proximal gonoduct; *pha* pharynx; *pro* prostate; *rad* radula; *rhg* rhinophoral ganglion; *rhi* rhinophore; *rhs* rhinophoral sheath; *sgl* salivary gland; *sin* sinus; *sp* sphincter; *sto* stomach; *vag* vagina; *vd* vas deferens; *ven* ventricle.

Receptaculum seminis wide, highly elongated, folded, leading to vagina distally. Bursa copulatrix small, rounded, saccular; placed at middle part of vaginal duct/receptaculum seminis. Vagina flattened, highly ciliated, leading outside by wide aperture; sharing wide atrium with mucus gland. We follow the functional terminology of Klussmann-Kolb (2001) for nidamental glands, composed of capsule gland, followed by membrane gland, leading into mucus gland. Capsule gland occupying right antero-lateral region of animal; composed by thin, columnar cells containing small microvilli in apical pole, basal nucleus, cytoplasm entirely composed of bluish granules, which become increasingly pinkish towards end of gland (Fig. 3F). Membrane gland lying ventrally under capsule gland, extending further posteriorly under gonad, composed by columnar cells filled with reddish granules (Fig. 3G). Mucus gland, the largest part of the nidamental gland, occupying ventrally about 2/3 of body, also extending anteriorly; composed of columnar cells containing basal nucleus, many small, ovoid, violet granules (Fig. 3H).

Nervous system (Fig. 2C). Oesophageal nerve ring composed of four ganglia. Cerebral ganglia fused to pleural ganglia into cerebropleural ganglia. Ganglia composed by cortical layer of neurones encircling central neuropil. From neuropil of cerebropleural ganglion one nerve connects to small rhinophoral ganglion, placed right at basal part of each rhinophore. Rhinophoral nerve short leading to top of rhinophore; short optic nerves connecting to eyes. Eyes containing large, spherical lens; retina showing melanin granules. Both cerebropleural ganglia nearly close together, no cerebral commissure visible. Pedal ganglia, interconnected by relatively long commissure, lying close to cerebropleural ganglia with short connectives. From these, one nerve running anteriorly, another down to foot passing through giant cells (GCs) and sinus. A total of 27 GCs measuring 168.7 ± 20.14 µm (mean ± sd) in maximum diameter, seen close to left cerebropleural and pedal ganglia and right salivary gland, extending posteriorly forming a circle in left anterior region of animal; only one GC lying under left pedal ganglion; cytoplasm remains mostly occupied by huge nucleus containing faint bluish fibrillar appearance (appearing shining in micro-CT like the cortical neurons of ganglia); several amoeboid-shaped nucleoli, staining dark blue, apparent (Fig. 3D). All GCs contact to each other, close to central hemolymphatic sinus and/or to small vessel-like sinus. The whole GC complex occupying one third of anterior body volume.

Circulatory and excretory systems (Fig. 2C). Pericardium wide, flattened; situated dorsally, behind anal papilla. Auricle connecting to small anterior lying ventricle. Vessel-like structure running from auricle anteriorly to edge of pericardium, reaching cerata. Kidney flattened, connecting ventrally on right side to pericardium; extending far into posterior part of animal, widening notably; nephroduct extending far into anal papilla, leading outside close to anus.

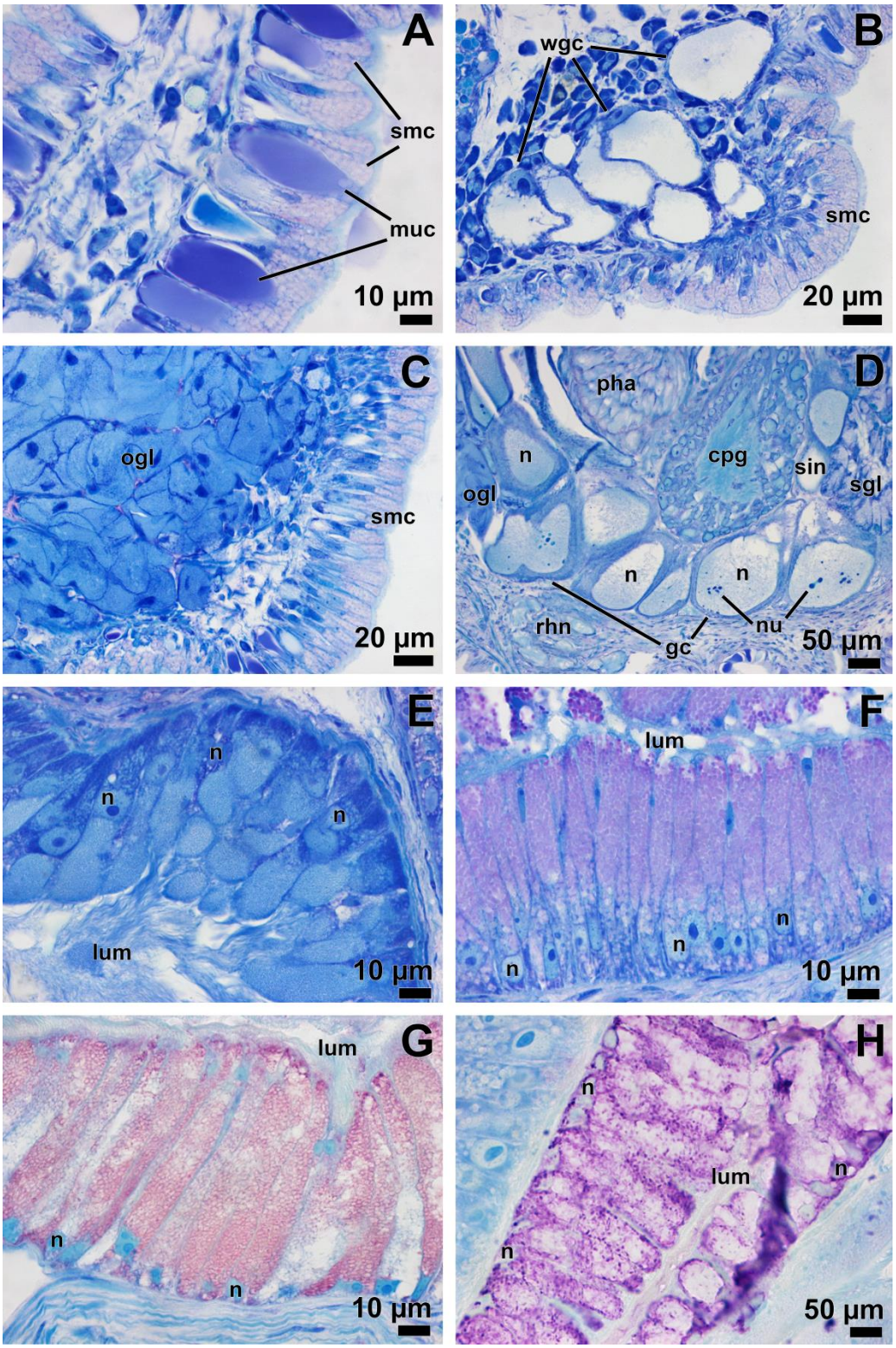

Figure 3. Histological slides of the glandular structures in *D. antarctica*. **A** Epidermis of the rhinophoral sheath. **B** Detail of one cerata tubercle showing defensive glandular cells (white punctuation in live animals). **C** Oral glands. **D** Giant neurones chain attached to the cerebropleural ganglion; a thin cortex, large nucleus, and nucleolus of each cell can be seen. **E** Detail of the prostate glandular cells. **F** Detail of the capsule glandular cells. **G** Detail of the membrane glandular cells. **H** Detail of the glandular mucus cells. *gc* giant cells; *lum* lumen; *muc* glandular mucus cell; *n* nucleus; *nu* nucleolus; *ogl* oral glands; *pha* pharynx; *rhn* rhinophoral nerve; *sin* sinus vessel; *sgl* salivary gland; *smc* specialised multivacuolised cell; *wgc* white glandular cells.

Epithelial glandular structures. Notal epithelium formed by unicellular layer of pinkish multivacuolized cells (specialised vacuolated epithelium), interspersed with mucus glandular cells (Fig. 3A). Multivacuolized cells prismatic in shape, having basal nucleus, presenting microvilli all over apical part. Mucus gland cells presenting one huge vacuole occupying whole cytoplasm, containing different shades of homogeneous violet-stained contents (acid mucopolysaccharides); ubiquitously seen in notum, rhinophores, and cerata. Subepithelial clusters of large, bluish, glandular cells observed in rhinophore tips, sheath, and cerata tubercles; probably responsible for whitish appearance in live animals; probably exuded their contents when animal disturbed, thus defensive function is proposed (Fig. 3B).

Egg mass (Fig. 1G). Oval, slightly bean-shaped, 3.5–4 x 3.5 x 2 mm (length:heigth:width); enveloped by thick membrane composed of four layers (Fig. 6). Outermost layer with maximal width of 3.3 µm measured, blue coloured; following outer layer 53.3 µm, pink, containing profuse wholes; inner layer 40.51 µm, pink; innermost 48.39 µm, purple. Egg mass presenting dorsal keel, of around 0.4 mm, extending far from one side to the other. Whole egg mass attached to substrate by twisted stalk placed in mid-lateral position. Dorsal keel and stalk composed mainly by first three layers. Egg capsules measured 336.95 ± 32.16 µm (mean ± sd); cytoplasm mainly composed by blue-staining protein platelets.

Ecology. The 19 specimens were found in benthic ecosystems at 65–500 m depth. Some specimens were found laying egg masses on two unidentified hydrozoans of the genera *Oswaldella* Stechow, 1919 (Plumularioidea: Kirchenpaueriidae) and *Antarctoscyphus* Peña Cantero, Garcia Carrascosa & Vervoort, 1997 (Sertulariidae).

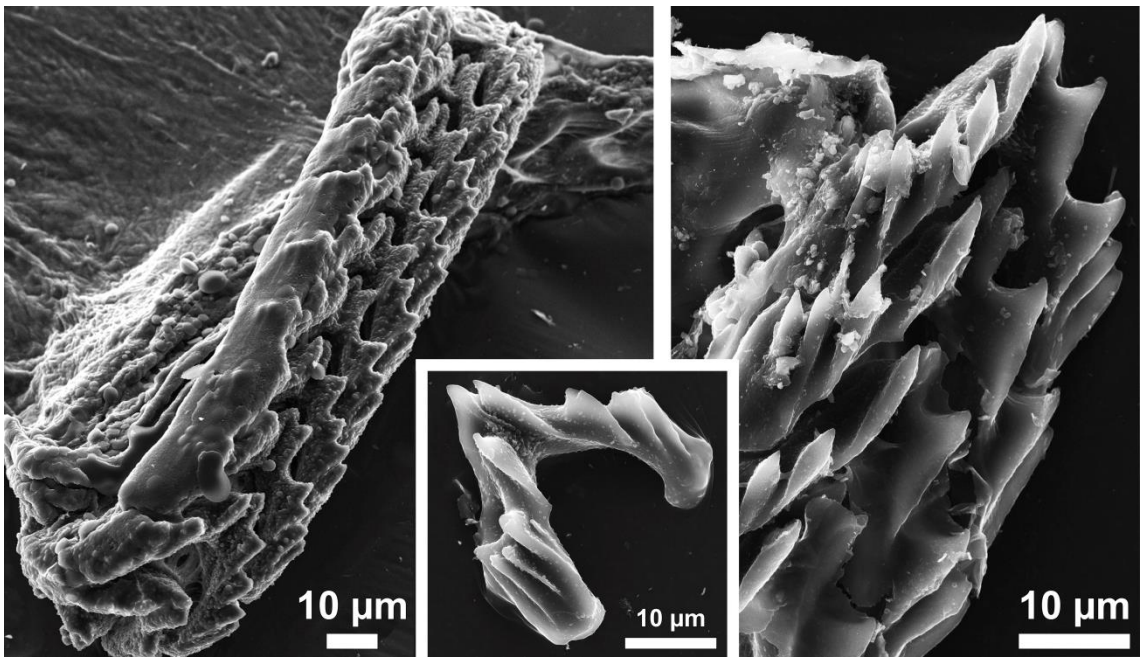

Figure 4. Scanning electron micrographs (SEM) of the radular teeth of *D. antarctica*.

***Doto carinova* Moles, Avila & Wägele n. sp.**

(Figures 1,2,5,6) (See 3D PDF of the reconstructed anatomy of the anterior region of the specimen in Supplementary Material 2)

http://zoobank.org/NomenclaturalActs/7023623D-B021-4D83-B216-B8C6ACDCDD19

Type locality. Eastern Weddell Sea (71° 6.44' S; 11° 27.76' W), 277 m depth.

Material studied. A single specimen collected during the Antarctic cruise ANT XXI/2 (see table 1). Deposited in SNSB Zoologische Staatssammlung München (Catalog number ZSM Moll 2016114).

External morphology (Fig. 1B, D, F). Body bulged anteriorly due to reproductive system; live specimen measured 13 mm in length, 10 x 4 x 5 mm (length:width:height) when preserved. Body and cerata creamy coloured; presenting bright white spots (glandular cells) in tip of rhinophores, along edge of rhinophoral sheath, and each tubercle in cerata. Velum short, rounded. Oral tentacular processes absent. Eight long, pointed cerata pairs, 6 mm high, narrow connections to body; apex long, lobulated; elongate tubercles on cerata disposed in 4–5 circlets with maximum of nine per circlet. Pseudobranchs absent. Rhinophores transparent, thin, smooth, blunt; rhinophoral sheath short, cylindrical, narrow (calyciform), slightly expanded anteriorly, being up to 1/2 of rhinophore size. Anal papilla large, placed dorsally in mid-right position. Genital apertures lying under right row of cerata, between 1st and 2nd. Foot narrow, rounded anteriorly, tapering posteriorly to short, blunt tail.

Digestive system (Fig. 2B, D). Mouth opening ventrally between oral veil and foot; oral tube relatively short, surrounded by follicular oral glands. Pharynx bulbous, containing thin cuticle. Radula not analysed since the specimen was not dissected. Paired salivary glands leading to middle part of pharynx, entering via long ducts; right one stretching along posterior part of cerebropleural ganglion, not reaching backwards as left one, which extends far posteriorly along oesophagus; both being long, saccular, widening progressively posteriorly (giving pyriform appearance). Oesophagus opening at posterior dorsal side of pharynx; widening, connecting to stomach. Stomach widening, flattening, opening into two digestive gland diverticula, which run into anterior cerata; third (posterior) branch of digestive gland opening posteriorly from stomach, running to posterior part of animal, branching off diverticula which reach into last cerata pairs. Digestive gland diverticula connecting to typical diffuse, racemose digestive gland, situated exclusively in cerata, occupying it almost entirely. Intestine cylindrical in cross section, opening from posterior part of stomach, leading outside by long anal papilla.

Reproductive system (Fig. 2F). Diaulic. Gonad occupying whole posterior body region, reaching far until mid-longitudinal section on dorsal side. Smaller gonoducts uniting into anterior gonoduct, leading into large, elongated ampulla; the latter lying dorsally underneath gonad, close to mucus and membrane glands. From distal part of

ampulla thin distal gonoduct extending anteriorly to prostate and oviduct. Prostate long, isodiametric, folded, leading to thin, contorted distal vas deferens, connecting to penis. Penis long, conical, unarmed; placed in ovoid penial sheath; tip of penis seen outside penial pore in preserved specimen. Genital openings lying in antero-right position.

Oviduct starting with a sphincter, widening, branching into distal oviduct, leading directly into nidamental glands and vaginal duct, opening directly into receptaculum seminis (flow through system). Bursa copulatrix attached to widened part of receptaculum seminis. Both placed close to and below prostate; oviduct separated from bursa copulatrix again by additional sphincter. Bursa copulatrix wide, highly elongated, folded, leading distally to vagina. Receptaculum seminis small, rounded, saccular; attached to vaginal canal in middle position. Vagina short, flattened, leading outside by wide aperture; sharing wide atrium with nidamental glands.

Nidamental glands composed of: 1) capsule gland occupying right antero-lateral region, 2) membrane gland placed right under capsule gland, extending further posteriorly below gonad, 3) highly folded mucus gland reaching ventrally far into anterior and posterior part of body.

Nervous system (Fig. 5). Oesophageal nerve ring composed of four ganglia. Cerebral ganglia fused to pleural ganglia into cerebropleural ganglia; standing close without distinct commissure; also standing close to pedal ganglia, the latter interconnected by relatively long commissure. Each cerebropleural ganglion sending one nerve to small rhinophoral ganglion, placed right at bottom of each rhinophore; from there, a short nerve leading to top of rhinophore. From pedal ganglia, one nerve running down to foot, through giant cells (GCs) and sinus. Forty interconnected GCs can be seen (appearing shining in micro-CT like the cortical neurons of ganglia), some close to cerebropleural and pedal ganglia and right salivary gland; extending posteriorly, forming a circle in left anterior region of animal body; only two GCs found under pedal ganglia; all GCs attached to large hemolymphatic sinus; whole GCs complex occupying one third of body volume in anterior part.

Circulatory and excretory systems (Fig. 2D). Pericardium wide, flattened, occupying dorsal part of body right behind anal papilla. Heart composed by large auricle and anterior lying ventricle, both placed in longitudinal axis. Several sinuses connecting to pericardium, eventually to auricle, receiving oxygenated hemolymph from each ceras; running close to digestive gland diverticula (only two at left and one at right side could be seen and depicted in micro-CT reconstruction). Kidney placed ventrally of pericardium; directly connected in anterior right position through small duct; nephroduct leading to anal papilla; nephropore close to anus.

