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ORIGINAL PAPER

Giant embryos and hatchlings of Antarctic nudibranchs (Mollusca: Gastropoda: Heterobranchia)

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Abstract *Bathydoris hodgsoni* and *Doris kerguelenensis* are two of the largest Antarctic nudibranchs. They are both common circumpolar species with broad bathymetric distributions, although *B. hodgsoni* is restricted to deep waters in the Antarctic high latitude. Egg masses and juveniles of these species were collected over multiple years (1998–2012) in the eastern Weddell Sea and the South Shetland Islands, and here new data are provided about egg mass characteristics and ontogeny using histological techniques. The egg mass of *B. hodgsoni* has a maximum length of 12.4 cm with one or two egg capsules with a mean diameter of 4.9 cm. The capsules either contained non-developing eggs or ready-to-hatch juveniles up to 2.9 cm long. The egg mass of *D. kerguelenensis* is a semicircular ribbon-like structure including 1,500–2,400 oval capsules (~1.7 × 1.2 mm) containing various stages of development up to ready-to-hatch juveniles 2.5 mm in length. Based on their morphology and development in egg masses maintained in the laboratory, the embryonic period for *B. hodgsoni* is estimated to be up to 10 years, and for *D. kerguelenensis* 13 months. Thus, *B. hodgsoni* has the largest egg capsules and probably the largest hatchlings of any mollusc. Chemical analyses of *D. kerguelenensis* egg masses showed no trace of terpenoid acylglycerols, although these compounds were present in field-collected juveniles and adults. None of four sponges that likely serve as food for *D. kerguelenensis* had the glycerides, or their precursors, found in the nudibranch.

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Introduction

Isolation of the Antarctic continent and the formation of the Antarctic Circumpolar Current allowed benthic species to co-evolve in habitats characterised by low and relatively stable temperatures (Clarke 1992; Dayton et al. 1994; Clarke et al. 2004). Benthic fauna inhabiting the cold waters of the Antarctic Ocean grow more slowly, live longer, are often very large, and have a delayed age of maturity relative to benthic species in warmer oceanic regions (Pearse et al. 1991; Clarke 2003; Peck et al. 2007; Moran and Woods 2012). Low temperatures and/or differences in seasonal availability of organic matter favour intracapsular development as a common strategy among Antarctic species to protect early stages of their life cycles (Wray and 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 and Doyle

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1981; Hain and 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, the Subantarctic cephalaspidean snail *Antarctophiline gibba* (Heterobranchia; Chaban 2016) hatches after 120 d from eggs up to 429 µm in diameter (Seager 1979), while *Philine* spp. from warmer waters present shorter embryonic periods and smaller eggs (Schaefer 1996). Usually, high-latitude Antarctic heterobranch development takes longer. For instance, late veliger larvae of the Antarctic *Antarctophiline alata* hatched after 180 days (Hain and Arnaud 1992), and *Bathyberthella antarctica* (Pleurobranchomorpha) hatched after 100 days from eggs with a diameter up to 1.2 mm (Wägele 1996).

Among Antarctic nudibranchs, *Bathydoris hodgsoni* Eliot (1907) is one of the largest nudibranchs in the world, with eurybathic (i.e., 152–2,757 m depth) and circumpolar Antarctic distributions (Valdés 2002). Only three egg masses of *B. hodgsoni* have been found to date. They contained 2–4 oval, elongated, big, flat, egg capsules each, arranged in a line (Wägele 1996). The whole and the largest egg mass was 100 × 62 × 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 that this species may have an embryonic period of at least 2.5 years. Similarly, *Doris kerguelenensis* (Bergh 1884) 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 × 12–18 mm (length:width) and contained about 1,280–2,380 egg capsules. Late-in-development embryos of 2–4 mm were described in the capsules of a single egg mass, leading Gibson et al. (1970) to suggest that *D. kerguelenensis* has direct development. In fact, eggs of 500 µm and advanced developmental stages of 900 µm were observed inside 1.2–1.9 mm egg capsules of *D. kerguelenensis* (Wägele 1989a, 1996; Hain and Arnaud 1992). 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 an aquarium. Overall, none of these studies had sound evidence for either metamorphic or ametamorphic intracapsular development in either species so only non-planktonic, intracapsular development has been reported to date.

Both nudibranchs have broad diets. *Bathydoris hodgsoni* is a generalist predator feeding on a wide variety of organisms, including foraminiferans, sponges, cnidarians, bryozoans, polychaetes, molluscs, crustaceans, and echinoderms (Wägele 1989b; Avila et al. 2000). *Doris kerguelenensis*, on the other hand, 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 by McDonald and Nybakken 1997).

