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REPRODUCTIVE ANALYSIS OF WARM AFFINITY FISHES IN A BIOGEOGRAPHIC TRANSITION ZONE (GALICIAN WATERS, NORTH-EAST ATLANTIC OCEAN)

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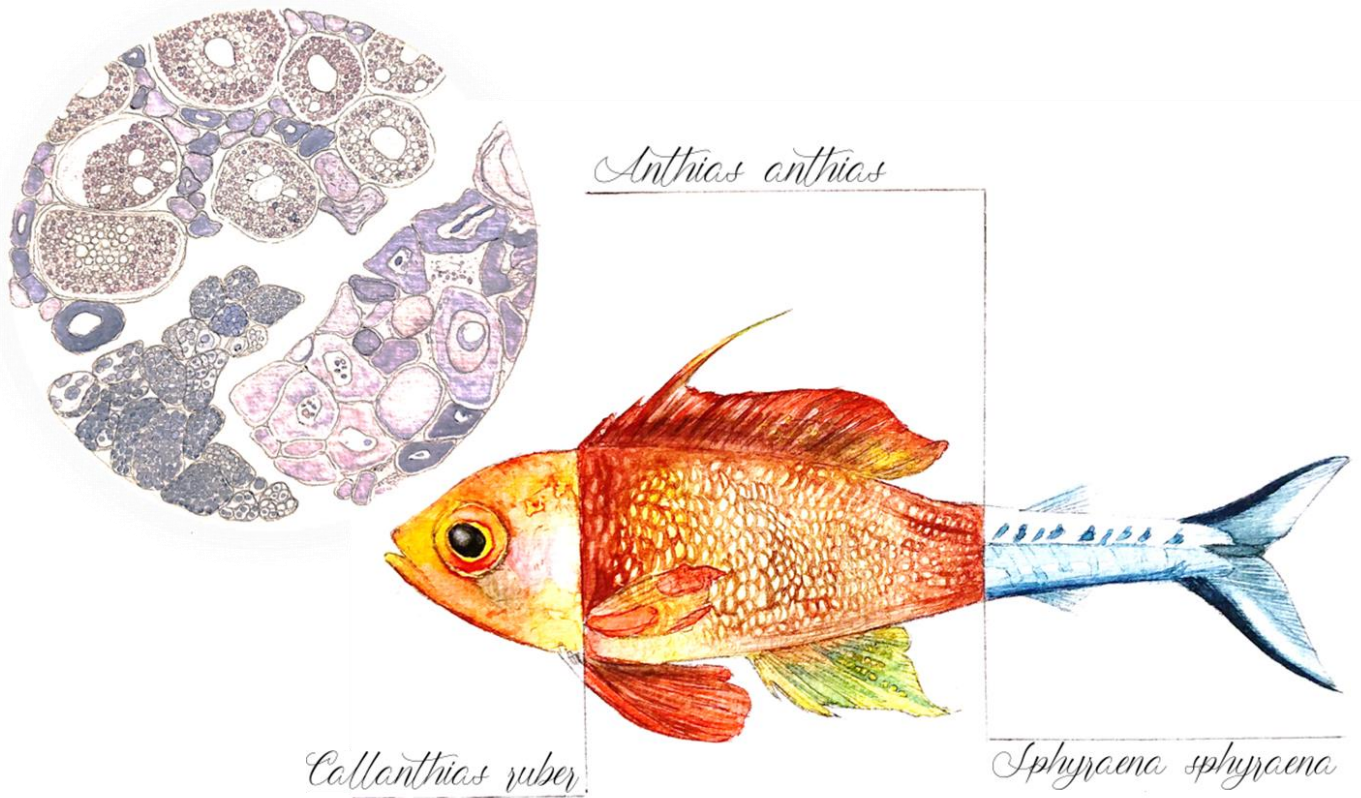
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Table of Contents

Executive summary.....	5
Abstract.....	6
1. Introduction & Aims.....	7
2. Material & Methods	
2.1 Study area.....	10
2.2 Biological sampling.....	11
2.3 Histological processing and examination.....	13
2.4 Oocyte measurements and statistical analysis.....	14
3. Results	
3.1 Biological sampling.....	14
3.2 Reproductive analysis.....	15
4. Discussion.....	23
5. Conclusion & Future perspectives.....	29
6. Acknowledgements.....	30
7. References.....	30
Supplementary material.....	38

EXECUTIVE SUMMARY

Spread of non-native species threatens marine biodiversity worldwide, pushed by global warming. Increasing temperature on marine ecosystems induces the displacement of warm affinity species towards the poles, common in fish, mostly poikilothermic organisms and therefore sensitive to heating. This scenario can trigger changes in species assemblage composition of an area, which could potentially affect its native taxa. Unusual records of *Anthias anthias* (Serranidae), *Callanthias ruber* (Callanthiidae), *Dactylopterus volitans* (Dactylopteridae), *Halobatrachus didactylus* (Batrachoididae), *Seriola rivoliana* (Carangidae), and *Sphyraena sphyraena* (Sphyraenidae) were found in Galician waters (North-eastern Atlantic Ocean), considered biogeographic transition zone and an area with a likely significant impact in terms of colonization due to global warming.

The assessment of fish reproductive biology is essential to understand colonization processes of non-native species and their ability to settle and constitute populations. Through histological techniques, we analysed the potential of the species mentioned above to spawn locally and establish in the area by determining the reproductive developmental phase of the specimens studied. Furthermore, a detailed histological examination of gonadal tissue allowed determining biological traits of reproductive strategies of these species. Here, gonadal histology has been documented for the first time in *A. anthias*, *C. ruber*, and *D. volitans*. For *C. ruber*, *D. volitans*, and *S. sphyraena*, oocyte diameter measurements showed significant differences among oocyte development stages. Also, gonadosomatic index (GSI) was calculated for some individuals. Both, oocyte sizes and GSI values, represent a baseline for further comparative studies in these species, although larger sample sizes are required.

Our results revealed signs of potential spawning capability in male of *A. anthias* and *H. didactylus*, and female of *C. ruber*, which suggests the potential of these species to settle in Galician waters. On the other hand, no evidence of recent/imminent spawning in the area were detected for *D. volitans* or *S. sphyraena*. The assignment of reproductive strategies was carried out when possible, determining that *C. ruber* female was an asynchronous batch spawner. Lobular testis structure with cystic spermatogenesis was described in *C. ruber*, *A. anthias*, as well as in *H. didactylus* males. Other finding to highlight is the identification of

melanomacrophage centres in *A. anthias* and *C. ruber* males, potential histological signal of protogyny. Although further studies and dedicated samplings that confirm the settlement of these unusual species in Galician coasts are required, this study represents the first evidence of potential reproductive activity of some of these warm affinity fishes in the area.

ABSTRACT

Changes in the fish fauna composition of North-eastern Atlantic Ocean have been recorded based on the occurrence of warm affinity species. Individuals of *Anthias anthias* (Serranidae), *Callanthias ruber* (Callanthiidae), *Dactylopterus volitans* (Dactylopteridae), *Halobatrachus didactylus* (Batrachoididae), *Seriola rivoliana* (Carangidae), and *Sphyraena sphyraena* (Sphyraenidae) have been found along Galician waters, considered unusual species in this biogeographic transition zone. To classify them as seasonal migrant visitors or local spawners with settlement potential, gonad histological analyses were performed, showing that at least *A. anthias*, *C. ruber*, and *H. didactylus* were spawning capable when present in the area.

In turn, this work sheds light on the reproductive biology of these species, of which gonadal histological data were limited or non-existent. In fact, this study represents the first gonad histological analyses of *A. anthias*, *C. ruber*, and *D. volitans*. Our results described important traits of the reproductive strategies of the species analysed, having classified a *C. ruber* female as asynchronous batch spawner, and identifying lobular type and cystic spermatogonial testis in *A. anthias*, *C. ruber*, and *H. didactylus* males. Males of *A. anthias* and *C. ruber* presented potential histological evidence of protogyny with the description of melanomacrophage centres in both species.

Keywords: Exotic species · Gonadal histology · Protogyny · Reproductive biology · SW Europe · Unusual fish

1. INTRODUCTION & AIMS

There is an increasing concern over the consequences that climate change and global warming have on the state of marine ecosystems (Brander, 2010; Hoegh-Guldberg *et al.*, 2010). Climate change may potentially affect survival, growth, reproduction, and distribution of the individuals of a species (Jackson *et al.*, 2009), what could have an impact at population and ecosystem levels (Brander, 2007). Fish are mostly poikilothermic species, thus particularly sensitive to warmth. In fact, water temperatures above the optimum or over the tolerance range of the species may alter metabolism, schooling behaviour, patterns of resource use, reproductive success, and generate active movement and changes in migratory patterns (Roessig *et al.*, 2004; Perry *et al.*, 2005). Thus, one of the most obvious effects of ocean warming is a shift in species abundance and distribution ranges, triggering dramatic changes in assemblages and trophic webs (Horta e Costa *et al.*, 2014; Punzón *et al.*, 2016).