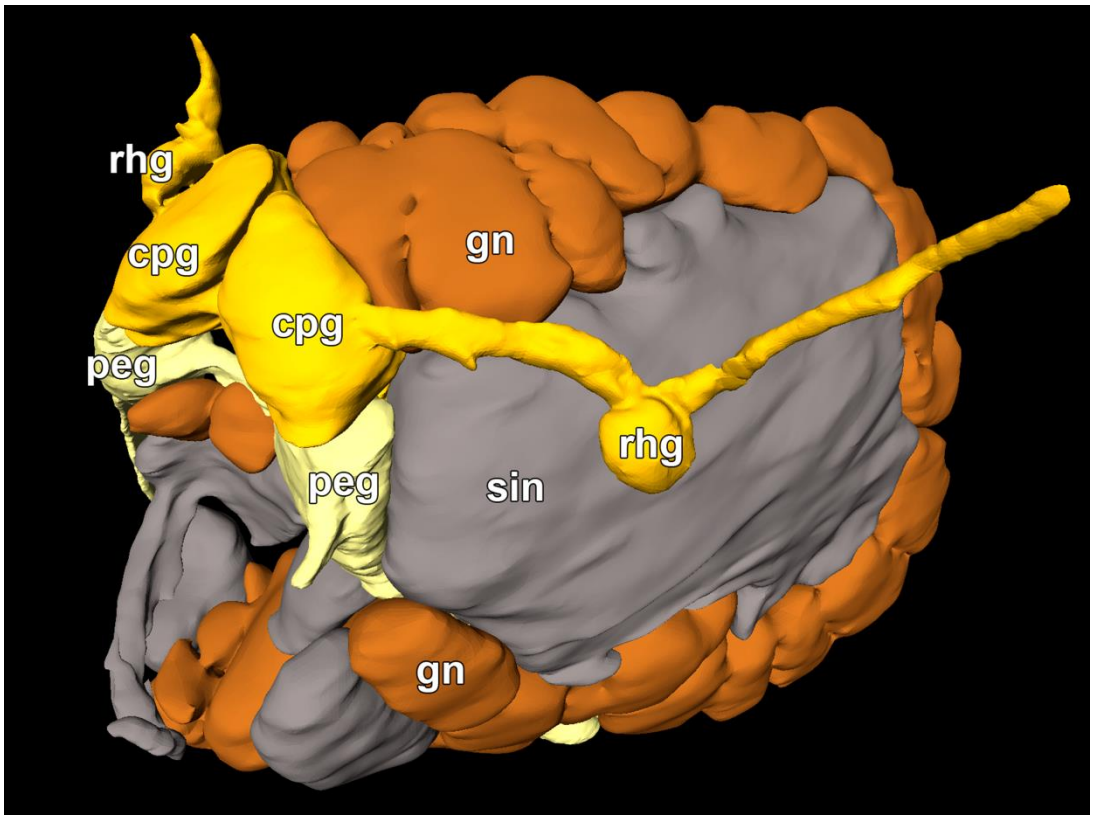

Figure 5. Left antero-lateral view of the micro-CT reconstruction of the nervous system of *D. carinova* Moles, Avila & Wägele n. sp. *cpg* cerebropleural ganglion; *gc* giant cells; *peg* pedal ganglion; *rhg* rhinophoral ganglion; *sin* sinus.

Egg mass (Fig. 1H). Reniform, bean-shaped egg masses measured 5.5–8.4 x 3.5–6 x 2–2.5 mm (length:heigth:width); slightly asymmetrical in transverse section, being bulged in one side; enveloped by thick membrane, composed by four layers. Outermost layer staining blue; 3 µm in width; following outer layer 33.3 µm, pink, containing abundant wholes; inner layer 23.71 µm, pink; innermost 27.7 µm, purple (Fig. 6); dorsal keel and stalk only composed by the three outer layers. Dorsal keel measuring 0.8–1 mm in length by 118 µm in width at wider base, running from one side to the other. Whole egg mass attached to substrate by one twisted stalk placed in mid-lateral position. Live egg capsules white, becoming orange when preserved, measuring 357.48 ± 29.8716 µm (mean ± sd); cytoplasm mainly composed by blue-staining protein platelets.

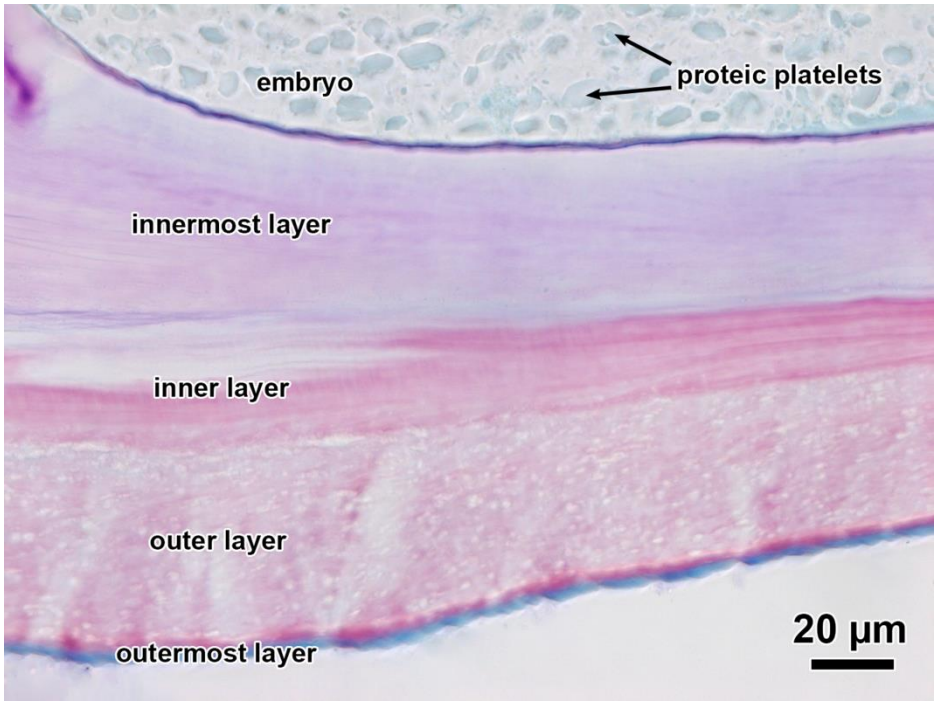

Figure 6. Histological slide of outer surface of the egg mass in *Doto carinova* Moles, Avila & Wägele n. sp.

Ecology. *Doto carinova* n. sp. was collected from muddy bottoms at 277 m depth in a benthic community with abundant gorgonians (*Thouarella*, Primnoidae), sponges, colonial tunicates, hydrozoans, bryozoans, amphipods, ophiuroids, and molluscs. The animal was found laying an egg mass on the Isididae gorgonian *Primnoisis antarctica* (Studer, 1878), close to other three additional egg masses.

Etymology. In the name *Doto carinova* n. sp., the specific epithet is an apposition derived from the words *carina* (=keel) and *ova* (=eggs) in Latin, referring to the pronounced keel observed in the egg mass.

Remarks (Table 2). Externally *D. carinova* n. sp. differs from the sympatric *D. antarctica* in having a longer and paler body, a shorter velum, eight (versus six) longer cerata with less and more pronounced tubercles, a short and circular rhinophoral sheath, and a slightly larger anal papilla. The salivary glands differ notably from these of *D. antarctica,* by being longer, presenting longer salivary ducts, and presenting the right salivary gland reaching far posteriorly. The ampulla is folded and presents a proximal bean-shaped diverticulum in *D. antarctica*, while it is elongated and isodiametric in *D. carinova* n. sp. Moreover, the prostate is longer and folded in *D. carinova* n. sp., while in *D. antarctica* it is short, wide proximally, and forms a pronounced loop. The penis is also longer in the new species. More GCs (40) can be seen in the new species than in *D. antarctica* (27).

Table 2. Differential characters among *D. carinova* Moles, Avila & Wägele n. sp. and *D. antarctica*.

	***Doto carinova* n. sp.**	***Doto antarctica* Eliot, 1907**
External morphology		
colour	yellowish, creamy, pale	brownish
rhinophoral sheath	rounded, calyciform	elongated anteriorly, "calla lilly"-shaped
velum	narrow	broad
cerata	8	6
tubercles	up to 9, long (6 mm), lobulated	up to 12, short (3–4 mm), rounded
Digestive system		
salivary glands	pyriform, elongated, right extending back over oesophagus	saccular, roundish, not extending back
Nervous system related		
giant cells	40	27
Reproductive system		
ampulla	elongated	convoluted; round, bean-shaped proximal diverticulum, elongated distal part
prostate	convoluted, long, isodiametric	widened proximally, pyriform, short, arranged in a loop
penis	long, thin	short, conical
Egg mass		
shape	bean-shaped, transversally assymetrical	rounded, transversally symmetrical
dorsal keel	broad (0.8–1 mm)	narrow (0.4 mm)
Substrate	gorgonians (*Primnoisis antarctica*)	hydrozoans (*Oswaldella* sp., *Antarctoscyphus* sp.)

The egg clutches of *D. carinova* n. sp. are more elongated, slightly asymmetrical in transverse section, and possess a higher dorsal keel (1 mm) than those of *D. antarctica*. However, there are no histological differences in the mucus layers of the egg masses of the two species. Egg masses of both species were found in different cnidarian substrates, being perhaps different prey items. While *D. antarctica* was found on hydrozoans of the genera *Antarctoscyphus* and *Oswaldella*, *D. carinova* n. sp. was found on the gorgonian *Primnoisis antarctica*.

Phylogenetic analysis

The total data set contained 62 species of *Doto* and 11 outgroup species. The aligned genes comprised 2,283 characters. The maximum likelihood (ML) and Bayesian (BI) trees are similar (Fig. 7). The family Dotidae resulted monophyletic. *Kabeiro* species are the sister group of all *Doto* species. Within *Doto*, *D. pinnatifida* was sister group to all other species. The two newly sequenced specimens of *D. antarctica* from the Weddell

Sea clustered together and with the specimens from the Ross Sea. *Doto antarctica* is sister group of Philippine and Papua New Guinea specimens (*D. ussi*, *D. greenmayeri*, and other unidentified species). Thus *D. antarctica* is more closely related to Pacific *Doto* species than to Atlantic (northern and southern hemispheres) species.

Regarding the additional Mediterranean species sequenced herein, all of them clustered with species from the Northern Sea and Eastern North Atlantic. *Doto floridicola* specimens clustered with the Mediterranean specimen accessed in GenBank (Wollschield-Lengeling *et al*, 2001). The specimens of *D. koenneckeri* grouped with the previous Mediterranean and Welsh specimens. However, two supposed *D. dunnei* specimens (#1 and #3) used in this study clustered also within the *D. koenneckeri* individuals. An additional *D. dunnei* (2) from the same locality was not found to be related to the previous specimen sequenced from Wales, but instead clustered with the three Mediterranean specimens of *D. coronata* sequenced here. However, these latter specimens did not cluster to any *D. coronata* specimens sequenced from the Netherlands, North Sea, USA, nor Wales. Although *D. coronata* has been also recorded in the Mediterranean, it is possible that this species is an undescribed species of *Doto*, since it presents morphological characters that differ from those of the original description (author's unpub. data). Finally, *D. paulinae* was found to be closely related to *D. eireana*, although with low support.

DISCUSSION

In this study, we added molecular evidence for the circumpolar distribution of *D. antarctica*, as well as a detailed anatomical and histological description of the species and its egg masses. A thorough anatomical description is highly important since the species of *Doto* have often been misidentified due to the lack of clear external diagnostic characters. External descriptions available until now for *D. antarctica* clearly coincide with the description presented herein (Eliot, 1907; Odhner, 1934). However, Eliot noted a ridge in front of the rhinophoral sheaths, which might be a fixation artefact (Odhner, 1934). Also, the number of rows of radular teeth in our dissected specimen was smaller than in previous observations (Eliot, 1907; Odhner, 1934), although this may be an age-dependent character. The shape of the rachidian tooth is consistent with the descriptions within the genus (Thompson *et al.*, 1990). Since radulae are variable within species and among teeth of the same radula (usually bilaterally asymmetrical), this character is not considered relevant for distinguishing *Doto* species (Odhner, 1936; Marcus, 1961).

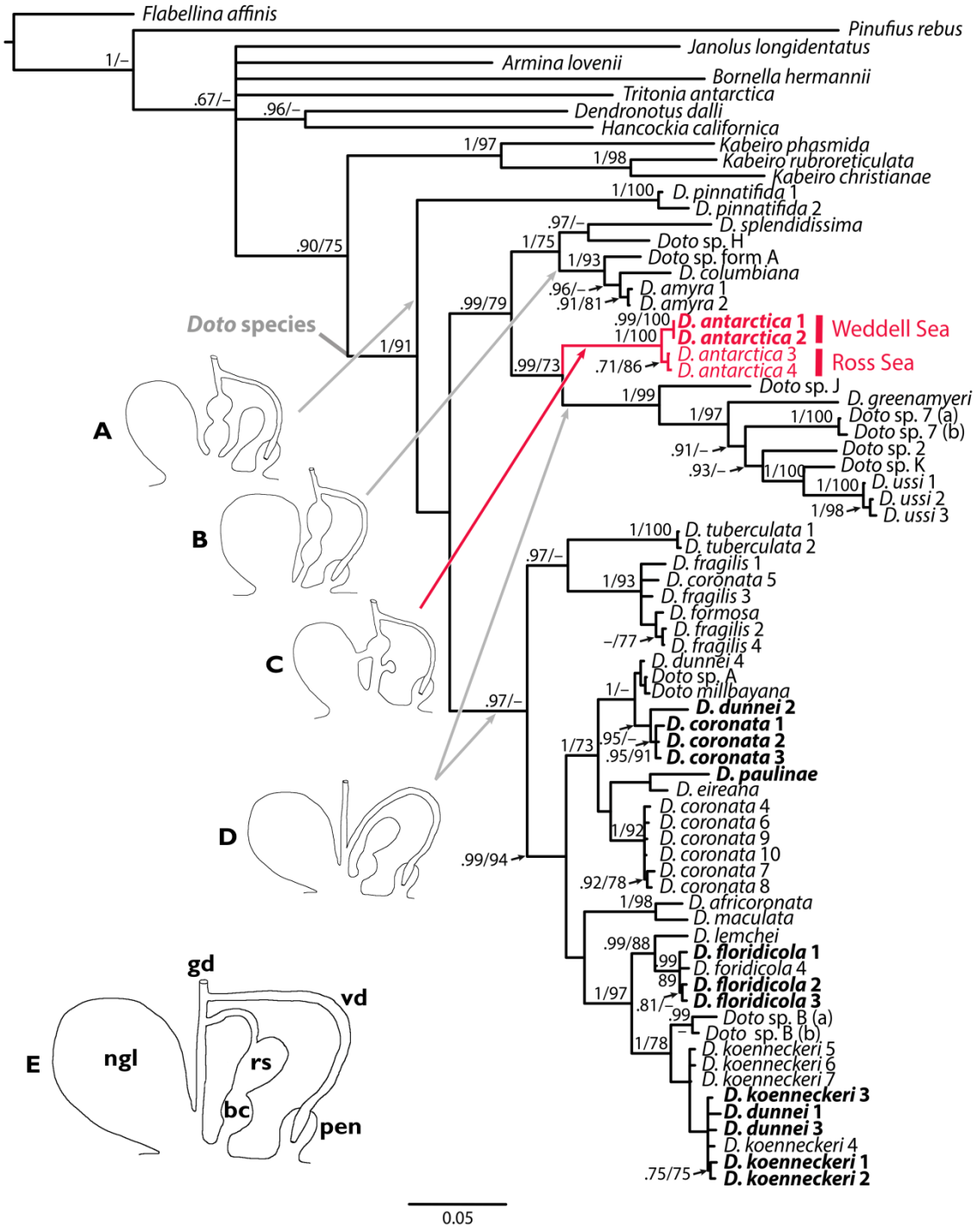

Figure 7. Phylogenetic tree of *Doto* species based on the combined COI, 16S, and H3 genes using Bayesian inference (BI) and maximum-likelihood (ML). Numbers on nodes indicate posterior probability values (BI) and bootstrap support values (ML). Specimens sequenced are in bold; *Doto antarctica* specimens are coloured in red. Schematic drawings of the reproductive system of *Doto* species are depicted (A–D), as well as the unsequenced *D. uva* Marcus, 1955 (E). *bc* bursa copulatrix; *gd* gonoduct; *nigl* nidamental glands; *pen* penis; *rs* receptaculum seminis; *vd* vas deferens.

Histological sections of *D. antarctica* showed the typical notal epithelium of cladobranchs, composed by multivacuolised cells and mucus glandular cells (Wägele *et al.*, 2006; Moles *et al.*, 2016). The former cells protect the slug against cnidocysts (Greenwood, 2009), the latter are rather typical for Dendronotida (Wägele *et al.*, 2006; Affeld *et al.*, 2009). Subepithelial clusters of single gland cells staining light blue (white in live specimens) were found in the most exposed parts of the animal, *i.e.*, cerata and rhinophores. We propose a defensive function of these glands due to the strategic location in exposed parts, and because they everted their content when molested. These single glandular cells are commonly suggested to be defensive glandular cells (Baba, 1971; Wägele *et al.*, 2006), where the animal stores defensive compounds obtained from prey or *de novo* biosynthesised by the slug, such as terpenoids (Putz *et al.*, 2011).

Doto carinova Moles, Avila & Wägele n. sp. was found in sympatry with *D. antarctica* in the Weddell Sea and firstly described herein. Although sequencing of the new species from chemically fixed material was impossible, morphological characters help to differentiate both species. Externally, *D. carinova* n. sp. exhibits a paler colouration, a higher number and more elongated cerata, and calyciform rhinophoral sheaths (see remarks above). Remarkably, the egg masses of both species described herein are unique in shape among *Doto* species. Usually they are ribbon like structures deposited on the substrate in a zig-zag folded way (Kress, 1975; Lemche, 1976; Ortea & Urgorri, 1978; Picton & Brown, 1981; Fischer *et al.*, 2006), whereas in the Antarctic species they have a bean-like shape, possessing a keel, and being attached to the substrate by a twisted stalk. However, the egg mass of *D. carinova* n. sp. is more pronouncedly reniform and has a wider keel. Furthermore, both species were found on different cnidarian species, albeit being collected from the same locality in the Weddell Sea. Different substrates might represent different food sources for the species, a fact that should be considered as an additional character for distinguishing among species (Lemche, 1976). Descriptions presented herein will be useful to identify the undetermined species of *Doto* collected in different Antarctic regions (Thiele, 1912; Schiaparelli *et al.*, 2006; Ghiglione *et al.*, 2013), and probably to expand the distribution of *D. carinova* n. sp.

