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ORIGINAL PAPER

Reproductive aspects of *Notothenia rossii* and *N. coriiceps* (Perciformes, Nototheniidae) at Potter Cove, 25 de Mayo (King George) Island during austral summer

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Abstract Several fish species of the suborder Notothenioidei (Perciformes) predominate in the Antarctic Convergence Zone; nevertheless, reproductive studies are scarce due to difficulties on regular sampling. This study takes the research area of reproductive biology of notothenioids to a new level by providing, for the first time, data on sex hormone and vitellogenin detection in the blood of females of *Notothenia rossii* and *N. coriiceps* and correlates this data with morphological maturity indices as well as ovarian histology. Fish were captured during the Antarctic summer at Potter Cove, 25 de Mayo (King George) Island, and blood and ovary were collected. Histological analysis revealed that females of both fish possess group synchronous ovarian development with two distinct clutches of oocytes: a more advanced batch of vitellogenic oocytes ready for spawning and a second batch of previtellogenic oocytes for the next spawning event. Since liver vitellogenin synthesis is stimulated by estradiol produced by the ovaries, gonadal development, estradiol levels, and vitellogenin showed that both species were at a more advanced stage of maturation in March than in January. On the other hand and irrespectively of the month, gonadosomatic index and plasma estradiol levels of *N. coriiceps* were higher than those of *N. rossii*. Furthermore, females of *N. coriiceps* showed an advanced stage of vitellogenesis or were ready for spawn, contrary to results of previous studies. Our results indicate the successful use of gonadal morphology, estradiol, and vitellogenin detection for the estimation of sexual maturity stage of female adults.

Keywords Nototheniidae · Vitellogenin · Group synchronous ovarian development · Sex steroids · Potter Cove

Introduction

Among fish, the suborder Notothenioidei is the most common and conspicuous group in Antarctic waters. Notothenioids have been overwhelmingly successful in the Southern Ocean, including many species that remain in the inshore waters of the Antarctic continent and sub-Antarctic islands year round, and no other oceanic ecosystem is so dominated by a single taxonomic group of fish (DeWitt 1971; Eastman and Grande 1989; Knox 2006). This suborder is composed of eight families: Nototheniidae, Channichthyidae, Bathydraconidae, Artedidraconidae, Harpagiferidae, Bovichtidae, Eleginopidae, and Pseudaphritidae (Eastman 2005).

According to the distribution of fish in the Antarctic marine ecosystem, three major zones can be recognized in the Southern Ocean. These are, from south to north, the High-Antarctic Zone, the Seasonal Pack-Ice Zone, and the Ice-Free Zone (Kock 1992; Knox 2006). The Antarctic Peninsula and the region of 25 de Mayo (King George) Island lie within the intermediate Seasonal Pack-Ice Zone and represent one of the three areas of southern waters with the greatest ecological productivity, being an extremely

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rich and diverse environment (Garrison and Buck 1989; Marrari 2008; Siciński et al. 2011). The fish fauna of this zone accounts for 99 % of the biomass in this region and is mostly represented by the Channichthyidae and Nototheniidae families (Tiedtke and Kock 1989; Skora and Neyelov 1992). Within the latter family, *Notothenia coriiceps* Richardson and *N. rossii* Richardson are particularly found in shelf areas of the Scotia Arc, in the Seasonal Pack-Ice Zone and the islands north of it (Casaux et al. 1990; DeWitt et al. 1990; Clarke and Johnston 1996). Studies carried out in Potter Cove, 25 de Mayo (King George) Island, indicate that both species have similar ecology in the fjords, living predominantly in inshore waters from 5 to 50 m depth on rocky bottoms with macroalgae beds (DeWitt et al. 1990; Barrera-Oro 2002; Barrera-Oro and Casaux 2008) and also found that some specimens of *N. coriiceps* spend its entire life inshore (Casaux et al. 1990).

Upon the establishment of the Convention for the Conservation of Antarctic Marine Living Resources in 1982, a series of conservation measures were enacted in order to promote recovery of overexploited fish species in many Antarctic zones. Although fin fishing has been banned in the South Shetlands-Antarctic Peninsula area since 1991, the local population of *N. rossii* is still recovering after more than three decades of intense fishing for commercial use in the late 1970s (Barrera-Oro and Marschoff 2007; Marschoff et al. 2012). On the other hand, an increase in the population of *N. coriiceps* due to the decrease in interspecific competition and consequent expansion of its ecological niche was evident (Barrera-Oro et al. 2000).