Nudibranchs usually possess bioactive molecules to ensure their survival against potential predators (Blunt et al. 2016 and previous reviews of the series; Avila et al. in press.). These natural products may be de novo biosynthesized by the slug or derived from its diet (Fontana 2006; Cimino and Ghiselin 2009). In *B. hodgsoni* and *D. kerguelenensis*, the de novo biosynthesis has been suggested for the compounds with a terpene skeleton that 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), repels the sympatric sea star predator *Odontaster validus* (Avila et al. 2000). *Doris kerguelenensis* possesses a wide variety of terpene acylglycerols in the notum (Gavagnin et al. 1995, 1999a, b, 2003a, b; Diyabalanage et al. 2010; Cutignano et al. 2011; Maschek et al. 2012), some of which have anti-predatory activity against *O. validus* (Iken et al. 2002). The metabolites of *D. kerguelenensis* are synthesised through diverse metabolic routes with a remarkable variability among even individuals within a population (Cutignano et al. 2011), thus indicating syntopic coexistence in the sense of Rivas (1964). This, in combination with molecular phylogenetic analyses, led Wilson et al. (2009, 2013) to suggest that this species is, in fact, a species complex, with speciation driven by predation. 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) describe 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, through histological methods; and (2) unravel the defensive strategies in early stages of *D. kerguelenensis* by rearing the embryos for several months and analysing the presence/absence of their defensive chemicals at different ontogenetic stages. Finally, further information on the origin of the compounds was gained by analysing the NPs in four sponges that are the possible prey of *D. kerguelenensis*.

Materials 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 using Agassiz and bottom trawls during the ANT XV/3 (1998) and ANT XXI/2 (2003–2004) cruises on board the RV Polarstern. Additional samples of *D. kerguelenensis* were collected by SCUBA diving at Livingston Island (South Shetland Islands), during the ACTIQUIM-3 cruise on the BIO Las Palmas in 2012. They were preserved in 4% formalin/seawater and subsequently transferred into 70% EtOH for histological analysis. During the 2003–2004 cruise, four egg masses of *D. kerguelenensis* were maintained alive in aquaria and afterwards reared in the laboratory until 2005 (see Online Resource 1). The egg masses were kept separated in seawater tanks at −2 °C, and water was changed every 2–3 days. Moreover, four sponges, where we found *D. kerguelenensis* feeding, 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 an alcohol series and subsequently embedded in HEMA (Kulzer's method, see Wägele 1997). Serial Sects. (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.

Estimation of embryonic periods

To estimate the embryonic development 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, as follows:

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

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

Chemical analyses

Egg masses and early developmental stages (including eggs, embryos, and juveniles) of *D. kerguelenensis* were extracted individually, while adults were first dissected into mantle and viscera. After grinding in a mortar with a pestle (thrice), the samples were immersed in acetone and extracted by ultrasonic bath (∼1 min). Extracts were concentrated under vacuum, and the resulting aqueous suspension was partitioned with diethyl ether (thrice). Comparative TLC analysis of the lipid extracts was carried out in light petroleum/diethyl ether (1:1). Purification of the extracts was performed on a silica gel column using an increasing gradient of diethyl ether in petroleum ether as eluent. We followed the procedures of Cutignano et al. (2011) for isolation and characterisation of the compounds. All ether extracts and purified fractions were analysed by LC-APCI/MS and/or NMR spectroscopy.

The four sponges collected as putative dietary sources were also ground separately in a mortar with pestle and extracted (thrice) with methanol after ultrasonic bath (∼5 min). The alcoholic extracts were evaporated in vacuo, and the resulting aqueous suspensions were partitioned into diethyl ether (thrice). Ether extracts were analysed on SiO_2-TLC with petroleum ether/diethyl ether (8:2, 1:1, 2:8) by UV and cerium sulphate detection. This material was then purified on silica by 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 were referenced to the residual $CHCl_3$ 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 5 µm, 100A, 250 × 4.6 mm, flow 1 ml min^{-1}) on Alliance HPLC system (Waters) coupled with a QTof*micro* (Waters) equipped with an APCI probe operating in positive ionisation mode.

Results

Bathydoris hodgsoni egg masses and embryos

Material examined (Online Resource 1)

Four egg masses collected from the eastern Weddell Sea during ANT XXI/2: two with a single egg capsule and non-developed embryos, one with two egg capsules containing hatchlings, and another with two empty egg capsules.

Egg mass (Fig. 1a)

Maximum size of 124 × 68 × 14 mm (length:width:height). One or two egg capsules, elongated, flat, large, yellowish, slightly iridescent; measuring 48.8 ± 3.6 × 44.6 ± 0.55 × 12.6 ± 0.9 mm (mean ± SD; length:width:height). Egg mass thick, membranous, semi-transparent. A single egg capsule containing extra-

embryonic, cream, crescent-shaped body within basal part, shining through capsule wall. One late juvenile observed inside each capsule.External morphology (Fig. 1b)

Late juveniles found inside egg capsules 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 (Fig. 2a, b)

Epidermis consisting of specialised vacuolated cells, mucous 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, with 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 (Fig. 2c–e)