Regarding the internal anatomy, the shape and arrangement of the salivary glands, ampulla, prostate, and penis are clearly diagnostic among both Antarctic species (see remarks above). A proximal blind diverticulum of the ampulla was only present in *D. antarctica*. The prostate is shorter and wider in *D. antarctica*, while it is longer in *D. carinova* n. sp. Both species present sphincters to separate ampulla, oviduct, and the allosperm vesicles (receptaculum seminis and bursa copulatrix), similarly to *D. uva* (Fischer *et al.*, 2006) and many other *Doto* species. The general outline of the reproductive system in *D. carinova* n. sp. is similar to *D. antarctica*. The diaulic reproductive system of both Antarctic species presents an oviduct leading into the receptaculum seminis (and bursa copulatrix annexed), which connects into a separate folded area of the nidamental glands (Fig. 7C). Although the connection of the oviduct

with the nidamental glands lies more internally in the Antarctic species, this arrangement of ducts is very similar to the more closely related *D. amyra* and *D. columbiana* (Fig. 7B; Marcus, 1961). A similar arrangement can be found in several other *Doto* species not included in our phylogenetic analyses (*D. bella, D. caramella, D. chica, D. divae, D. doerga, D. ganda, D. japonica, D. kya, D. varians*, and *D. wara*; Marcus, 1959, 1961; Baba, 1971; Schmekel & Portmann, 1982). Another feature in which the Antarctic species differ from the latter species is the distinct bursa copulatrix attached to the vaginal duct.

Doto pinnatifida was recovered basal in our phylogenetic tree, displaying a similar arrangement to the Antarctic species, but with a bursa inserting close to the vaginal opening (Fig. 7A; Schmekel & Kress, 1977). In addition, a fourth, quite spread, reproductive system is depicted; this displays an oviduct not connected proximally to the receptaculum (Fig. 7D). This fourth system is found in a lineage close to *D. antarctica* and composed by *D. greenmayeri* and *D. ussi* (Shipman & Gosliner, 2015). Likewise, a second linage represented by *D. africoronata*, *D. coronata*, *D. floridicola*, *D. formosa*, *D. fragilis*, and *D. paulinae* also presented this system (Dreyer, 1911; Baba, 1938; Marcus & Marcus, 1963; MacFarland, 1966; Marcus, 1972; Schmekel & Kress, 1977; Schmekel & Portmann, 1982; Shipman & Gosliner, 2015). Additionally, according to Fischer *et al*. (2006) a triaulic reproductive system is present in *D. uva* (Fig. 7E), albeit Marcus (1959) considered it similar to that of *D. pinnatifida*. Summarizing, our phylogenetic analyses show a trend towards the reduction of bursa copulatrix and the separation of the vaginal duct from the oviduct (Fig. 7). This implies that the "flow through system" of the oviduct into vaginal duct is changing into a separate outleading duct for eggs and a shift of the fertilization chamber towards the distal part of the female genital system.

Histological analysis revealed gigantic gland cells around the salivary glands of *D. bella*, *D. japonica*, and *D. uva* (Baba, 1971; Fischer *et al.*, 2006). They have been traditionally considered accessory glandular cells to the salivary glands. In our histological and tomographic analyses these giant cells resemble neuronal cells, similar to ganglionic cortical neurones, but larger in size. Moreover, these are not exclusively located close to the salivary glands but are in close contact to the ganglionic complex (see Fig. 2 C–D). Therefore, we consider them to be giant neurones as recorded in anaspideans and pulmonates (Weiss & Kupfermann, 1976), as well as in cladobranch and doridacean nudibranchs (Newcomb & Katz, 2007). They are located in the ganglionic mass (*i.e.*, metacerebral cells), related to external sensory input from the head and considered homologous within these groups (Weiss & Kupfermann, 1976). These giant neurones have been found to be polyploid by increasing the DNA content step-wise as the animal grows (Boer *et al.*, 1970). This has been suggested to be related to a major hormone secretory function, responsible for behavioural responses such as crawling (Newcomb & Katz, 2007). However, in *D. antarctica* and *D. carinova* n. sp., although being connected to the cerebropleural ganglia, they form a circle extending posteriorly (Fig. 5). Since our GCs, like other describedgiant neurones, possess a huge

(active) nucleus, we speculate that neurosecretory hormones might be secreted into the hemolymphatic sinus located within this circle. Our study seems to be the first description of a complex and asymmetrical neuronal/secretory arrangement in heterobranchs. Furthermore, it is possible that the number of GCs could be used as a diagnostic character for discriminating among *Doto* species. For instance, Antarctic species of *Doto* have larger (170 µm) and more abundant (27–40) GCs than the South American *D. uva* (150 µm, N=12) (Fischer *et al.*, 2006).

The phylogenetic analyses recovered trees with similar topologies than others recently published (Shipman & Gosliner, 2015; Pola & Gosliner, 2015). However, contrary to Pola and Gosliner (2010), *Pinufius* is not part of the Dotidae clade. *Doto antarctica* specimens from the Weddell Sea were closely related to those from the Ross Sea, as suggested morphologically herein. Contrary to the morphological similitudes mentioned by Eliot (1907) when describing *D. antarctica,* and comparing to *D. fragilis* (Forbes, 1838), phylogenetic analyses revealed that these species are not closely related. Based on external appearance, Odhner (1934) stated later that *D. antarctica* was more closely related to *D. formosa* Verrill, 1875. However, in our analyses, *D. fragilis* and *D. formosa* were closely related to each other, sharing a similar reproductive system, but were dissimilar to *D. antarctica.* Once more, descriptions merely based on external characters seem insufficient to establish phylogenetic relationships, or even to identify and describe *Doto* species. A closer phylogenetic relationship was found among *D. antarctica* and the Indo-Pacific species, and altogether with the rest of Southern species of *Doto.* This relationship could be related to the Antarctic origin of the group, and the older origin of the Indo-Pacific species respect to the Atlantic ones. However, the majority of the sequenced species so far are from the northern hemisphere. Thus, there is a need for sequencing more species from the Austral oceans to assess possible phylogeographic relationships among *Doto* species. Further sampling efforts should be conducted to collect and sequence *D. carinova* n. sp. and other undetermined species around Antarctica to increase the knowledge of Dotidae, with only two species found in the Southern Ocean to date.

CONCLUDING REMARKS

New Dotidae species have been usually described based only on external morphological and radular characters. Nonetheless, internal organ organisation and egg mass structure is desirable for describing *Doto* species. Micro-CT and histology has demonstrated to be very useful techniques to reconstruct the internal anatomy of these two *Doto* species. Two new occurrences of *D. antarctica* were recorded in Bouvet Island and the eastern Weddell Sea. These specimens are morphologically and genetically characterised herein and appeared related to *D. antarctica* from the Ross Sea, which strongly suggests a circumpolar distribution. We also described *D. carinova* n. sp. occurring in sympatry with *D. antarctica* in the Weddell Sea. Although some

distinguishing characters can be size-related, the lower number of tubercles on the cerata, the different form of the rhinophoral sheath, the shape and arrangement of the salivary glands, ampulla, and prostate in the large specimen of *D. carinova* n. sp., as well as differences in the egg masses and cnidarian substrate indicate separate evolutionary lineages.

A phylogenetic hypothesis including various species of *Doto* from various regions showed a trend towards the reduction of bursa copulatrix and distal connection of oviduct to the nidamental glands with separate pathways for eggs and allosperm. Furthermore, we identified and described the nervous system of *Doto* species that contains accessory giant cells that might represent neurones with neuronal/secretory function. Future studies may unravel the properties and function of these peculiar giant cells. Moreover, further studies should revisit and check the identification of *Doto* species collected in former Antarctic cruises, because the present study provides new characters that may allow distinguishing among species.

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Supplementary Table 1. Species included in the phylogenetic analysis. Species sequenced in this study are in **bold**.

Species	**Locality**	**Voucher**	**CO1**	**16S**	**H3**	**Reference**
Armina lovenii	Kattegat, North Sea		AF249781	AF249243	–	Wollscheid-Lengeling *et al.*, 2001
Bornella hemannii	Malaysia: Tokong Kamundi South	CASIZ175743	HM162705	HM162625	HM162531	Pola & Gosliner, 2010
Dendronotus dalli	USA, North Atlantic		AF249800	AF249252	–	Wollscheid-Lengeling *et al.*, 2001
Doto africoronata	South Africa	CASIZ176278	HM162734	HM162657	HM162566	Pola & Gosliner, 2010
Doto amyra 1	California, USA	CASIZ179473b	KJ486702	KJ486767	KJ486670	Shipman & Gosliner, 2015
Doto amyra 2	California, USA	CASIZ181213	KJ486703	KJ486768	KJ486674	Shipman & Gosliner, 2015
***Doto antarctica* 1**	N Kapp Norvegia, E Weddell Sea, Antarctica	ANTXV/3 48/033-91	**KX274295**	**KX274324**	**KX274308**	This study
***Doto antarctica* 2**	N Kapp Norvegia, E Weddell Sea, Antarctica	ANTXV/3 48/033-92	**KX274294**	**KX274325**	**KX274310**	This study
Doto antarctica 3	Ross Sea, Antarctica		GQ292025	–	–	Shields *et al.*, 2009
Doto antarctica 4	Ross Sea, Antarctica	CASIZ190213	KJ486705	KJ486765	KJ486686	Shipman & Gosliner, 2015
Doto columbiana	Washington, USA		GQ292026	–	–	Shields *et al.*, 2009
***Doto coronata* 1**	Blanes, Spain, Mediterranean Sea	CBL1	**KX274285**	**KX274321**	**KX274305**	This study
***Doto coronata* 2**	Blanes, Spain, Mediterranean Sea	CBL5	**KX274287**	**KX274323**	**KX274306**	This study
***Doto coronata* 3**	Palamós, Spain, Mediterranean Sea	CFO2	**KX274286**	**KX274322**	**KX274307**	This study
Doto coronata 4	Kattegat, North Sea		AF249794	–	–	Wollscheid-Lengeling *et al.*, 2001
Doto coronata 5	North Sea		KR084788	–	–	Barco *et al.*, 2016
Doto coronata 6	Eastern Scheldt, Netherlands	CASIZ190710a	KJ486720	KJ486763	KJ486655	Shipman & Gosliner, 2015
Doto coronata 7	Pembrokeshire, Wales	Mn33146	KJ486722	KJ486764	KJ486652	Shipman & Gosliner, 2015
Doto coronata 8	Tuskar Rock, Skomer,	Mn33135	KJ486723	KJ486762	KJ486653	Shipman & Gosliner, 2015

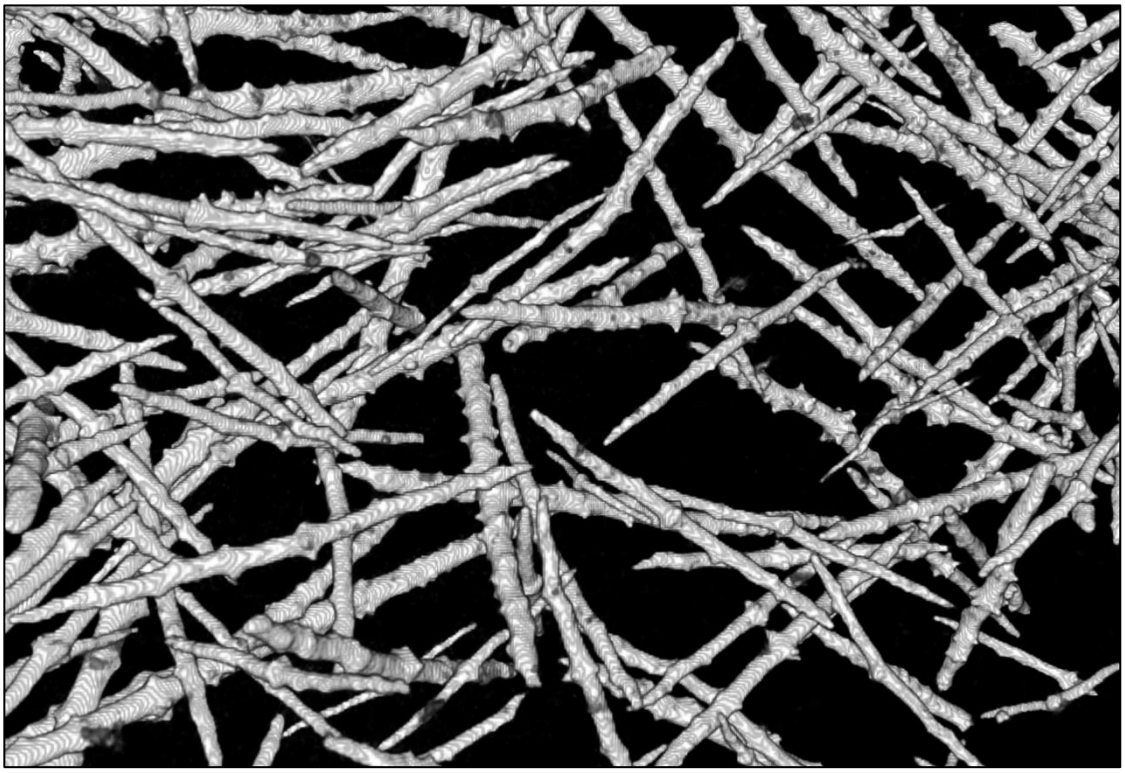
	Wales					
Doto coronata 9	Eastern Scheldt, Netherlands	CASIZ190710b	KJ486721	KJ486761	KJ486656	Shipman & Gosliner, 2015
Doto coronata 10	Maine, USA	CASIZ183936	KJ486719	KJ486760	KJ486654	Shipman & Gosliner, 2015
***Doto dunnei* 1**	Cap de Creus, Spain, Mediterranean Sea	DPG1	**KX274292**	**KX274318**	**KX274300**	This study
***Doto dunnei* 2**	Cap de Creus, Spain, Mediterranean Sea	DPG2	**KX274293**	**KX274319**	**KX274301**	This study
***Doto dunnei* 3**	Cap de Creus, Spain, Mediterranean Sea	DME3	**KX274291**	**KX274320**	**KX274299**	This study
Doto dunnei 4	Pembrokeshire, Wales	Mn33147	KJ486725	–	KJ486659	Shipman & Gosliner, 2015
Doto eireana	Spain, NE Atlantic	CASIZ190544	KJ486657	AF249248	–	Wollscheid-Lengeling *et al*., 2001; Shipman & Gosliner, 2015
***Doto floridicola* 1**	Cap de Creus, Spain, Mediterranean Sea	FTN4	**KX274290**	**KX274313**	**KX274303**	This study
***Doto floridicola* 2**	Cap de Creus, Spain, Mediterranean Sea	FTN5	**KX274288**	**KX274312**	**KX274302**	This study
***Doto floridicola* 3**	Cap de Creus, Spain, Mediterranean Sea	FTN6	**KX274289**	**KX274314**	**KX274304**	This study
Doto floridicola 4	Spain, Mediterranean Sea		AF249820	–	–	Wollscheid-Lengeling *et al*., 2001
Doto formosa	Maine, USA	CASIZ183923	–	–	KJ486667	Shipman & Gosliner, 2015
Doto fragilis 1	Sweeden		–	AJ223392	–	Thollesson, 1999
Doto fragilis 2	North Sea		KR084559	–	–	Barco *et al*., 2016
Doto fragilis 3	Ferrol, Spain: Atlantic Coast		–	KJ486754	–	Shipman & Gosliner, 2015
Doto fragilis 4	Pembrokeshire, Wales	Mn33151	KJ486735	KJ486755	KJ486668	Shipman & Gosliner, 2015
Doto greenamyeri	Papua New Guinea	CASIZ185101	KJ486715	KJ486769	KJ486683	Shipman & Gosliner, 2015
***Doto koenneckeri* 1**	Palamós, Spain,	KFO5	**KX274283**	**KX274316**	**KX274297**	This study

	Mediterranean Sea					
***Doto koenneckeri* 2**	Palamós, Spain, Mediterranean Sea	KFO6	**KX274284**	**KX274315**	**KX274296**	This study
***Doto koenneckeri* 3**	Palamós, Spain, Mediterranean Sea	KFO7	**KX274282**	**KX274317**	**KX274298**	This study
Doto koenneckeri 4	Spain, NE Atlantic		AF249797	AF249249	–	Wollscheid-Lengeling *et al*., 2001
Doto koenneckeri 5	Thorn Rock, Skomer, Wales	Mn33141	KJ486732	KJ486752	KJ486665	Shipman & Gosliner, 2015
Doto koenneckeri 6	Thorn Rock, Skomer, Wales	Mn33140	KJ486730	KJ486751	KJ486666	Shipman & Gosliner, 2015
Doto koenneckeri 7	Mediterranean Sea	CASIZ176815	KJ486729	KJ486750	KJ486664	Shipman & Gosliner, 2015
Doto lemchei	Thorn Rock, Skomer, Wales	Mn33144	KJ486727	KJ486749	–	Shipman & Gosliner, 2015
Doto maculata	Pembrokeshire, Wales	Mn33143	–	KJ486757	KJ486661	Shipman & Gosliner, 2015
Doto millbayana	Tuskar Rock, Skomer, Wales	Mn33145	KJ486726	KJ486759	KJ486660	Shipman & Gosliner, 2015
Doto paulinae	Mataró, Spain, Mediterranean Sea	J33-1	**KX274281**	**KX274311**	**KX274309**	This study
Doto pinnatifida 1	Spain, NE Atlantic		AF249797	AF249250	–	Wollscheid-Lengeling *et al*., 2001
Doto pinnatifida 2	Tuskar Rock, Skomer, Wales	Mn33137	KJ486736	KJ486748	KJ486689	Shipman & Gosliner, 2015
Doto sp. 2	Philippines	CASIZ177543	HM162737	HM162660	HM162569	Pola & Gosliner, 2010
Doto sp. 7 (a)	Philippines	CASIZ177542	HM162738	HM162661	HM162570	Pola & Gosliner, 2010
Doto sp. 7 (b)	Philippines	CASIZ181291	KJ486711	KJ486771	KJ486685	Shipman & Gosliner, 2015
Doto sp. A	Thorn Rock, Skomer, Wales	Mn33136	KJ486724	KJ486758	KJ486658	Shipman & Gosliner, 2015
Doto sp. B (a)	Açores Islands, Portugal	CASIZ178247	HM162735	HM162658	HM162567	Pola & Gosliner, 2010
Doto sp. B (b)	Açores: Sao Miguel Island	CASIZ178248	KP940456	KP940451	KP940461	Shipman & Gosliner, 2015