The success and geographical expansion of native warm water biota has been called by some authors “meridionalization”, different from “tropicalization”, defined as the process caused by the incoming and spread of exotic species with tropical or subtropical origin (Azzurro, 2010; Punzón *et al.*, 2016). Therefore, in recent years, many studies examine climate-related effects in the composition of marine fish assemblages, and illustrate variable patterns in species distribution shifts in response to fishing pressure and changing temperature (Genner *et al.*, 2010; Lenoir *et al.*, 2011; Kleisner *et al.*, 2016). Increasing temperature on marine ecosystems induces quick poleward shifts in distributions of fish and plankton in regions such as the North-east Atlantic, where temperature change has been rapid (Brander, 2007). In this scenario, it may happen that the response of the community to warming is characterized by a diminution in the abundance of cold-water species rather than an increase of warm-water species (Auber *et al.*, 2017). However, the majority of documented tropicalization and meridionalization events involve the increase of thermophilic fish species, as in the study of Punzón *et al.* (2016), who evidenced spatiotemporal increase of warm water affinity demersal fish species richness in the southern Bay of Biscay (North of Iberian Peninsula), pointing at climate change as the most plausible explanation (Punzón *et al.*, 2016).

Located on the Atlantic coast to the northwest of the Iberian Peninsula, Galician waters could be considered a biogeographic transition zone in a marine temperate system (Castro *et al.*, 1997; Horta e Costa *et al.*, 2014) with a strong upwelling period from March-April to September-October and downwelling for the rest of the year (Álvarez-Salgado *et al.*, 2002). Biogeographic transition zones are areas of intense biotic interaction located on the boundaries between biogeographic regions promoted by ecological changes that allow the mixing of different biotic components (Morrone, 2004). They have been described as vulnerable in a global warming scenario in which cyclical oscillations of oceanographic conditions could be disrupted (Horta e Costa *et al.*, 2014), being able to imminently experience the colonization of new taxa and changes in the assemblages of species.

Coastal warming trend along Atlantic Arc ranges from $3.5\text{ }^{\circ}\text{C century}^{-1}$ at latitudes close to 48°N to $1.2\text{ }^{\circ}\text{C century}^{-1}$ at latitudes close to 37°N , observed to be reflected in sea surface temperature increments which heighten northward in the Atlantic area (Gómez-Gesteira *et al.*, 2008). Concretely, since 1974, there has been a significant increasing trend in land and sea surface temperatures of 0.5 and $0.24\text{ }^{\circ}\text{C decade}^{-1}$ respectively in the Northwestern Iberian Peninsula (Gómez-Gesteira *et al.*, 2011). At the same time, the presence of fish species with tropical affinities in European Atlantic waters has been recorded since the 1960s (Quero *et al.*, 1998), with many examples of unusual fish species along Galician coast the last 20 years (e.g., Bañón *et al.*, 2002; Bañón *et al.*, 2011; Bañón *et al.*, 2017). These facts have been supported by the hypothesis of an expansive movement of the species with a marked latitudinal component from the equator toward the poles, related to a gradual displacement of the thermal gradient in this area (Bañón *et al.*, 2002). Cuesta *et al.* (2016) points out that in Galician waters, a global warming scenario will facilitate the establishment of non-indigenous species both favoring the northward expansion of tropical species (tropicalization process) and the changes in environmental conditions (meridionalization process).

Effective conservation and management of coastal ecosystems requires the routine monitoring of potentially invasive species in coastal waters and the persistent collection of baseline data on species ecology and distribution under a climate change scenario (Cheung *et al.*, 2012; Bañón *et al.*, 2019). Increasing knowledge of biological traits of non-native species would help to understand colonization processes (Keller *et al.*, 2011). The invasive potential

of the species may depend on their biological plasticity and reproductive strategies (Arias *et al.*, 2013), vital for establishing in an area and expanding their range of distribution. Understanding fish reproductive biology is crucial for discovering the species' life history and seasonal reproductive cycle (Ismail *et al.*, 2018). Studies of reproductive traits of fish have mainly focused on fisheries management, as an accurate assessment of reproductive strategies is an essential component to understand reproductive success and promote an effective fisheries management and conservation (Alonso-Fernández *et al.*, 2011; Brown-Peterson *et al.*, 2011). However, information about these aspects still remain unknown for many species (Tomkiewicz *et al.*, 2003). Therefore, the reproductive analysis of unusual fish individuals is a necessary tool to evaluate their potential to reproduce and settle in an area, either due to the establishment of a self-maintained population or due to their presence because of migratory fluxes (Azzurro, 2010), in addition to providing information about their reproductive strategies.

Gonad maturation process in teleost fish is a very sensitive phase and suboptimal conditions of a new environment may have an impact on the final stages of ovarian maturation (Azzurro, 2010). Thus, the study of reproductive biology and, specifically, the reproductive maturation stage of fish colonizers seems to be revealing to project early settled populations. Routinely used for sex identification and assessment of reproductive developmental phase, histology offers a powerful tool for reproductive works (Blazer, 2002). Although time consuming, histology allows the application of robust criteria to study main traits of fish reproductive biology (Alonso-Fernández *et al.*, 2011). Microscopic gonadal staging by histology provides a direct snapshot of developmental stages of individual gametes within the gonads (Zeyl *et al.*, 2014), having been described as the only safe mean for identifying and consequently quantifying the various stages of very small oocytes such as primary growth oocytes and post-ovulatory and atretic follicles (Ganias, 2013). In addition to be helpful in the assessment of reproductive capacity and potential of fish, useful for classifying them as seasonal migrant visitors or local spawners established in an area, gonad histology provides information about the reproductive strategies of the species. A clear example is its particularly crucial utility for verification and classification studies of hermaphroditic fish species (Ismail *et al.*, 2018), since details of internal gonad structure are only visible when

using histological techniques (Sadovy & Shapiro, 1987; Sadovy & Domeier, 2005; De Mitcheson & Liu, 2008).

Based on the above mentioned, the hypothesis of our study is that individuals of non-native teleosts Swallowtail seaperch *Anthias anthias* (Linnaeus, 1758) (Serranidae), Parrot seaperch *Callanthias ruber* (Rafinesque, 1810) (Callanthiidae), Flying gurnard *Dactylopterus volitans* (Linnaeus, 1758) (Dactylopteridae), Lusitanian toadfish *Halobatrachus didactylus* (Bloch & Schneider, 1801) (Batrachoididae), Longfin yellowtail *Seriola rivoliana* Valenciennes, 1833 (Carangidae), and European barracuda *Sphyraena sphyraena* (Linnaeus, 1758) (Sphyraenidae) are potentially reproductively active fish ready to spawn in Galician waters, what makes them not only seasonally or occasionally visitors but species able to settle in this area of the North East Atlantic Ocean.

In order to test this hypothesis, the main objective of this work was to assess histologically the reproductive developmental phase of individuals belonging to six unusual teleost species (*A. anthias*, *C. ruber*, *D. volitans*, *H. didactylus*, *S. rivoliana*, and *S. sphyraena*) in Galician waters (North-east Atlantic Ocean) to identify local spawners with settlement potential in the area. A secondary objective was to contribute with novel information about the reproductive strategies (mainly ovarian and testicle development organization, spawning pattern, and sexual pattern) of these poorly studied species.

2. MATERIAL & METHODS

2.1 Study area

Galicia (Northwest of Iberian Peninsula) (Figure 1) is a territory of Spain with 1,498 km of coastline (Alonso-Fernández *et al.*, 2019) bathed by the Atlantic Ocean to the west and the Cantabrian Sea to the north. It is located between Eo (43°32'N, 7°01'W) and Miño (41°50'N, 9°40'W) rivers. Galicia is the main fishing region for coastal species of Spain and one of the most important in Europe with around 4,000 small fishing boats (López *et al.*, 2003; Alonso-Fernández *et al.*, 2019).