Digestive system generally well developed, completely filled with lipid-rich yolk, homogeneously staining dark-blue. Oral tube extremely short, labial disc lying in mouth region. Oral glands few, not developed. Jaws present. Radula present, with several rows of teeth, and several teeth per row. Pharynx surrounded by 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

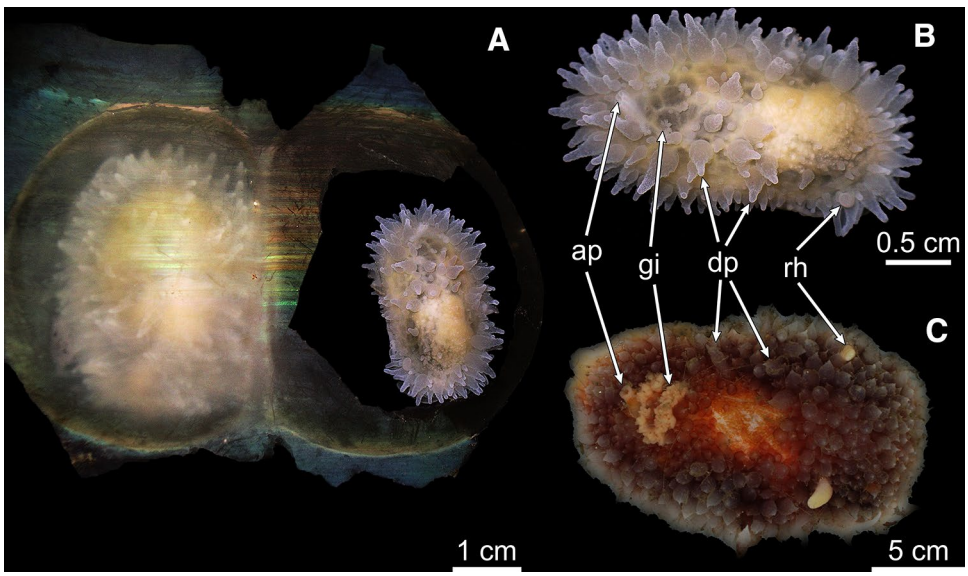


Fig. 1 Developmental stages of *Bathydoris hodgsoni*. **a** Egg mass with two egg capsules containing late-developed embryos; one capsule artificially opened. **b** Detailed view of 29 mm-long, late-developed embryo. **c** Adult. *ap* anal papilla, *dp* dorsal papillae, *gi* gills, *rh* rhinophores

heterochromatin surrounding central axis. Statocysts developed. Nerves within dorsal papillae present.

Circulatory and excretory systems (Fig. 2b, f–h)

Pericardium with muscular ventricle and thin, non-muscular auricle developed. A gland composed of numerous follicular glands (as described in Wägele 1989c) present inside of pericardium, but also occurring outside, very close to auricle (Fig. 2g); differing from large excretory cells lying under the epithelium (Fig. 2b). Kidney developed, forming saccular to fold-like structure, lying on top of the digestive gland; folds intermingled with digestive gland; cells small, containing distinct nucleus and non-staining vacuole. Syrinx internally highly folded, with long cilia (Fig. 2f).

Doris kerguelenensis egg masses, embryos, and juveniles

Material examined (Online Resource 1)

Juveniles were classified as (1) early, artificially hatched in the laboratory; (2) early, naturally hatched; and (3) late, found in the sea, larger and more developed.

Egg masses (Figs. 3a–c, 4a)

Ribbon-like, surrounded by transparent membrane, partially-closed circle; yellowish; measuring 115 ± 64 × 27 ± 6 × 3. 5 ± 0.7 mm (mean ± SD; length:width:height); containing 1,500–2,400 egg capsules (25–28 eggs cm^{-2}) (Fig. 3a). Egg capsules measuring 1,740 ± 684 × 1,222 ± 395 µm (mean ± SD; length:width), capsule thickness 219 ± 54 µm; yellowish; cuboid- or rhomboid-shaped (Fig. 3b, c). Egg capsules containing spherical spaces (Fig. 4a) increasing in number and size throughout development; thinner when juveniles hatch. Two- to eight-cell and morula stages visible inside egg capsules (Fig. 3b). Subsequent stages not visible through egg capsule.

Embryonic development (Figs. 3, 4)

Histological sections showed an advanced developmental stage with a distinct shell, statocysts, and foot (Fig. 4a); closely related to capsule elements (Fig. 4b). Visual observations of living material showed slight variations along the egg mass; juveniles observed inside capsule (Fig. 3c).