Doto sp. form A	California, USA	CASIZ182040	KJ486704	KJ486766	KJ486673	Shipman & Gosliner, 2015
Doto sp. H	Mexico	LACM174964	HM162740	HM162663	HM162572	Pola & Gosliner, 2010
Doto sp. J	Sardinia, Italy	CASIZ175711	HM162742	HM162665	HM162574	Pola & Gosliner, 2010
Doto sp. K	Philippines	CASIZ177460	HM162575	HM162666	HM162575	Pola & Gosliner, 2010
Doto splendidissima	South Africa	CASIZ176123	HM162742	HM162664	HM162573	Pola & Gosliner, 2010
Doto tuberculata 1	Pembrokeshire, Wales	Mn33142	KJ486734	KJ486756	KJ509924	Shipman & Gosliner, 2015
Doto tuberculata 2	Spain: Atlantic Coast	CASIZ190542	KJ486733	–	KJ486669	Shipman & Gosliner, 2015
Doto ussi 1	Philippines	CASIZ177438	HM162736	HM162659	HM162568	Pola & Gosliner, 2010
Doto ussi 2	Philippines	CASIZ182893	KJ486706	KJ486780	KJ486675	Shipman & Gosliner, 2015
Doto ussi 3	Philippines	CASIZ177514	KP940457	KP940452	KP940462	Pola & Gosliner, 2015
Flabellina affinis	Menorca, Spain, Mediterranean Sea	MNCN15.05/53696	HQ616753	HQ616716	HQ616782	Carmona *et al.* 2011
Hancockia californica	Costa Rica	CASIZ175722	HM162702	HM162621	HM162257	Pola & Gosliner, 2010
Janolus longidentatus	South Africa: A-Frame: Western False Bay, Cape Prov. Philippines:	CASIZ176320	HM162749	HM162673	HM162582	Pola & Gosliner, 2010
Kabeiro christianae	Philippines	CASIZ185993	–	KJ486782	KJ486691	Shipman & Gosliner, 2015
Kabeiro phasmida	Philippines	CASIZ177545	HM162739	HM162662	HM162571	Pola & Gosliner, 2010
Kabeiro rubroreticulata	Philippines	CASIZ177726	KJ486739	KJ486791	KJ486697	Shipman & Gosliner, 2015
Pinufius rebus	Philippines	CASIZ177763	HM162744	HM162667	HM162576	Pola & Gosliner, 2010
Tritonia antarctica	Bouvet Island, Sub-Antarctica	CASIZ171177	HM162718	HM162643	HM162550	Pola & Gosliner, 2010

Chapter 7

Bipolarity in sea slugs: On the description of *Doridunculus punkus* n. sp. (Nudibranchia, Onchidoridoidea) from Antarctica



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Chapter 7. Bipolarity in sea slugs: A new species of *Doridunculus* (Mollusca: Nudibranchia: Onchidoridoidea) from Antarctica

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ABSTRACT

Bipolar distributions of benthic taxa have intrigued many biologists since the first Antarctic expeditions. Records of taxa, either at species or higher taxonomic levels, encompassing this peculiar distribution have been regularly reported since then. Moreover, the study of heterobranch molluscs from remote areas, such as Antarctica, is essential for systematics, since they may help to untangle phylogenetic conundrums by providing key taxa so far unknown. We describe here a new species of nudibranch from the eastern Weddell Sea using micro-computed tomography (micro-CT), namely *Doridunculus punkus* n. sp. The new species belongs to a North Polar genus of the family Akiodoridididae, thus expanding the distribution of this genus to the Southern Ocean for the first time. This peculiar disjunct distribution is discussed in the frame of the current literature. We provide an extensive description of morphological and anatomical characters of *D. punkus* n. sp., thereby offering new insights into the organ systems of the hitherto understudied family Akiodoridididae. An extensive comparison of the Akiodoridididae described species and the new species shows that the latter exhibits intermediate characters between Akiodoridididae and other Onchidoridoidea families (i.e. hook-shaped innermost lateral teeth). Furthermore, the detailed study of its reproductive system suggests a close relationship of Akiodoridididae and Goniodoridididae families. We suggest an Antarctic origin of Akiodoridididae followed by an either vicariant or a transequatorial dispersion, and a subsequent speciation in the North Pole.

Key words: Akiodoridididae; Phanerobranchia; Heterobranchia; Weddell Sea; disjunct distribution; micro-CT

Capítulo 7. Bipolaridad en las babosas de mar: una nueva especie de *Doridunculus* (Mollusca: Nudibranchia: Onchidoridoidea) de la Antártida

RESUMEN

Las distribuciones bipolares de taxones bentónicos han intrigado a muchos biólogos desde las primeras expediciones a la Antártida. Varios taxones con esta peculiar distribución, ya sea a nivel de especie o niveles taxonómicos superiores, han sido descritos periódicamente desde entonces. El estudio de los moluscos heterobranquios de zonas remotas, como la Antártida, es esencial en sistemática, ya que pueden ayudar a desentrañar enigmas filogenéticos, así como proporcionar taxones clave hasta ahora desconocidos. En este estudio describimos una nueva especie de nudibranquio mediante tomografía micro-computarizada (micro-CT), del este del mar de Weddell, que hemos denominado *Doridunculus punkus* n. sp. La nueva especie pertenece a un género del Polo Norte de la familia Akiodoridídae, ampliando de esta manera por primera vez la distribución de este género al Océano Antártico. Se analizan las posibles razones de esta peculiar distribución disjunta en el marco de la literatura actual. Proporcionamos una extensa descripción de los caracteres morfológicos y anatómicos de *D. punkus* n. sp., lo que ofrece nuevas perspectivas en la anatomía de la familia Akiodoridídae, hasta ahora poco estudiada. La extensiva comparación entre las especies descritas de Akiodoridídae y nuestra nueva especie nos muestra caracteres intermedios entre Akiodoridídae y otras familias de Onchidoridoidea (*i.e.*, diente lateral interior en forma de gancho). Por otro lado, el estudio detallado de su sistema reproductivo nos sugiere una estrecha relación entre las familias Akiodoridídae y Goniodoridídae. Por todo ello, se sugiere aquí un posible origen antártico de Akiodoridídae, seguido de una dispersión o bien vicariante o transecuatorial, y una posterior especiación en el Polo Norte.

Palabras clave: Akiodoridídae; Phanerobranchia; Heterobranchia; mar de Weddell; distribución disjunta; micro-CT

INTRODUCTION

A disjunct distribution of sister taxa covering the northern and southern hemispheres is a phenomenon known as bipolarity (Stepanjants *et al*., 2006). Bipolar distributions can occur either at the species, genus or higher taxonomic levels (Allcock and Griffiths, 2015). In molluscs, approximately 30 % of living Antarctic bivalve and gastropod families are bipolar, including heterobranch genera such as *Philine* Ascanius, 1772 and *Toledonia* Dall, 1902 (Rudman, 1972; Warén, 1989; Dell, 1990; Crame, 1993). The wide fossil record of molluscs suggests at least three paleontological periods in which bipolar and/or amphitropical (*i.e.*, on both sides of the tropics) events occurred: Late Jurassic (~150 Mya), Paleogene-Neogene (~23 Mya), and Neogene-Pleistocene (~2.6 Mya; Crame, 1993). Current disjunct distributions might be the result of transequatorial dispersal during glacial maxima cooling or, alternatively, a prior cosmopolitan species isolated vicariantly in high latitudes during interglacial periods (Allcock and Griffiths, 2015). Vicariant cases imply that species once placed in the tropics might have sheltered in deep waters during interglacial periods, a phenomenon called equatorial submergence (Stepanjants *et al*., 2006). This is applicable for *Philine* for example, which is distributed in deep waters of all world oceans (OBIS, 2016). However, evidence of deep equatorial species is often lacking, probably due to the scarce sampling done so far in deep tropical waters (Allcock and Griffiths, 2015).

Antarctic glacial and inter-glacial periods during the late Cenozoic (~65 Mya) triggered species migration towards warmer temperatures in lower latitudes or in deep-sea shelters (Thatje *et al*., 2005). The Antarctic final breakup during the Early Cenozoic allowed the formation of the Antarctic Circumpolar Current (~25 Mya). This thermal and hydrographic barrier hampered marine organisms' dispersion from north to south and above 1,000 m depth at the Southern Ocean (SO; Barker and Thomas, 2004). However, deep-sea organisms from the Weddell Sea may have dispersed through the Antarctic bottom water, which flows as part of the global thermohaline circulation system (Stepanjants *et al*., 2006; Pawlowski *et al*., 2007). Thereby, organisms with high dispersal capabilities, either by planktonic larvae or by dispersal attached to floating debris, may colonise distant regions (Raguá-Gil *et al*., 2004), even contemporarily (Stepanjants *et al*., 2006; Pawlowski *et al*., 2007; Jun *et al*., 2012).

Wägele *et al*. (2008) suggested that basal members of some major Nudibranchia lineages may have an Antarctic origin. Several families and genera are only found in the Southern Ocean, being crucial for understanding the evolution of heterobranch lineages. Nudibranchia, with approx. 35 described species from Antarctica, is the most speciose heterobranch lineage (De Broyer *et al*., 2016). Among them, the Onchidoridoidea Gray, 1827 of the SO are understudied phanerobranch dorids (*i.e.*, unable to fully retract their gills). This taxon comprises five families, namely Akiodoridídae Millen & Martynov, 2005; Calycidorididae Roginskaya, 1972; Corambidae Bergh, 1871; Goniodorididae Adams & Adams, 1854; and Onchidorididae Gray, 1827 (WoRMS, 2015). They all exhibit a buccal pump, hence they formerly

belonged to Suctoria (Bergh, 1892). Akiodorididae is considered to be a basal family within Onchidoridoidea, presently related to Goniodorididae based on the reproductive system (Hallas and Gosliner, 2015). In fact, Akiodorididae was recently erected to embrace genera previously placed in Goniodorididae and Onchidorididae (Millen and Martynov, 2005). The family Akiodorididae currently contains five genera, all of them restricted to polar waters. *Akiodoris* Bergh, 1879 confined to the N Pacific, *Armodoris* Minichev, 1972 from the SO, and *Doridunculus* Sars, 1878 from the N Pacific and N Atlantic, have each two described species; and *Echinocorambe* Valdés & Bouchet at the Norwegian Sea, 1998 and *Prodoridunculus* Thiele, 1912 from the Davies Sea, are both monotypic. The latter genus was described based on juvenile specimens and it was suggested to be the senior synonym of *Armodoris antarctica* Minichev, 1972 (Millen and Martynov, 2005). The main synapomorphies of Akiodorididae are a smooth, thin, lip disk; two or more inner lateral teeth; and rectangular, reduced outer laterals (Millen and Martynov, 2005). Externally, these species have a spiculated notum covered by tubercles. The gills are dorsal and arranged in a semicircle, with the anus behind; gills are not placed into a pocket, as it happens in Onchidorididae (Hallas and Gosliner, 2015). Exceptionally, gills are reduced to one simple leaf and the anus is ventral in *Echinocorambe* (Valdés and Bouchet, 1998). When present, the rachidian tooth ranges from a small plate to a wide arch-shaped structure, sometimes with a central cusp. The inner lateral teeth have a strong cusp directed downwards, with several denticles along the margins. The marginal teeth are rectangular and progressively decrease in size towards the edge of the radula.

The genus *Doridunculus* was first described by Sars (1878) from Risvær (Norway) at 100 m depth based on a single specimen, named *D. echinulatus* Sars, 1878. The species presents two dorsal keels lying side by side and is covered by conical spiculated tubercles over the dorsum. Later, Odhner (1907) described *D. pentabranchus* Odhner, 1907 from further south Norway in muddy bottoms of Skagerrak, at 335 m depth. This species differs from the type species only by having five gills instead of three. Therefore, Odhner (1922) himself further considered *D. pentabranchus* as a probable junior synonym of *D. echinulatus*. Decades later, a new species, named *D. unicus* Martynov & Roginskaya, 2005, was described from abyssal waters (3000–3620 m) off the Sea of Japan (Martynov and Roginskaya, 2005). Contrary to *D. echinulatus*, *D. unicus* lacks the dorsal keels, presents more gills, larger oral tentacles, and a rachidian tooth. Presently, only these two species of *Doridunculus* have been described and are considered valid. Both species are exclusively distributed in the northern hemisphere.

Here, we describe *Doridunculus punkus* n. sp., collected in the eastern Weddell Sea (Antarctica), based on a specimen collected in Austasen at 228 m depth. Description of new nudibranch species requires dissection, radula preparation, and internal anatomy description, thus, usually at least one specimen is almost completely destroyed in doing that. Since holotype specimens should remain intact for deposit in a museum after description, we performed 3D reconstruction analysis, by using micro-

CT techniques, to describe the new *Doridunculus* species. Thereby, unique type material from regions difficult to survey, like this one, is investigated in a non-destructive way. Moreover, we provide a comparative anatomical description between *Doridunculus* and the rest of Akiodorididae genera. A discussion on the bipolar distribution of this enigmatic family is also included, considering other examples found in the literature.

MATERIAL AND METHODS

Sample collection

One specimen of *Doridunculus punkus* n. sp. was collected in the eastern Weddell Sea (Antarctica) during the Antarctic cruise ANT XXI/2 (November 2003–January 2004) of RV Polarstern (Alfred Wegener Institute, Bremerhaven, Germany). The specimen was collected with Agassiz trawl in Austasen at 228 m depth (PS65/280-1), later photographed alive and transferred to 70% ethanol for morphological analysis.

Morphological analysis

For micro-CT analysis, *Doridunculus punkus* n. sp. was contrasted with 1% iodine metal (I_2) dissolved in 100% ethanol (I2E) for 24 h, transferred to 100% ethanol, and mounted on a pipette tip-specimen arrested, mounted on pin with superglue. Three X-ray tomography scans were performed with an XRadia Micro XCT-200 (Carl Zeiss X-ray Microscopy Inc.). For the macro-scan, the 0.4x object lens unit was used, at 40 kV and 200 µA, with a pixel size of 15.35 µm. For scanning the anterior body region, the 4x object lens unit, at 90 kV and 88 µA was used, with a pixel size of 3.70 µm. For the micro-scan of the radula and spicules, the 10x object lens unit, at 40 kV and 200 µA, was used with a pixel size of 2.02 µm. All scans were performed by using Binning 2 (summarizing 4 pixels) and subsequently reconstructed by using Binning 1 (full resolution). Tomography projections were then reconstructed using the reconstruction software XMReconstructor software (Carl Zeiss Microscopy GmbH), resulting in image stacks (Tiff format). The software platform Amira® 5.4. (FEI, Visualization Science Group) for image segmentation was used.

RESULTS

Systematics

Euctenidiacea Tardy, 1970
Doridacea Thiele, 1931
Onchidoridoidea Gray, 1827
Akiodorididae Millen & Martynov, 2005

Doridunculus Sars, 1878

Type species. *Doridunculus echinulatus* Sars, 1878

Diagnosis. Colour translucent white. Body elongated, notum rounded anteriorly, posteriorly tapered, slightly shorter than tail. Velum not clearly separated from ventral notum side. Oral tentacles small, conical or folded. Notal longitudinal keel(s) present or absent. Notum covered by numerous conical tubercles. Tail ridged. Gills dorsal, minute, three to ten, disposed in semicircle. Network of elongated spicules visible through epidermis. Radular formula 1–10.1.(0-1).1.10–1, (with or without rachidian); inner lateral hooked-shaped or with pronounced, big cusp pointing downwards; rectangular marginal teeth forming interlocking pointed plates, decreasing in size towards edge.

The genus contains only three species: *D. echinulatus* Sars, 1878, *D. unicus* Martynov & Roginskaya, 2005, and *D. punkus* n. sp. described herein.

Doridunculus punkus n. sp.

(Figures 1–5) (See 3D PDF of the reconstructed anatomy of the whole specimen and a most detailed anterior part in Supplementary Material 1 and 2, respectively)

http://zoobank.org/NomenclaturalActs/69A41F49-FAEC-4A6F-ADD6-0657084A5745

Holotype. Adult specimen, 16 mm. Austasen, eastern Weddell Sea (71° 7.15' S, 11° 26.23' W) collected by Agassiz trawl on 29/12/2003, at 228 m depth (PS65/280-1). Deposited in SNSB Zoologische Staatssammlung München (Catalog number ZSM Moll 2016113).

Type locality. Austasen, eastern Weddell Sea (Antarctica).

External morphology (Fig. 1). Live animal measured 16 mm, when preserved 15 x 6 x 6 mm (length:width:height). Colour translucent white, brownish digestive gland seen postero-laterally by transparency. Body high, elongated, pentagonal in transverse section; mantle rim protruding; oral veil rounded, slightly folded laterally; oral tentacles grooved; notum rounded posteriorly; foot narrow, notched anteriorly, tapering posteriorly, mid-dorsal ridge present in tail. Posterior part not covered by notum. Rhinophores bearing seven diagonal lamellae, retractile within smooth cavities. Notum dorsally keeled, starting right in front of rhinophores, extending posteriorly to gills, forming a protuberance tilted towards left side of gills; average height of keel about 2 mm. Dorsal notum heterogeneously covered by pointed, conical, spiculated papillae; smaller in notal periphery and at sides of dorsal keel. Gills five, slightly pinnate, arranged in semicircle; anal papilla small, placed just behind gills. Genital pore small, situated antero-laterally beneath notum rim. Spicules conspicuous, fusiform, irregularly multi-knobbed, sometimes with median crown of knobs (Fig. 2D); densely lying within notum, imparting rough texture, forming dense network towards epidermis (Fig. 2C).

