New evidence of brooding in the deep-sea brittle star *Astrotoma agassizii* Lyman, 1876 from a South Western Atlantic Canyon

Juan José Berecoechea[a,*], Martín I. Brogger[b], Pablo E. Penchaszadeh[a]

[a] Laboratorio de Ecosistemas Costeros, Museo Argentino de Ciencias Naturales "Bernardino Rivadavia" – CONICET, Av. Ángel Gallardo 470, C1405DJR Buenos Aires, Argentina
[b] Laboratorio de Reproducción y Biología Integrativa de Invertebrados Marinos, Instituto de Biología de Organismos Marinos – CONICET, Bvd. Brown 2915, U9120ACD Puerto Madryn, Argentina

ARTICLE INFO

ABSTRACT

The reproduction of the brittle star *Astrotoma agassizii* was studied from deep waters of the South Atlantic Ocean, based on samples collected in August 2012, and May and September 2013. Ten samples from 800 to 1400 m depths off Mar del Plata Canyon were studied. The species was found to be a brooding simultaneous hermaphrodite. Hermaphroditic gonads contained testis and ovaries inside the same sacs. Both, ovary and testis contained different stages of gametogenesis development simultaneously. Gonads contained several stages of oocytes in different stages of gametogenesis. The largest oocyte recorded was 800 µm diameter. Free spermatozoa were observed in the lumen of the testis, together with spermatogenic columns. Five individuals, from a total of 30 examined, resulted brooding, and most contained mature ovotestis at the same time. Incubation occurs in five of the ten bursal sacs, containing 15–20 young juveniles each. Maximum disc diameter recorded for a brood was 1100 µm. Herein we hypothesize that *Astrotoma agassizii* could be continuous breeder species in the deep-sea.

1. Introduction

Echinoderms are among the most abundant and diverse organisms of the megafauna in the deep sea (Grassle et al., 1975; Gage and Tyler, 1991; Tyler, 2003; Rex and Etter, 2010). The reproductive biology of some deep-sea echinoderms has been investigated through the histological observations of their gonads (Tyler, 1988; Gage and Tyler, 1991; Hendler and Tran, 2001). However the life history of deep-sea organisms in general remains poorly studied. In addition, many of the difficulties in deep-sea studies are associated with low access for taking regular samples or high maintenance required for culture such as the high pressure conditions needed to keep live specimens in sufficient numbers and in good health for live examination of embryonic development or feeding behavior (Gillespie and McClintock, 2007; Mercier and Hamel, 2008). The mode of reproduction of these organisms has been inferred mostly from the approximate relationship between egg size and type of development (Mortensen, 1921; Thorson, 1934; Billett et al., 2013). The idea that nonpelagic development was the dominant mode of reproduction by benthic marine animals for high latitudes and cold-water, was postulated by Thorson (1950). Mileikovsky (1971) formalized this into the Thorson´s Rule hypothesis, which argued that invertebrate species living in the Artic, Antarctic and in the deep-sea have high proportion of non- pelagic larval development. Thorson´s Rule was discussed by some authors considering distribution of brooding species and different conditions of habitats. They concluded that exceptions to this rule must be taken into account, although predation and be swept away from suitable settling sites tend to favored lecithotrophic development (Gillespie and McClintock, 2007; Pearse et al., 2009).

The Mar del Plata Canyon is located off La Plata River, beginning with a terrace at 500 m depth. It has a typical V-shape configuration with a length of 100 km and a 25 km width and a maximum depth of 3700 m (Violante et al., 2010). Class Ophiuroidea is well represented in Argentinean seas with a total of 32 species recorded including 20 genera and 9 families (Brogger et al., 2013a). Nevertheless, Mar del Plata Canyon has been particularly scarce studied: Challenger Expedition reported a single station, of 1098 m depth, from this deep-sea area (Lyman, 1869). However, some recent studies on the reproduction of other echinoderms, collected in the Mar del Plata Canyon, have been published (Rivadeneira et al. 2017; Martinez and Penchaszadeh, 2017).