External morphology of juveniles (Figs. 3d–g, 5a, b)

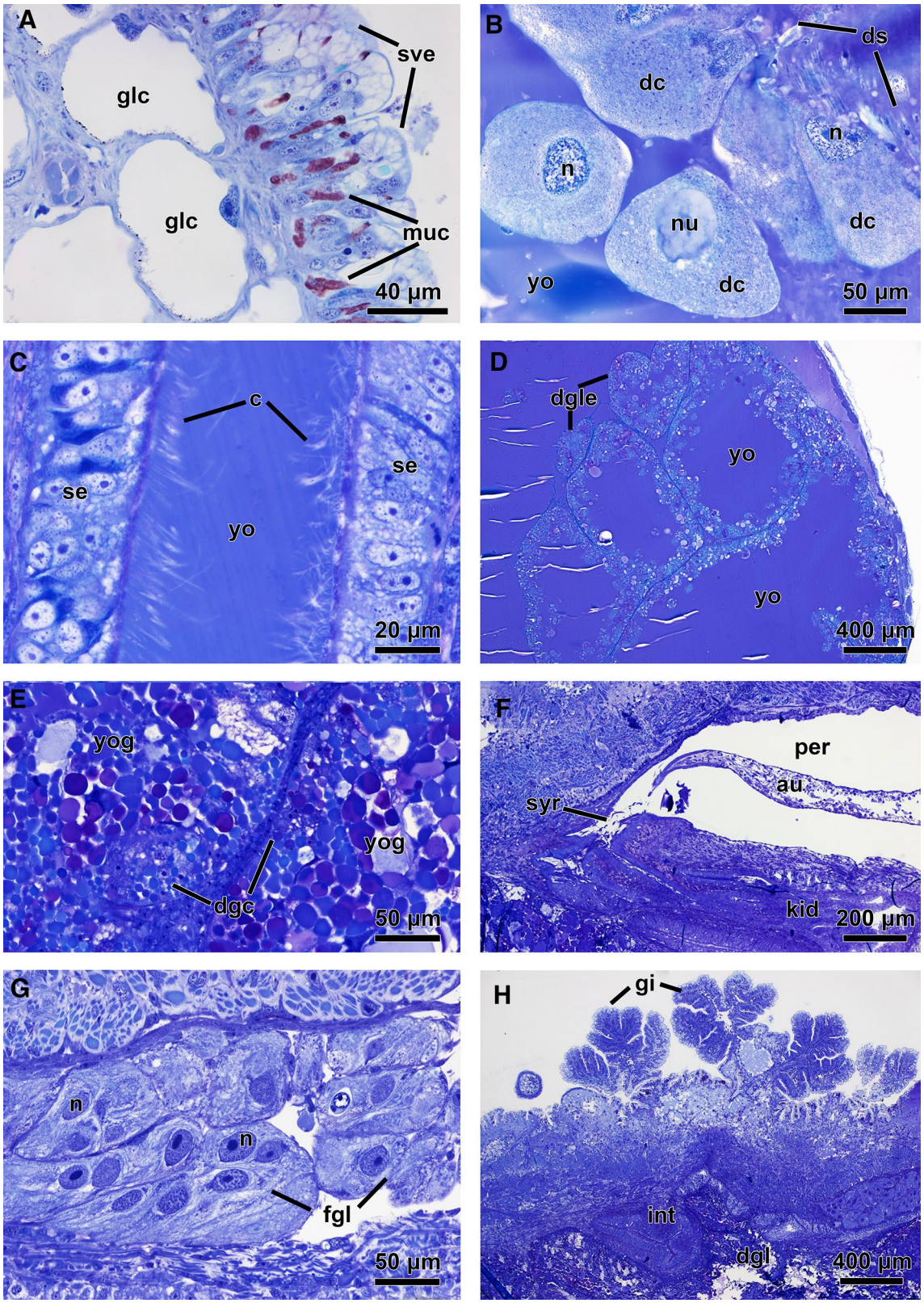
Early, artificially hatched, rounded juvenile filled with lipids (Fig. 3d); moving actively inside egg capsule. Early, hatching juveniles (36 individuals analysed) measuring 2.91 ± 0.33 x 1.93 ± 0.28 mm (length:width) on average, some 4.7 mm long; crawling actively. Notum covered by small, conical tubercles, which increased in size while cultivated in aquarium; subepithelial spicules few, interspersed (Fig. 3d, e). Rhinophores present, laminae scarce, less distinct than in late juveniles (Fig. 5a, b). Eyes visible through transparent notum (Fig. 3d, e). Oral tentacles present. Foot developed (Fig. 3f). 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*.

General anatomical and histological considerations (Figs. 3d, 4c–f)

Organs and tissues less well developed in mid-section compared with anterior and posterior regions (Fig. 4c, d). Specialised 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 sparsely distributed in epithelium. Connective tissue cells, muscle fibres, and spicules present in connective tissue of notum wall; spicules more abundant in late juveniles. Hemolymphatic cavity distinct, above digestive system. Foot glands developed in anterior and posterior regions of early, artificially hatched juveniles; homogenously spread along foot in early, naturally hatched and late juveniles. Yolk homogeneously in structure, stained dark purple, mainly ventral, between digestive tract and foot; voluminous, especially in middle region of body (giving rounded appearance to early, artificially hatched juveniles; Figs. 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; yolk completely lacking in late juveniles.