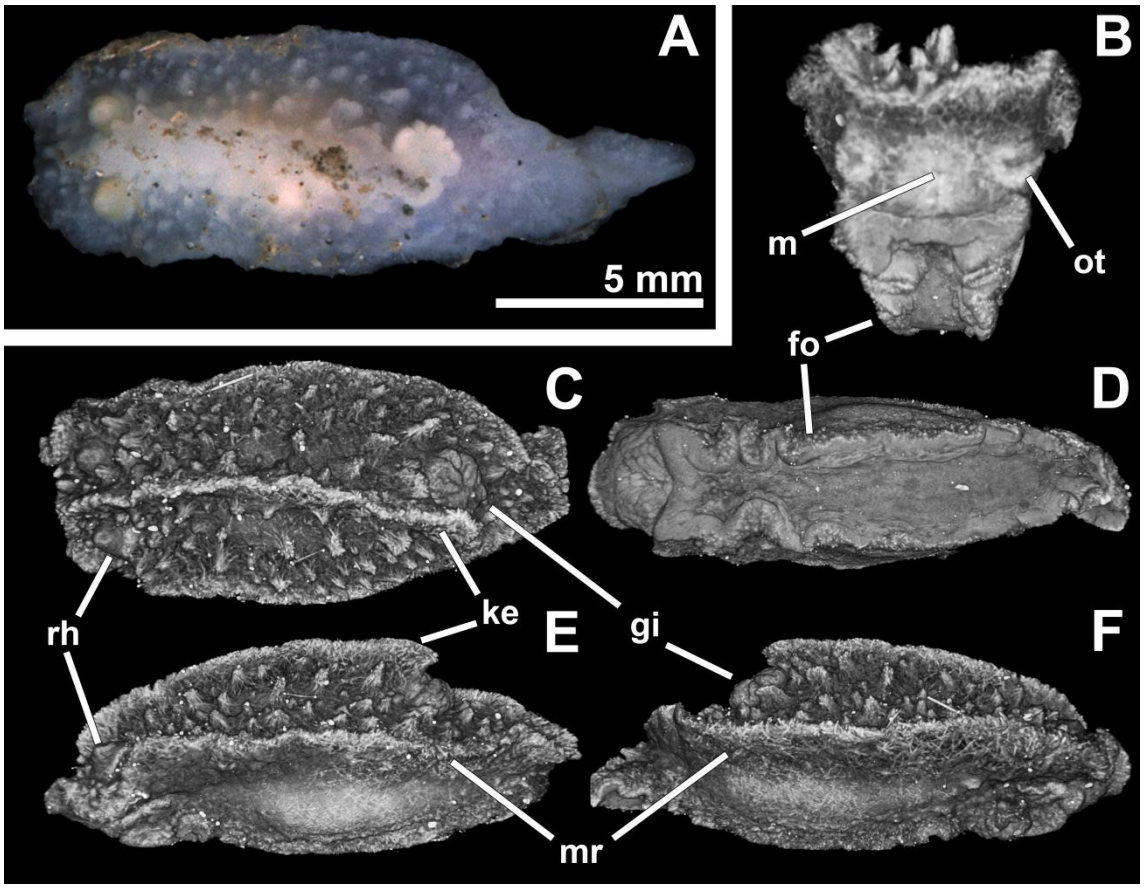

Figure 1, Live photograph (A) of *Doridunculus punkus* n. sp. and volume rendering (B–F) of the preserved animal. **A –** Dorsal view. **B –** Frontal view. **C –** Dorsal view. **D –** Ventral view. **E –** Left-lateral view. **F –** Right-lateral view. *fo* foot; *gi* gills; *ke* keel; *m* mouth; *mr* mantle rim; *ot* oral tentacles; *rh* rhinophores.

Digestive system (Fig. 2A, B; 3). Mouth lying ventrally, opening at base of oral veil, right in front of foot edge, in vertical furrow. Oral tube pyriform, surrounded by follicular oral glands. Oral disc thin, smooth, cuticular; demarcating transition into pharynx. Pharynx bulbous ventrally; two thick longitudinal muscles arise from postero-lateral part reaching gonad laterally (Fig. 4C). Jaws ear-shaped, without distinct ornamentation. Odontophore placed at rear part of pharynx. Radular formula 41 x 10.1.0.1.10 (Fig. 2A, B). Innermost lateral hook-shaped, with one longitudinal denticle at middle part (see white arrows in Fig. 2B), flatened at base. Marginal teeth romboid in shape, presenting outer pointed cusp; decreasing in size towards lateral rim. Rachidian not observed. Salivary glands paired, saccular, small; lying behind pharynx, connected to it through small, thin, tubular ducts (Fig. 3B). Oesophagus thin, originating from posterior part of pharynx; passing through nerve ring, widening towards ventral side, reaching stomach far posteriorly; partly covered by gonad. Stomach completely surrounded by holohepatic digestive gland, situated in posterior third of viscera. Digestive gland presenting reticulated pattern, more pronounced towards periphery. Intestine thin, originating mid-dorsally from stomach, widening, becoming compressed, forming pronounced loop to right side, returning towards left side, ending in anal papilla.

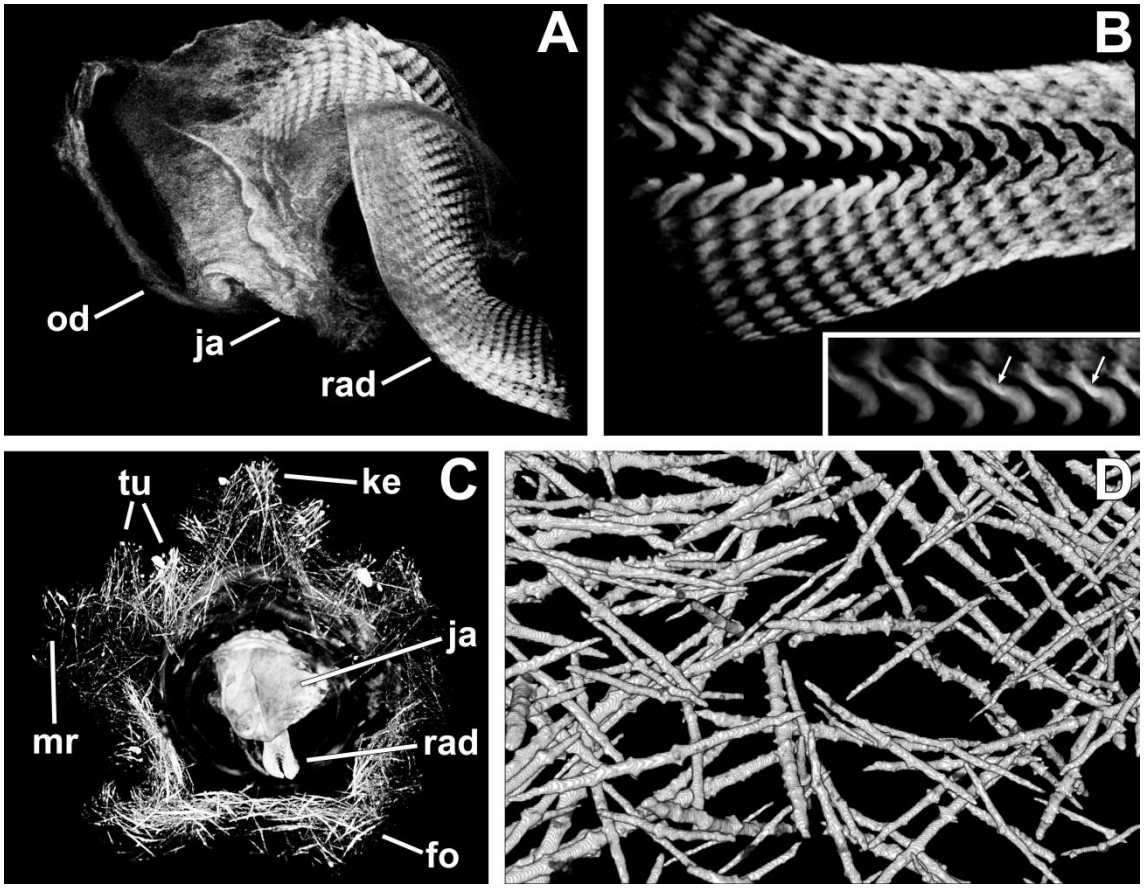

Figure 2 Micro-CT photographs of sclerotized structures of *Doridunculus punkus* n. sp. **A –** Lateral view of the buccal mass. **B –** Distal part of the radula; close up showing inner lateral teeth, note longitudinal denticles (white arrows). **C –** Transverse section of the cephalic region showing spicules' arrangement in the body wall. **D –** Detail of the notal spicules. *fo* foot; *ja* jaw; *ke* keel; *mr* mantle rim; *od* oral disc; *rad* radula; *tu* tubercles.

Reproductive system (Fig. 4A–C; 5). Triaulic. Gonad placed in middle region of body in front of digestive gland; globular in appearance, presenting up to 60 follicles. Gonoducts large connecting in front of gonad, converging into thin, tubular ampulla. Ampulla lying on gonad, extending ventrally. Vas deferens originating from distal gonoduct close to ampulla, leading into big, folded prostate; prostate making counter-clock wise loop alongside body wall, connecting dorsally to thick distal vas deferens, proximally making short loop. Penis small, short, apparently unarmed; placed inside large, globe-shaped, muscular, penial sheath, lying close to genital atrium in right antero-lateral side of body.

Vaginal duct originating from proximal oviduct, close to ampulla forming a very short uterine duct, entering saccular receptaculum seminis. Bursa copulatrix saccular, roundish, originating from thin duct distally from vagina, placed in central transverse position. Vagina relatively short, opening into genital atrium. Oviduct short, placed at distal gonoduct leading to capsule gland. Capsule gland extending far posteriorly into gonad. Membrane and mucus glands not clearly distinct from each other, embracing capsule gland, extending latero-ventrally close to body wall.

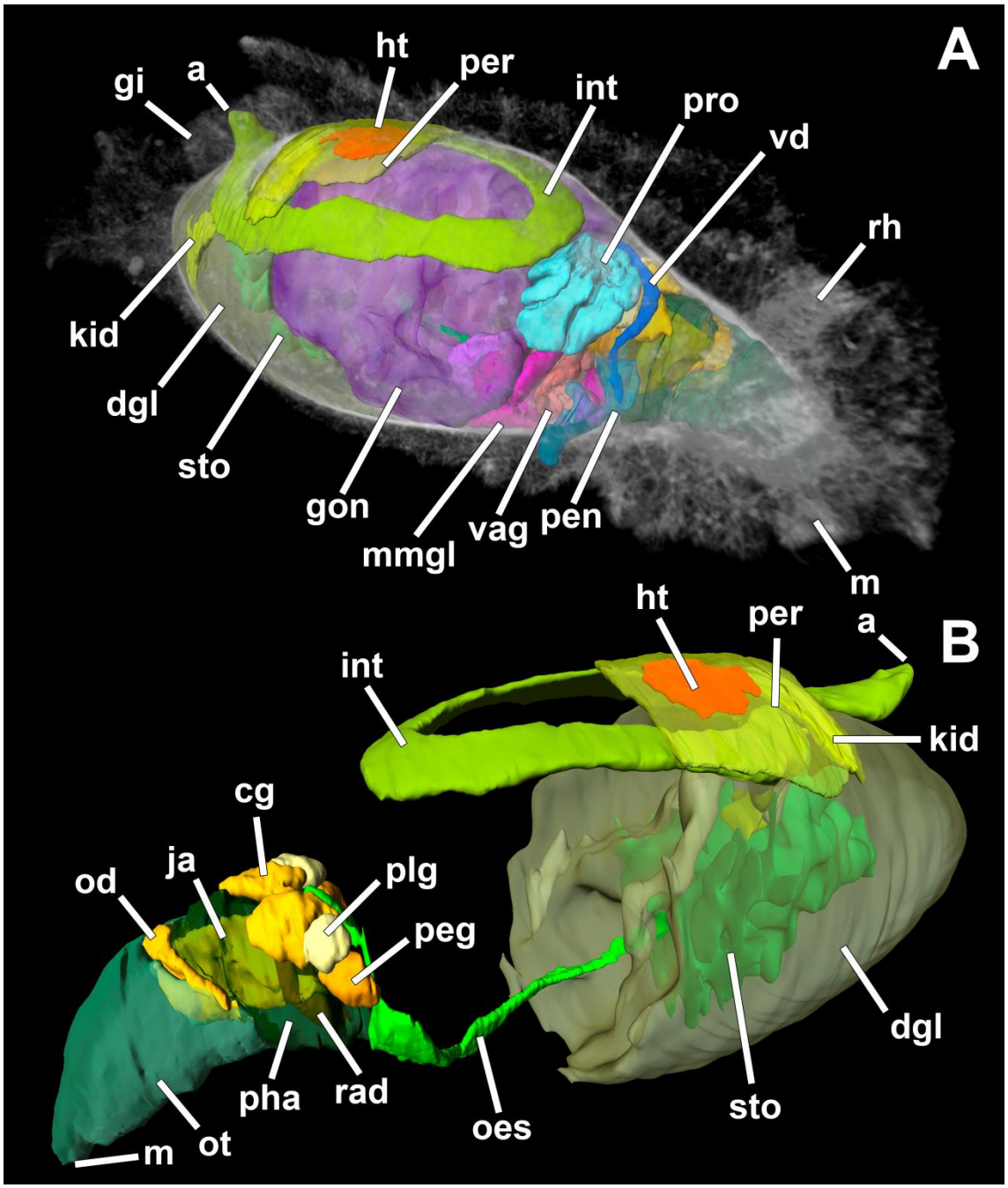

Figure 3. Macroscan (0.4x) reconstruction of *Doridunculus punkus* n. sp. **A –** Right-lateral, general view of all reconstructed organs. **B –** Left-lateral view of the digestive system. *a* anus; *cg* cerebral ganglia; *dgl* digestive gland; *gi* gills; *ht* heart; *int* intestine; *ja* jaws; *gon* gonad; *kid* kidney; *m* mouth; *mmgl* membrane + mucus glands; *od* oral disc; *oes* oesophagus; *ot* oral tube; *pen* penis; *peg* pedal ganglia; *per* pericardium; *pha* pharynx; *plg* pleural ganglia; *pro* prostate; *rad* radula; *rh* rhinophore; *sto* stomach; *vag* vagina; *vd* vas deferens.

Nervous system (Fig. 4D). Cerebral ganglia large, interconnected by short commissure; laterally oral nerve (N1) reaching mouth; labial nerve (N2) reaching anterior part of oral tube; rhinophoral nerve (N3) connected through small rhinophorial ganglia to cerebral ganglia, innervating rhinophores. Neither optical nerve, optic ganglion, nor eyes detected. Pleural ganglia attached to cerebral ganglia, without

visible connectives. Statocysts present on ventral side of pleural ganglia, close to pharynx. Pedal ganglia large, interconnected by long commissure, two lateral nerves originating, running to ventral side. Two buccal ganglia, small, neighbouring, lying at base of salivary glands, partly surrounded by them.

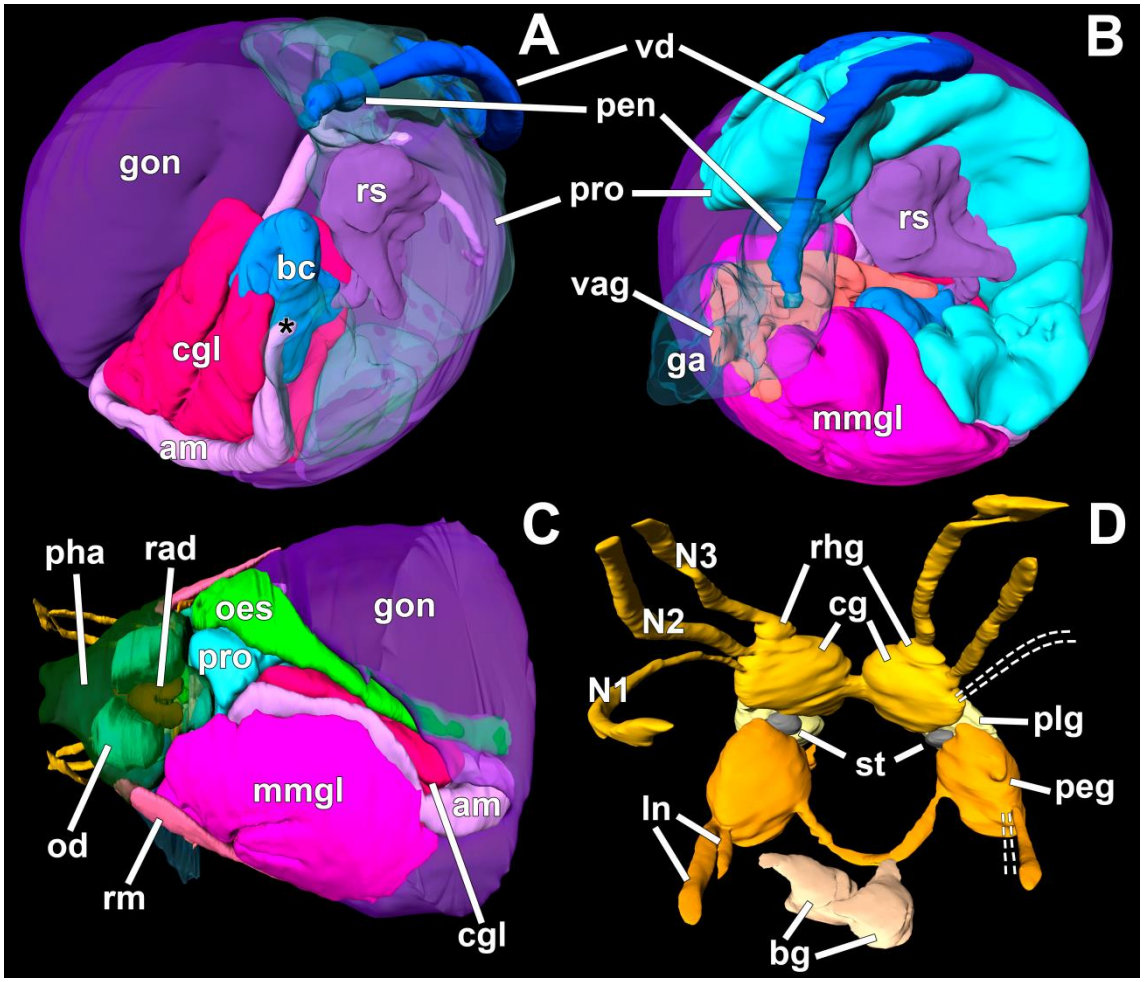

Figure 4. Micro-CT reconstruction of the anterior body region of *Doridunculus punkus* n. sp. (4x). **A –** Left antero-lateral view of the reproductive system; membrane + mucus glands and vagina not depicted; asterisk showing connection between distal ampulla and prostate. **B –** Frontal view of the reproductive system. **C –** Ventral view of digestive, nervous, and reproductive systems. **D –** Frontal view of the nervous system; missing nerves are depicted with white-dashed lines. *am* ampulla; *bc* bursa copulatrix; *bg* buccal ganglia; *cg* cerebral ganglia; *cgl* capsule gland; *ga* genital atrium; *gon* gonad; *ln* lateral nerves; *mmgl* membrane + mucus glands; *N1* oral nerve; *N2* labial nerve; *N3* rhinophoral nerve; *od* odontophore; *oes* oesophagus; *pen* penis; *peg* pedal ganglia; *pha* pharynx; *plg* pleural ganglia; *pro* prostate; *rad* radula; *rhg* rhinophorial ganglia; *rm* retractor muscles; *rs* receptaculum seminis; *st* statocist; *vag* vagina; *vd* vas deferens.