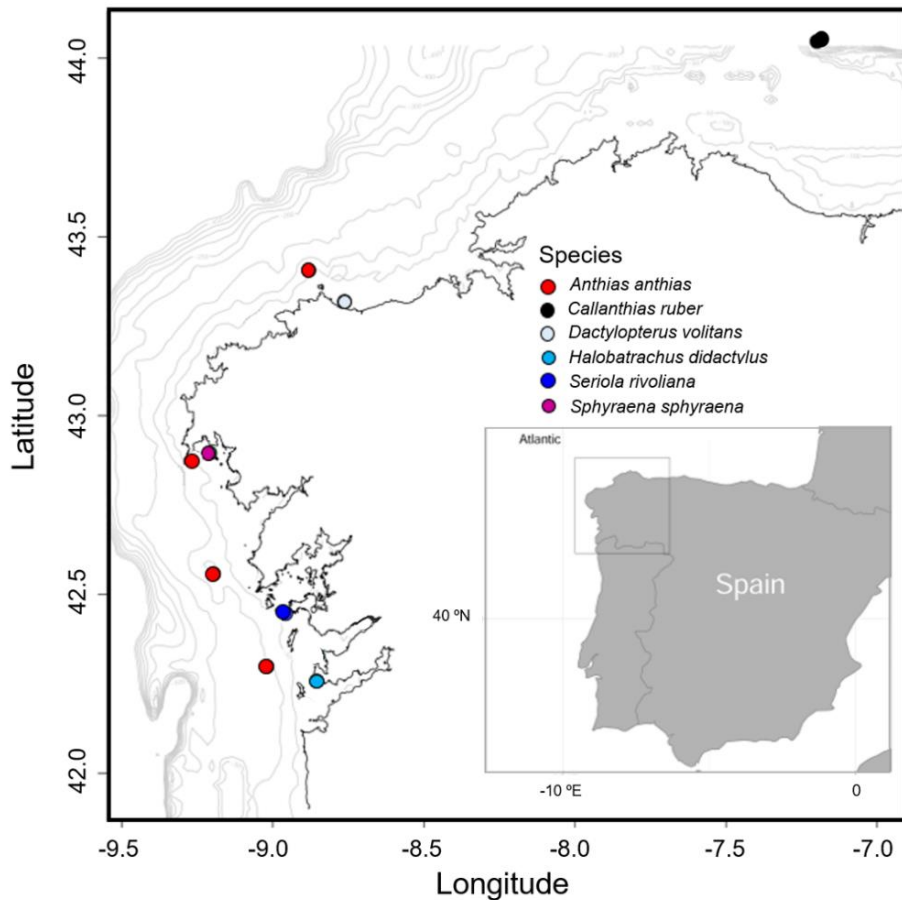


Figure 1. Distribution of the capture locations of the studied specimens in Galician waters (NE Atlantic and NW Iberian Peninsula).

2.2 Biological sampling

Ten specimens belonging to six teleost species (*A. anthias*, *C. ruber*, *D. volitans*, *H. didactylus*, *S. rivoliana*, and *S. sphyraena*) (Figure 2) have been recorded between 2017 and 2019 (Table 1). Fish were found along the Galician coast (Figure 1) by artisanal fishers who provided us with both specimens and data on the dates and locations of the catches. Once captured, the individuals were firstly preserved fresh or frozen until being identified in the laboratory to species level (Bañón *et al.*, Under review). Of these observations, *D. volitans* specimen represented the first record of the species in the study area, while the rest of the species have been considered as rare or scarce (Bañón *et al.*, Under review).

In this study, the following parameters were recorded for each fish: total length (± 1 mm), total weight (± 0.01 g), and gonad weight (± 0.01 g); as well as sex, reproductive developmental phase (and subphase, if it proceeded), and oocyte sizes (± 0.01 μm) (in the case that it was a female). Oocyte size may be used as a predictor of developmental stage if size ranges of the various stages are known (West, 1990). Thus, current oocyte diameter measurements could be considered potentially useful hereafter to assign a reproductive developmental phase to female individuals of these species. When possible, gonadosomatic index (GSI) was estimated as an index of reproductive investment, using gonad weight (GW) (g) and total weight (TW) (g) as follows: $\text{GSI} = 100 \times [\text{GW} / \text{TW}]$.

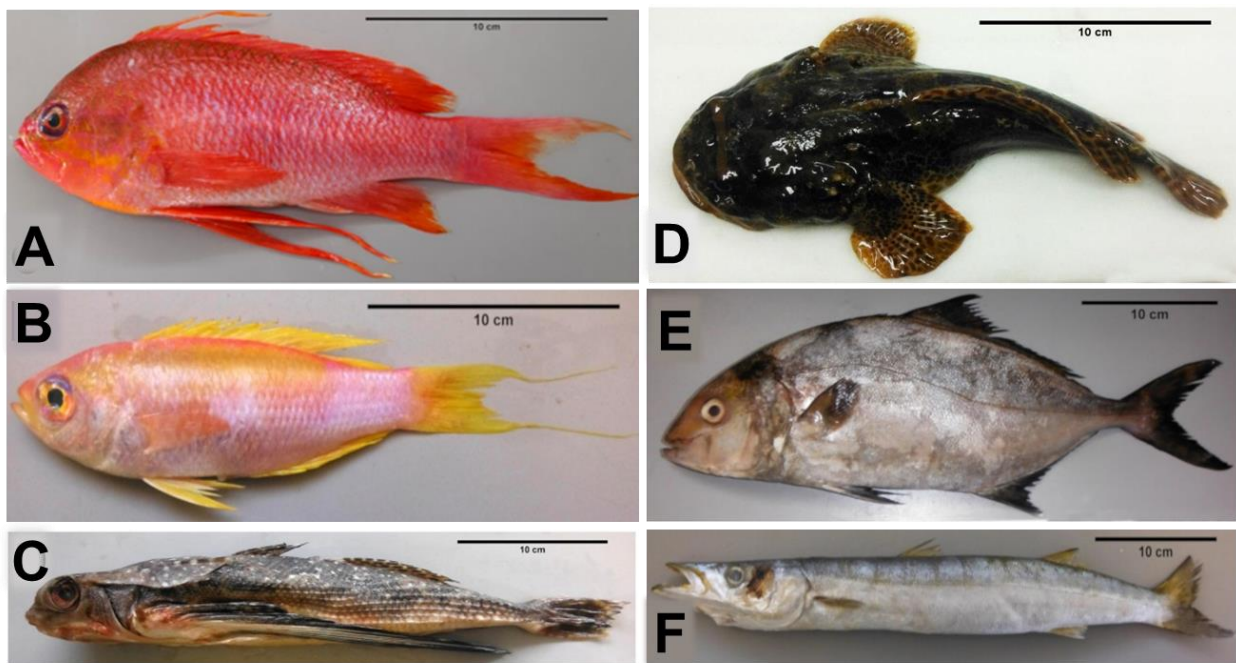


Figure 2. Specimens of the unusual species found in Galician waters (North-east Atlantic Ocean). A) *Anthias anthias*, 280 mm TL; B) *Callanthias ruber*, 245 mm TL; C) *Dactylopterus volitans*, 392 mm TL; D) *Halobatrachus didactylus*, 258 mm TL; E) *Seriola rivoliana*, 415 mm TL; F) *Sphyraena sphyraena*, 465 mm TL.

2.3 Histological processing and examination

We used histological methods applied to reproductive tissue to classify the specimens according to sex, reproductive developmental phase, and reproductive strategies. Gonads were removed and immediately fixed in 4% formaldehyde buffered with $Na_2HPO_4 \times 2H_2O$ (0.046 M) and $NaH_2PO_4 \times H_2O$ (0.029 M). A subsample from the central section of both lobes of the gonad of each individual was extracted, dehydrated, and embedded in paraffin for being sectioned at 3 μ m and stained with haematoxylin-eosin for its examination under microscope.

Histological examination of the reproductive tissue was performed using QWin software (© Leica Imaging Systems) on a PC (AMD Athlon XP 3000+) connected to a video camera (Leica IC A) on a stereo microscope (Leica MZ6). The accurate identification of cellular structures present in the gonads helped us determine sex and reproductive developmental phase for each of the specimens. Follicles of each ovary were classified into stages of development according to histological criteria: primary growth, cortical alveoli, early and advanced vitellogenesis, germinal vesicle migration, and hydration (West, 1990; Tyler & Sumpter, 1996; Brown-Peterson *et al.*, 2011). Atretic oocytes and post-ovulatory follicles (POFs) were also detected in ovarian histological samples. Testicular tissue germ cells were classified into the following stages of development: spermatogonia, spermatocytes, spermatids, and spermatozoa (Grier, 1981). Based on the presence of the cell structures described above, fish females and males of each of the species were classified into one of the following reproductive phases: immature, developing (with early developing subphase, if applicable), spawning capable (with actively spawning subphase to females, if applicable; or early, mid, or late germinal epithelium subphase to males, if applicable), regressing, and regenerating (Brown-Peterson *et al.*, 2011). In some cases, the quality of histological slides did not allow the reproductive phase classification due to freezing prior to the extraction of the gonads (i.e., *A. anthias* 280 mm TL and *S. rivoliana* 451 mm TL).

Histological examination of reproductive tissue was used to describe and identify the main traits of reproductive strategies: ovarian and testicular development organization, spawning pattern, and sexual pattern (Murua & Saborido-Rey, 2003; Brown-Peterson *et al.*, 2011).

2.4 Oocyte measurements and statistical analysis

In order to explore the size range for each oocyte developmental stage identified in all the females found, *C. ruber* (182 mm TL), *D. volitans* (392 mm TL), and *S. sphyraena* (465 mm TL), mean diameter for each oocyte stage ($n = 10$ oocytes per stage) was calculated as the average between the major and minor axes in the histological sections making use of the tools of QWin software (© Leica Imaging Systems). Using R software version 3.5.1. (R Development Core Team, 2018), Shapiro-Wilk test was applied to test the assumption of normality and Levene's test was executed to check the homogeneity of variances (Zar, 1996). If both assumptions were met, one-way analysis of variance (ANOVA) test was performed to explore the differences in diameter among the oocyte stages within each of the species studied. Conversely, when homoscedasticity assumption was violated, data were analysed with Generalized Least Squares (GLS) statistical technique. To deal with heterogeneity, variances were added to the model with GLS, which prevented data transformation (Zuur *et al.*, 2010). Then, post-hoc Tukey's range test was applied to each species to find out which specific oocyte stages means were significantly different from each other in terms of diameter. Statistically significant differences were considered if $P < 0.05$.