Although there is considerable life-history information for these species including an age and rate of growth estimate, analysis of diet, and mark and recapture data, there is still no consensus about the spawning season of *N. coriiceps* and previous studies indicate a wide variation of this period between different localities and conditions (Everson 1970; Casaux et al. 1990; Kock and Kellermann 1991; Barrera-Oro and Casaux 1996; Casaux and Barrera-Oro 2002). In the case of *N. rossii*, spawning occurs between April and June (Freytag 1979; Burchett 1983). Indeed, most studies on the reproduction of these species have concentrated on analyses of macroscopic gonadal appearance, egg counts, and sizes at first reproduction, but histological information is so far nonexistent or scarce (Everson 1977; Kock and Kellermann 1991; Sapota 1999). Despite the fact that macroscopic staging is a practical and quick method in field work, validation by histology is necessary to support data accuracy (Holden and Raitt 1974; Macchi and Barrera-Oro 1995; Rae and Calvo 1995). It is important, as well, to consider changes in circulating reproductive hormones, since gonadal development in female teleosts is known to be dependent on several steroid hormones (Fostier et al. 1983; Tveiten et al. 1998; Dahle et al. 2003). Among these steroids, estrogens induce the synthesis of vitellogenin (VTG) produced by the liver. VTG is transported through bloodstream to the ovaries and incorporated into the growing oocytes. Inside these cells, VTG is cleaved into yolk proteins, which participate both in the physiology of the ovulated egg and the nutrition of developing embryos (Mommsen and Walsh 1988; Brooks et al. 1997; Babin et al. 2007).In female teleosts, plasma VTG detection has been widespreadly used as an indicator of sexual maturity (Scott et al. 1984; Kobayashi et al. 1988; Harmin et al. 1995; Webb et al. 2002; Kumar et al. 2015). However, synthesis of female-specific proteins such as VTG has been poorly studied in notothenioid fish (Canapa et al. 2007).

Because little is known about the reproductive status of *N. rossii* and *N. coriiceps* in Potter Cove; the aim of this work was to provide a new insight in the reproductive biology of both species during the austral summer. To our knowledge, this is the first report of sex hormones and VTG detection in females of Antarctic fish related to maturity indices and ovarian histology.

Materials and methods

Animals

Specimens of *N. rossii* and *N. coriiceps* were collected during the austral summer (January 2012 and 2013, and March 2014). Sampling was carried out at Potter Cove, 25 de Mayo (King George) Island, South Shetland Islands, close to the Scientific Station Carlini—formerly Jubany Station (62°14′S; 58°40′W). In this area, fish were caught with trammel nets (length 25 m; width 1.5 m; mesh 2.5 cm) set on the bottom between 15 and 40 m water depths.

Fish were transported to laboratory aquaria, measured (total length), and weighed. Blood samples were obtained by caudal puncture with a heparin-coated syringe coupled with a 23-G needle. Blood samples were equally divided in two different 1.5-mL polypropylene tubes, one containing 10 µL of protease inhibitor cocktail (Sigma-Aldrich) for Western blot analysis and the other for measurement of steroids. Both tubes were centrifuged at 3000 rpm for 15 min at 4 °C, and plasma was stored at −20 °C, until use. Total protein content of each sample was determined by Lowry's method using bovine serum albumin (BSA) as a standard (Lowry et al. 1951).

After dissection, fish were sexed and subsequently confirmed by histological analysis. For this study, only females were selected. Ovaries and liver were removed and

weighed for determination of the gonadosomatic (GSI, gonad weight × body weight^{-1} × 100) and hepatosomatic (HSI, liver weight × body weight^{-1} × 100) indices. A mid-portion of the gonad was fixed in Bouin's solution during 18 h for later histological processing.

Vitellogenin detection

Plasma samples with equal amount of proteins (40 μg) were mixed with loading buffer (120 mM Tris–HCl, pH 6.8, 3 % sodium dodecyl sulfate, 10 % glycerol, 2 % bromophenol blue, and 1 % β-mercaptoethanol), boiled for 5 min, and briefly spun down before loading them into polyacrylamide gel wells. Molecular weight standards were loaded in a separate well (SeeBlue Plus2 Pre-Stained Standard, Invitrogen Corporation, USA). A sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE followed by Western blot), as described by Laemmli (1970), was performed at constant 100 V using 5 % stacking and 8 % separating gel (Mini-Protean III, Bio-Rad, USA) and 124 mM Tris–HCl, pH 8.8 running buffer. Transference to nitrocellulose membranes (ECL Amersham Biosciences, UK) was done at 100 V for 90 min, in 25 mM Tris, 187 mM glycine, and 20 % methanol. Subsequently, membranes were soaked with TTBS (100 mM Tris–HCl, 0.9 % NaCl, 0.1 % Tween-20, pH 7.5), and endogenous peroxidases were blocked with 2 % H_2O_2 30 vol in TTBS for 5 min. Afterward, unspecific binding sites were blocked with 3 % skimmed milk and 3 % BSA in TTBS overnight at 4 °C. For vitellogenin (VTG) immunodetection, membranes were incubated with different primary VTG antisera (see Table 1) for 90 min at room temperature, followed by sequential 60-min incubations with biotinylated anti-rabbit secondary antibody 1:10,000 (Sigma) and streptavidin–peroxidase 1:3000 (Sigma). The antigen antibody complex was visualized using 3,30-diaminobenzidine (DAB) solution (1 mM DAB, 50 mM Tris–HCl buffer pH 7.6, and 0.006 % H_2O_2). Omission of primary antibodies was also performed (not shown). Finally, membranes were scanned and molecular weights of VTG bands were estimated using SigmaGel software (Jandel Scientific software 1.0, USA).

Histological analysis

Ovaries subsamples were dehydrated and embedded in Paraplast (Oxford, USA). Then, they were sectioned at 7 μm, stained with Masson's trichrome, dehydrated, and mounted in DPX medium. Photomicrographs were taken with a Microphot FX (Nikon) microscope coupled with a Coolpix 5400 digital camera (Nikon, Japan). Histological analysis of ovarian tissue was performed to determine the reproductive development status of individual female fish.