Circulatory and excretory systems (Fig. 3). Pericardium flattened, placed at posterior part of body, above kidney, intestine, and digestive gland. Heart placed in longitudinal axis, no obvious distinction of auricle or ventricle. Aorta and blood gland not observed. Kidney lying above pericardium, extending back behind digestive gland; no nephroduct observed.

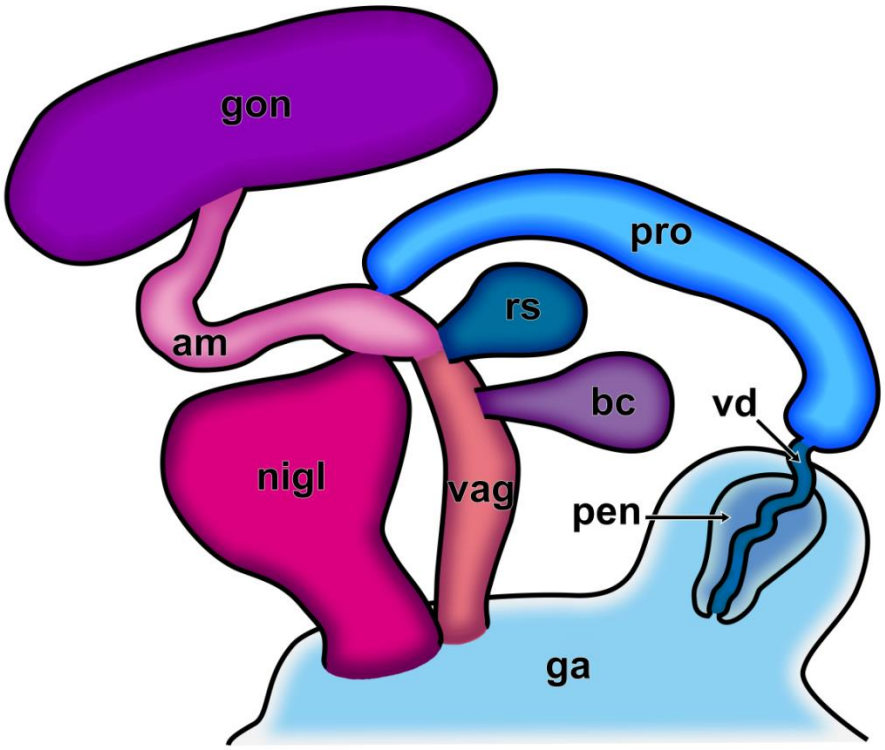

Figure 5. Schematic outline of the reproductive system of *Doridunculus punkus* n. sp. *am* ampulla; *bc* bursa copulatrix; *ga* genital atrium; *gon* gonad; *nigl* nidamental glands; *pen* penis; *pro* prostate; *rs* receptaculum seminis; *vag* vagina; *vd* vas deferens.

Ecology. The single specimen of *D. punkus* n. sp. was found in a detritus-rich benthic bottom at 228 m depth. The community was dominated by sessile phyla, such as sponges (*Cinachyra*, *Clathria*, *Isodyctia*, *Iophon*, *Tedania*), bryozoans (*Alcyonidium*, *Carbasea*, *Isoschizoporella*, *Notoplites*, *Reteporella*), gorgonians (*Thouarella*, *Primnoisis*), ascidians (*Aplidium*, *Cnemidocarpa*), and pterobranchs (*Cephalodiscus*). Vagile fauna such as nudibranchs (*Doris*, *Doto*, *Tritonia*, *Tritoniella*), sea cucumbers, and polychaetes were also collected at the same station.

Etymology. *Doridunculus punkus* n. sp. is named after the Mohican hairstyle of the punks, referring to the presence of one keel in the dorsum.

Remarks

Doridunculus punkus n. sp. differs from its congeners by the small folded oral tentacles, rounded (instead of bilobed) posterior notum, and by having a notal rim not covering the foot. The new species only presents one dorsal keel, while *D. echinulatus* presents two keels, more or less separated (Sars, 1878; Odhner, 1907), while they are lacking in *D. unicus* (Martynov and Roginskaya, 2005). Both *D. punkus* n. sp. and *D. echinulatus* lack a rachidian tooth. In *D. unicus* the rachidian is present and similar to that of *Akiodoris* and *Armodoris* (Millen and Martynov, 2005). A hook-shaped inner lateral is an autapomorphy of the herein described species, not found in any of the described species of Akiodoridae. The stomach of *D. punkus* n. sp. is completely enveloped by the digestive gland, while it is fully free in *D. unicus*. Cerebral and pleural ganglia are

separated in *D. punkus* n. sp., similarly to *Armodoris* (Millen and Martynov, 2005). In *D. punkus* eyes were not observed, this character was not specified in *D. echinulatus* (Sars, 1878; Odhner, 1907), while *D. unicus* possess eyes (Millen and Martynov, 2005).

DISCUSSION

Doridunculus punkus n. sp. is the first record of the genus in the southern hemisphere, since it was only recorded previously in the Norwegian Sea and the Sea of Japan (Sars, 1878; Martynov and Roginskaya, 2005). It seems plausible that the whole family is restricted to either polar and/or deep waters. We were able to reconstruct both hard and soft tissues of the single specimen collected using micro-CT techniques. The new species is placed within the genus *Doridunculus* since it conforms with most of the characters of the described species hitherto (see Table 1). For instance, *D. punkus* n. sp. and its congeners exclusively possess a dorsal ridge in the foot tail. Their notum does not cover the foot posteriorly, as in *Armodoris* and *Prodoridunculus* (Minichev, 1972; Valdés and Bouchet, 1998). However, *D. punkus* n. sp. differs from congeners in having the posterior part of the notum rounded, like in *Prodoridunculus*, and not bilobed as in *D. echinulatus, D. unicus*, and *Echinocorambe*. Moreover, *D. punkus* n. sp. presents a notal rim extending laterally, and not ventrally, as the flaps seen in all other Akiodoridídae. A single dorsal keel similar in position and height to the two present in *D. echinulatus* is also a diagnostic character of the new species (named after it). The gills are arranged in a semicircle in all akiodoridids and also in *D. punkus* n. sp. The lack of a branchial pocket to withdraw the gills, a feature also typical for the new species, was considered a synapomorphy of Akiodoridídae, separating this group from other Onchidoridoidea (Millen and Martynov, 2005).

In the buccal apparatus, a thin and smooth lip disk is shared among akiodoridids. The radular structure is quite different in *D. punkus* n. sp.; it lacks a rachidian tooth, although a rudimentary thin plate could have possibly been unnoticed in the micro-CT scan. In fact, the other species lacking the rachidian teeth were not analysed using scanning electron microscopy (SEM), i.e. *D. echinulatus* and *P. gaussianus* (Sars, 1878; Thiele, 1912), thus this absence is not well documented (Martynov and Roginskaya, 2005). Remarkably, *D. punkus* n. sp. is the first member of the Akiodoridídae that possess a large hook-shaped inner lateral tooth, suggesting that multiple inner lateral teeth evolved once within Onchidoridoidea (Hallas and Gosliner, 2015). Indeed, the inner lateral tooth of most akiodoridids still presents a strong curved cusp, thus resembling a rudimentary hook. Likewise, the whole family seems to

Table 1. Comparative table of diagnostic characters of the Akiodorididae genera, including all the species of *Doridunculus*. *n.s.* not specified.

	***Doridunculus punkus* n. sp.**	***Doridunculus echinulatus* G.O. Sars, 1878**	***Doridunculus unicus* Martynov & Roginskaya, 2005**	***Akiodoris* Bergh, 1879**	***Armodoris* Minichev, 1972**	***Echinocorambe* Valdés & Bouchet, 1998**	***Prodoridunculus* Thiele, 1912**
Notum	elevated	elevated	elevated	elevated	elevated	flattened	flattened
rim	expanded laterally	expanded ventrally	expanded ventrally	expanded	expanded ventrally	expanded ventrally	expanded ventrally
tail	not covered	not covered	not covered	not covered	covered	not covered	covered
posteriorly	rounded	bilobed	bilobed	rounded	rounded	bilobed	rounded
dorsal tubercles	conical	elongated, conical	elongated, cylindro-conical	elongated or rounded	rounded	elongated	small and big, conical in 4 longitudinal rows
ridge	one, mid-dorsal	two, mid-dorsal	absent	absent	absent	absent	absent
spicules	rod-like, tuberculate	present	rod-like, normally hollow	rod-like or quadrate	rod-like, straight	absent	present
Rhinophores	conical	large	large	conical	short, wide	conical	n.s.
sheath	smooth	smooth	few tubercles	tuberculated	tuberculated	smooth	n.s.
Gills	5, semicircle	3–5, semicircle	6–10, semicircle	4–17, semicircle	5, semicircle	1	n.s.
position	dorsal	dorsal	dorsal	dorsal	dorsal	ventral	n.s.
shape	pinnate	pinnate	uni- and bipinnate	bi-, tri- or quadripinnate	unipinnate	smooth	n.s.
Tentacles	small, folded	club-shaped	large, flattened	rounded or flap-like	rounded	large	n.s.
Radular formula	10.1.0.1.10	1–6.0.6–1	4–9.1.1.1.1.1.9–4	3–13.2.1.2.13–3	4–8.4–6.1.4–6.8–4	4.3.1.3.4	2.2.0.2.2
rachidian	absent	absent	trapezoidal, long cusp	wide, arch-shaped or very small	plate-like, with or without central cusp	cusp-less plate	absent
inner lateral(s)	hook-shaped	inner cusp, 4–5 denticles	inner cusp, 2–6 denticles	inner cusp, 2–3 denticles	inner rounded cusp, 2–4	inner cusp, 3–4 denticles	inner cusp, 3–4 denticles

					denticles		
marginal	squared, one large outer cusp	squared, one large outer cusp	squared, one large outer cusp	squared	Inner cusp, 6–0 denticles	squared, one large outer cusp	squared, one large outer cusp
Stomach	fully free from digestive gland	n.s.	partly covered by digestive gland?	fully free from digestive gland	partly covered by digestive gland	n.s.	n.s.
Reproductive system	Triaulic	n.s.	Triaulic	Triaulic	Triaulic	n.s.	n.s.
penis	unarmed	n.s.	unarmed	armed	armed	unarmed	n.s.
ampulla	tubular	n.s.	voluminous, club-shaped	tubular	tubular or voluminous bean-shaped	n.s.	n.s.
gonad	free, not covered by digestive gland	n.s.	partly covered by digestive gland?	partly covered by stomach and digestive gland	partly covered by stomach and digestive gland	n.s.	n.s.
bursa copulatrix	saccular	n.s.	flattened, saccular	wide, saccular	oval, large	n.s.	n.s.
receptaculum seminis	saccular	n.s.	long and narrow	small	small or long	n.s.	n.s.
Cerebro-pleural ganglia	separated	n.s.	fused	fused	separated	n.s.	n.s.
eyes	not observed	n.s.	present	present	present	absent	n.s.
Distribution	Eastern Weddell Sea (Antarctica)	Norwegian Sea	Sea of Japan	Sea of Okhotsk and British Columbia (N Pacific)	Davies Sea, Ross Sea, and South Shetland Islands (Antarctica)	Norwegian Sea	Davies Sea (Antarctica)
Depth range (m)	228	80–100	3000–3620	10–780	25–40	2538–3016	n.s.
Reference	present study	Sars (1878)	Martynov and Roginskaya (2005)	Millen and Martynov (2005)	Millen and Martynov (2005), Valdés *et al.* (2011)	Valdés and Bouchet (1998)	Thiele (1912)

have squared marginal teeth decreasing in size towards the outer edge. The radula of *D. punkus* n. sp., as well as many Goniodorididae taxa, is similar to that of the Polyceridae, although the latter are lacking the innermost reduced lateral tooth (*e.g.*, Vallès *et al.*, 2000). Therefore, Wägele and Willan (2000) postulated that the innermost lateral tooth of the Goniodorididae actually could represent the second lateral of the Polyceridae. This might also be the case in *D. punkus* n. sp.

Traditionally, studying the internal anatomy of Akiodoridídae species has required dissection, but histological or tomographic analyses were always missing. Regarding the digestive system of *D. punkus* n. sp., the pharynx is bulged, forming a buccal pump similar to that of Akiodoridídae, although the presence of two wide longitudinal retractor muscles at each side of the pharynx was never reported in this family before. The stomach is completely enclosed by the digestive gland in *D. punkus* n. sp., contrary to *Akiodoris* and *Armodoris* (Millen and Martynov, 2005) and probably to *D. unicus* as depicted by Martynov and Roginskaya (2005), albeit not reported therein. The intestine is dorsal and forms a pronounced loop onwards, as in all studied species of Akiodoridídae. The anus is dorsal in all species, except for the aberrant *Echinocorambe brattegardi* (Valdés and Bouchet, 1998), where it is ventral, and thus, strongly resembling the species of the genus *Corambe* (Martynov and Schrödl, 2011). The digestive gland is overlapping the gonad in *Doridunculus*, *Akiodoris*, and *Armodoris.* However, only in *D. punkus* n. sp. the gonad seems to be restricted to the mid-longitudinal section of the animal, while it seems spread through the viscera in, at least, *Armodoris.* However, this might be attributed to the ontogenetic stage of the animal we found. In the new species, the ampulla is thin and tubular, and connects with a receptaculum seminis by a short uterine duct, similarly to *Akiodoris* (Millen, 1985). This, in turn, leads to a vaginal duct which has a saccular bursa copulatrix placed distally. The prostate can be tubular and club-shaped and it is always wide and voluminous in Akiodoridídae. The penis is normally unarmed, except in *Akiodoris* and *Armodoris,* where it is densely covered with spines (Millen and Martynov, 2005).

Thus, *Doridunculus punkus* n. sp. shares most of the characters with Akiodoridídae. However, it also looks similar externally to *Aegires albus* Thiele, 1912 found in the same waters (Wägele, 1987a). Both species are of similar colour, size, and shape, presenting dorsal, irregularly-scattered tubercles and spicules (Wägele, 1987b). Nevertheless, *D. punkus* n. sp. presents a rather squared anterior part, the mantle rim protruding laterally, and a dorsal keel. Similarly, the external appearance of the new species (*i.e.*, elongated notum, trailing ridged foot) strongly resembles that of the genus *Diaphorodoris* Iredale & O'Donoghue, 1923 (Millen, 1985). *Diaphorodoris* is presently assigned to the family Calycidoridídae, together with the Arctic monotypic genus *Calycidoris* Abraham, 1876 (Hallas and Gosliner, 2015). But *D. punkus* n. sp., as well as all akiodoridids, lacks a semicontractible branchial pocket in which gills can be retracted, a feature typical for Calycidoridídae and some Onchidoridídae (Fahey and Valdés, 2005; Martynov *et al.*, 2009). Morphological and phylogenetic studies placed Akiodoridídae as sister group of Goniodorididae, sharing a receptaculum seminis that

is connected to the uterine duct (Hallas and Gosliner, 2015). Instead, in Calycidorididae the uterine duct is situated independently on the vagina, and in Corambidae and Onchidorididae the receptaculum is doubly connected to the vagina and the uterine duct (Millen, 1985; Fahey and Valdés, 2005; Millen and Martynov, 2005; Martynov and Schrödl, 2011).

With our finding of a representative of *Doridunculus* in the SO the existence of a bipolar distribution of the genus is proved, and, by extension, the presence of the family Akiodoridídae in Antarctica. Current bipolar disjunct distributions may have been the result of periods of dispersal and/or vicariant isolation, which have occurred several times in Earth's history (Crame, 1993). Meridional deep flows were stronger in periods of climate cooling, and may have formed dispersion bridges of animals from temperate and cold zones of one hemisphere to another (Vinogradova, 1997). Alternatively, a prior cosmopolitan distribution in cooler times may have caused vicariant isolation during interglacial periods (Crame, 1993; Allcock and Griffiths, 2015). Nudibranchs, as well as other heterobranch taxa, may have originated in Antarctica (Wägele *et al*., 2008; Göbbeler and Klussmann-Kolb, 2010). This assumption is based on the presence of basal members of several heterobranch lineages in these waters. Therefore, it is plausible to think about the Antarctic origin of Akiodoridídae, and the posterior dispersion out of Antarctica in glacial periods, a common pattern observed for other different species, such as cnidarians, priapulids, polychaetes, amphipods, copepods, isopods, tanaidaceans, holothuroids, and ophiuroids (Vinogradova, 1997; Stepanjants *et al*., 2006; Brandt *et al*., 2007; Clarke, 2008; Allcock and Griffiths, 2015). These abrupt cooling events implied periods of environmental stress for Antarctic fauna, resulting in a dramatic decrease in diversity (Zinsmeister, 1982). Shelf fauna was completely impoverished by grounded ice masses during glacial maxima, inducing the sheltering migration into marine oasis (polynyas) and deep-sea waters (Thatje *et al*., 2005, 2008). Consequently, species could have migrated using deep-water gateways, such as the Antarctic bottom water, as a part of the global thermohaline circulation system (Stepanjants *et al*., 2006; Pawlowski *et al*., 2007). The lack of molecular data of Akiodoridídae precludes answering the mechanisms of such distribution. Therefore, a thorough taxon sampling in remote areas and deep-sea waters, as well as molecular clock analyses, become essential for revealing the phylogeographic history, including origin, dispersion, and speciation of Akiodoridídae.

