Comparative embryonic development patterns in three deep-water skates from the southwest Atlantic

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Abstract
Embryonic development is a vulnerable and key period during the life cycle of an oviparous elasmobranch. Captivity studies are difficult to carry out on non-coastal species; hence, embryonic development can be analysed from samples collected from the seabed. Here, embryonic development of three shelf and deep-water skates from the southwest Atlantic Ocean, Bathyraja brachyurops, B. macloviana and Amblyraja doellojuradoi was studied. Egg cases containing embryos in different stages were collected from 84 to 1006 m depth in the northern part of the Argentinean continental shelf and continental slope (36°S-41°S), including the Mar del Plata Canyon (38° S). Common development patterns were observed among the three species and also with other skate species previously studied. Anatomical structures and embryo features were similar among species in initial, early and middle stages. Advanced and pre-hatching embryos showed species-specific differences regarding pigmentation and spinulation. This study sheds light on the identification of neonates of shelf and deep-water species, which could be useful to recognize specific nursery areas in the deep ocean. In addition, this embryological comparative study expands the existing database on the different chondrichthyan lineages, thus making a step forward towards understanding of their phylogenetic relationships.

Keywords
Amblyraja, Bathyraja, Deep-sea, Embryonic stages, Nursery areas, Pre-hatching embryos.

1. Introduction
Skates comprise a diverse order (Rajiformes) of benthic and demersal chondrichthians. They are oviparous, a reproductive mode also found in three families of sharks (Heterodontidae, Scyliorhinidae, and Hemiscylliidae) and holoccephalans (Hamlett, 2005). Eggs are fertilized and encapsulated in a structurally complex capsule and then laid onto the sea floor, where the embryo develops by feeding on the yolk until hatching. Skates play important ecological roles in marine ecosystems, acting as energetic linkers between different habitats and as bioturbators by removing the sediments when feeding (Martins et al., 2018). In addition, they play key trophic roles in benthic communities since they consume a wide range of prey items, but also are preyed on by several organisms (Ebert and Bizarro, 2007; Barbini et al, 2018; Martins et al., 2018). Among members of this order, genera *Bathyraja* and *Amblyraja* (Families Arhynchobatidae and Rajidae, respectively) are distributed worldwide and are by far the most common skates in the deep-sea ocean (Last and Yearsley, 2002), occurring on continental shelf and slope, reaching depths of more than 3100 m (Last et al., 2016; Weigmann, 2016).

Even though deep-water marine environments are difficult to access, much progress has been made into the field of taxonomy and biology of deep-water skates (Ebert, 2015; 2016; Last et al., 2016). Even more, valuable information in deep-water skates has recently been obtained by means of *in situ* observations using remotely operated and autonomous underwater vehicles (ROVs and AUV’s) (Hoff, 2010; Amsler et al., 2015; Salinas-de-León et al., 2018; Kuhnz et al., 2019). However, the available information regarding some aspects of life history is still fragmentary. Embryonic development, for instance, is almost unknown for most shelf and deep-sea skates.

Embryonic development is a vulnerable and key period during the life cycle of an organism that determines survivability of species. Skate egg cases have anti-microbial and antifouling properties (Kormanik, 1993; Thomason et al., 1996) and also provide mechanical protection to the developing embryo. However, some predators such as gastropods, feed on egg cases, which is the most widely reported mortality source in skate embryos (Cox and Koob, 1993; Lucifora and García, 2004). Early embryonic stages are more susceptible to predators than later stage embryos, given the fact that a more voluminous and nutritive yolk is present at the beginning of development. Early embryos have a large external yolk sac and are embedded in a jelly matrix, whereas later stage embryos are bigger and the yolk sac dramatically shrinks in size. Some *Bathyraja* embryos absorb yolk slowly during early development, and much more rapidly...
at later stages (Hoff, 2009). Predators could indeed prefer freshly deposited egg cases over those in advanced stages since these have thinner walls and, therefore, are easier to bore into. Mortality caused by predation is likely to differ among skate species as predation rates do (Lucifora & García, 2004). Understanding embryonic development as a whole, including internal processes and external threats can help shed light on early-life mortality, which is relevant to fish recruitment. Also, a clear understanding of all chondrichthyan life stages can lead to implement better and more successful management and conservation strategies.