Hormone analysis

Estradiol levels were measured in fish plasma by ECLIA (electrochemiluminescence immunoassay) (Cobas analyzer, Roche), with a detection limit of 5 pg/mL. Androgens levels were assayed by RIA (DSL-4000 ACTIVE-Coated-Tube Radioimmunoassay Kit), with a limit of detection of 0.08 ng/mL. All analyses were carried out according to the manufacturer's instructions and a standard curve was run for each steroid.

Statistical analysis

GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA, USA) was used for statistical analysis of indices and steroid levels. Values were expressed as mean ± SE (standard error). Comparisons between groups were performed with Student's *t*-test, while nonparametric test (Mann–Whitney) was used when data did not conform to assumptions of homogeneous variances. The normality of the data was tested using GraphPad InStat v3.06 (GraphPad Software, San Diego, CA) and data were log-transformed in cases where assumption of normality was not fulfilled. For comparisons of more than two means, 1-way ANOVA was performed followed by Tukey's multiple comparison

Table 1 Data of antisera used for vitellogenin detection in Western blot technique

Antiserum	Raised against	Dilution	Provided by
Anti-seabream VTG	*Sparus aurata* (Teleostei, Perciformes, Sparidae)	1:2000	Biosense Laboratory, Norway
Anti-Arctic char VTG	*Salvelinus alpinus* (Teleostei, Salmoniformes, Salmonidae)	1:5000	Dr. Helge K. Johnsen, Tromsø University, Norway
Anti-mummichog VTG	*Fundulus heteroclitus* (Teleostei, Cyprinodontiformes, Fundulidae)	1:3000	Dr. Akio Shimizu, National Research Institute of Fisheries Science, Fisheries Research Agency, Kanazawa, Yokohama, Japan

test. Means were considered statistically different at $p < 0.05$.

Results

The fish caught at Potter Cove included eighteen females of *N. rossii* ranging 27.5–43 cm in total length (TL) and 334.18–1125.5 g in body weight (BW), and twelve females of *N. coriiceps* ranging 22.5-46 cm in TL and 153.8–1364.43 g in BW. Table 2 summarizes size and organ weight data for all the animals collected.

Gonadal histology and somatic indices

In females of *N. rossii*, the gonadosomatic (GSI) and hepatosomatic (HSI) indices did not show significant differences between the sampling months (Table 2).

Histological studies revealed that the ovaries were surrounded by a thick tunica albuginea, comprised of collagen fibers, nerves, and blood vessels, which sends septa into the organ forming ovarian lamellae. The epithelia covering of the lamellae is the germinal epithelium where germ cells and follicles are located (Fig. 1a).

During January, the ovaries showed oogonia forming nests and a predominance of previtellogenic oocytes of increasing size, such as early primary oocytes and late primary oocytes (Fig. 1b, c). Follicles containing primary oocytes had a thin *zona pellucida* and a layer of squamous follicular cells. Early primary oocytes exhibited an eccentric nucleus with dense clumps of condensed chromatin while in late primary oocytes the nucleus became central with evenly distributed chromatin and a large number of peripherally located nucleoli. At this stage, it was possible to distinguish a large basophilic area called Balbiani body which was located in an asymmetrical position near the nucleus, composed of endoplasmic reticulum, mitochondria, Golgi, RNA, and proteins (Fig. 1c). As oogenesis proceeds, the Balbiani body became more dispersed and progressively displaced toward the oocyte cortex.

In March, females had gonads at a more advanced maturity stage. Histologically, the ovaries contained not only primary but also secondary oocytes characterized by the appearance of large lipid droplets in the ooplasm (Fig. 1d). The nucleus was central and several nucleoli remain close to the nuclear envelope. In the oocyte periphery, the *zona pellucida* became conspicuous and the follicular cells assumed cuboidal shape (Fig. 1e).

In females of *N. coriiceps*, the GSI increased from 1.03 ± 0.14 in January to 3.04 ± 0.80 in March; however, no significant differences in GSI or between HSI could be seen. The mean BW of females collected in January had the greatest standard error due to one female whose weight was twice as the others (1364.43 g).

Females sampled in January possessed ovaries with different stages of oocyte development with a predominance of secondary oocytes (Fig. 2a, b). In these ovaries, it was also possible to distinguish vitellogenic oocytes (Fig. 2c). At this stage, the oocyte contained an eccentric nucleus with numerous peripheral nucleoli, while the ooplasm was filled with spherical yolk platelets, and conspicuous lipid droplets peripherally located. The *zona pellucida* was more developed than that in secondary oocytes, and the follicular cells presented a cuboidal shape. Outside the follicle, the theca cells had similar shape to fibroblasts (Fig. 3a). Two female ovaries also exhibited hydrated oocytes with irregular shape and translucent ooplasma (Fig. 2a).

All the mentioned stages were also present in ovary samples of females collected during March. Furthermore, vitellogenic oocytes were mostly observed during this month (Figs. 2c, d, and 3b).

When mean values of GSI were compared between species, *N. coriiceps* presented a higher index both in January ($p = 0.061$) and in March ($p < 0.0001$), being these twice and six times greater, respectively (Table 2). In the same manner, plasma levels of estradiol were higher in *N. coriiceps* than in *N. rossii* not only in January but also in March (Fig. 4a, b). In both species, hormonal analyses reveal an increase in mean estradiol levels from January to March (Fig. 4c, d). On the contrary, androgen levels showed no significant differences between species or sampling periods. The values for *N. rossii* were 0.097 ± 0.003 ng/mL in January and 0.11 ± 0.01 ng/mL in March; for *N. coriiceps*, androgen levels were 0.13 ± 0.03 and 0.19 ± 0.07 ng/mL for January and March, respectively.