Digestive system (Figs. 4c, d, 5e–h)

Oral tube developed (Fig. 4c); oral glands rather few in number 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 development (Fig. 5e, f). Salivary glands paired, increasing in size 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 posteriorly and ending in the anus. Anus posteroventral between posterior notum and foot in early, artificially hatched juveniles (Fig. 3d); dorsal in early, naturally hatched and late juveniles (Fig. 3e, f).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◂**Fig. 2** Histological sections of *Bathydoris hodgsoni* recently hatched juveniles. **a** Detail of epithelium of dorsal papillae, containing vacuolized epidermal cells and large, non-staining subepithelial gland cells. **b** Large dorsal cells in dorsal tissue within large yolk mass. **c** Detail of stomach containing yolk. **d** Digestive gland with yolk in lumens of tubes. **e** Detail of digestive gland epithelium. **f** Cross section through heart region; syrinx connects excretory and circulatory systems. **g** Follicles of glandular cells lying in and close to the auricle. **h** Transverse section of the posterior region of the body, showing intestine close to dorsal gills. *au* auricle, *c* cilia, *dc* dorsal cell, *dgc* digestive gland cells, *dgle* digestive gland epithelium, *dgl* digestive gland, *ds* dorsal septum, *fgl* follicular glands, *gi* gills, *glc* glandular cells, *int* intestine, *kid* kidney, *muc* mucous glandular cells, *n* nucleus, *nu* nucleolus, *per* pericardium, *se* stomach epithelium, *sve* specialized vacuolated epithelium, *syr* syrinx, *yo* yolk, *yog* yolk granules

Reproductive system

Not developed in any of the juveniles analysed.

Nervous system (Fig. 5c, d)

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, with statocysts in between. Cortex clearly differentiated from neuropile in the ganglia; cortical neurones 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; ventral in early juveniles, dorsal 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 water in the eastern Weddell Sea ($n = 2$) and shallow waters at Livingston Island ($n = 2$) do not reveal the detectable presence of terpenoid acylglycerols. Intracapsular ontogenetic stages of the three cultured egg masses were extracted individually and compared by LC–MS with known terpenoid acylglycerols, but there was no trace of these products. On the other hand, TLC and LC–MS ($M\text{-}H_20 + H^+$ at *m/z* 361 and 403, respectively) analysis revealed the presence of mono-terpenoyl glycerols and terpenoyl-acetyl glycerols in eight juveniles (<10 mm long) collected in the field (eastern Weddell Sea). Extracts of the analysed adults also contained terpenoid glycerols only in mantle tissues: those from the Weddell Sea had monoacyl- and diacyl- terpene glycerides with 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 sponges analysed showed the typical glycerides isolated from *D. kerguelenensis*, or their precursors.

Discussion

Although inhabiting similar ecosystems, both *Bathydoris hodgsoni* and *Doris kerguelenensis* differ considerably in their reproductive strategies, egg capsule size and number. Egg capsules of *B. hodgsoni* are few (1–4), and larger (52.4 × 45.2 × 13.5 mm; length:width:height) than in any other mollusc taxon (Online Resource 2), containing the largest embryos yet reported for any marine invertebrate. On the other hand, *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 nudibranchs studied (Type 1; Hain and Arnaud 1992), with crawling juveniles 29 and 3 mm in length after hatching for *B. hodgsoni* and *D. kerguelenensis*, respectively. Extra-embryonic yolk uptake occurs either at embryonic or juvenile stages, thus allowing the growth of such large embryos. Moreover, 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 size and adult size seems unlikely since larger heterobranch species than *B. hodgsoni*, such as some *Aplysia* spp. (up to several kgs in mass), have 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*.

According to our estimates, the two nudibranchs studied here seem to have the longest embryonic periods 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 equation of Thompson and 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 benefits for such long embryonic periods are unknown. In fact, long developmental times may be a consequence of slow metabolism in the cold, highly stable environments of the Southern