Ophiuroidea is composed mostly by unbranched species of brittle stars with a few generally larger species called basket stars. Ophiuroids contribute significantly to biomass in marine environments, particularly in the deep-sea benthos (Gage and Tyler, 1991). Approximately
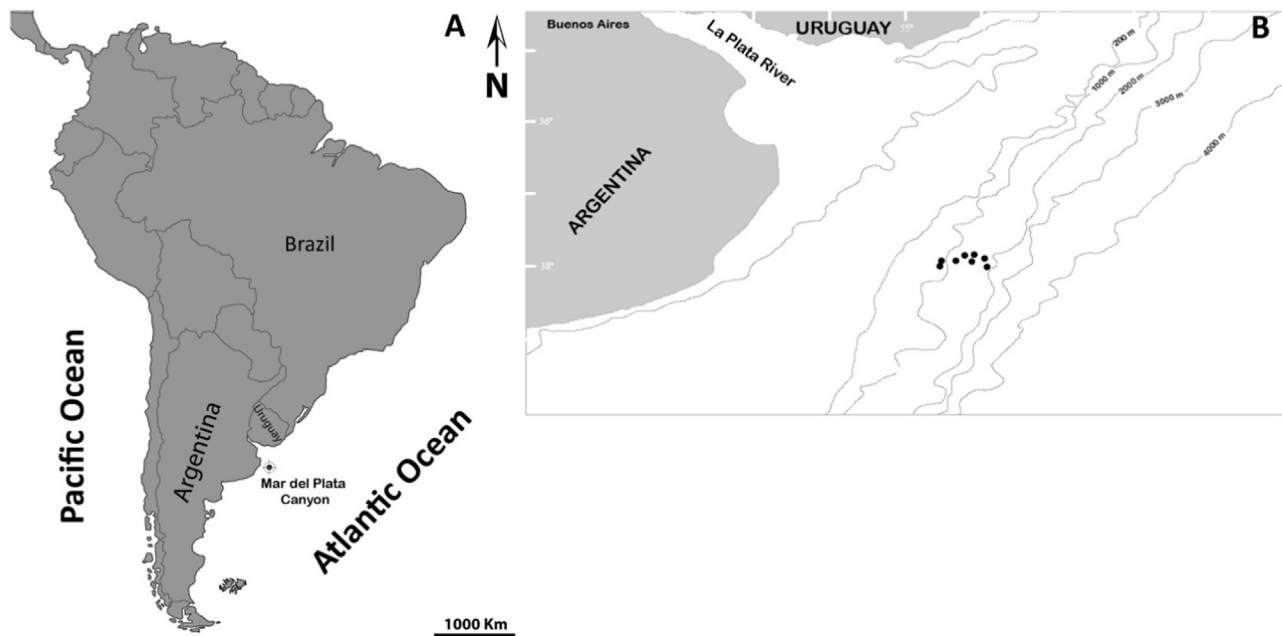
Fig. 1. A Map of South West Atlantic Ocean showing approximate location of Mar del Plata Canyon. **B** Trawls stations at Mar del Plata Canyon area.

2000 brittle stars species are known worldwide (Stöhr et al., 2012). Despite their abundance and diversity, the reproductive biology has been documented for only 4% of all ophiuroid species (Hendler, 1975).

The majority of brittle stars are gonochoric bearing a set of paired gonads in each inter-radial area of the central disc. The position of the gonads is variable, but in most species they are closely associated with the coelomic surface of the bursae (Hyman, 1955). At least 43 species of ophiuroids are recognized as hermaphrodites (Table 2 in Hendler, 1991), mostly small brooding forms. Among hermaphrodite species, gonads are either differentiated into a set of ovary and testis, or may occur as ovotestis (Fell, 1946; Hyman, 1955; Hendler, 1991). Although 10 species have been reported as protandric, only few of them can be corroborated with certainty (Hendler, 1979; Tyler and Gage, 1982).

Bursae are distinctive to the Ophiuroidea and function in reproduction, serving among others things as common outlet for multiple gonoducts and brooding chambers. Most ophiuroids have two bursae at the base of each arm. Fused bursae are commonly found in broadcast spawning species, and are more rarely found in brooding species (Hendler, 1991). Parental care among ophiuroids involves retention of the eggs in the bursae and incubation of the embryos in this brood chamber (Hendler and Littman, 1986). Brooding brittle stars may have viviparous or ovoviviparous development. Viviparous species such as *Amphipholis squamata* invest considerable post vitellogenic reserves into embryogenesis (Fell, 1946). Ovoviviparous species have large eggs containing sufficient yolk reserves to support complete embryogenesis (Byrne, 1989). The majority of brooding ophiuroids are ovoviviparous (Hendler, 1991).