Table 2 Morphometric variables, hepatosomatic (HSI), and gonadosomatic (GSI) indices of females of *N. rossii* and *N. coriiceps* collected at Potter Cove

Month	January		March	
Species name	*N. rossii*	*N. coriiceps*	*N. rossii*	*N. coriiceps*
n	8	4	10	8
Body weight ± SE (g)	602.5 ± 99.31	736.6 ± 210.2	589.3 ± 111.3	697.0 ± 108.6
Total length ± SE (cm)	35.19 ± 2.35	36.25 ± 3.28	34.35 ± 2.18	34.81 ± 2.07
HSI	2.34 ± 0.50	3.60 ± 0.66	1.63 ± 0.16	2.595 ± 0.48
GSI	0.40 ± 0.03	1.031 ± 0.14	0.46 ± 0.036	3.043 ± 0.80

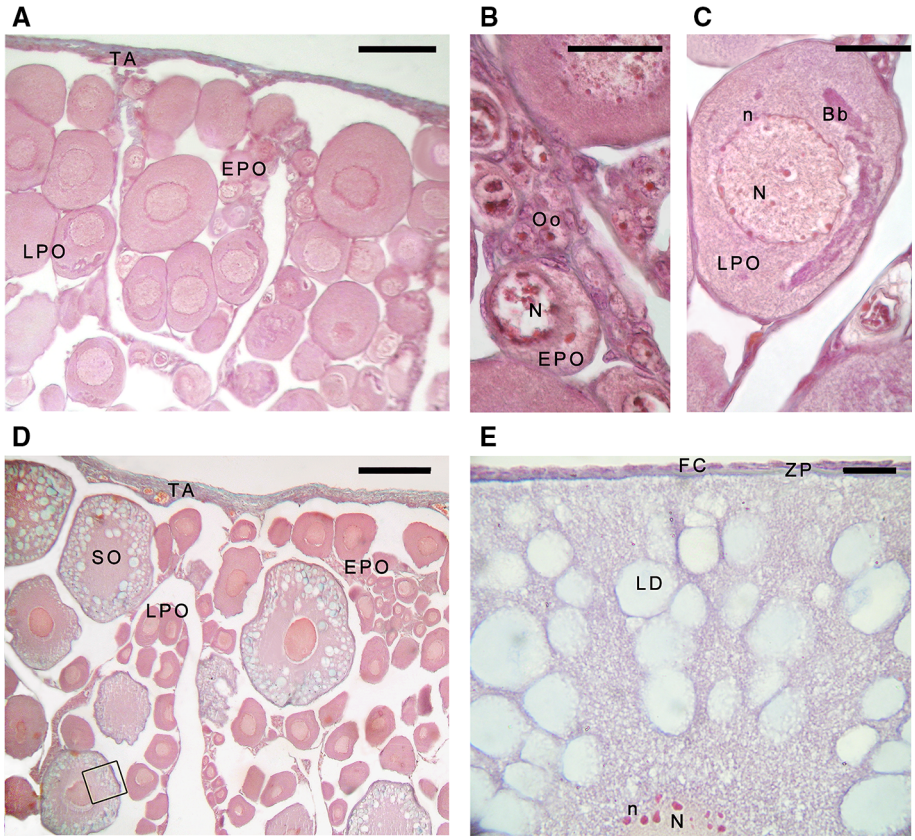

Fig. 1 Histological sections of *N. rossii* ovaries, sampled during Antarctic summer. **a** Cross section of ovarian lamellae filled with early primary oocytes and late primary oocytes from a representative female collected in January. **b** Oogonia forming nests near an early primary oocyte. **c** Detail of a late primary oocyte with peripheral nucleoli and cytoplasmic basophilic areas corresponding to Balbiani bodies. **d** General aspect of the ovary from a representative female collected in March, showing ovarian lamellae filled with primary and secondary oocytes. **e** Magnification of a secondary oocyte. *Bb* Balbiani bodies, *FC* layer of follicular cells, *LP* lipid droplet, *n* nucleoli, *N* nucleus, *Oo* oogonia, *EPO* early primary oocyte, *LPO* late primary oocyte, *SO* secondary oocyte, *TA* tunica albuginea, *ZP* *zona pellucida*. *Scale bars* **a**, **d** (200 μm); **b**, **c** (25 μm); **e** (50 μm). Stain: Masson's trichrome

Vitellogenin detection

Analysis of *N. rossii* plasma samples collected during January revealed a faint band with weight of 106.8 ± 1.1 kDa when seabream antiserum was used (Fig. 5a, lane 1). The Arctic char antiserum demonstrated affinity to a band of 77 kDa (Fig. 5b, lane 1). For both antibodies, VTG immunodetection was only observed in 25 % of the specimens. With the mummichog antiserum, there was no detection of VTG (Fig. 5c, lane 1). Contrary to January results, females collected in March presented no VTG in plasma samples when seabream antiserum was used (Fig. 5a, lane 3). The Arctic char antiserum revealed a VTG band of 106.7 ± 1.1 kDa in 30 % of the females (Fig. 5b, lane 3). On the other hand, mummichog antiserum showed a band of 72 kDa in 50 % of the females (Fig. 5c, lane 3).