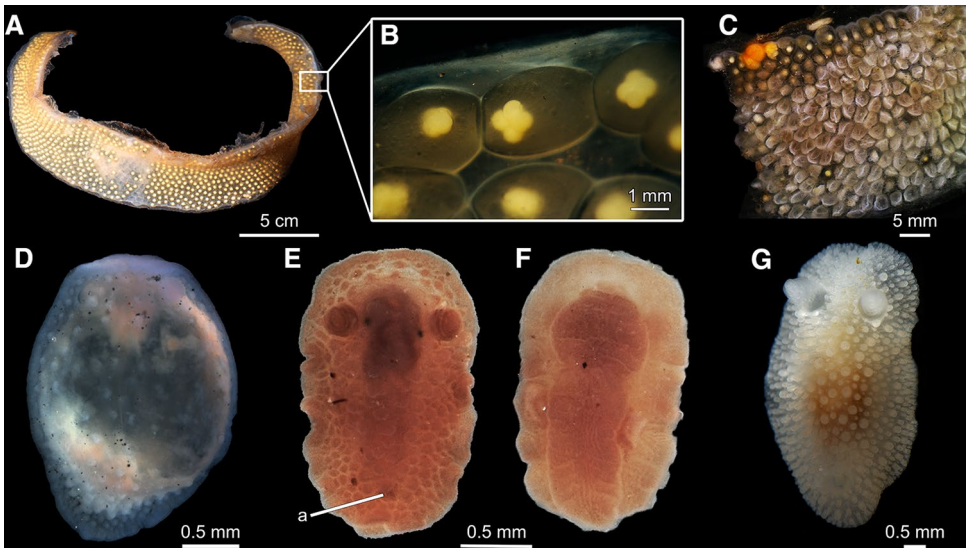


Fig. 3 Developmental stages of *Doris kerguelenensis*. **a** General view of egg mass. **b** Close-up of egg mass edge showing 4-cells embryos. **c** Detail of egg mass with early, ready-to-hatch juveniles inside egg capsules. **d** Early, artificially hatched juvenile (2.5-mm long) with rounded appearance, note absence of anus on the dorsal side. **e** Dorsal view of early, naturally hatched juveniles (3-mm long), with dorsal anus. **f** Ventral view of early, naturally hatched juvenile. **g** Late, well-developed juvenile (5-mm long) found inside hexactinellid sponge *Rossella* cf. *fibulata*, gills present

Ocean, and this might also partially explain the gigantic dimensions of this species (Moran and Woods 2012). The equation to calculate this, however, was not formulated for such large egg capsules (Thompson and Jarman 1986) and, therefore, these estimations should be considered with caution. Our data support the assumption, though, that *B. hodgsoni*, like other Antarctic invertebrates (Pearse et al. 1991), is distinguished by great longevity, comparable to the cephalopods *Nautilus* spp. (up to 20 years, Saunders 1984), bivalves such as *Arctica islandica* or *Tridacna* spp. (up to 100 years; Ungvari et al. 2012), or heterobranch eupulmonates such as *Helix pomatia* (up to 20 years; Animal Base Project Group 2016).

Extended intracapsular development requires large amounts of yolk, and even their own capsules may provide additional nutrition, as previously suggested for both species (Wägele 1989a, 1996). The capsule wall is formed by packing the usually viscous albumen into a compact layer, which provides greater structural integrity to the egg capsule (Klussmann-Kolb and Wägele 2001). Here, our histological observations on *D. kerguelenensis* confirm former results on the uptake of capsule elements (Wägele 1989a). Hence, this species may consume the capsule during the embryonic and advanced developmental stages. In this way, juveniles of *D. kerguelenensis* have more food available and can hatch more easily from the egg capsules, when the walls are thinner. In the mid-section of early juveniles of *D. kerguelenensis*, a large reservoir of yolk seems to be present along the longitudinal axis, containing peripheral connective cells that might uptake and transport yolk granules. The 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 in more anterior or posterior regions. These are the central nervous system and the anterior and posterior parts of the digestive, excretory, and circulatory systems. We observed delayed development at least in the rhinophores, eyes, radula, and digestive gland (Fig. 5). In the case of *D. kerguelenensis*, the anus is subventral in earlier post-embryonic stages and later migrates to the dorsum, as suggested for cryptobranch nudibranchs (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), but there are many exceptions to this rule (Pearse et al. 1991; Palmer 1994; Levin and Bridges 1995; Clarke 2008), including some nudibranchs (Clark and 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 for the two nudibranchs studied here, since they have broad diets and most of their prey species are long-lived (McDonald and Nybakken 1997; Avila et al. 2000; Iken et al. 2002). Survival of these species may not be compromised by the limited dispersal of embryos, living in a very stable environment with high predictability and food availability. Since long developmental times for