Most skate development studies have been carried out by keeping individuals in captivity (Luer and Gilbert, 1985; Koop, 2005; Harahush et al., 2007; Jañez and Sueiro, 2007; Mabragaña et al., 2015). In these conditions, detailed monitoring of embryonic development is possible from the day of laying until hatching. Species that live at great depths (beyond 200 m) are especially adapted to low temperatures, low light intensity and high hydrostatic pressure (Marshall, 1979; Herring, 2000), conditions that cannot be easily achieved in experimental environments. Information on embryonic development in these species is sparse and comes from studying egg cases collected from the seabed (Stehmann and Merrett, 2001; Scenna, 2011; Henry et al. 2016). First studies focused on marine life inhabiting the depths of the Argentine sea began only a few years ago, with 4 cruises sampling up to 3500 m depth (Flores et al., 2019; Penchaszadeh et al., 2019). During these expeditions egg case nursery sites were identified for several oviparous chondrichthyans, including those of genera *Bathyraja* and *Amblyraja* (Vazquez et al., 2016). However, embryonic stages from these egg cases were not analysed.

Here, as part of a major study delving deeper into the southwest Atlantic (SWA) shelf and slope habitat, stages of embryonic development of the broadnose skate *B. brachyurops*, the Patagonian skate *B. macloviana* and the southern thorny skate *A. doellojuradoi* are described, focusing on similarities and differences between initial, early, middle, advanced and pre-hatching development stages. This study sheds light on the identification of neonates of shelf and deepwater species, which can be useful to recognize specific nursery areas in the deep ocean. This paper can help to elucidate phylogenetic relationships through the comparison of the morphological changes that occur throughout embryonic development in different chondrichthyan lineages.

2. Materials and methods
2.1 Sample collection

Sampling was carried out in the northern part of the Argentinean continental shelf and continental slope (36°S-41°S), including the Mar del Plata Canyon (38° S). Depth range surveyed was from 50 to 3447 m (Figure 1). Samples came from eight research cruises carried out on board of the Argentinean O/V Puerto Deseado (OVPD) and commercial vessels between 2009 and 2014. Samples from research cruises were collected using two bottom trawls, a shrimp net (50 mm mesh in the wings, and 20 mm in the cod end; vertical height 1 m, horizontal opening 4 m) and a bottom trawl net (135 mm mesh in the wings, and 60 mm in the cod end; vertical height 3.7 m, horizontal opening 10 m). Two dredges (horizontal openings 0.6 and 0.8 m) were also used. Those from commercial vessels were collected using a bottom trawl net (mesh size 120 mm, horizontal opening 22 m).

Figure 1. Location of samples collected in the northern part of the Atlantic continental shelf and continental slope (36°S-41°S), including the Mar del Plata Canyon (38°S). Black circles: sites with egg cases with embryos; empty circles: sites with empty egg cases; crosses: sites with no catches.