Concerning to *N. coriiceps*, in one plasma sample collected during January (46 cm TL, 1364.43 g BW), it was possible to recognize a pattern of seven major bands: 143.4 ± 0.5, 121.9 ± 0.5, 106.7 ± 1.1, 95.5 ± 1.7, 71.9 ± 0.6, 60.4 ± 0.2, and 46.1 ± 0.4 kDa with seabream antisera (Fig. 5a, lane 2). The same pattern was detected when Arctic char antiserum was used, except for the 143 kDa and the addition of 50 kDa band. In the rest of females, both antisera revealed a pattern with one or more of the mentioned bands. The mummichog antiserum revealed two major VTG bands of 55.2 ± 0.3 and 50.5 ± 0.4 kDa (Fig. 5c, lane 2). One of the females exhibited a slightly different pattern with 84.19 ± 0.9, 55.2 ± 0.3, and 50 kDa bands (Fig. 5c, lane 3).

In March, VTG was detected in 50 % of plasma samples of *N. coriiceps* using the seabream antiserum, where in addition to the aforementioned VTG bands detected in January, another band was present (84.19 ± 0.9 kDa; Fig. 5a, lane 4). The other females exhibited a different pattern sharing one or more mentioned bands, and the smallest females (22.5 and 32 cm TL) did not show any

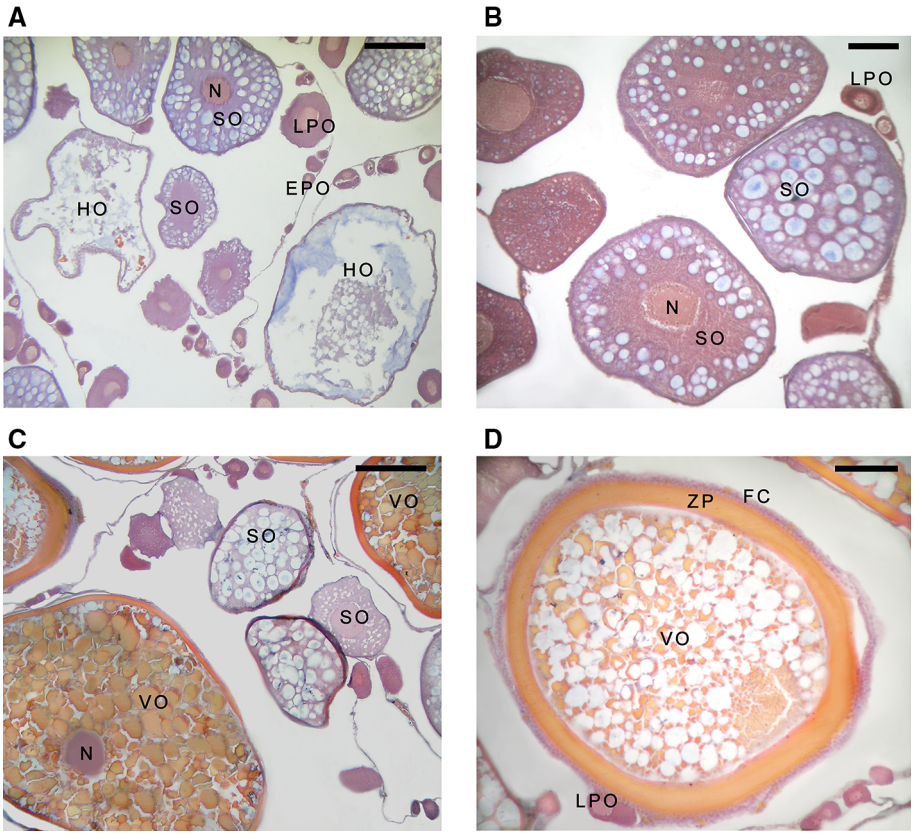

Fig. 2 Histological sections of *N. coriiceps* ovaries sampled during Antarctic summer. **a** General aspect of the ovary of a representative female collected in January where early primary oocytes, late primary oocytes, secondary oocytes, and hydrated oocytes are evident. **b** Detail of secondary oocytes filled with lipid droplets. **c** Cross section of the ovary of a representative female collected in March. Vitellogenic oocytes and secondary oocytes are observed. **d** Magnification of a vitellogenic oocyte with lipid droplets and yolk platelets within the ooplasm. *FC* follicular cells, *HO* hydrated oocytes, *N* nucleus, *EPO* early primary oocyte, *LPO* late primary oocyte, *SO* secondary oocyte, *VO* vitellogenic oocyte, *ZP zona pellucida*. *Scale bars* **a**, **c** (200 μm); **b**, **d** (100 μm). Stain: Masson's trichrome