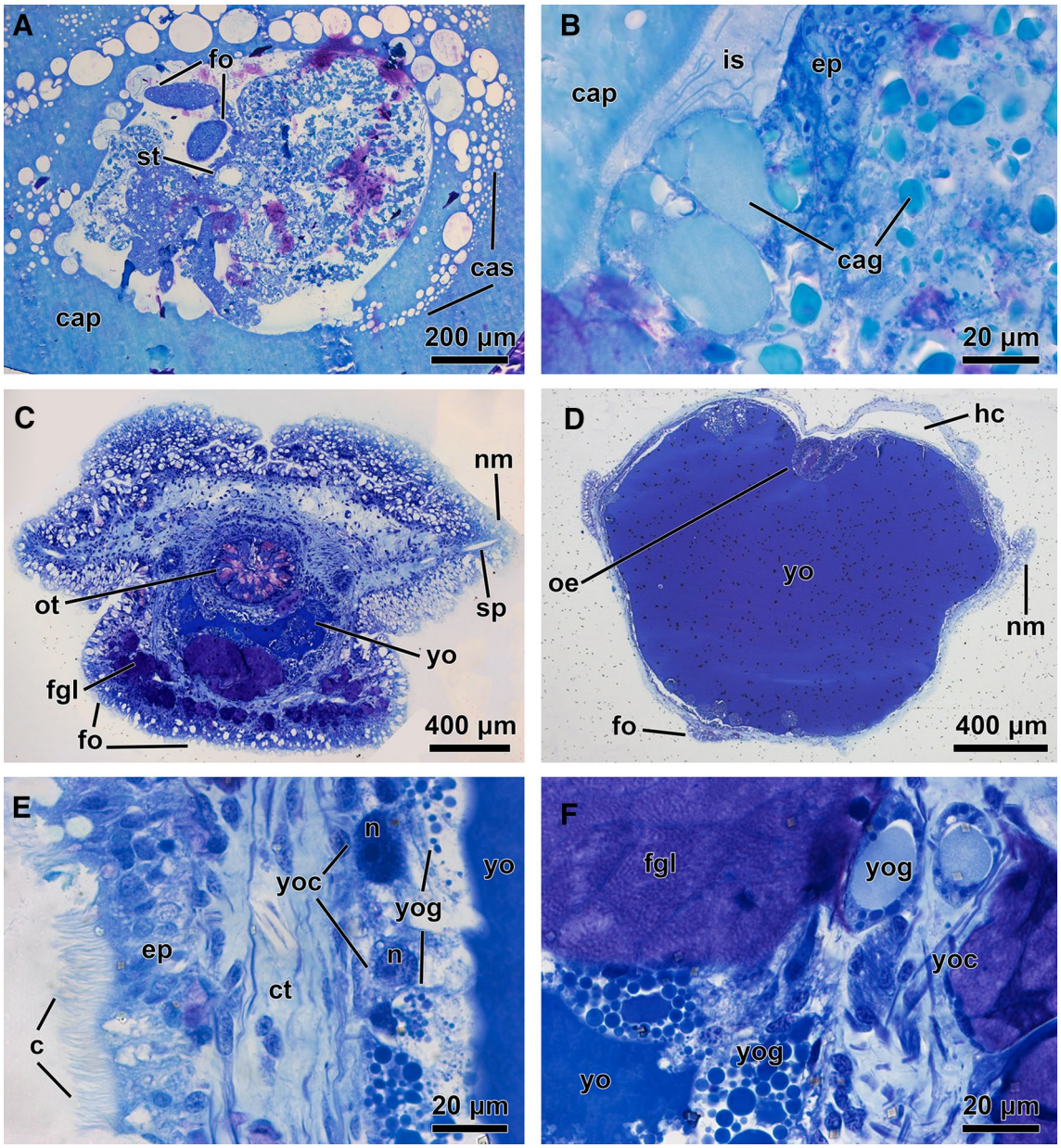
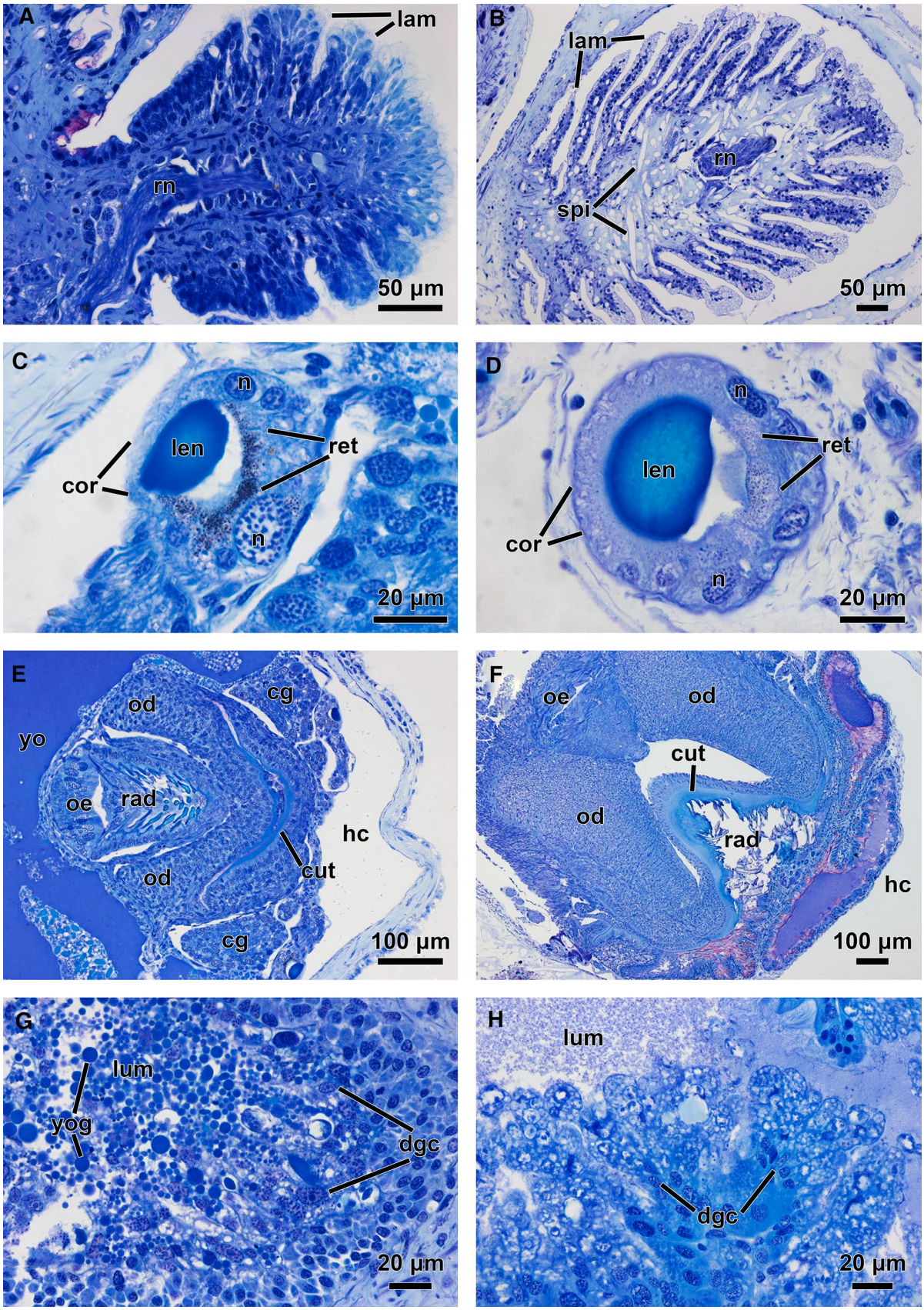