3. RESULTS

3.1 Biological sampling

In this study, samples from 2017 to 2019 of fish captured at different times of the year were analysed. *Anthias anthias* was the only species found consecutively in different seasons (Table 1): October, 2017; June, 2018; and April, 2019. Specimens of *C. ruber* and *D. volitans* were fished in January, 2019, and *S. sphyraena* individual during January, 2018. *Halobatrachus didactylus* specimen was found in December, 2018, and *S. rivoliana* during June, 2017. As for the GSI values, they were calculated in some of the individuals. The three female specimens (*C. ruber*, *D. volitans*, and *S. sphyraena*) presented values around 2, with *C. ruber* being the highest (GSI = 2.672). On the other hand, GSI values of *C. ruber* and *H. didactylus* male individuals were much lower, of 0.125 and 0.221 respectively (Table 1).

Table 1. Summary table of biological sampling of the unusual fish individuals studied captured in Galicia waters. Capture date of the specimen and its preservation method (Sample status) are indicated. Sex, reproductive developmental phase and (subphase) are listed. Total length (TL, +1 mm), total weight (TW, +0.01 g), gonad weight (GW, +0.01 g), and gonadosomatic index (GSI) are present.

Species	Capture date	Sample status	Sex	Reproductive phase	TL (mm)	TW (g)	GW (g)	GSI
<i>A. anthias</i>	09/10/2017	Frozen	Male	Developing	267	NA	NA	NA
<i>A. anthias</i>	10/10/2017	Frozen	Male	Developing	290	NA	NA	NA
<i>A. anthias</i>	12/06/2018	Frozen	Male	Spawning capable (Late germinal epithelium)	258	NA	NA	NA
<i>A. anthias</i>	28/04/2019	Fresh	Male	NA	280	174.6	NA	NA
<i>C. ruber</i>	16/01/2019	Frozen	Female	Spawning capable (Actively spawning)	182	65.5	1.75	2.672
<i>C. ruber</i>	16/01/2019	Frozen	Male	Developing	295	80.23	0.10	0.125
<i>D. volitans</i>	04/01/2019	Frozen	Female	Developing (Early developing)	392	538	12.72	2.364
<i>H. didactylus</i>	24/12/2018	Frozen	Male	Spawning capable	258	330	0.73	0.221
<i>S. rivoliana</i>	13/06/2017	Frozen	NA	NA	451	1068	NA	NA
<i>S. sphyraena</i>	16/01/2018	Frozen	Female	Developing	465	376	8.03	2.136

3.2 Reproductive analysis

Reproductive activity

Histological examination of the reproductive tissue of the fish individuals sampled revealed that *A. anthias* male (258 mm TL, 12/06/2018), *C. ruber* female (182 mm TL, 16/01/2019), and *H. didactylus* male (258 mm TL, 24/12/2018) were spawning capable at the moment of the catch.

Anthias anthias (258 mm TL) showed cysts throughout the testicular tissue presenting all stages of an active spermatogenesis (spermatogonia, spermatocytes, spermatids, and spermatozoa, this last being the fastest to be identified). Abundant sperm was observed in the lumen of lobules. *Halobatrachus didactylus* specimen also had a large number of

spermatozoa in the lumen of lobules of its testicular tissue, and all the spermatogenic stages were clearly observed in cysts. In *C. ruber* ovarian tissue, we found clear signs of past and recent or imminent spawning activity (actively spawning subphase), like POFs and germinal vesicle migratory and hydrated follicles.

The rest of the specimens analysed did not show any evidence of recent/imminent reproductive activity (Table 1).

Reproductive traits

Anthias anthias

All the specimens histologically analysed of *A. anthias* were males. The individuals of 267 mm and 290 mm TL presented spermatocytes, spermatids, and some spermatozoa but lacked sperm in lumen of lobules. Thus, they were classified as males in developing phase, although in an advanced state within this phase because few clusters of spermatozoa were observed (Figure 3 A, B). We identified a potential spawning capable male (258 mm TL) (Figure 3 C, D), since it showed abundant sperm in lumen of discontinuous lobules, therefore, probably at late germinal epithelium subphase. Prominent pink pale to yellowish teardrop shape (Figure 3 D) and ovoid structures (Figure 4 A) were identified randomly distributed among the germinal cells and gonadal stroma of both 258 mm TL spawning capable and 290 mm TL developing males respectively, identified as melanomacrophage centres.

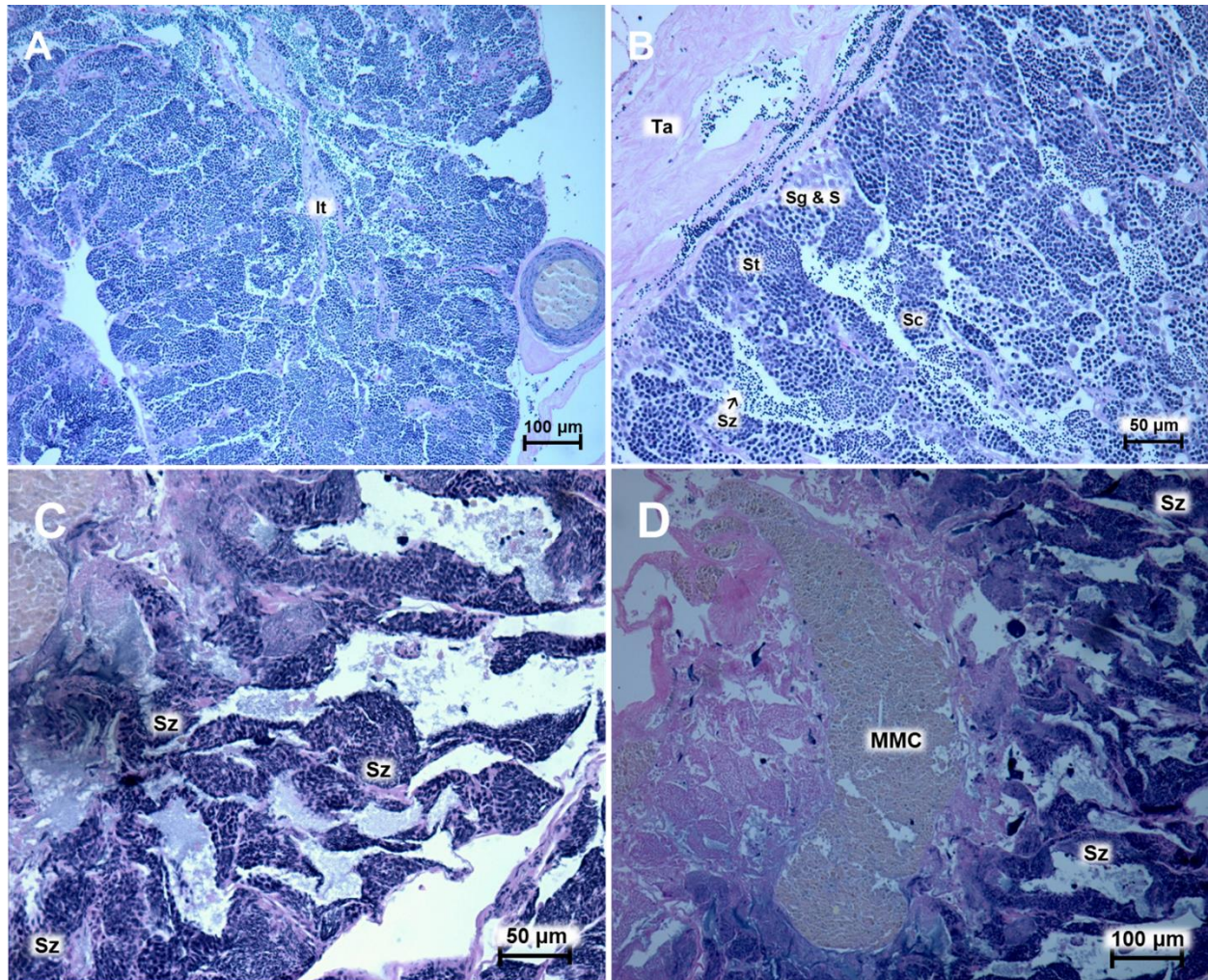


Figure 3. Photomicrographs of *Anthias anthias* testicular tissue. A, B) Testicular tissue of a developing male (290 mm TL); C, D) Testicular tissue of a spawning capable (late germinal epithelium subphase) male (258 mm TL). Interstitial tissue (It), tunica albuginea (Ta), spermatogonia and Sertoli cells (Sg & S), spermatocytes (Sc), spermatids (St), spermatozoa (Sz), and melanomacrophage centres (MMC) were identified.

The histological structure of the testis of *A. anthias* seemed to be lobular, with spermatogonial development along the germinal epithelium of hollow lobules in reproductively active fish. Spermatogenesis is cystic, since developing germ cells are enclosed within germinal cysts formed by enveloping Sertoli-cell processes; a synchronous maturation of germ cells within each cyst was determined.