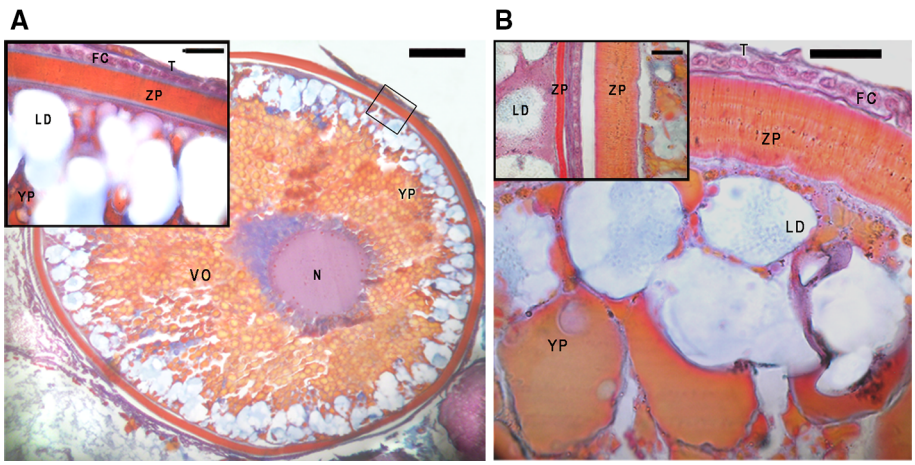

Fig. 3 **a** Vitellogenic oocyte of a female of *N. coriiceps* collected in January. The nucleus had migrated to the animal pole of the oocyte; the ooplasm is filled by yolk platelets and lipid droplets. *Inset*: detail of the thick *zona pellucida*, cuboidal follicular cells and thecal layer surrounding the oocyte. **b** Vitellogenic oocyte of a female collected in March. The ooplasma is filled with lipid droplets and yolk platelets, and the *zona pellucida* is even more developed and exhibits conspicuous radial striations formed by the interdigitations between the oocyte and the follicle cells. *Inset* see the two adjacent vitellogenic oocytes showing the differential development of *zona pellucida* (the space between the follicular layer and the thickest *zona pellucida* is due to an artifact). *FC* follicular cells, *LP* lipid droplet, *N* nucleus, *T* theca, *VO* vitellogenic oocyte, *YP* yolk platelets, *ZP zona pellucida*. *Scale bars* **a** (100 μm); *Inset* (25 μm); **b** (20 μm). *Inset* (20 μm). Stain: Masson's trichrome

band (data not shown). The Arctic char antiserum revealed at least one band in all female samples and 50 % of them showed the pattern obtained with seabream, except for the 84 kDa and the addition of 50 kDa band (Fig. 5b, lane 4). The two smallest females presented no VTG, as seen with the seabream antiserum. VTG was detected in all females

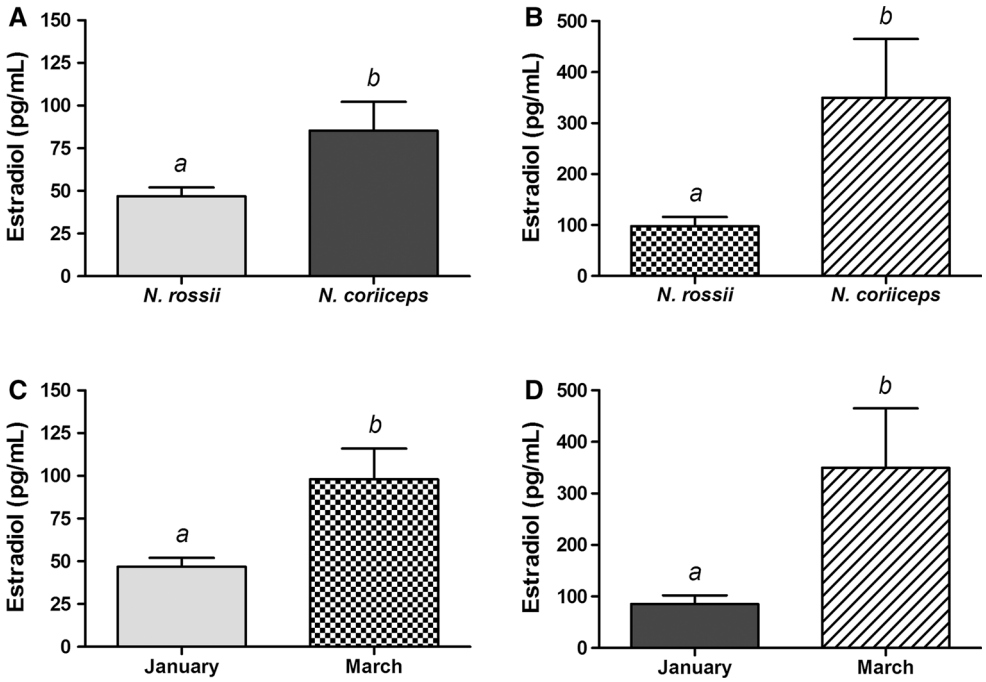

Fig. 4 Mean plasma estradiol levels. **a** Females of *N. rossii* and *N. coriiceps* sampled during January ($p = 0.0274$). **b** Females of *N. rossii* and *N. coriiceps* sampled during March ($p = 0.0005$). **c** Mean plasma levels of *N. rossii* collected in January and March ($p = 0.0252$). **d** Mean plasma levels of *N. coriiceps* collected in January and March ($p = 0.0061$). *Different letters* indicate significant differences at $p < 0.05$

of *N. coriiceps* using the mummichog antiserum and it revealed a pattern with one or more of the aforementioned bands (Fig. 5c, lane 5).

Discussion

In the last decades, the scientific community has demonstrated increasing interest in the Antarctic Peninsula region, where Potter Cove is located, as it is the area that has experienced the most rapidly changing climate and accelerated warming in the Southern Hemisphere (Meredith and King 2005; Clarke et al. 2007; Vaughan 2008; Bromwich et al. 2013; Turner et al. 2013). Considering the effects that these changes could have on the aquatic organism physiology and ecology, both at individual and population levels, the establishment of long-term scientific programs to monitor the biological components of these regions has been proposed (ATCM 2007; Pankhurst and Munday 2011; Raga et al. 2014; Moon et al. 2015). The information provided by our study enhances the knowledge of the reproductive biology of two successful fish species, *N. coriiceps* and *N. rossii*.