2.2 Egg cases identification and description of development stages

Most samples came from research cruises and were frozen on board. Those egg cases from commercial vessels were fixed in formalin 4% since it was not possible to freeze them on board. Egg cases were examined in the laboratory. They were identified following Mabragaña et
Egg case length without horns (ECL) and maximum width (MAW) were recorded to the nearest 0.5 mm. Egg cases were opened and the following measurements and features were recorded for the embryos: embryo position relative to the egg case, embryo total length (TL) and disc width (DW), external and internal (if present) yolk-sac diameter, and sex. A relative development stage was assigned to each embryo following Ballard et al. (1993) and Caldeira Brant (2003), who proposed a total of 34 stages (in sharks) and 35 (in skates), respectively. These authors consider the first 17 stages as microscopic. In addition, a recent embryo scale of five stages (I to V) proposed by Luer et al. (2007) was considered. Morphological features such as pharyngeal arches, external gill filaments, development of pectoral and pelvic fins, formation of eyes and spiracles, pigmentation and spinulation patterns were recorded for each species. All these features allowed assignment of the embryonic development stage. Each embryo was photographed using a digital camera or microscope camera (Biotraza) when needed. Embryonic stages were grouped into initial (< 17), early (18 to 24, I to III), middle or intermediate (25 to 29, III to IV), advanced (30 to 34, IV to V) and pre-hatching (35, V) for subsequent comparison between species. Egg cases and embryos were fixed in formalin 4% and stored in alcohol 70% in the Fish Collection of Instituto de Investigaciones Marinas y Costeras (IIMyC), Mar del Plata, Argentina (IIMyC-CEGAR 12, 13, 16, 21, 41, 71-74, 89, 105-110, 112, 114, 117, 160-163, 166-169, 172, 174-184, 186, 187, 190-192, 195-197, 212-215 and 221). Given the difficulty of obtaining deep-water samples, a complete collection of all stages of embryonic development of each deep-water skate species was not possible. Descriptions of development were realized by comparing similar stages, and only the best representative photos of them are presented.

Given that egg cases of *Amblyraja doellojuradoi* were collected over a wide depth range (95 to 1006 m), small pieces of tissue sample were taken in some embryos in order to check that they corresponded to the same species. Samples were preserved in 96% ethanol at -20°C for genetic analysis. DNA extraction, polymerase chain reaction (PCR), and sequencing of the 5′ region of the COI gene were performed following standard DNA barcoding protocols (Ivanova et al., 2006) coupled with primers and primer cocktails developed for fishes (Ward et al., 2005; Ivanova et al., 2007). DNA extraction and amplification were performed at the Argentine International Barcode of Life Laboratory reference (IIMyC, CONICET, Mar del Plata, Argentina). Sequencing was performed in Advanced Analysis Centre’s Genomics Facility (College of Biological Sciences, University of Guelph, Ontario Canada) and the Canadian Centre...
for DNA Barcoding (CCDB) at the Biodiversity Institute of Ontario, (University of Guelph, Ontario, Canada). Using the library of sequences available on BOLD (Barcode of Life Data Systems), the closest matches to our DNA sequences were obtained.

3. Results

3.1. Collected samples

Fifty two egg cases containing embryos were collected from 84 to 1006 m depth: 19 of *Bathyraja brachyurops* (ECL range: 76.9-107.4 mm, MAW range: 51.7-64.7 mm), 18 of *Bathyraja macloviana* (ECL range: 68.2-95.1 mm, MAW range: 40.5-50.1 mm) and 15 of *Amblyraja doellojuradoi* (ECL range: 60.9-81.6 mm, MAW range: 36.1-52.9 mm). When compared to the BOLD “Species Level Barcode Records” database, all deep-water *Amblyraja*-like samples (IIMyC-CEGAR 160, 162 and 163) matched *A. doellojuradoi* with a similarity range of 99.85-100% supporting that all samples belonged to this skate species. Information about development stage of each embryo, number of embryos analysed and depth range is summarized in Table I.