Astrotoma agassizii is a large and abundant brittle star in cold temperate waters of the South Atlantic Ocean, and has a widespread distribution through the Southern Atlantic Ocean and Antarctica; it occurs between depths of 80–1200 m (Bartsch, 1982; Ferrari and Dearborn, 1989). This brittle star species is one of the 13 ophiuroid species shared between Antarctica and South America (Fell et al., 1969). It has been described as a cryptic species (Hunter and Halanych, 2008), composed by three genetically distinct lineages. *Astrotoma agassizii* has been reported as a viviparous species, with brooding occurring in the ovary (Bernasconi, 1965; De la Serna De Esteban, 1966; Bartsch, 1982). Nevertheless, it has been hypothesized that there is an alternative reproductive mode for the Antarctic populations of *Astrotoma agassizii* where the larvae are released from the bursae into the plankton (Heimeier et al., 2010). This work contributes to such debate in order to enrich the discussion on the possibility of different lineages of *Astrotoma agassizii* with alternative reproductive modes.

Here we study the reproductive biology of *Astrotoma agassizii* from Argentinean deep sea, particularly from off Mar del Plata area (38°S), through histological analysis of the gonads and the morphology of the embryos examined by Scanning Electron Microscopy (SEM).

2. Materials and methods

Specimens were collected on board the R/V "Puerto Deseado" in the Mar del Plata Canyon area (Fig. 1), between 800 and 1400 m depth (Fig. 1b), in August 2012, and May and September 2013. A dredge trawl and fishing net were used for collecting the specimens. Individuals were fixed in 96% ethanol. Digital images of specimens were taken using a Canon PowerShot SX 110. Out of a total of 64 stations, specimens from stations 5, 15, 16, 31, 36, 38, 42 and 59 were analyzed (Table 1).

Specimens were dissected in order to examine the gonads, and the broods and embryos were removed from bursae of brooding individuals. Herein we will be calling brood/s to young ophiuroids with their disc and arms completely developed; and embryos to rudimentary and early stages of development, usually with one or two arms, that are incubated inside the bursae. The presence of embryos and/or broods was recorded on dissections. Individuals were decalcified between 24 and 48 hs, depending on size, using non diluted solution of Histodecal Extra® Biopack.

For histological examinations the gonadal tissues were dehydrated, embedded in plastic resin (Historesin® Leica) and sectioned with a Leica microtome at 5–7 µm. All sections were stained with hematoxylin and eosin. Gonad sections were examined under a Zeiss Axio Imager Z1 microscope, and photographed using an Axiocam HRc digital camera. Gonadic maturity stages and size-frequency counts of the oocytes were determined by image analysis of ovary sections. All measurements were done on transversal stained sections, using the software Axio Vision version 4.4.

Internal broods were removed from the bursae, treated with hypochlorite sodium solution, rinsed in distilled water several times, air dried and transferred to aluminum stubs, metal sputter coated and observed under SEM (Philips ×30).

Table 1
Specimens examined from Mar del Plata Canyon.

Site	Latitude (S)	Longitude (W)	Depth (m)	Date	Number (n) of individuals	Disc diameter range (cm)	Disc diameter Mean ± SD (cm)	Number (n) of brooding specimens
5	37° 58.651'	55° 9.104'	528	10 Aug 2012	1	3.30	3.30	0
15	38° 0.500'	54° 25.069'	1200	12 Aug 2012	3	2.8–3.40	3.16 ± 0.32	1
16	37° 57.288'	54° 23.456'	1308	12 Aug 2012	3	0.65–2.20	1.65 ± 0.86	1
31	38° 1.499'	54° 44.171'	819	16 Aug 2012	3	0.97–1.35	1.23 ± 0.20	1
36	37° 57.508'	54° 23.989'	1298	25 May 2013	5	0.88–2.10	1.68 ± 0.49	1
38	37° 59.308'	54° 25.207'	1099	25 May 2013	8	0.48–2.75	1.53 ± 0.72	3
42	37° 59.110'	54° 41.136'	877	26 May 2013	4	0.87–1.92	1.32 ± 0.44	0
59	37° 49.688'	54° 5.236'	1398	10 Sep 2013	3	0.85–1.74	1.30 ± 0.44	1
					30			

3. Results

In total, thirty specimens were studied. The disc diameter of *Astrotoma agassizii* ranged between 0.48 cm and 3.40 cm. Brooding individuals were found in all analyzed months (Table 1). The smallest brooding individual recorded was 1.05 cm disc diameter. Smallest specimens lacked identifiable gonads and no embryos were found.

Brooding individuals presented mature gonads beneath the bursal sacs in the inter-radial space (Fig. 2C). Gonads of *A. agassizii* are located between each arm and stomach folds, at the abradial side of the genital slit (Fig. 2A, B, C). The maximum number of gonads per inter-radial found was nine.

The gonads are comprised of two sacs (an outer and inner sac), being the germinal epithelium the latter and separated by a coelom (Fig. 3A). No gonoduct was observed. Brooding adults contained round to elongate ovotestis, located at the inter-radial side of the bursal slit attached to the genital scales.

Histological examination of the hermaphrodite gonads revealed oocytes at different stages of development, with previtelogenic, midvitellogenic and late vitellogenic oocytes in every month sampled (Fig. 3A, B, E). Nourishing cells were observed between midvitellogenic oocytes (Fig. 3B, E). Ovotestis with mostly late vitellogenic oocytes and spermatozoa were orange to yellow in color and up to 1.80 mm diameter. The largest oocyte recorded in *Astrotoma agassizii* was 800 μm in diameter.

The color of ovotestis with mostly spermatozoas, but some midvitellogenic oocytes (Fig. 3C), appeared white or light yellow when dissected. The maximum size recorded for these ovotestis was 850 μm in diameter. Some ovotestis contained several stages of gametogenesis simultaneously. Spermatogonia proliferate at the basal line of the testes and give rise to spermatids columns that finally develop into spermatozoa (Fig. 3D).

Incubation of embryos and broods occurs inside the bursal sacs. These sacs occupy each inter-radial space between genital slits, although only five of ten bursae incubate the embryos (Fig. 2D). As the embryos develop, the walls of the bursae expanded to accommodate their growth. Brooding individuals were easily identified because of their distended discs. All the broods found inside the bursal sacs were at the same stage of development. This synchrony of development was seen in every brooding bursae examined. Between 15 and 20 broods grew tangled inside each bursal sac (Fig. 2E). Maximum disc diameter recorded for a young inside the bursal sac was 1100 μm.

4. Discussion

Many ophiuroids go through a pelagic larval stage, although as Mortensen (1936) stated, many echinoderms of the Antarctic region tend to undergo direct development. At least 19% of Antarctic species are known to be viviparous. Of the 13% known to be ovoviviparous, several have large yolky eggs, suggesting direct development. Mortensen (1920, 1936) also reports that among Antarctic ophiuroids there is a high incidence of hermaphroditism, a condition generally correlated with diminutive size and brooding (Strathmann and Strathmann, 1982).

Astrotoma agassizii is a hermaphroditic and brooding species (Bernasconi, 1965; De la Serna de Esteban, 1966). De la Serna de Esteban (1966) stated, based in 3 specimens analyzed, that this is a protandric hermaphrodite species; we found always hermaphrodite gonads in several stages of gametogenesis simultaneously. This author also stated that embryos are found inside ovaries. In thirty individuals examined in this work, we found no evidence of intra-ovarian embryos. De la Serna de Esteban (1966) refers the embryo as a mesenchymal cell mass, very vacuolated and full of yolk, within which no cellular limits are defined. Unfortunately images published are not clear enough to appreciate that statement. According to our observations, embryos and broods of *Astrotoma agassizii* develop and grow inside the bursal sacs,
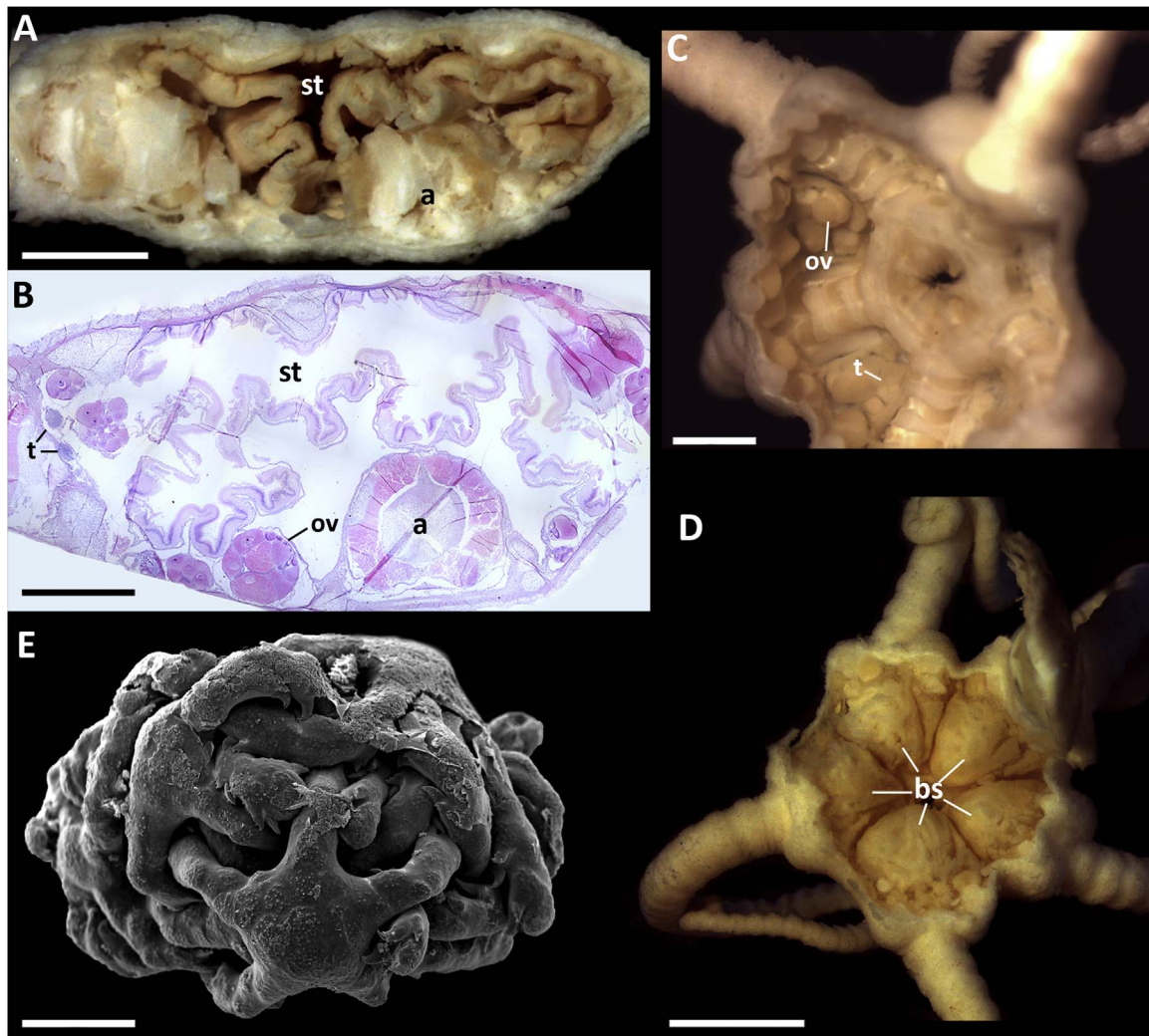
Fig. 2. A Transversal section of *A. agassizii* under stereoscope. **B** Stained section of *A. agassizii*. st, stomach; a, arm; ov, ovotestis with mostly vitellogenic oocyte; t, ovotestis with mostly spermatozoa. **C** Dissection showing developed ovotestis beneath the bursal sac in the inter-radial. ov, ovotestis with mostly vitellogenic oocyte; t, ovotestis with mostly spermatozoa. **D** Female with bursal sac (bs) incubating young broods. **E** Scanning Electron Microscope image of internal broods masses. Scale bars, **A, B** = 0.2 cm; **C, D** = 0.5 cm; **E** = 0.5 mm.

corresponding to an ovoviviparous species *sensu* (Strathmann and Rumrill, 1992).

According to Hunter and Halanych (2008) *Astrotoma agassizii* is a complex of at least 3 cryptic species in the Southern Ocean, with the Antarctic population genetically distinct and geographically isolated from *Astrotoma agassizii* in South America. Moreover, as it was reported, *Astrotoma agassizii* may have a planktonic larval history phase in the Antarctic, reinforcing the hypothesis of three lineages (Heimeier et al., 2010) and its wide distribution. Between samples analyzed by De La Serna de Esteban (1966) one individual could be considered as from the same locality as specimens analyzed in our work. Consequently, the statements about protandry and intraovarian brooding in *Astrotoma agassizii* may not be attributable to other lineages proposed by Heimeier et al. (2010). On the basis of the samples studied herein, the population from the Mar del Plata Canyon reinforced the idea of a brooding lineage of *A. agassizii* in the Southwestern Atlantic Ocean.

In some echinoderm species, parental care involves matrotrophy (direct feeding of the young from the female´s body) (McClary and Mladenov, 1990; Frick, 1998). Most ophiuroids that brood their young have large eggs and the embryos are not supplied by any supplementary nutrition (Byrne, 1991; Hendler, 1991). The egg of *Astrotoma agassizii* (850 μm diameter) is one of the biggest reported for brooding ophiuroids (Hendler, 1991). *Astrotoma agassizii* is a large species, with large gonads (1.80 mm diameter) and yolky eggs. The bursae can expand

inside the disc occupying considerable volume, increasing the brood capacity, which can reach the maximum of 20 juveniles per bursae, although only five of the ten bursae incubate the embryos, and mature ovotestis are found in brooder individuals.

Most ophiuroids from cold temperate waters are reported to be slow-growing species that take 2–3 years to attain maturity and then breed annually for several years (Mortensen, 1927; Fell, 1966; Bowner, 1982). Temperature and depth are important factors governing the distribution of ophiuroids. From these and other data it is suggested that the range of many ophiuroids is controlled by depth, and that shallow-water routes have been of great importance in the dispersal of echinoderms (Fell et al., 1969).

Geographic patterns of some deep-sea species are also explained by their reproductive strategies. For example, *Ophiura ljungmani* has a wide geographic range, from North to South Atlantic, which would be explained by its pelagic larvae that disperse in deep-sea (Schoener, 1972). *Astrotoma agassizii* is a broad distributed species through the South Atlantic Ocean and Antarctic. It has been recorded from Magellanic regions, the southern coast of Chile and Argentina, Burdwood Bank, East and West Malvinas Is.; and also presents an Antarctic circumpolar distribution: Ross Sea, McMurdo, Adelie and Wilkes Lands (Fell, 1961). Bathymetric distribution is also wide in this species, going from 80 to 1400 m (Bartsch, 1982; present work). From this distribution and based on samples analyzed herein, it is determined that *Astrotoma agassizii* is a
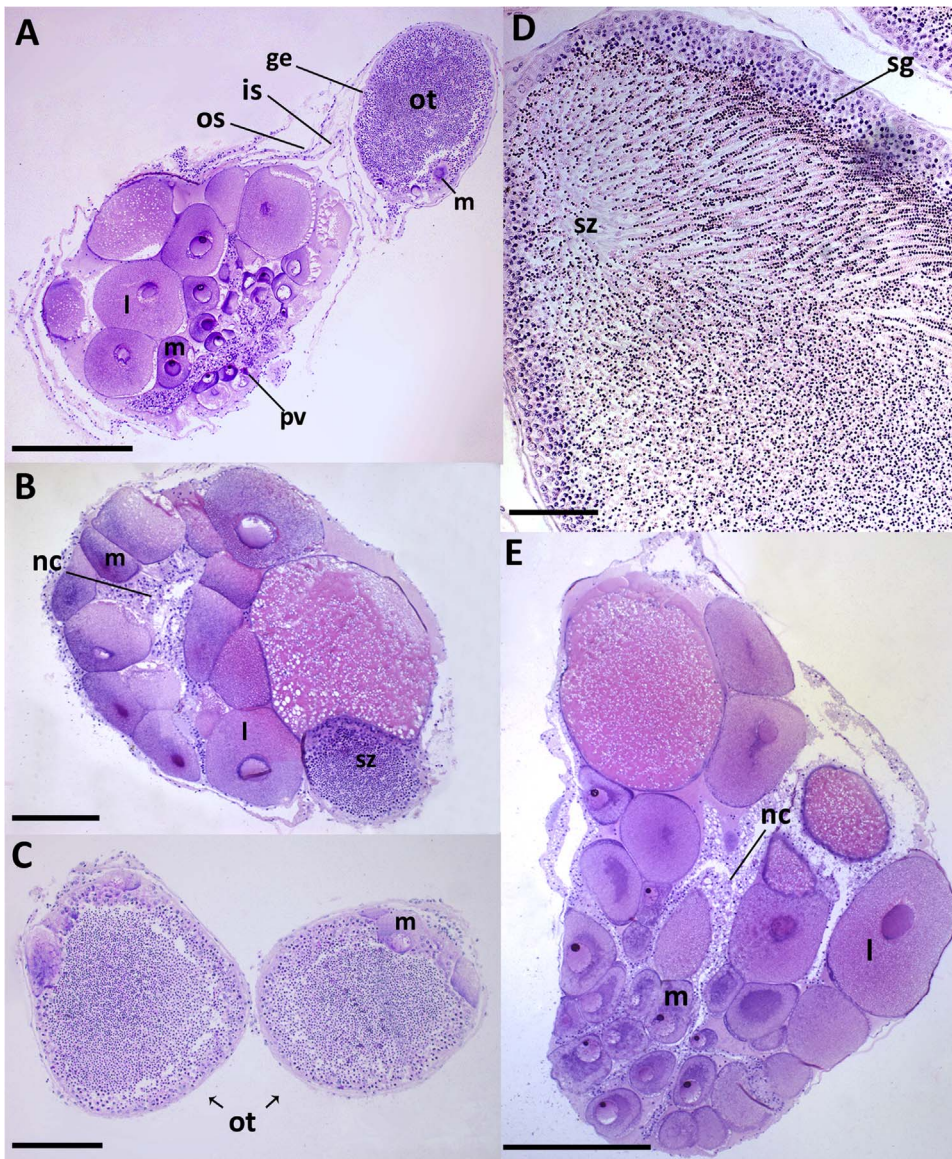
Fig. 3. Histology of *A. agassizii*. **A** Hermaphroditic gonads showing outer and inner sac (is, os) and germinal epithelium (ge). ot, ovotestis; pv, previtellogenic oocyte; m, midvitellogenic oocyte; l, late vitellogenic oocyte. **B** Developing hermaphroditic gonad with mid and late vitellogenic oocytes surrounded by nourishing cells (nc). **C** Ovotestis with midvitellogenic oocytes. **D** Detail of one ovotestis showing spermatogenic columns and free spermatozoa (sz) in the lumen. sg, spermatogonia. **E** Hermaphrodite gonad showing midvitellogenic and late vitellogenic oocytes. Scales bars, **A**, **B**, **E** = 0.5 mm; **C** = 0.2 mm; **D** = 0.1 mm.