To date, some reviews have summarized data on the reproduction of several notothenioids, indicating a prolonged gametogenesis, low fecundity, and large yolky eggs as common characteristics (Everson 1984; White and North 1987; Kock and Kellermann 1991; Duhamel et al. 1993; Christiansen et al. 1998). In the present study, two distinct clutches of oocytes can be easily distinguished and separated by size in the ovaries of mature females of *N. rossii* and *N. coriiceps*: one more advanced batch of vitellogenic or mature oocytes that will be spawned in the current season and a second batch composed of previtellogenic oocytes at several stages of development (i.e., early primary oocytes and late primary oocytes stages) forming the reserve stock for the next spawning season. According to the classification of Wallace and Selman (1981), these are characteristics of group synchronous ovarian development, which is a common feature among Antarctic fishes (Kock and Kellermann 1991).

Histological analysis of females captured in March indicated that both species were at an advanced stage of maturation. Although no statistical differences among the GSI of January and March could be found, there was a clear trend toward increase in GSI of *N. coriiceps*. Therefore, progress in gametogenesis and steroidogenesis has no major impact on the value of GSI, at least in March. The increment in GSI and oocyte development is associated with increase in 17β-estradiol circulating levels (Harmin et al. 1995; Lee and Yang 2002; Berg et al. 2004). Likewise, estradiol levels of *N. coriiceps* and *N. rossii* increased in March.

As it was previously noted by several authors (Mommsen and Walsh 1988; Karlsen et al. 1995; Dahle et al. 2003), body reserves must be used to meet the energy requirements just before spawning so a high lipid and protein consumption in the ovary is expected. A reduction in liver size is usually observed in spite of the fact that hepatocytes enlarge (hypertrophy) due to yolk production

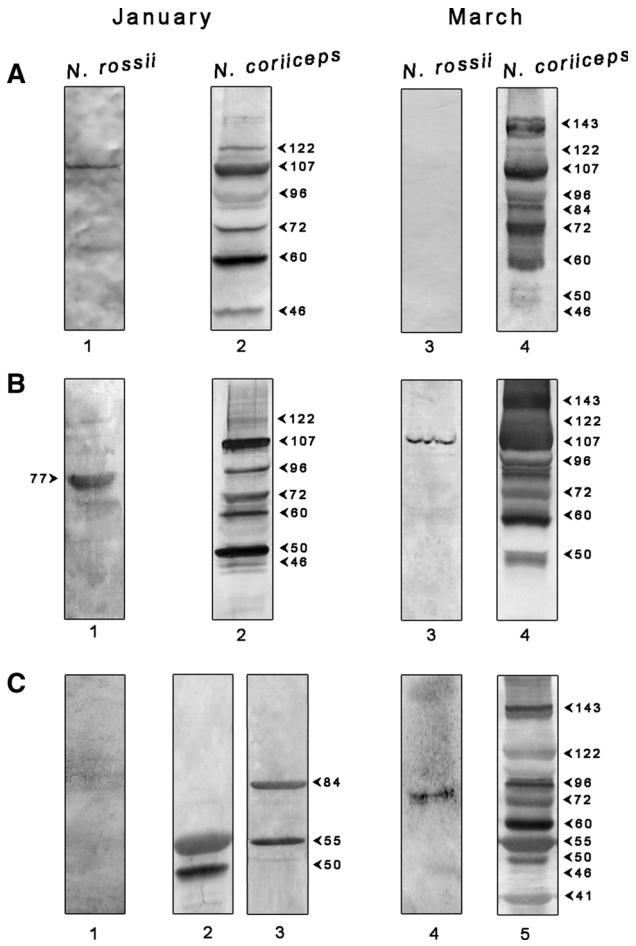

Fig. 5 Western blot analysis of vitellogenin (VTG) from plasma of *N. rossii* and *N. coriiceps*. Different patterns of VTG bands of females collected in January and March. **a** anti-seabream VTG antiserum, **b** anti-Arctic char VTG antiserum, and **c** anti-mummichog VTG antiserum were used for immunodetection. *Numbers* next to each subfigure represent molecular weight in kilodaltons (kDa)

(Chakrabarti and Chatterjee 2014). This could explain the slightly but not significant reduction in HSI that was seen in *N. coriiceps* and *N. rossii* in March.

Histological observations indicated that during the sampling period, adults of *N. coriiceps* females did not have ovaries at "rest" as it was reported by Sapota (1999); on the contrary, they were ready for spawning. However, our results well agree with those of Hureau (1970), who found spawning females in January at Terre Adelie with a GSI of about two, and with Casaux et al. (1990) who suggested that spawning occurs between February and May in Potter Cove. This different timing of gametogenesis for the same species is not surprising since, in the Antarctic environment, several variations have been reported in the reproductive period (Dearborn 1965). For the same species, even slight local variations in environmental conditions can have a determining role in dictating sexual activity, which might be, therefore, the result of adaptation to the specific environmental and ecological conditions of a given site (Everson 1970; Russo et al. 2000).