| Species          | Proposed staging | TL (mm) | DW (mm) | Caldeira Brant (2003) | Luer et al. (2007) | Total | Depth (m) |
|------------------|------------------|---------|---------|-----------------------|--------------------|-------|-----------|
| *Bathyraja brachyurops* | Initial | -         | -       | <17                   | I                  | 7     | 95-136    |
|                   | Early           | 21.4    | <5      | 23                    | III                | 1     | 98        |
|                   | Middle          | 23      | <5      | 24/25                 | III                | 1     | 104       |
|                   | Middle          | 30.5    | <5      | 25                    | III                | 1     | 104       |
|                   | Middle          | 58.3    | 11.4    | 28/29                 | IV                 | 1     | 95        |
|                   | Middle          | 59.8-62.9 | 11.5-12.3 | 29                  | IV                 | 2     | 98        |
|                   | Advanced        | 70.3    | 20.4    | 30                    | IV                 | 1     | 98        |
|                   | Advanced        | 90.2-92.9 | 26.2-26.8 | 32/33             | IV                 | 3     | 95-98     |
|                   | Advanced        | 108.4   | 39.5    | 33/34                 | IV                 | 1     | 136       |
|                   | Pre-hatching    | 155.5   | 82.5    | 35                    | V                  | 1     | 251       |

| Species          | Proposed staging | TL (mm) | DW (mm) | Caldeira Brant (2003) | Luer et al. (2007) | Total | Depth (m) |
|------------------|------------------|---------|---------|-----------------------|--------------------|-------|-----------|
| *Bathyraja macloviana* | Initial | -         | -       | <17                   | I                  | 6     | 97-110    |
|                   | Early           | 9.23    | <5      | 19                    | III                | 1     | 117       |
|                   | Middle          | 34.3-35.7 | 4.2-5.3 | 26                    | IV                 | 2     | 98-108    |
|                   | Middle          | 40      | 5       | 27                    | IV                 | 1     | 96        |
|                   | Middle          | 65.9    | 15.5    | 29                    | IV                 | 1     | 84        |
| Stages                  | TL      | DW      | Depth      | Stage | Total | Depth |
|-------------------------|---------|---------|------------|-------|-------|-------|
| Initial                 | -       | -       | <17        | I     | 8     | 95-852|
| Early                   | 17      | <5      | 19/20      | III   | 1     | 998   |
| Middle                  | 39.6    | 4.4     | 25/26      | III/IV | 1    | 998   |
| Middle                  | 63      | 11.8    | 29/30      | IV    | 1     | 110   |
| Advanced                | 74.3-76.5 | 22.9  | 31         | IV    | 2     | 852-1006 |
| Advanced                | 86.6    | 31.3    | 32         | IV/V  | 1     | 998   |
| Pre-hatching            | 97.3    | 44.4    | 34/35      | V     | 1     | 780   |

**Table 1.** Embryonic stages found for *Bathyraja brachyurops*, *Bathyraja macloviana* and *Amblyraja doellojuradoi* considering Caldeira Brant (2003) and Luer et al. (2007) staging table. TL: Embryo total length and DW: disc width. “Total” indicates the number of embryos analysed. “Depth” shows the depth range where egg cases with embryos were collected, in meters.

3.2. Initial embryonic stages

Initial stages are characterized by the presence of a large external yolk sac. The full content is embedded in a jelly matrix, which has a protective and supportive function. This matrix also blocks the opening of the respiratory canals, which are located along the lateral edge of each horn, towards the external environment (Figure 2a).

3.3. Early embryonic stages

Besides the aforementioned features a small embryo is visible. An anterior curvature in the trunk is present forming the head region, where optic vesicles and six pairs of pharyngeal arches are already developed. These arches are opened on their ventral side so that the mouth is not yet formed. The embryo is attached to the yolk sac through a long yolk stalk. Neither fins nor pigmentation are observed (Figure 2b).

In later stages (by stage 23), pharyngeal arches start to close ventrally delimiting the primitive mouth and behind them pectoral fins begin to emerge as small flaps on either side of the trunk (Figure 2c).
Figure 2. Egg case of Amblyraja doellojuradoi initial stage with a remarkable external yolk sac (a); early stages of Bathyraja macloviana (b) and B. brachyurops (c). EYS: external yolk sac, OV: optic vesicles, PA: pharyngeal arches. Embryonic stage (St.) corresponds to Caldeira Brant 2003 (arabic numbers) and Luer et al. 2007 (roman numbers in brackets).