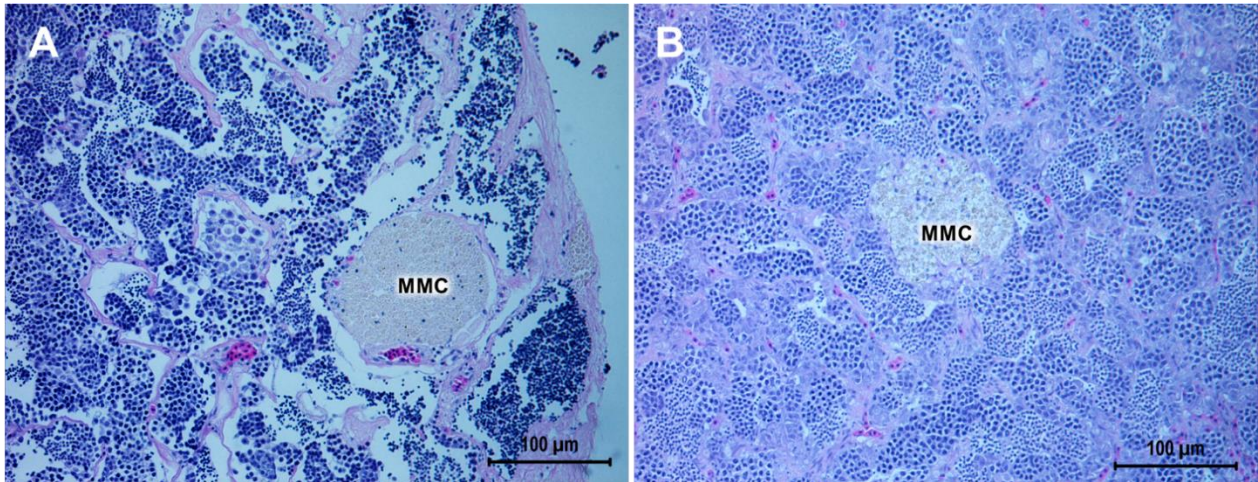


Figure 4. Melanomacrophage centers (MMC) in fish testicular tissue. A) Gonad of *A. anthias* developing male (290 mm TL); B) Gonad of *C. ruber* developing male (295 mm TL).

Callanthias ruber

We identified a *C. ruber* female (182 mm TL) and a male (295 mm TL) individuals. The female presented simultaneously all oocyte stages: primary growth (PG), cortical alveoli (CA), early vitellogenesis (EVT), advanced vitellogenesis (AVT), germinal vesicle migration (GVM), and hydrated follicles (H). This means that *C. ruber* exhibited an asynchronous development of secondary growth follicles, i.e., secondary growth follicles of all stages of development were present without a dominant population (Figure 5 A, B).

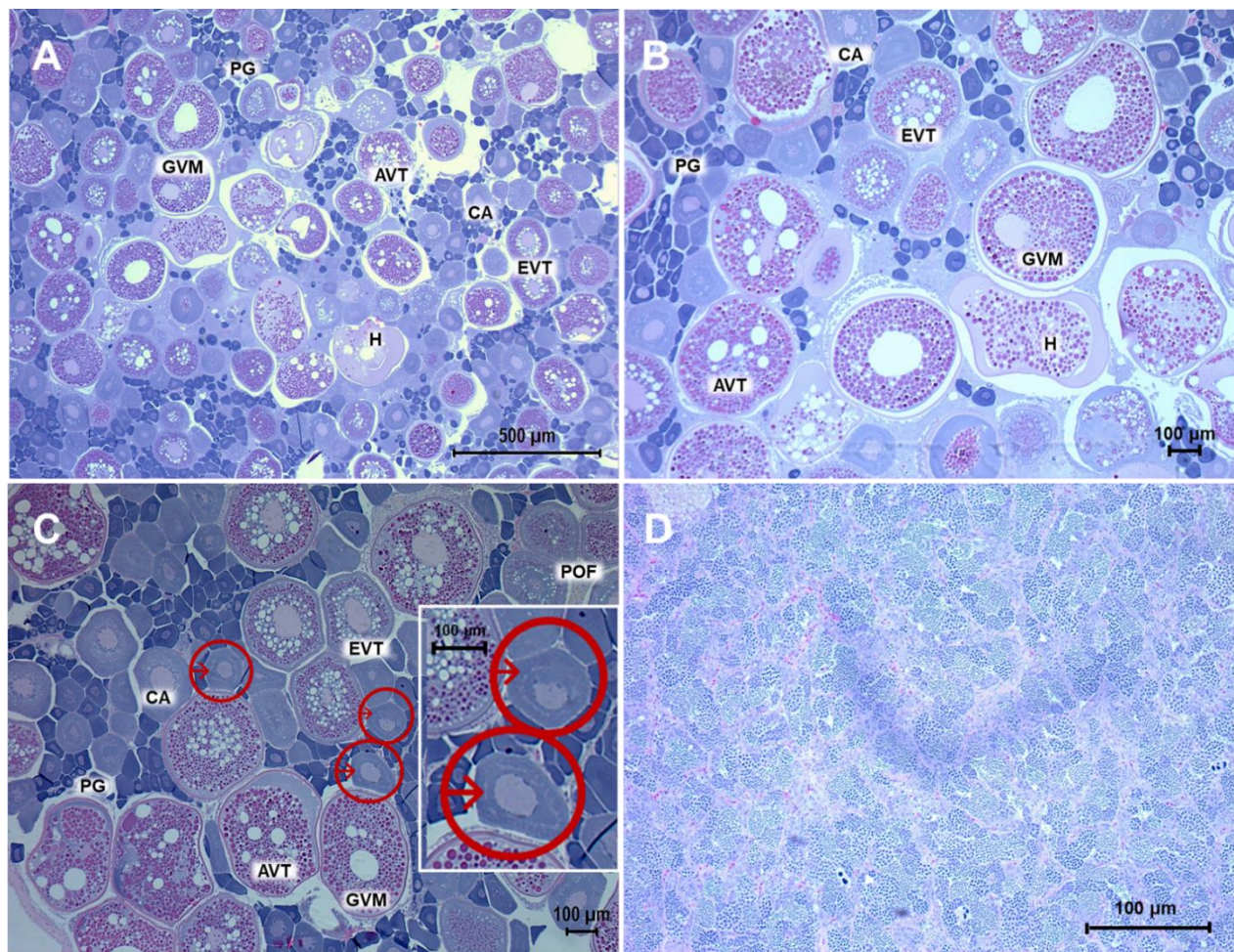


Figure 5. Photomicrographs of gonad histological sections of two *Callanthias ruber* specimens. A, B, C) Ovary of a spawning capable (active spawner) female. All the following oocyte stages can be differentiated in the photography: primary growth (PG), cortical alveoli (CA), early vitellogenesis (EVT), advanced vitellogenesis (AVT), germinal vesicle migration (GVM), hydration (H), and post-ovulatory follicles (POF). Circumnuclear rings in previtellogenic oocytes are highlighted in red; D) Appearance of testicular tissue of a developing male.

Measurements of oocyte size according to oocyte developmental stage in *C. ruber* ovarian tissue were taken and compared (Figure 6), showing significant differences in diameter among all the oocyte stages analysed ($P < 0.05$). PG oocyte diameter was $57.50 \pm 12 \mu\text{m}$, far apart from CA ($186 \pm 30.30 \mu\text{m}$), and reaching $382 \pm 35.90 \mu\text{m}$ in AVT stage (Table S1). In the cytoplasm of previtellogenic oocytes, circumnuclear rings were identified (Figure 5 C). We also found post-ovulatory follicles (POFs) (Figure 5 C), indication of past reproductive activity.

Due to the observation of the tissue traits here described, we classified this female in spawning capable phase (actively spawning subphase).