For females of both species, gonadal development and synthesis of vitellogenin (VTG) correspond with increasing estradiol levels registered in March, knowing that this hormone stimulates the synthesis and release of liver VTG and depends on ovarian maturation (Aida et al. 1973; De Vlaming et al. 1980; Yaron et al. 1980; Sundararaj and Nath 1981; Dahle et al. 2003). Furthermore, Western blot revealed major and more evident bands of VTG when compared with the VTG pattern of females captured in January. Faint bands were observed only in developing females of *N. rossii* with ovaries containing previtellogenic oocytes, whereas *N. coriiceps* females with vitellogenic and/or hydrated oocyte stages showed a pattern of several major bands of VTG. This difference was more pronounced in the immunoblotting with anti-VTG mummichog, with an increase in banding in samples obtained in March. This antiserum would be ideal to be used only if the study is focused on the reproductive cycle of *N. coriiceps*. However, Arctic char antiserum would be the adequate one to be chosen in future research if both species want to be compared. Due to the lack of immunoreactivity of seabream antiserum with *N. rossii* plasma samples of March, we consider that this antiserum would not be suitable in comparative studies. Moreover, for *N. coriiceps*, no major difference in the VTG pattern could be detected after using this latter antiserum.

Based on information gathered from hormonal profile, gonadal histology, GSI, and VTG detection, it is evident that for the same sampled period, both species were at different stages of the reproductive cycle. Androgen levels were similar irrespectively of the species or the sampling period, and near the limit of detection, probably due to the low sensibility of the method or low circulating levels of androgens. *N. coriiceps* females showed a higher GSI and estradiol levels than those of *N. rossii*. Similar results were obtained with VTG immunodetection, since in plasma of *N. coriiceps* several VTG bands of greater intensity could be detected compared to *N. rossii*, both in January and in March. The physiological significance of VTG and the primary derivatives is usually attributed to nutrition of the embryo in the form of yolk (Wahli 1988; Brooks et al. 1997; Le Menn 2002; Romano et al. 2004). However, several studies have suggested that differential expression of the VTG genes and proteolysis occurring during oocyte maturation is particularly important in marine species with spawning pelagic eggs. Free amino acids generated in this process produce an osmotic gradient and promote oocyte hydration, which is essential to increase egg buoyancy (Rønnestad et al. 1999; Patiño and Sullivan 2002;

Matsubara et al. 2003; Finn 2007; Williams et al. 2014). Hydrated oocytes observed in females of *N. coriiceps* indicate that these individuals were in spawning period, since this process occurs just prior to spawn as observed in several species (Hunter and Macewicz 1985; Kjesbu et al. 1990; Brickle et al. 2006; Prut'ko 2012). Accordingly, females whose ovaries had hydrated oocytes showed the greatest VTG immunoreactivity. On the other hand, earlier stages of oocyte maturation were observed in *N. rossii* females. Our results are consistent with those of Duhamel (1982) and Burchett (1983) who described life cycle and indicated that the later fish migrates to deeper coastal waters to spawn between April and May.

To our knowledge, no studies have been conducted to detect plasma vitellogenin in notothenioids. Our results reveal, in the case of *N. coriiceps* females, different patterns for VTG according to the antibody used, with five specific immunoreactive bands (122, 107, 96, 72, and 60 kDa) detected in both months with anti-VTG of seabream and Arctic char. In addition, other bands (143, 55, 50 and 46 kDa) could be immunodetected in various plasmas collected in March. These results are consistent with those of Nilsen et al. (1998) who suggested that the VTG molecule contains several immunologically distinct epitopes that are conserved to varying degrees in different fish species. Furthermore, earlier studies have demonstrated that some antibodies against VTG are able to cross-react with VTG of several fish species within the same order (Tyler et al. 1996) or even with species from phylogenetically distant orders (Heppell et al. 1995). In this study, in order to validate the use of heterologous antisera, we compared the results obtained with three different ones. The repetition of the pattern observed gives specificity to VTG detection, being this the first report of vitellogenin in Antarctic fish.

Our results also indicate that a small withdrawal of blood in large species such as *N. rossii* and *N. coriiceps* can distinguish between females with advanced sexual maturity and developing females, not being necessary to sacrifice the animal to determine its maturity. On the other hand, Western blot technique represents a fast and simple method to detect VTG to perform a regular biomonitoring because this protein has also been used as a sensitive biomarker of estrogenic contamination in aquatic environments (Jobling et al. 1998; Nilsen et al. 1998; Denslow et al. 1999). Future investigations might include vitellogenin detection in skin mucus samples, considering that the procedure is even easier and virtually harmless (Meucci and Arukwe 2005; Genovese et al. 2011).

Finally, ovarian study revealed that both species have group synchronous ovarian development. Our results on the reproduction of *N. rossii* are in good agreement with the existing information on the spawning period of the majority of species and populations in the seasonal Pack-Ice Zone and around the islands north of it, which shows a high proportion of autumn and winter spawning species (Kock and Kellermann 1991; Kock 1992). Nevertheless, *N. coriiceps* exhibits evidence of high vitellogenic activity and ready to spawn females during our sampled period. Estradiol profile and VTG detection in *N. rossii* and *N. coriiceps*, which were performed in this study, are the major contributions of this work to existing knowledge of these fish species. Therefore, we believe our results aid in addressing the timing of spawning for conservation and management of these species, as well as providing reference parameters for future comparisons to enable continuous evaluation of the status of the species involved.

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