3.4. Middle embryonic stages

Both rounded-shaped pectoral and pelvic fins are observed and the external yolk sac is slightly vascularised (Figure 3a). Buds of external gill filaments are visible on all arches and start to gradually grow except in the first arch, where spiracles are differentiated. The tail is dorsoventrally covered by a veil or membrane from which the two dorsal fins develop posteriorly, and the cloaca can be observed. In the head region, the mouth becomes circular and optic cups are visible (Figure 3b).

Later stages show a lateral expansion of the pectoral fins. The anterior end of these fins is not welded to the main axis initially but gradually begins to expand to form a complete disc. Pelvic fins have an initial middle notch and then acquire a triangular shape differentiating the anterior and posterior lobes. The first outlines of claspers can be observed in those posterior lobes in males. At this stage, nares are present, mouth becomes oval and then triangular and a ring of pigment is visible around the eyes. Gill filaments reach their maximum length and start to be absorbed (Figure 3c).

More advanced stages (older than stage 28) show mouth and nares in the neonatal position (Figure 3d).
Figure 3. Middle embryonic stages of *Bathyraja brachyurops* (a, c and d) and *B. macloviana* (b). Embryonic stage (St.) corresponds to Caldeira Brant 2003 (arabic numbers) and Luer et al. 2007 (roman numbers in brackets).

3.5. Pre-hatching and advanced embryonic stages

Advanced stages of embryonic development start with the formation of a complete disc (Figure 4a). Pelvic fins increase in size and gill filaments begin to reabsorb until they disappear. Eyes are completely pigmented (Figure 4b). A small internal yolk sac in the abdominal cavity is observed and the external yolk sac is greatly reduced (Figure 4c), with a little remaining external yolk at the time of hatching. Males can be easily recognized by the presence of claspers which are absent in females (Figure 4d).
Figure 4. Advanced embryonic stages of *Bathyraja brachyurops* (a), *B. macloviana* (c and d) and *Amblyraja doellojuradoi* (b). EYS: external yolk sac, IYS: internal yolk sac. Embryonic stage (St.) corresponds to Caldeira Brant 2003 (arabic numbers) and Luer et al. 2007 (roman numbers in brackets).

Spinulation and body pigmentation patterns are set, and remarkable differences between the three species are found. In *Bathyraja brachyurops*, primordia of 17 caudal thorns can be observed along the tail (from the middle of the pelvic fins) with an interdorsal primordium, which then hardens. Two nuchal thorns subsequently emerge in the midline. The whole embryo dorsal surface is covered with small dermal denticles, which are less abundant in the posterior margin of the disc. There is a gap between the nuchal and the midline thorns covered with denticles (Figure 5a). The first signs of pigmentation appear as small dark dots across the medial disc zone. Pigmentation progressively becomes more intense in the central area until it reaches a bright dark brown, with several small white circular spots scattered all over the disc (Figure 5b).
The spinulation pattern in *Bathyraja macloviana* starts with 29 primordia in the tail (from the nuchal region), followed by one or two interdorsal primordia. Subsequently, two ocular primordia appear in the inner margin of each eye and one primordium of the scapular thorn on each side of the midline of disc. Dermal denticles are present covering all the dorsal side of the disc (Figure 5c and d). Body pigmentation begins with brown spots in the centre, then becomes more intense and covers the entire embryo. Several white spots are distributed on the disc, of which the two biggest occur in the posterior third of the disc, near the pelvic fin notch (Figure 5d).