Fig. 4 Histological sections of *Doris kerguelenensis*: advanced developmental stage and one early, artificially hatched juvenile. **a** Advanced developmental stage inside egg capsule, note large spherical spaces in capsule and close contact with embryonic tissue with and within walls spaces. **b** Detail of advanced developmental stage, note capsule digestion and granule uptake. **c** Transverse section of the anterior region of an early juvenile. **d** Transverse section of mid-region of same early juvenile. **e** Detail of yolk cells uptake close to the external epithelium. **f** Detail of yolk cells' uptake close to foot gland. *c* cilia, *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, *st* statocyst, *yo* yolk, *yoc* yolk cells, *yog* yolk granules

embryos of both species increase exposure to predators (Pearse et al. 1991; Wägele 1996), embryos of both species might rely on the physical defence of such thick egg capsules (2 mm in *B. hodgsoni* and 0.3 mm in *D. kerguelenensis*; Wägele 1989a, 1996).

In agreement with our previous investigations, we found that the four diet sponges of *D. kerguelenensis* evaluated lacked the defensive chemicals (and precursors), as did all the egg masses and the embryos within the eggs. However, small juveniles collected from a sponge already had distinct traces of diacylglycerols. In addition, recent phylogenetic analyses show a high prevalence of cryptic speciation in *D. kerguelenensis*, with several lineages discovered to date (Wilson et al. 2009,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◂**Fig. 5** Comparative histological sections of *Doris kerguelenensis*: early, artificially hatched (2.5-mm long; a, c, e, g), and late, hatched in the sea (5 mm-long; b, d, f, h), juveniles. **a, b** Rhinophore, note the fewer number of lamellae in the smaller individual. **c, d** Eye, note less developed lens and cornea. **e, f** Pharynx. **g, h** Detail of the digestive gland with many yolk granules still present in a smaller individual, while absent in 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

2013). This might be behind the different array of compounds found in the species (Cutignano et al. 2011), so our conclusions are preliminary.

Unfortunately, we do not have any information about *B. hodgsoni* juveniles after hatching, but 9.5 cm-long individuals do contain defensive terpenes (Avila et al. 2000). The hodgsonal identified in these individuals was found in similar concentrations to large adults (0.08% dry weight in the mantle), and it has been suggested to be biosynthetic in origin (Avila et al. 2000). This provides good evidence that hatched juveniles of both species rely on de novo biosynthesized compounds as a chemical anti-predator strategy, as previously suggested for other Antarctic nudibranchs (Moles et al. 2016).

Overall, we suggest that both nudibranchs compensate for the low numbers of juveniles produced by reducing mortality through a physical defence (very thick egg capsules) during intracapsular development, and by a chemical defence as soon as the nudibranchs hatch. In conclusion, the complementarity of developmental, defensive, and trophic strategies becomes essential in the cold, stable, and seasonal 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 among Antarctic heterobranch molluscs.

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Compliance with ethical standards

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Informed consent "Informed consent was obtained from all individual participants included in the study."

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