eurybathic species.

Seasonal variations of food supply and environmental conditions in deep-sea waters have been of much interest to explain periodical physiological processes of deep-sea species (Tyler, 1988). However, continuous breeders may exploit a more uniform food source in the deep-sea which could maintain their growth constantly (Tyler, 1988). *Astrotoma agassizii* is known as a predator species with prevalence of copepods on its stomach contents (Dearborn et al., 1986; Ferrari and Dearborn, 1989), although limited data is available for deeper populations.

Reproductive periodicity has been described in the deep-sea species *Ophiomusium lymani*, *Ophiocten gracilis* and *Ophiura ljungmani* (Schoener, 1968; Lightfoot et al., 1979; Gage and Tyler, 1981; Gage, 1982). Our results showed presence of hermaphroditic gonads containing spermatozoa and oocytes at different stages of development, revealing a simultaneous hermaphroditism in *Astrotoma agassizii*. This pattern is similar to the gonochoric species *Ophioplocus januarii* which reproduces continuously throughout the year in shallow Northern Patagonia, Argentina, revealing ovaries containing oocytes at different stages of development and spermatogenesis simultaneously (Brogger et al., 2013b). The synchrony in the development of the hermaphroditic gonad of *Astrotoma agassizii* could be indicative of continuous

reproduction for this species in the deep waters from Southwestern Atlantic Ocean. This type of reproductive strategy that includes large eggs and lack of seasonality has been suggested in some studies from warm-water to deep-sea echinoderm species (Gage and Tyler, 1982; Byrne, 1989; Rivadeneira et al., 2017).

We conclude that *Astrotoma agassizii* is a simultaneous hermaphroditic species, with a maximum oocyte diameter of 850 µm recorded in the ovary; no evidence was found of intraovarian embryo development; at least 20 broods can be incubated inside one bursal sac; all the embryos found were at the same developmental stage; maximum size recorded of a young brood was 1100 µm disc diameter and only five of ten burse contained embryos. Based on our findings we hypothesize that *Astrotoma agassizii* from Mar del Plata Canyon´s area might be a continuously breeding species. It is hoped that further sampling campaigns along the year at Mar del Plata Canyon area will provide more data to answer this question.

Acknowledgements

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