Fourteen primordia of thorns can be seen along the tail in *Amblyraja doellojuradoi*, from the nuchal region to the first dorsal fin (two nuchal plus twelve midline thorns, Figure 4b). Three primordia of ocular thorns appear in the inner margin of each eye and finally three pairs of primordia of scapular thorns are arranged on each side of the midline. All of these primordia will harden to form strong thorns. Dorsal body surface is completely covered with small dermal denticles. First signs of pigmentation appear as dark irregular spots scattered in the dorsal surface, except at the edges of the pectoral fins. These spots gradually become more abundant and fill the entire dorsal surface, thus forming a uniform dark brown colouration pattern. Ventral face is white (Figure 5e).
Figure 5. Pre-hatching embryonic stages of *Bathyraja brachyurops*, showing the spinulation pattern (a) and colouration (b); *B. macloviana*, showing spinulation (c) and colouration (d); and *Amblyraja doellojuradoi*, showing both patterns (e). Embryonic stage (St.) corresponds to Caldeira Brant 2003 (arabic numbers) and Luer et al. 2007 (roman numbers in brackets).
Embryos can freely move throughout development since they have enough space within the egg case (Figure 6a). In pre-hatching embryos space becomes limiting so they fold their pectoral fins dorsally over the body and place the long tail over the edge, with a tail filament (whip-like extension) next to the respiratory region (Figure 6b). Shortly before hatching, the embryo faces its rostrum towards the posterior end of the egg case preparing to hatch (Figure 6c).

Figure 6. Embryo arrangement inside the egg case. Amblyraja doellojuradoi, embryo with enough space to freely move inside the egg case (a); Bathyraja brachyurops, space becomes limiting so embryo folds its pectoral fins dorsally over the body and place the long tail over the edge (b); and B. macloviana, the embryo faces its rostrum towards the posterior end of the egg case and is ready to hatch (c).

4. Discussion

Early stages of the life cycle of oviparous chondrichthians (skates, holocephalans and some sharks) include the release of leathery egg cases on the seafloor, where the embryo fully develops until hatching. Embryonic development time (also called incubation period) may take from several months to years, depending on the species and environment conditions
(Berestovskii, 1994; Hoff, 2007; Jañez and Sueiro, 2007; Mabragaña et al., 2015). Even though complete sets of embryological data are difficult to obtain in non-coastal chondrichthyans, a comparative description of different embryo stages of three shelf and deep-water skates from the SWA was performed here.

ECL and MAW showed a relatively wide range within each of the species under study. Previous studies have correlated egg case size in skates with female’s length, showing that the larger the female, the bigger the egg case laid (Templeman, 1982; Scenna, 2011). As the size at maturity within each species studied here is wide (Paesch and Oddone, 2008; Delpiani, 2016), it is likely that females of different length lay egg cases of different sizes.

Early development features observed here are similar to those reported previously for other skate species. Early embryos show the typical curvature of the trunk present in the ontogeny of all vertebrates, and there are no marked differences with embryos of different chondrichthyan lineages (Ballard et al., 1993; Didier et al., 1998; Caldeira Brant, 2003; Luer et al., 2007; Maxwell et al., 2008; Onimaru et al., 2018). Development of sharks and skates can be differentiated at the time when pectoral fins start to emerge as little flaps in the lateral sides, forming the typical batoid disc. In Raja eglanteria (= Rostroraja eglanteria) the pectoral fins and mouth appear first than gill filaments (Luer et al., 2007), the opposite of what was observed for the three skate species studied here, in which the gill filaments develop first. However, the period of time between these two events seems to be very short and insignificant, since in Sympterygia acuta mouth appears shortly before pectoral fins and gill filaments (Caldeira Brant, 2003).