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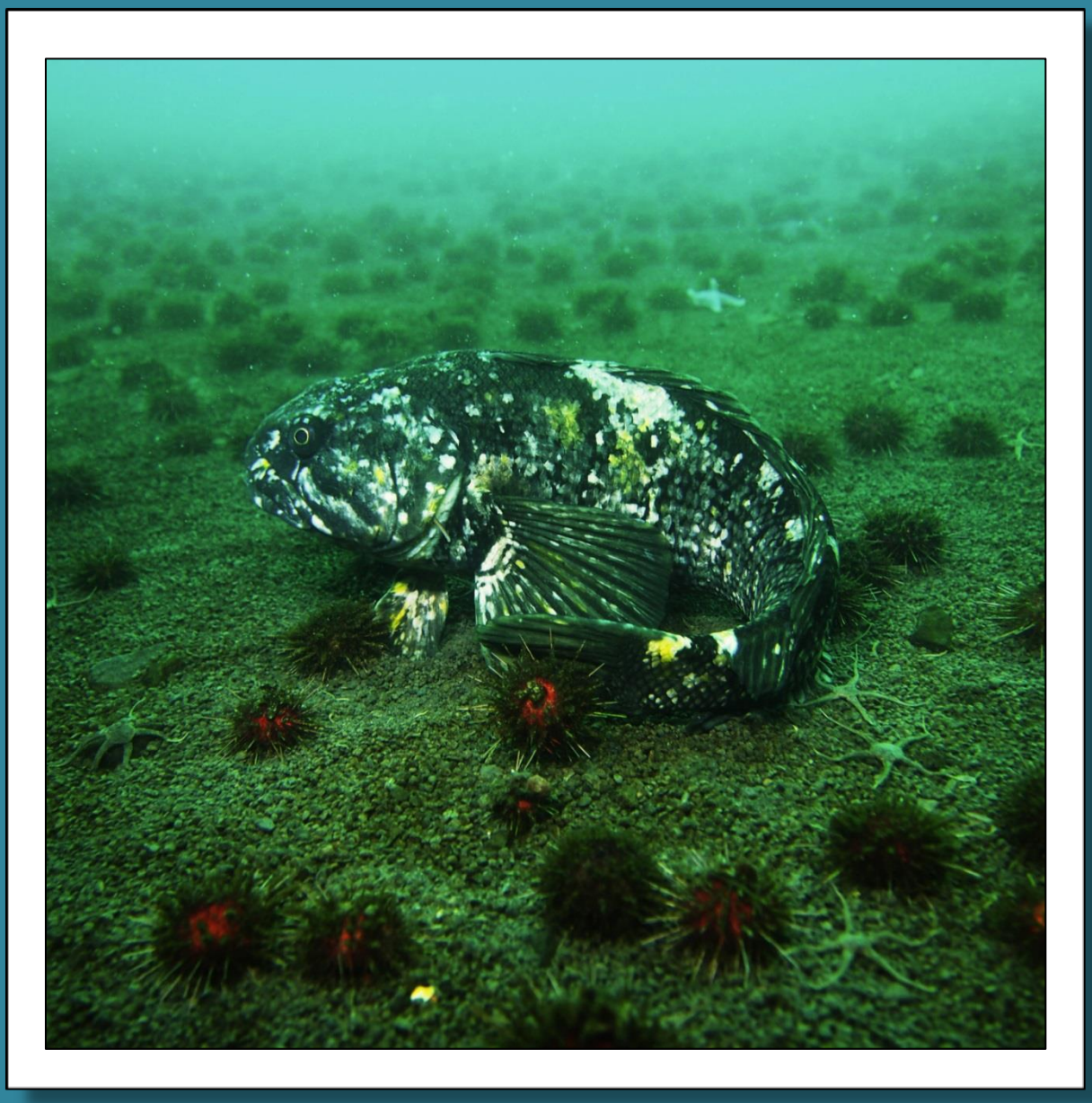
General Discussion and Conclusions



GENERAL DISCUSSION

This PhD thesis deals with three relevant aspects of **Antarctic heterobranch molluscs**: ecology, taxonomy, and systematics. In our Antarctic expeditions we were able to collect several species of marine heterobranchs from a broad area using a wide array of methodologies, including scuba diving and trawling. Our samples include some unknown or understudied species of **sea slugs**, from which we intended to gain further insight into their biology and ecology.

Sea slugs are distributed all over the world oceans, from the tropics to the poles (WoRMS, 2016). But while their taxonomy and ecology in temperate and tropical waters is better assessed, polar seas are far for being explored yet. Antarctic and Subantarctic heterobranch diversity has been surveyed in several campaigns during the XIX and XX centuries (reviewed in Willan & Bertsch, 1987), and large monographs have been published with taxonomic descriptions of numerous new species. Since then, various authors have revisited these descriptions and have contributed to accurate redescriptions, performing anatomical dissections and using histology, and to the reassessment of type material and newly collected specimens. Some of these studies synonymise and/or describe new species, including nudibranchs (Wägele, 1987, 1989a,b,c, 1990a,b, 1995a,b; Ballesteros & Avila, 2005; Wilson *et al.*, 2009, 2013; Valdés *et al.*, 2010, 2011), pleurobranchomorphs (Willan & Bertsch, 1987; Wägele & Hain, 1991; García *et al.*, 1994, 1996; Wägele & Willan, 1994), and cephalaspideans (Linse & Schiøtte, 2002; Chaban, 2016). This thesis work has also contributed to enlarge the knowledge on Antarctic heterobranchs by integrating molecular, histological, microscopic, and tomographic techniques, overall evidencing that much more remains still underexplored (see Table 1).

Table 1. Complete species list of Antarctic heterobranchs described after this Thesis (blue colored).

"LOWER HETEROBRANCHS"
Acteonidae
Acteon antarcticus Thiele, 1912
Neactaeonina edentula (Watson, 1883)
Neactaeonina fragilis Thiele, 1912
Mathildidae
Turritellopsis gratissima Thiele, 1912
Turritellopsis latior Thiele, 1912
Omalogyridae
Omalogyra atomus (Philippi, 1841)
Orbitestellidae
Microdiscula subcanaliculata (E. A. Smith, 1875)
Microdiscula vanhoeffeni Thiele, 1912
Pyramidellidae
Streptocionella pluralis Dell, 1990
Rissoellidae
Rissoella notabilis (Thiele, 1912)
Rissoella powelli Ponder, 1983

CEPHALASPIDEA
Cylichnidae
Cylichna cumberlandiana (Strebel, 1908)
Cylichna gelida (E. A. Smith, 1907)
Cylichna georgiana (Strebel, 1908)
Toledonia elata Thiele, 1912
Toledonia globosa Hedley, 1916
Toledonia limnaeaeformis (E. A. Smith, 1879)
Toledonia major (Hedley, 1911)
Toledonia palmeri Dell, 1990
Toledonia parelata Dell, 1990
Toledonia punctata Thiele, 1912
Toledonia striata Thiele, 1912
Diaphanidae
Diaphana anderssoni (Strebel, 1908)
Diaphana inflata (Strebel, 1908)
Diaphana paessleri (Strebel, 1905)
Diaphana pfefferi (Strebel, 1908)
Newnesiidae Moles, Wägele, Schrödl & Avila, 2016
Newnesia antarctica E. A. Smith, 1902
Newnesia joani Moles, Wägele, Schrödl & Avila, 2016
Philinidae
Philine antarctica E. A. Smith, 1902
Philine apertissima E. A. Smith, 1902
Philine kerguelensis Thiele, 1925
Philinorbidae
Antarctophiline alata (Thiele, 1912)
Antarctophiline amoena (Thiele, 1925)
Antarctophiline gibba (Strebel, 1908)
Scaphandridae
Kaitoa scaphandroides Powell, 1951

PTEROPODA
Cliidae
Clio piatkowskii van der Spoel, Schalk & Bleeker, 1992
Clio pyramidata Linnaeus, 1767
Clionidae
Clione limacina (Phipps, 1774)
Limacinidae
Limacina helicina (Phipps, 1774)
Limacina rangii (d'Orbigny, 1834)
Limacina retroversa (Fleming, 1823)
Thielea helicoides (Jeffreys, 1877)
Peraclidae
Peracle reticulata (d'Orbigny, 1834)
Pneumodermatidae
Spongiobranchaea australis d'Orbigny, 1836

NUDIBRANCHIA
Aegiridae
Aegires albus Thiele, 1912
Akiodoridae
Armodoris antarctica Minichev, 1972
Armodoris anudeorum Valdés, Moran & Woods, 2011
Doridunculus punkus Moles, Wägele & Avila, 2016
Prodoridunculus gaussianus Thiele, 1912
Bathydoridae
Bathydoris hodgsoni Eliot, 1907
Prodoris clavigera (Thiele, 1912)
Cadlinidae
Cadlina affinis Odhner, 1934
Cadlina georgiensis Schrödl, 2000
Cadlina kerguelensis Thiele, 1912
Cadlina magellanica Odhner, 1926
Charcotiidae
Charcotia granulosa Vayssière, 1906
Pseudotritonia antarctica (Odhner, 1934)
Pseudotritonia gracilidens Odhner, 1944
Pseudotritonia quadrangularis Thiele, 1912
Doridiidae
Doris kerguelenensis (Bergh, 1884)
Dotidae
Doto antarctica Eliot, 1907
Doto carinova Moles, Avila & Wägele, 2016
Eubranchidae
Eubranchus glacialis (Thiele, 1912)
Eubranchus adarensis Odhner, 1934
Galvinella antarctica Eliot, 1907
Notaeolidiidae
Notaeolidia gigas Eliot, 1905
Notaeolidia schmekelae Wägele, 1990
Notaeolidia depressa Eliot, 1907
Tergipedidae
Cuthona crinita Minichev, 1972
Cuthona elioti (Eliot, 1907)
Cuthona georgiana (Pfeffer in Martens & Pfeffer, 1886)
Cuthona giarannae Valdés, Moran & Woods, 2012
Cuthona modesta (Eliot, 1907)
Guyvalvoria francaisi Vayssière, 1906
Guyvalvoria paradoxa (Eliot, 1907)
Tergipes antarcticus Pelseneer, 1903
Tritoniidae
Tritonia challengeriana Bergh, 1884
Tritonia dantarti Ballesteros & Avila, 2006
Tritonia vorax (Odhner, 1926)
Tritoniella belli Eliot, 1907

PLEUROBRANCHOIDEA
Pleurobranchidae
Bathyberthella antarctica Willan & Bertsch, 1987 *Bathyberthella orcadensis* (García, García-Gómez, Troncoso & Cervera, 1994)
Bathyberthella tomasi (García, Troncoso, Cervera & García-Gómez, 1996)
Tomthompsonia antarctica (Thiele, 1912)

PULMONATA
Siphonariidae
Siphonaria lateralis Gould, 1846

Ecological interactions in sea slugs

Under the effects of anchor ice and ice scouring, Antarctic benthic communities are highly structured and biologically accommodated (Dayton *et al.*, 1974; Arntz *et al.*, 1994). Accordingly, effective **defence** mechanisms come to be crucial for the survival of the species (Taboada *et al.*, 2013; Moles *et al.*, 2015b). Our results on the chemical ecology of sea slugs are in agreement with this (**Chapters 1**, **2**, and **4**), leading to the rejection of the old latitudinal hypothesis, as expected. Such hypothesis originally predicted a negative gradient in the occurrence of predation and chemical defences from the tropics to the poles (Bakus *et al.*, 1986), but it is no longer accepted for the Southern Ocean (SO) benthic environment (Amsler *et al.*, 2000; Avila *et al.*, 2008; Núñez-Pons & Avila, 2015). Although several studies of Antarctic sea slugs' chemical ecology have been performed (*e.g.*, McClintock *et al.*, 1994; Bryan *et al.*, 1995; Avila *et al.*, 2000; Iken *et al.*, 2002), in general, Antarctic chemical ecology has been poorly studied (Avila *et al.*, 2008). This is due to the challenges in reaching those areas and collecting samples there, although this has gradually improved over the years. Although still much more has to be investigated, several patterns in chemical ecology of heterobranchs can be observed. Interestingly, our work on the nudibranchs ***Charcotia granulosa***, ***Bathydoris hodgsoni***, and ***Doris kerguelenensis*** shows how organisms from remote high latitudes possess similar defensive strategies to those from temperate and tropical areas (**Chapter 2** and **4**). In fact, natural products (NPs), such as the sesquiterpene **hodgsonal** of *B. hodgsoni,* and the **terpene acylglycerols** of *D. kerguelenensis*, present a chemical structure similar to that found in related nudibranch species from other areas around the world. For instance, the structural analogies between hodgsonal and the bioactive compounds of *Dendrodoris* species (Avila *et al.*, 1991) leads us to suggest that defense in nudibranch molluscs might be achieved by **similar strategies**, even when defending against very different kinds of **predators** (Avila *et al.*, *in press*). On the one hand, tropical and temperate water sea slugs are usually predated by demersal fishes and decapod crustaceans, and accordingly anti-predatory assays have been performed involving such taxa (Avila & Paul, 1997; Mollo *et al.*, 2008). On the other hand, the SO fauna is characterized by the poor presence of fish and decapods as either competitors or predators (Clarke *et al.*, 2004; Gili *et al.*, 2006). In these areas, **echinoderms** are the dominant vagile megafaunal taxa in terms of abundance and diversity, and they have a predominant role in structuring benthic communities (Dayton *et al.*, 1974; Clarke & Johnston, 2003; Moles *et al.*, 2015a). Hence, many organisms have developed mechanisms to deter them (Avila *et al.*, 2008; Moles *et al.*, 2015b; Núñez-Pons & Avila, 2015), including sea slugs (Bryan *et al.*, 1998; Avila *et al.*, 2000; Iken *et al.*, 2002). Thereby, **ecological interactions** between SO sea slugs and non-visual, chemosensory predators such as sea stars might have driven the absence of **aposematic colouration** (*i.e.*, warning), a noteworthy phenomenon in almost all marine, worldwide heterobranchs, otherwise unnecessary for Antarctic heterobranchs.

Defensive patterns involving the NP granuloside in *C. granulosa*, although being similar, appear unique for marine environments (**Chapter 1**). Among NPs, **sesterterpenes** are a group of secondary metabolites uncommon in nature, but typically found in a few genera of terrestrial and marine organisms, including nudibranchs (Alvi and Crews, 1992, Bergquist *et al.*, 1999, Fontana *et al.*, 2000). In **Chapter 1** we reported **granuloside** as the first example of a linear homosesterterpene ever described in nature, representing also the first report from marine heterobranchs. Beyond the apparent absence of complexity, this product evokes intriguing biosynthetic questions, since the **origin** of **homoterpenes** in nature is largely unknown. The structural novelty of granuloside and the absence of previous chemical studies on the genus *Charcotia* indicated that further investigations to establish the function of this natural product were warranted. To this aim, feeding experiments and ecological tests were performed in **Chapter 2** to address the origin, location, and the potential defensive role of granuloside. A multidisciplinary approach to **chemical ecology** with microscopic, ultrastructural, ecological, and chemical methods on this Antarctic nudibranch provided evidence of using natural products as a defensive strategy against the sympatric sea star predator, *Odontaster validus* (**Chapter 1** and **2**). An uneven distribution between external and internal organs, as well as the absence of granuloside in *C. granulosa*'s bryozoan prey, leads us to suggest a non-dietary origin of the homosesterterpene granuloside in this charcotiid species, which is likely *de novo* biosynthesised in early juvenile stages and on. In contrast to many investigated cladobranch groups, **Charcotiidae** belongs to the few taxa that, such as Dotidae and Tethydidae, seem to rely on ***de novo* biosynthesis** of natural compounds (Cimino & Ghiselin, 2009; Putz *et al.*, 2010, 2011). *De novo* biosynthesis allows the species to be independent from diet for obtaining their defensive compounds (Avila, 1995; Kubanek *et al.*, 2000).

Apart from the scarce chemo-ecological studies against predation in Antarctic heterobranchs also macro-ecological **ectosymbiont** and **parasitic interactions** had never been reported hitherto. We describe here a new copepod, ***Anthessius antarcticus* n. sp.**, living in association with *C. granulosa* (**Chapter 3**). This is the first record of this type of association in Antarctica, suggesting that although sea slugs possess anti-predatory mecanisms, ectosymbiotic relationships are possible. As this association occurred at a very low rate (it was only detected in one specimen), we cannot suggest specificity of the copepod to a single host species. It seems plausible that many species of *Anthessius* probably remain to be discovered there, since this is the first species described to date from Antarctica. In fact, two additional specimens of *Anthessius* were recently found crawling in the notum of the Antarctic dendronotid *Tritoniella belli* (J. Moles unpubl. data), but taxonomical analyses have not been performed yet. Moreover, our histological investigations in the cephalaspid ***Newnesia joani* n. sp.** also reveal a high incidence of endoparasitic relationships (**Chapter 5**). Therefore, we firstly report a macro-**symbiotic relationship** in Antarctic

Heterobranchia, which, unlike heterobranchs from temperate and tropical waters, were never reported in the SO waters to date.

Another understudied topic in Antarctic Heterobranchs is **reproduction** (Wägele, 1989, 1996; Schaefer, 1996). Antarctic cold temperatures and/or differences in seasonal availability of food (*i.e.* algal production) favour protected **intracapsular development** as a common strategy among Antarctic heterobranchs, to protect early stages of their life cycle (Wray & Raff, 1991; Peck *et al.*, 2006). Although most adult nudibranchs studied in the field present secondary metabolites, their specific ontogenetic origin in species which *de novo* biosynthesise NPs is not assessed. We provide here the first description of *C. granulosa* egg mass, showing the absence of granuloside or any other NP on it. A physical protection of the clutch together with a fast development is assumed to be the strategy to protect early intracapsular development, reducing the exposition time to predators. Furthermore, we suggest that the thick **egg capsules** of the anthobranchs *Bathydoris hodgsoni* and *Doris kerguelenensis* might act as a physical defensive strategy for embryos, while hatched and more 'vulnerable' juveniles might rely on chemical defences (**Chapter 4**), as the adults do (Avila *et al.*, 2000; Iken *et al.*, 2002). Therefore, both anthobranch species might compensate the low numbers of juveniles produced by reducing the mortality during embryonic, juvenile, and adult stages. This fact is especially relevant since the long developmental times for embryos of both species thrives the exposure to predators (Pearse *et al.*, 1991; Wägele, 1996; **Chapter 4**). All our data provide good evidence that hatched juveniles of the three nudibranch species studied herein already rely on biosynthesized NPs as an anti-predatory strategy. Additionally, the thick egg mass clutch of the Antarctic *Doto* species may act as a physical defence against predation, although chemical protection of these species remains still unstudied (**Chapter 6**).

Histological methods have proved to be very useful for disentangling the defensive ecological role of several species studied herein. A structural protection in the form of masses of intracellular grains in vacuolated epithelial cells, together with mucous secretions, may be a first physical protection in *C. granulosa* against parasites, microbes, and even cnidarian attacks (Avila and Durfort, 1996; Wägele *et al.*, 2006; Martin *et al.*, 2007). The number and location of **Mantle Dermal Formations** (MDFs) in the most vulnerable parts of *C. granulosa* (*i.e.*, rhinophores, notal edges) suggested a defensive role against predators (following the postulates of the ODT; Rhoades and Gates, 1976). MDFs store natural products for defensive purposes (*e.g.*, García-Gómez *et al.*, 1990; Avila, 1995; Avila and Paul, 1997), and this might also be the case in *C. granulosa*. Notwithstanding single glandular cells are commonly found and widespread in the family Charcotiidae, no evidence of MDFs has been found so far in members of the genera *Pseudotritonia* and *Leminda*. Similarly, histological sections of ***Doto antarctica*** showed a typical cladobranch notal epithelium composed by multivacuolised cells and mucus glandular cells (**Chapter 6**). The former cells protect the slug against cnidarian cnidocysts (Greenwood, 2009), which are their prey; the latter are rather typical for **Dendronotida** (Wägele *et al.*, 2006; Affeld *et al.*, 2009).

Subepithelial clusters of single gland cells were found in the most exposed parts of *D. antarctica*, *i.e.*, cerata and rhinophores. We also propose a **defensive function** for these glandular cells in *D. antarctica* due to their strategic location and since they everted their content when the animals were molested. Single glandular cells are commonly suggested to be defensive in *Doto* (Baba, 1971; Wägele *et al.*, 2006), in which the animals store defensive compounds obtained from its prey or *de novo* biosynthesised by the slug, such as terpenoids (Putz *et al.*, 2011). Moreover, we found two follicular and multicellular repugnatorial glands in the new cephalaspidean family **Newnesiidae n. fam.**, described herein (**Chapter 5**). These repugnatorial glands might represent modified Blochmann's glands, a gland type that is seen in other heterobranch species too (Brenzinger *et al.*, 2013). These glandular organs are surrounded by musculature helping to release the contents outside, probably in a similar way as in the MDFs of doridoideans from temperate waters (Avila & Durfort, 1996), the Antarctic *C. granulosa* (**Chapter 2**), and other heterobranchs distributed worldwide (Wägele *et al.* 2006). However, its follicular arrangement and the presence of distinct secreting ducts lead us to conclude that these are not in fact true MDFs, in contrast to previous interpretations (Wägele *et al.* 2006), but a distinct glandular organ only found in the family Newnesiidae to date. Nonetheless, this adds more evidence to the current hypothesis of Wägele *et al.* (2006), suggesting that complex glandular structures (*i.e.*, MDF and MDF-like) may have constraints concerning structure—and therefore function—since they are found widespread in completely unrelated heterobranch taxa.

Overall, we have proved that the general defensive strategies of marine heterobranchs are not that different between polar and warmer waters, even if the predators are remarkably different. Effective protection from potential enemies thus, is achieved by **similar patterns of chemical defensive strategies** in very different ecosystems (Avila *et al.*, *in press*). Our results highlight the need of multidisciplinary approaches entailing ecological interactions in key organisms structuring Antarctic benthic ecosystems, such as heterobranchs. Beyond biological interactions, there is still a lack of taxonomical studies, otherwise vital for the comprehension of ecosystem functioning.

Beyond taxonomy and systematics: Towards the past and present of Heterobranchia

The diversity of marine heterobranchs in the SO seems low so far, although comprehensive surveys are still lacking for remotes areas such as the areas studied herein, *i.e.*, Bouvet Island and the Weddell Sea, and others such as Amundsen, Bellinghausen, and Davies Seas. Our new data reflect the need of more taxonomic and biogeographic studies in Antarctic areas, where major sampling effort is still necessary (see Table 1). Interdisciplinary taxonomic and systematic studies involving histological,

tomographic, molecular, and electron-microscopic techniques facilitated the description of three new heterobranch species (**Chapter 5**, **6**, and **7**). In both **Chapters 5** and **6**, molecular and morphological data have proved to provide complementary, useful information regarding the position of the species in a phylogenetic context. We give integrative taxonomical evidence for the description of a new species of Cephalaspidea, ***Newnesia joani* n. sp.**, from the Drake Passage (**Chapter 5**). The genus *Newnesia* forms a distinct lineage at the base of the Cephalaspidea, and we thus consider it to represent a discrete family named Newnesiidae n. fam. encompassing a **circumpolar** and **eurybathic** distribution. The new family presents some shared morphological characters with the genera originally assigned to the **Diaphanidae**, which may be interpreted as **homoplasic adaptations** to epifaunal habits and suctorial feeding (Jensen, 1996). In fact, based on morphological evidence (Odhner, 1926; Warén, 1989), several subfamilies have been proposed within Diaphanidae *s. l.*, some of which have been supported as distinct families in recent molecular phylogenies (Oskars *et al.*, 2015; **Chapter 5**). The basal position of Newnesiidae based on molecular analyses is also reflected by the presence of such a broad array of **plesiomorphic** morphological features not found in any other cephalaspidean groups. With all the evidence given in **Chapter 5** we hypothesise an **Antarctic origin** of the Cephalaspidea. This has been also suggested for Nudipleura (Nudibranchia + Pleurobranchoidea) according to the scarce diversification of these taxa in Antarctica, as well as for the presence of basal members of several major lineages (Wägele *et al.*, 2008; Martynov & Schrödl, 2009; Göbbeler & Klussmann-Kolb, 2010). Likewise to Nudipleura species, cephalaspideans may have dispersed through deep-sea waters thanks to the **Antarctic Bottom Water**, as a part of the global thermohaline circulation system (Stepanjants *et al.*, 2006; Pawlowski *et al.*, 2007). Migration through deep waters from Antarctica to the Atlantic and Pacific Ocean basins might have occurred during **glacial maxima**, similarly to what happens in other benthic phyla, such as cnidarians, priapulids, polychaetes, amphipods, copepods, isopods, tanaidaceans, holothuroids, and ophiuroids (Vinogradova, 1997; Stepanjants *et al.*, 2006; Brandt *et al.*, 2007; Clarke, 2008; Allcock & Griffiths, 2015). This is also supported by the occurrence of other basal lineages such as *Diaphana* and *Toledonia* in Antarctic and deep-water areas (Marcus, 1976; Schiøtte, 1998).

Moreover, with our finding of a representative of the genus ***Doridunculus*** in the SO, the existence of a bipolar distribution of this genus is proved (**Chapter 7**). Current bipolar disjunct distributions may have been the result of periods of dispersal and/or vicariant isolation, which have occurred several times in Earth's history (Crame, 1993). Meridional deep flows were stronger in periods of climate cooling, and may have formed **dispersion bridges** of animals from temperate and cold zones of one hemisphere to another (Vinogradova, 1997). Alternatively, a prior cosmopolitan distribution in cooler times may have caused **vicariant isolation** during interglacial periods (Crame, 1993; Allcock & Griffiths, 2015). As for Newnesiidae species, it is plausible to suggest the Antarctic origin of Akiodoridídae. The abrupt cooling events implied periods of environmental stress for Antarctic fauna, resulting in a dramatic

decrease in **diversity** (Zinsmeister, 1982). Shelf fauna was completely impoverished by grounded ice masses during glacial maxima, inducing the migration into sheltered marine oasis (polynyas) and deep-sea waters (Thatje *et al.*, 2005, 2008). Consequently, species of cephalaspideans and nudibranchs could have migrated using deep-water gateways (Stepanjants *et al.*, 2006). Even so, a thorough taxon sampling in remote areas and deep-sea waters, as well as molecular clock analyses, become essential for revealing the phylogeographic history, including origin, dispersion, and speciation of Antarctic heterobranch families.

Our taxonomic surveys provide two new species of nudibranchs described in **Chapter 6** and **7**, where we showed **micro-CT** analysis as an extremely valuable tool when describing new species with restricted availability of specimens. Furthermore, we recorded two new occurrences of *D. antarctica* in Bouvet Island and the eastern Weddell Sea (**Chapter 6**). These specimens are morphologically and genetically characterised herein, and appear related to *D. antarctica* from the Ross Sea, which strongly suggests a **circumpolar distribution**. Although species in Dotidae have been regularly described based only on external anatomy and radula, the data on internal organ organisation and egg mass structure is desirable for describing *Doto* species. In **Chapter 6** micro-CT and histology have been demonstrated again to be very useful techniques to reconstruct the internal anatomy of two *Doto* species. Although some distinguishing characters between the two *Doto* species can be size related, the lower number of tubercles on the cerata, the different form of the rhinophoral sheath, the shape and arrangement of the salivary glands, ampulla, and prostate in the large specimen of ***Doto carinova* n. sp.**, as well as differences in the egg masses indicate separate evolutionary lineages. A thorough taxonomic description of *D. carinova* n. sp. and its single Antarctic congener *D. antarctica* reveals intriguing questions regarding the evolution of the reproductive system of this worldwide specious genus (**Chapter 6**). We provided a **phylogenetic hypothesis** including various *Doto* species from several regions, showing a trend towards the reduction of bursa copulatrix and distal connection of oviduct to the nidamental glands with separate "flow-through" systems for eggs and allosperm. Moreover, detailed three-dimensional anatomical reconstructions further disclose newly discoveries in the nervous system of the genus, so far unknown. We identified and described the nervous system of both *Doto* species, and identified **giant cells** putatively being neurons, which were never recognised before (Baba, 1971; Fischer *et al.*, 2006). Giant neurones located in the ganglionic mass (*i.e.*, metacerebral cells) are related to external sensory input from the head, and are considered homologous in anaspideans and pulmonates (Weiss and Kupfermann, 1976), as well as for cladobranch and doridoidean nudibranchs (Newcomb and Katz, 2007). These neurones have been found to be **polyploid** (Boer *et al.*, 1970), which has been suggested to be related to a major **hormone secretory function**, responsible for behavioural responses such as crawling (Newcomb and Katz, 2007). Since they possess a huge (very active) nucleus, we speculate that neurosecretory hormones might be secreted into the

hemolymphatic sinus that is apparent within this circle. However, more detailed anatomical and histochemical studies of Dotidae are needed to test this assumption. Additionally, micro-CT techniques let us reconstruct the internal soft and hard tissues of another new species of nudibranch, ***Doridunculus punkus* n. sp.** (**Chapter 7**). The new species is comprised in the family **Akiodorididae** by sharing several morphological and anatomical characters. For instance, gills are arranged in semicircle in all akiodoridids and *D. punkus*, lacking a branchial pocket to withdraw them. This character was suggested as an autapomorphy of Akiodorididae, distinguishing this family from other families within the **Onchidoridoidea** (Millen and Martynov, 2005). Remarkably, *D. punkus* is the first member of the Akiodorididae that possess one large hook shaped inner lateral tooth in the radula, suggesting that multiple inner lateral teeth evolved once within Onchidoridoidea (Hallas and Gosliner, 2015). *Doridunculus punkus* n. sp., as well as many Goniodorididae taxa, has a similar radula to that of the Polyceridae, although lacking the innermost reduced lateral tooth (e.g., Vallès et al. 2000). Therefore, Wägele and Willan (2000) postulated that innermost lateral of the Goniodorididae actually could represent the second lateral of the Polyceridae, similarly to what might happen in *D. punkus.* Overall, we demonstrate the great utility of 3D reconstruction using micro-CT technique, in a **non-destructive** way, to study and describe unique type material from regions difficult to survey, like the Southern Ocean.

Concluding remarks

In conclusion, the results of this thesis highlight the need to apply multidisciplinary methodologies to study the ecological and diversity patterns of Antarctic marine sea slugs. Integrative taxonomical descriptions of four Antarctic species contributed to the worldwide systematics of different taxa herein. This came to be crucial when trying to disentangle phylogenetic conundrums of understudied taxa in the era of biodiversity and molecular tools. Although a major effort in recent decades lead to a better knowledge of the SO benthic ecosystems diversity and functioning, additional sampling, ecological, and taxonomical efforts are still needed. In this Thesis, the knowledge in the field of chemical ecology in Antarctic sea slugs has been enlarged; altogether, the studies reveal similar patterns of anti-predatory defence in heterobranchs from around the world. Global ecological and distribution patterns of heterobranchs are now better understood when including less assessed areas such as the SO. Despite the present day knowledge of chemical and taxonomical diversity of heterobranchs, essential baseline data on biodiversity and biogeography are still lacking for most regions of the SO (Kaiser *et al.*, 2013). This is urgently required to identify biological responses to predicted environmental changes in Antarctica. Among the main current threats of Antarctic ecosystems, climate warming in regions such as the Antarctic Peninsula may have significant ecological implications. Marine species in this region are specially sensible to very small shifts in ocean temperature (Meredith, 2005); a fact that may cause a decrease in species numbers, special permeability to alien

invasions (Fox, 2012), and overall, a restructuration of the benthic ecosystem (Walther *et al.*, 2002).

FINAL CONCLUSIONS

The conclusions of this PhD thesis are included below:

1. The newly characterised molecule granuloside, from *Charcotia granulosa*, is the first record of this type of natural products in the marine realm, and it evokes intriguing biosynthetic questions.

2. *Charcotia granulosa* possessess similar defensive strategies to other nudibranchs from temperate and tropical areas, which are active against the keystone sympatric predator, the sea star *Odontaster validus*. Glandular structures, similar to the Mantle Dermal Formations, probably accumulate granuloside as a defensive stratagem against sympatric predators. We hypothesise the *de novo* biosynthesis of granuloside in early stages of the development of the nudibranch.

3. A new species of ectosymbiont copepod, named *Anthessius antarcticus* n. sp., is described here from *C. granulosa*. Remarkably, the new species is the first record of the family in the Southern Ocean, as well as the first occurrence of this genus in a nudibranch.

4. The two anthobranchs *Bathydoris hodgsoni* and *Doris kerguelenensis* exhibit a similar ontogenetic development, but differ in egg capsule size and number. Their long developmental periods thrives the production of highly-yolked eggs and consumable, thick capsules. The early, intracapsular, embryonic stages might take advantage of the thick egg capsule to be protected from predators. They might compensate the low number of juveniles produced by reducing the mortality during the embryonic stages, while early hatched juveniles already rely on chemical defence, as adults do.

5. By describing the new family Newnesiidae and the new species *Newnesia joani* n. sp., we provide integrative evidence for the Antarctic origin of Cephalaspidea. The shared characters with the former Diaphanidae are interpreted as homoplasies and symplesiomorphies. Cephalic characteristics, as well as the follicular repugnatorial glands, are the main synapomorphic features defining the new family.

6. The 3D reconstruction of *Doto antarctica* and the comparison to the new species *D. carinova* n. sp. revealed novel characteristics in their anatomy, such as an asymmetrical arrangement of giant cells, probably neurones. Thorough phylogenetic analyses of *Doto* reveal trends in the reproductive system towards the reduction of bursa copulatrix and distal connection of oviduct to the nidamental glands with separate pathways for eggs and allosperm.

7. Further evidence for the bipolar distribution of the family Akiodoridídae is given with the description of the new species *Doridunculus punkus* n. sp. The comparative analyses using non-destructive techniques disclose novel data on the anatomy of this poorly studied family.

8. Overall, this PhD thesis highlights the need of integrative and multidisciplinary approaches for assessing the ecology, taxonomy, and systematics of the intriguing heterobranchs, still understudied in the Southern Ocean so far.

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This thesis covers three important aspects of Antarctic heterobranchs: ecology, taxonomy, and systematics. The first section deals with ecological interactions of several nudibranchs. In Chapter 1, we chemically characterize a new natural product (a homosesterterpene) called granuloside, from *Charcotia granulosa* Vayssière, 1906; remarkably, this is the first record of this type of compound in marine organisms. In Chapter 2, we assess the origin, function, and distribution of granuloside in this nudibranch; we found glandular structures probably responsible for storing granuloside, as a defensive mechanism against predators, like the sympatric starfish, *Odontaster validus* Koehler, 1906. We also hypothesize that granuloside is de novo biosynthesized by *C. granulosa*. This chapter reflects how organisms from polar latitudes have similar defensive strategies to those of temperate and tropical zones. In Chapter 3, a new species of ectosymbiont copepod, *Anthessius antarcticus* n. sp., living on *C. granulosa* is described. This is the first record of such association in Antarctica and the first time that this copepod genus has been found living on a nudibranch. In Chapter 4, we study the development of two anthobranchs, *Doris kerguelenensis* (Bergh, 1884) and *Bathydoris hodgsoni* Eliot, 1907, both with intracapsular development; we provide new data on the egg masses characteristics, and embryos morphology and anatomy, throughout their development; we also studied at which ontogenetic stage their natural products appear. We concluded that both nudibranchs exhibit developmental periods of up to several years; their embryos are physically defended by a thick egg capsule, while juveniles already rely on de novo biosynthesized defensive compounds. In the second section of this thesis, our interdisciplinary taxonomic and systematic studies, including histology, tomography, electron microscopy, and molecular tools, allowed us to describe three new species of heterobranchs. In Chapter 5, we provide integrative taxonomic evidence for the establishment of a new family (Newnesiidae), and the description of a new species of Cephalaspidea (*Newnesia joani* n. sp.) with eurybathic and circumpolar distribution; this discovery traces the origin of the cephalaspideans (distributed worldwide) to Antarctica. In Chapter 6, we performed a three-dimensional (3D) anatomical reconstruction and compared the two nudibranchs *Doto antarctica* and the new species *Doto carinova* n. sp.; their phylogeny reveals intriguing questions concerning the development of the reproductive system in this genus; 3D reconstructions reveal also the presence of probable giant neurons associated with the nervous system, which were unknown in this genus so far. Finally, in Chapter 7 we provide new evidence of bipolar geographic distributions by describing a new species of nudibranch, *Doridunculus punkus* n. sp., using only non-destructive tomographic techniques. Our results highlight both the need and the relevance of multidisciplinary approaches to study biodiversity and ecological interactions in heterobranch molluscs from a poorly studied area of the planet, such as Antarctica.