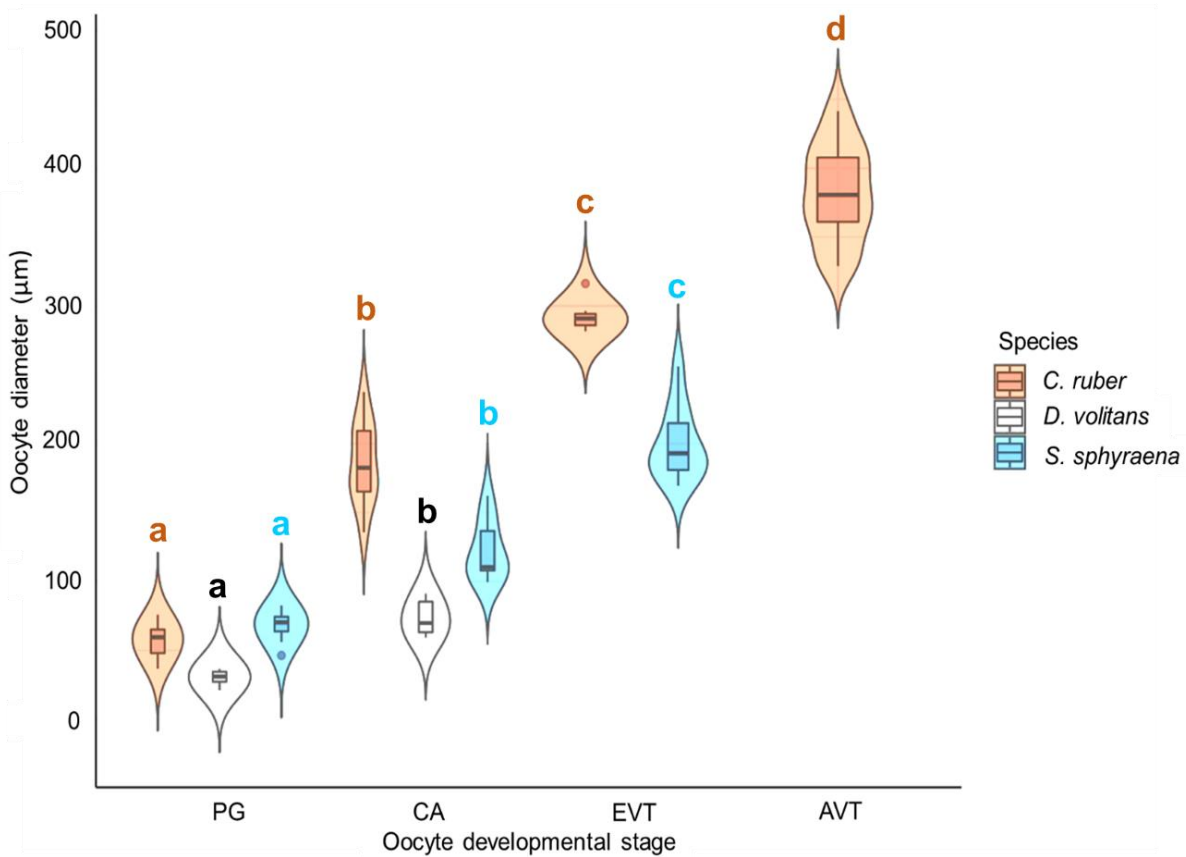


Figure 6. Violin plot of the oocyte mean diameter according to their stage of development in *Callanthias ruber* (n = 1), *Dactylopterus volitans* (n = 1), and *Sphyræna sphyræna* (n = 1) female specimens. Primary growth oocytes (PG) (n = 10), cortical alveoli oocytes (CA) (n = 10), early vitellogenic oocytes (EVT) (n = 10), and advanced vitellogenic oocytes (AVT) (n = 10) were measured when it was possible for each individual. Statistically significant differences ($P < 0.05$) in diameter among oocyte stages per each of the species are indicated by different letters after being examined by one-way ANOVA or GLS, and post-hoc Tukey's range test. The shape of the distribution indicates data concentration around the median. Inside each density curve there is a small box plot, with the rectangle showing the ends of the first and third quartiles; the median is represented as a central line; extreme values appear as small dots out of the small boxes.

The specimen of 295 mm TL was a male (Figure 5 D) with presence of spermatogonia, spermatocytes, and spermatids, but lacking on spermatozoa in lumen of a lobular testis type. Due to the set of the cellular structures mentioned, we classified this individual as a typical developing male. Spermatogenesis seemed to be cystic in this species, since developing germ cells are enclosed within germinal cysts formed by enveloping Sertoli cells. We observed pink pale to yellowish ovoid structures among the germinal cells and gonadal stroma of the developing male, recognized as melanomacrophage centres (Figure 4 B).

Dactylopterus volitans

In female specimen of *D. volitans* (392 mm TL) we could identify primary growth and cortical alveoli oocytes (Figure 7 A). Both oocyte stages showed significant differences in diameter measurements ($P < 0.05$) (Figure 6), and CA did not exceed 91 μm in diameter (Table S1). Old remainder atretic follicles were present (Figure 7 A, B). Therefore, and although it was difficult to confirm it due to the apparent poor stage of preservation of the sample, this female was likely at developing phase (potentially at early developing subphase due to lack of vitellogenesis), without any evidence of recent reproductive activity.

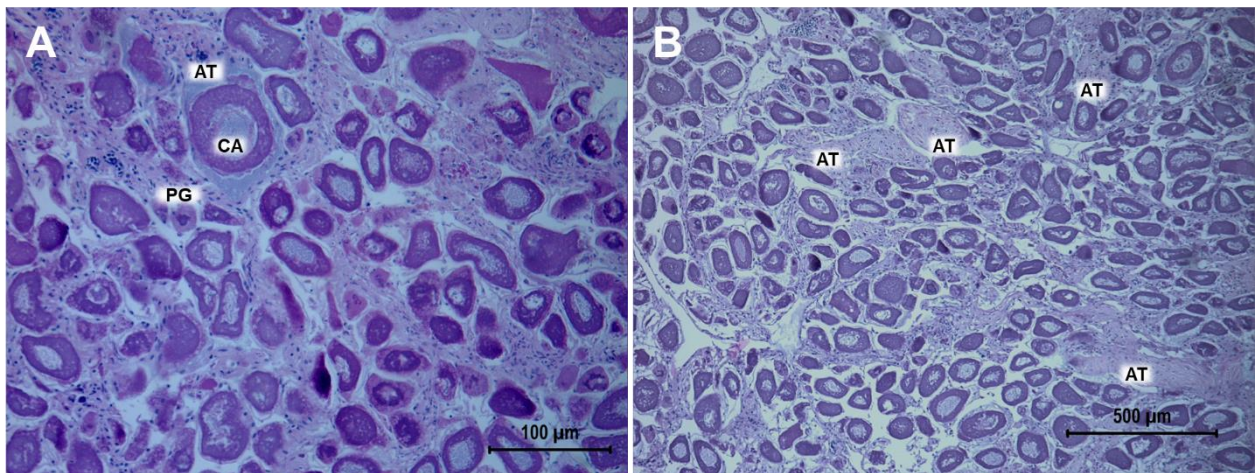


Figure 7. Photomicrographs of the oocyte stages present in developing (early developing subphase) *Dactylopterus volitans*. A) Primary growth oocytes (PG), cortical alveoli oocytes (CA), and atretic oocytes (AT) were identified; B) Old atretic oocytes (AT) can be appreciated.

Halobatrachus didactylus

Halobatrachus didactylus specimen was a male of 258 mm TL. All the spermatogenic stages were identified in cysts (spermatogonia, spermatocytes, spermatids, and spermatozoa). Many spermatozoa were also observed into the lumen of lobules (Figure 8 A). Thus, this specimen was classified as spawning capable. Spermatogonia were distributed along the lengths of the lobules (Figure 8 B). Lobules located at periphery contained abundant cysts with germinal cells, while the lobules near the efferent ducts contained less cysts with those cells.

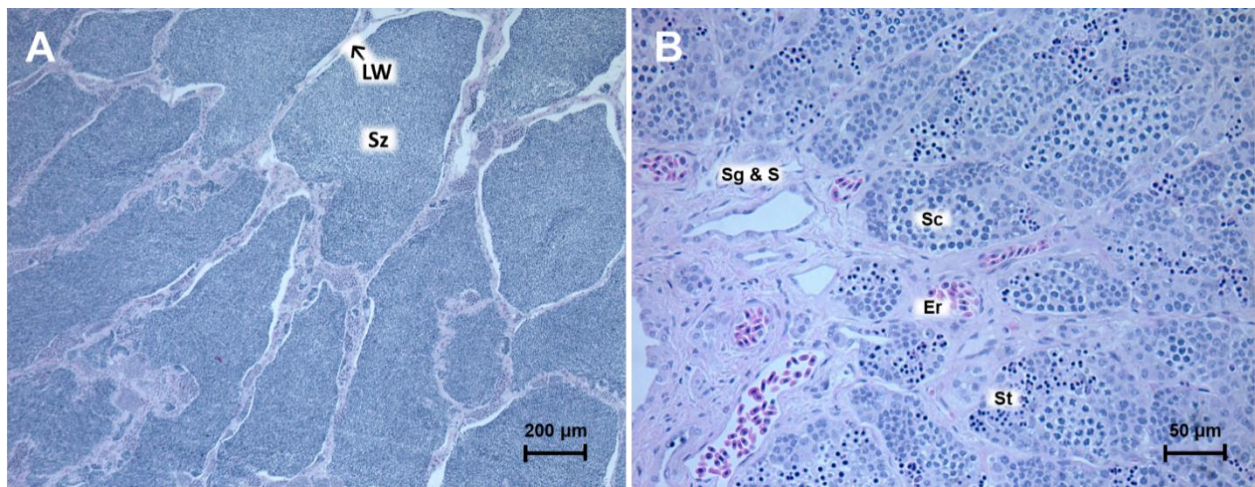


Figure 8. Photomicrographs of testicular tissue of a *Halobatrachus didactylus* spawning capable male. A) Lobules full of spermatozoa (Sz). Lobule wall (LW) is indicated; B) Cell identification near the periphery of the testis. Spermatogonia and Sertoli cells (Sg & S), spermatocytes (Sc), spermatids (St), and erythrocytes (Er) were observed.

Sphyaena sphyaena

Sphyaena sphyaena individual was identified as a female of 465 mm TL. It mostly presented primary growth and cortical alveoli oocytes (Figure 9 A, B), as well as few and dispersed early vitellogenic oocytes. Diameter measurements of PG, CA and EVT oocytes were carried out (Figure 6), being significantly different ($P < 0.05$) among them. EVT oocytes, the most developed oocyte stage, reached $199 \pm 26.50 \mu\text{m}$ (Table S1). Signals of atresia were also present in the gonadal tissue (Figure 9 A, B). Thus, this individual was classified as a developing female initiating the annual sexual cycle.

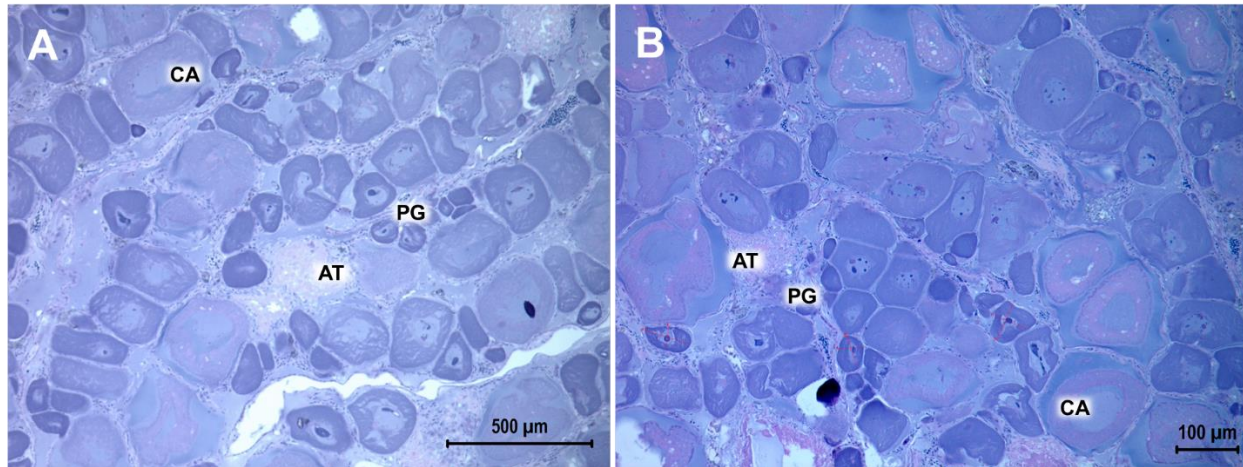


Figure 9. Photomicrographs of the developing gonad of *Sphyaena sphyaena* female. A, B) Primary growth oocytes (PG) and cortical alveoli oocytes (CA) can be identified in the images. Atresia (AT) was also observed.

3. DISCUSSION

The six teleost species presented in this work are considered non-native in the study area (Bañón *et al.*, Under review). Therefore, studying their potential settlement to form stable populations is of interest due to the impacts that this can have on the ecosystem, essential for sustainable management of its marine resources (Montero-Serra *et al.*, 2015). Reproductive aspects of fish are important cues that can provide information regarding the settlement and constitution of new populations (Bañón *et al.*, 2019), since colonizers that reach the maturation and become active spawners are key in such a process (Azzurro, 2010). However, recruits and juveniles, usually the main drivers for changing distribution limits of marine species, may shift more rapidly in response to warmer conditions than older, settled adults (Horta e Costa *et al.*, 2014). Thus, although our focus is on mature, reproductively active fish, the presence of young individuals should not be played down.

In Eastern Atlantic Ocean, the range of distribution of *A. anthias* had been defined close to the Azores southward to northern Namibia, including off-lying islands and the Mediterranean Sea (Smith-Vaniz & Iwamoto, 2015). According to this distribution, our study described for the first-time reproductive features of the species at histological level in the

northernmost records to date (Bañón *et al.*, Under review). The distribution of *C. ruber* ranged from the English Channel (occasionally) to Mauritania, including certain offshore islands (i.e., Azores, Madeira, and Canaries) (Anderson, 2016). Also, the first microscopic description of the ovary and testis for this species appears to be collected in this thesis. *Dactylopterus volitans* has been found in the Eastern Atlantic from Irish waters (Quigley *et al.*, 2004) to Angola, as well as in the Mediterranean (Smith-Vaniz, 2016). However, we processed the ovary of the first record of the species in Galician waters (Bañón *et al.*, Under review), and as far as we know, it is the first documented reproductive histological analysis in this species. Regarding *H. didactylus*, it has been described in Eastern Atlantic, in Norway (vagrant specimen) and from the northwest of Spain to the south to Ghana and, perhaps, Nigeria, as well as in the Western Mediterranean (Bañón *et al.*, 2019). The distribution of *S. sphyraena* in the eastern side of the Atlantic Ocean ranges from the Bay of Biscay to Angola and the Mediterranean Sea (Bañón & Garazo, 2006).

From the *A. anthias* analysed, only the male specimen of 258 mm TL captured in June, 2018 seemed to be a potential spawning capable fish (late germinal epithelium subphase) while the two specimens captured in October, 2017 (267 mm TL and 290 mm TL) were at the beginning of their sexual cycle, under developing phase. Micarelli & Barlettani (2005) place the breeding season between June and September of *A. anthias* caught in the Mediterranean and transferred to aquarium under natural conditions, which is consistent with our results, since the reproductively active individual was the only one captured within this season. *Anthias anthias* has been the only species from which we had individuals extracted in different years (from 2017 to 2019) and seasons (April, June and October). However, as no females were recorded to date, its settlement cannot be verified based on local spawning activity. Nevertheless, we present for the first-time evidence of potential reproductive activity in *A. anthias* in the possible extension of the distribution range towards the north of the Iberian Peninsula for this species.

Cystic spermatogenesis in a lobular testicular tissue (i.e., randomly distributed spermatogonia along the entire length of the lobule in reproductive active fish (Takashima & Hibiya, 1995)) was described for this species, which is equivalent to unrestricted spermatogonial type, typical of most teleosts (Grier, 1981). We identified melanomacrophage centres (MMC) randomly distributed along the gonadal tissue in developing (290 mm TL) and

spawning capable (258 mm TL) specimens of *A. anthias*. The basic function of MMC is to remove by phagocytosis foreign particles or products from cell degradation, occurring only in higher teleosts (Ravaglia & Maggese, 1995). MMC are frequent after sex transition in hermaphroditic fish species as a result of female tissue degeneration through phagocytosis during sex reversal period (Ravaglia & Maggese, 1995; Lo Nostro & Guerrero, 1996; Costa *et al.*, 2016). It is suggested that MMC are the result of late regressive stages of follicular atresia (Blazer, 2002), while Sadovy & Shapiro (1987) point also to other processes as capable of producing these structures, such as parasitic encystation, sperm degeneration, or non-specific tissue degeneration. Certain investigations already described serranid *A. anthias* as protogynous (Micarelli & Barlettani, 2005; Erisman & Hastings, 2011; Pla-Quirante, 2019) in a family in which protogyny has been considered as the ancestral sexual system (Erisman & Hastings, 2011). In Serranidae, 19 out of 326 species analysed are protogynous, and 54.4% of the species follow the sexual pattern of hermaphroditism (Pla-Quirante, 2019). Thus, the presence of MMC could point to the evidence of vestiges of ovarian tissue, being for the first time described and supported with histology for this species in this thesis.

Female specimen of *C. ruber* (182 mm TL) was classified into spawning capable phase, actively spawning subphase. Histological indicators of spawning differ in their duration. These differences determine how they should be interpreted. Ovulation and recent POFs can be extremely short-lived histological indicators and they have been used as the most conservative way to assess spawning time and location. For example, in Spotted seatrout *Cynoscion nebulosus* (Cuvier, 1830), ovulation can last from six to 14 hours (Brown-Peterson, 2003). However, it has to be minded that both ovulation induction (Stagey *et al.*, 1979) and recent POFs presence greatly depend on water temperature, as POFs can remain identifiable for months in species with cold water affinities or being reabsorbed in several days in warmwater species (Lowerre-Barbieri *et al.*, 2011). Therefore, oocytes undergoing germinal vesicle migration or hydration indicate imminent spawning, and the presence of neoteric POFs reflects very recent spawning (Lowerre-Barbieri *et al.*, 2009). Consequently, the female individual of warm water affinity *C. ruber* could be considered a local spawner, since the presence of germinal vesicle migration and hydrated follicles (Brown-Peterson *et al.*, 2011), as well as POFs (Pizzicori *et al.*, 2000; Brown-Peterson *et al.*, 2011) indicates imminent/recent spawning activity close to the capture location.

Instead, *C. ruber* male (295 mm TL) seemed to be at developing stage, supported by a low GSI value of 0.125. According to Anderson *et al.* (2015), *C. ruber* is reproductively active in December and January in the Mediterranean, period in which the individuals of our study were captured (both in January, 2019). Therefore, it would be interesting to study a potential settlement of this species in Galician waters due to these indications of full reproductive faculties by the female analysed, confirming our hypothesis.

Callanthias ruber female showed evidence of asynchronous oocyte development, what is associated with a batch spawning type, producing successive batches of oocytes several times during the spawning season (Brown-Peterson *et al.*, 2011). Frequently, this is associated with an indeterminate fecundity type (Murua & Saborido-Rey, 2003), although we cannot conclude it. Oocytes of *C. ruber* female presented circumnuclear rings at previtellogenic phases. Circumnuclear rings, a concentration of RNA and multiple cytoplasmic organelles in previtellogenic oocytes (Serrat *et al.*, 2019), have been reported in other teleosts such as Atlantic herring *Clupea harengus* Linnaeus, 1758 (McPherson & Kjesbu, 2012) or European hake *Merluccius merluccius* (Linnaeus, 1758) (Serrat *et al.*, 2019) as potential early marker of sexual maturation, since they appear before the onset of oocyte development (i.e., cortical alveoli) in many invertebrate and vertebrate organisms. They have been linked to the beginning of the oocyte development, operating as a centre for the transport of cell biosynthesis apparatus before yolk generation (McPherson & Kjesbu, 2012).

Lobular type was identified in *C. ruber* male testicular tissue. As in *A. anthias*, we also observed MMC in the gonadal tissue of *C. ruber* male. As explained above, it could be evidence of sex transition, indicating that the individual was a mature female able to spawn that ended up becoming a functional male prior to its capture. Anderson *et al.* (2015) documented the presence of residual oocytes throughout the testes of the related species *Callanthias legras* Smith, 1948, suggesting that it may be a protogynous hermaphrodite. Furthermore, our finding supports previous works that frame *C. ruber* as protogynous (Tortonese, 1972; Pla-Quirante, 2019), following the only reproductive strategy described in species of the Callanthiidae family so far (Pla-Quirante, 2019).

We found a female of *D. volitans* (392 mm TL) caught in January, 2019 which was in developing phase, probably, in early developing subphase, as females with CA oocytes as the

most advanced oocyte type are considered to be in this stage (Brown-Peterson *et al.*, 2011). In spite of the fact that atresia is mostly frequent during postspawning period, it can be observed in all stages of the reproductive cycle (Miranda *et al.*, 1999), including developing phase. Key (2015) placed the spawning season of the species in the Mediterranean Sea in June and July, months after the capture of the specimen analysed, which is in accordance with a period of oocyte development. Thus, there is no current evidence of reproductive activity for *D. volitans* in Galician waters.

Halobatrachus didactylus specimen (258 mm TL), caught in December, 2018, was identified as a spawning capable male mainly due to the observation of spermatozoa in lumen of lobules. Our individual presented a GSI of 0.221, similar to the one presented in Modesto & Canário (2003) for December in Ria Formosa (south of Portugal) in the case of type I males, below 0.3. Morphotypes I and II have been determined according to the energy invested in growth to the detriment of reproduction, apparently higher in type I males, presenting differences in the relative size of testis, testicular accessory glands and swim bladder, as well as androgen levels among them (Modesto & Canário, 2003). It was described that this index began to increase in January, peaking in May with a GSI close to 1.5 in morphotype I males and close to 10 in type II males (Modesto & Canário, 2003). First sexual maturation size (L_{50} , size at which half of the population reaches maturity) of *H. didactylus* is close to 302 mm TL in Cádiz Bay (south coast of Spain and Atlantic-Mediterranean transition) for males (Palazón-Fernández *et al.*, 2001), which is slightly higher than our fish sampled. However, the smallest size previously recorded for a mature male was 160 mm TL (Palazón-Fernández *et al.*, 2001), below 258 mm TL of our specimen.

This study may evidence the spawning capability of *H. didactylus* in Galician waters. However, no females were analysed, so its settlement cannot be confirmed based on local spawning activity. This means that we cannot conclude that a population of this species is currently reproducing in Galician coasts only with a spawning capable male individual. Nevertheless, Bañón *et al.* (2019) previously documented the presence of a *H. didactylus* male individual in Galician waters which also seemed to be in spawning capable condition in July, 2018 (five months before the capture of the specimen from our study). The explanation why both individuals appeared to be spawning capable in very distant months may be because spawning periods for *H. didactylus* males are not as marked as those for females (Palazón-

Fernández *et al.*, 2001). Despite the fact that this species shows difficulties in the dispersal and colonization of new territories due to its ecological characteristics (i.e., low fecundity, presence of benthic eggs and larvae, parental care of the offspring, and marked sedentary lifestyle of the adults) (Bañón *et al.*, 2019), further research focused on its potential establishment and population formation is required, since there are consecutive indications of males with the ability to reproduce in the area.

Histological analysis of the testicular tissue in *H. didactylus* also allowed to reveal that it presents lobular structure (unrestricted spermatogonial type (Grier, 1981)) and cystic spermatogenesis, both described in Modesto & Canário (2003) for this species.

We found a mature female of *S. sphyraena* (465 mm TL) caught in January, 2018 in developing phase. Chemmam-Abdelkader *et al.* (2007) place first sexual maturation size in 261.3 mm for *S. sphyraena* females in Tunisian coasts, shorter than the female in our work. Value of GSI (2.136) was low because this specimen seemed to be at the onset of gonad development within the reproductive cycle. Allam *et al.* (2004) quantified an even lower female GSI in January, below 0.5, and of 9.95-10.02 during May-June, when spawning season takes places in Egyptian Mediterranean waters off Alexandria (Allam *et al.*, 2004), in Tunisian coast and, generally, in the Mediterranean and the Atlantic (Chemmam-Abdelkader *et al.*, 2007). Females of the related species *Sphyraena jello* Cuvier, 1829 have a similar GSI (2.02) in January, also prior the spawning season, recorded from August till late September for this species (Hosseini *et al.*, 2009). Therefore, as we were unable to verify reproductive activity at the time of the capture, we cannot sustain that *S. sphyraena* is able to reproduce in the study area.

Regarding oocyte sizes registered in *S. sphyraena* specimen, cortical alveoli oocytes ranged from about 100 μm to 162 μm , in line with Villegas-Hernández *et al.* (2014), which describes diameters from 100 μm to 250 μm for this stage. Early vitellogenic oocytes of *S. sphyraena* were between 169 and 256 μm , smaller than the 300-400 μm described as immature by Allam *et al.* (2004) but similar to 200-350 μm reported by Villegas-Hernández *et al.* (2014) for this early vitellogenesis stage, both studies placed in the Mediterranean Sea.

5. CONCLUSION & FUTURE PERSPECTIVES

In this thesis, we identified individuals of non-native fishes with signs of local spawning capability/activity in Galician waters (i.e., *A. anthias* and *H. didactylus* males, and *C. ruber* female), confirming our hypothesis for these species, what could lead to potential settlement processes in the area. Furthermore, this work presents the first histological analysis of reproductive tissue for some species (i.e., *A. anthias*, *C. ruber*, and *D. volitans*), establishing a former reference and baseline of reproductive data for these teleosts. We could determine that *C. ruber* female appears to be an asynchronous batch spawner, and that *C. ruber*, *A. anthias*, and *H. didactylus* males have lobular testis structure with cystic spermatogenesis. As we have seen in *A. anthias* and *C. ruber*, the presence of melanomacrophage centres in the testicular tissue of individuals of both species may be an indicator of protogyny, what, as far as we know, has not been previously confirmed by histological procedures for any of the taxa. Nevertheless, more research effort is required.

It is important to comment that the fact that we have not found spawning capable individuals of *D. volitans* or *S. sphyraena* does not mean that these species could not be establishing in the area, since the samples of this thesis were obtained from discards and accidental fishing. Thus, sample size per species was too low (only one specimen for most of the species analysed) and should be increased in coming studies to come up with stronger conclusions. We suggest that proper sampling of gonadal tissue for reproductive analyses should be a common practice when dealing with records of exotic species. All this research effort could be linked to the future study of the ecological effects that these species could have on the colonized ecosystem, as tropicalization and meridionalization of coastal fish assemblages could involve ecological and socio-economic impacts in the near future in temperate and transition zones.

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SUPPLEMENTARY MATERIAL

Table S1. Mean diameter of oocytes per developmental stage (n = 10) ± standard deviation (SD) in micrometres (µm) of *Callanthias ruber*, *Dactylopterus volitans*, and *Sphyraena sphyraena*. Total length (TL) in millimetres (mm) of the fish and minimum (MIN) and maximum (MAX) oocyte diameter (µm) per stage and individual are also included. Diameter figures of primary growth oocytes (PG), cortical alveoli oocytes (CA), early vitellogenic oocytes (EVT), and advanced vitellogenic oocytes (AVT) are indicated when they were present in each case.

Species	TL (mm)	Stage	MIN (µm)	MAX (µm)	Mean (µm)	SD (µm)
<i>C. ruber</i>	182	PG	36.80	75.90	57.50	12
<i>C. ruber</i>	182	CA	136	238	186	30.30
<i>C. ruber</i>	182	EVT	282	316	292	9.96
<i>C. ruber</i>	182	AVT	329	441	382	35.90
<i>D. volitans</i>	392	PG	21.10	36.50	30.60	4.86
<i>D. volitans</i>	392	CA	59.30	91	73.40	12.40
<i>S. sphyraena</i>	465	PG	46.30	82.60	67.80	10.60
<i>S. sphyraena</i>	465	CA	99.70	162	122	20.90
<i>S. sphyraena</i>	465	EVT	169	256	199	26.50