Middle stages do not exhibit major differences compared with those observed by Maxwell et al. (2008) for Leucoraja ocellata, Luer et al. (2007) for R. eglanteria and Caldeira Brant (2003) for S. acuta. In these skate species gill filaments appear for the first time from stages 24-25. This is also seen in shark and chimaera embryos (Ballard et al., 1993; Didier et al., 1998; Onimaru et al., 2018). Dorsal fins can be distinguishable at late stage 26 and early stage 27 as also happens in other skate species (Caldeira Brant 2003; Maxwell et al., 2008) and the shark Chiloscyllium punctatum (Onimaru et al., 2018). In other shark species such as Scyliorhinus canicula (Ballard et al., 1993) and holoccephalans (Didier et al., 1998), dorsal fin development seems to be delayed, appearing at stage 30 and 28, respectively. Copulatory organs or claspers in males appear at the same stage 29-30 in skates (Caldeira Brant, 2003; Maxwell et al., 2008; present work) and in C. punctatum (Onimaru et al., 2018) whereas in S. canicula (Ballard et al.,
1993) claspers are recognized from stage 31. A delay in claspers development is already observed in chimaeras (Didier et al., 1998) appearing from stage 35. When all features are compared, it seems to be a high concordance in early and middle development stages of several skates (*L. ocellata, R. eglanteria, S. acuta* and species studied here), and also in some shark species (*C. punctatum*). The Chimaera lineage shares many early developmental events with elasmobranchs, but start to diverge when reaching late middle stages (Ballard et al., 1993; Didier et al., 1998; Caldeira Brant, 2003, Maxwell et al., 2008).

Advanced stages start in a similar way in the three skate species studied here. From stage 30, pectoral fins begin to expand fusing to the head to form a whole disc, pelvic fins become triangular-shaped and a small yolk sac appears in the abdominal cavity of the embryo. These events were also reported, but in later stages, in *L. ocellata* (stage 31, Maxwell et al., 2008) and *S. acuta* (stage 32, Caldeira Brant, 2003). First signs of epidermis pigmentation are visible from stage 31 in *A. doellojuradoi* (present work) and *S. acuta* (Caldeira Brant, 2003), whereas in *L. ocellata* (Maxwell et al., 2008), *S. canicula* (Ballard et al., 1993), *B. brachyurops* and *B. macloviana* (present work) they begin to be noticeable from stage 32. However, in *C. punctatum* the typical stripe pigmentation starts from stage 34 (Onimaru et al., 2018). Gill filaments completely disappear from stages 32 and 33 in the present work as was also reported in *L. ocellata* (Maxwell et al., 2008) and *S. canicula* (Ballard et al., 1993), whereas in *S. acuta* it happens from stage 34 (Caldeira Brant, 2003).

More remarkable differences between the three species became evident in the advanced stages. Colour and spinulation patterns resembled those of juveniles and adults of *B. brachyurops* reported by Cousseau et al. (2007). The advanced embryos do not have irregular brown spots on the ventral side of the tail (present work), as does occurs in juveniles and adults. Spinulation and colouration patterns in *B. macloviana* advanced embryos agreed with those described for juvenile and adults of this species (Cousseau et al., 2007). Pre-hatching embryos of *B. macloviana* and *B. brachyurops* can be easily distinguished by the high number of midline thorns and the presence of scapular and ocular thorns in the former. In addition, *B. brachyurops* pre-hatching embryos were larger (in both TL and DW) than those of *B. macloviana*, as also happens in adults of these species (Cousseau et al., 2007). Spinulation and colouration patterns in *A. doellojuradoi* embryos corresponded with those reported by Cousseau et al. (2007) for juvenile and adult specimens of this species. Dorsal surface of disc was dull dark brown without
the characteristic white spots ahead of the eyes and posterior ends of the pectoral fins. Pre-hatching embryos of *A. doellojuradoi* were shorter (in both TL and DW) than those of *B. brachyurops* and *B. macloviana*. This is consistent with maximum sizes reported for adults (Cousseau et al., 2007; Delpiani, 2016).

As observed in skate species studied here, similar behaviours of pre-hatching embryos inside the egg case have been reported by Luer and Gilbert (1985) for *Raja eglanteria*. The proximity of the tail filament to the gill region seems to be related to more efficiently circulate seawater through the respiratory canals to facilitate the embryo respiration, as also suggested for *R. eglanteria*. After hatching, the remaining external yolk sac will be absorbed in the first weeks.

Unlike coastal areas, deep water habitats are relatively constant environments, with little variability in physical conditions such as temperature, salinity and oxygen content. Embryonic development is mainly dependent on physical conditions of water, and temperature plays a key role in duration of the incubation period. Coastal skate species are exposed to higher temperatures than those of deeper waters, and incubation periods tend to be shorter (few months, Luer et al., 2007; Mabragaña et al., 2015). Conversely, shelf and deep-water skates are exposed to colder temperatures and have longer incubation periods. Neonates of some species of *Bathyraja* and *Amblyraja* can take up to 3.5 years to hatch at temperatures of 4.4 °C in the deep ocean (Berestovskii, 1994; Hoff, 2008). If incubation temperature increases only 0.5°C, incubation period can be reduced by 16% (six months) in *Bathyraja parmifera* (Hoff, 2008). Temperature also affects embryonic development itself since metabolism and rate of embryo development increases markedly with increasing temperature (Hume, 2019). However, no differences between coastal and shelf and deep-water species were noticed in relation to the order of appearance of morphological structures throughout development.

Skates lay their egg cases in areas known as egg case nurseries, where embryonic development and neonate hatching take place (Hitz, 1964; Hoff, 2007; 2016). Also, nursery areas are associated with the presence and occurrence of juveniles (Martins et al., 2018). Egg-laying sites for the species studied in this paper have recently been discovered in the SWA (Vazquez et al., 2016). However, areas where neonate and young-of-the-year skates concentrate are still unknown, possibly due to the difficulty in distinguishing neonates among these species. Characterization of colouration and spinulation patterns in pre-hatching or advanced embryos is crucial to unambiguously identify neonate and young skates. McEachran (1983) observed that
some species of the South American skate genus *Psammobatis* have conspicuous differences in spinulation and colour patterns when juveniles and adults are compared. Neonates of *Psammobatis normani* and *Psammobatis rudis* are different from juveniles and adults regarding colouration and spinulation patterns. This makes it difficult to recognize nursery areas for these species (Mabragaña, pers. com.). Identification of areas supporting critical life stages is of great importance in terms of conservation of these vulnerable species.

This work represents the first attempt to characterize embryonic development stages in three skate species occurring in the continental shelf and slope of the SWA. Furthermore, it constitutes a contribution to the knowledge of deep-water elasmobranchs since it is the deepest record of *Amblyraja* embryos worldwide. Other congeneric species of *Amblyraja* possibly co-occur with *A. doellojuradoi* in the slope. *Amblyraja frerichsi* (Krefft, 1968) was registered in the SWA from 600-2600 m (Last et al., 2016), *A. georgiana* (Norman, 1938) was recorded in Malvinas Islands up to 600 m (Agnew et al., 1999), and *A. taaf* (Meisner, 1987) was reported from northern Burdwood Bank at 1000 m depth (Coggan et al., 1996). Even though all these other species are larger than *A. doellojuradoi*, their egg cases are unknown. Based on molecular data, we could confirm that all *Amblyraja*-samples correspond to the same species supporting the wide depth range of egg-laying recognized by Vazquez et al. (2016) for *A. doellojuradoi*.

Chondrichthyan embryos have been used as models in the study of the origin of paired appendages in vertebrates (Cole and Currie, 2007). As a basally positioned gnathostome clade possessing paired fins, chondrichthians are important in terms of vertebrate evolution. Comparative embryological studies help to expand the available database on the different chondrichthyan lineages, and allow the ontogeny to be useful in determining phylogenetic relationships; these studies can also be used to explore homology and convergence issues (Zusi and Livezey, 2000). In this sense, the present paper contributes some steps towards better understanding of phylogenetic relationships among skates, the most diverse group of batoids.

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• The first record of skate egg cases with embryos in Southwest Atlantic deep waters is reported

• The embryo features were similar among species in initial, early and middle stages

• Species-specific differences between advanced and pre-hatching embryos were identified

• Spinulation in pre-hatching embryos is coincident with that recorded for adults
Declaration of interests

(X) The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

(    ) The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: