

Insights on the seasonal variations of reproductive features in the Eastern Atlantic Bluefin Tuna

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ABSTRACT

The Atlantic Bluefin Tuna (ABFT, *Thunnus thynnus*) is one of the most intensely exploited fisheries resources in the world. In spite of the years of studies on ABFT, basic aspects of its reproductive biology remain uncertain. To gain insight regarding the seasonal changes of the reproductive characteristics of the eastern stock of ABFT, blood and tissue samples were collected from mature specimens caught in the Mediterranean basin during the reproductive (May-June) and non-reproductive season (Oct-Nov). Histological analysis of the gonads of May-June samples indicated that there were females which were actively spawning (contained post-ovulatory follicles) and females that were not actively spawning that had previtellogenic and fully vitellogenic oocytes. In males, testis were at early or late stage of spermatogenesis during the reproductive season. In Oct-Nov, ovaries contained mostly previtellogenic oocytes as well as β and α atretic follicles while the testis predominantly contained spermatogonia and few cysts with spermatocytes and spermatozoa. Gonadosomatic index (GSI) in females was highest among the actively spawning individuals while in males GSI was higher in early and late spermatogenic individuals compared to those that were spent. Plasma sex steroids levels varied with the reproductive season. In females, estradiol (E_2), was higher in May-June while testosterone (T) and progesterone (P) did not vary. In males, E_2 and T were higher in May-June while P levels were similar at the two sampling points. Circulating follicle stimulating hormone (FSH) was higher in Oct-Nov than in May-June both in males and females. Vitellogenin (VTG) was detected in plasma from both males and females during the reproductive season with levels in females significantly higher than in males. VTG was undetected in Oct-Nov samples. Since choriogenesis is an important event during follicle growth, the expression of three genes involved in vitelline envelope formation and hardening was measured and results showed significantly higher levels in ovaries in fish caught in May-June with respect to those sampled in Oct-Nov. In addition, a set of genes encoding for ion channels that are responsible for oocyte hydration and buoyancy, as well as sperm viability, were characterized at the two time points, and these were found to be more highly expressed in females during the reproductive season. Finally, the expression level of three mRNAs encoding for different lipid-binding proteins was analyzed with significantly higher levels detected in males, suggesting sex-specific expression. Our findings provide additional information on the reproductive biology of ABFT, particularly on biomarkers for the assessment of the state of maturation of the gonad, highlighting gender-specific signals and seasonal differences.

1. Introduction

The Atlantic Bluefin Tuna (ABFT; *Thunnus thynnus*) is one of the most economically important fish species in the Mediterranean Sea. ABFT catches are regulated by the International Commission for the

Conservation of Atlantic Tuna (ICCAT), whose main goal is to ensure the sustainability of tuna fishery. Despite many studies (Berkovich et al., 2013; Corriero et al., 2009, 2007; Rosenfeld et al., 2012), knowledge regarding the reproductive biology of tunas is still limited and many aspects dealing with the life cycle and the reproduction of

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this species, particularly in the wild, are not fully understood. Efforts in this regard are hampered by the highly migratory behavior of ABFT, with known large-scale transoceanic movement for feeding and reproduction (Carruthers et al., 2018). In the Mediterranean Sea, size-dependent movements and spawning in different areas and periods have been documented (De Metrio et al., 2005; Heinisch et al., 2008; Karakulak et al., 2004; Rooker et al., 2007). Hence, for scientific investigations, sources of samples can be either from the wild, which can only be opportunistically collected from commercial catch, or from farms, which is limited during harvests.

Previous studies have described the histological characteristics of gonadal development in ABFT. The ABFT ovary is characterized by an asynchronous oocyte development, in which oocytes at different stages can be simultaneously found in reproductively active ovaries (Abascal and Medina, 2005). Ovaries are classified following four stages: resting (R), active non-spawning (ANS), active spawning (AS) and inactive mature (IM) (Abascal et al., 2004; Corriero et al., 2003; Medina et al., 2016; Schaefer, 1998). Males have an unrestricted spermatogonial testicular type, where spermatogonia occur along the greater part of the testicular tubules (Abascal et al., 2004). Testis maturity is classified as early spermatogenesis stage (ES), late spermatogenesis stage (LS), and spent (S) (Abascal et al., 2004; Corriero et al., 2007; Medina et al., 2016). Gonadal development of ABFT along their migratory pathways has been tracked by many authors (Addis et al., 2016; Fromentin and Powers, 2005; Fromentin and Lopuszanski, 2014) and stereological estimation indicated that the ovaries of spawning bluefin tuna from the Balearic Islands contained five-fold more highly yolked oocytes than bluefin tuna from Barbate (Medina et al., 2002). Other studies have described the expression of genes in ovaries that are involved in vitellin egg envelop (VE) formation, lipid accumulation and hydration (Gardner et al., 2012), as well as genes in the testis that have similar function as ion channel regulators, transporters or modifiers (Keresztes et al., 2003). In the present study, we investigated these genes as they are relevant to gamete quality.

As in other vertebrates, gonadal function in fish necessitates the full activation of the hypothalamus-pituitary-gonadal (HPG) axis (Berkovich et al., 2013). In teleosts, the GnRH neurons directly innervate gonadotropin-producing cells (gonadotrophs) in the pituitary, stimulating production and release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (Weltzien et al., 2004). The two gonadotropins, FSH and LH, stimulate growth and development of the gonads through gonadal biosynthesis of steroid hormones, mainly 17 β -estradiol (E₂), 11-ketotestosterone (11-KT) and 17 α ,20 β -dihydroxy-4-pregnene-3-one (17 α ,20 β -P) (Miura et al., 1991; Nagahama and Yamashita, 2008; Wallace, 1985) and growth factors (Levavi-Sivan et al., 2010; Lubzens et al., 2010). In female oviparous species, E₂ directly stimulates transcription in the liver of the vitellogenin (VTG) gene (Hara et al., 2016). VTG is then transported via the bloodstream to the follicular layer and then endocytosed by oocytes and used as the precursor of the yolk proteins (Sullivan and Yilmaz, 2018). Plasma VTG concentration is directly correlated with E₂ and its precursor testosterone (T), both of which rapidly increase during vitellogenesis and decline after spawning (Pousis et al., 2011; Rosenfeld et al., 2012; Susca et al., 2001b,a). Therefore, in the present study, circulating levels of FSH, gonadal sex steroids and VTG were determined at the two sampling points. Although FSH is generally known to regulate the early stages of gonadal development particularly in salmonids (Yaron et al., 2003), there are observations in other teleosts showing consistent plasma FSH levels throughout the breeding and non-breeding seasons (Molés et al., 2012; Nyuji et al., 2016). In males, it has been previously shown that both FSH and LH are equally potent in stimulating the production of androgens (Levavi-Sivan et al., 2010). As ABFT has been previously characterized by an asynchronous oocyte development, in which oocytes at different stages can be simultaneously found in reproductively active ovaries (Abascal and Medina, 2005; Berkovich et al., 2013), we aimed to confirm the relevance of circulating FSH

levels during the spawning and non-spawning season. Previous studies in ABFT have reported pituitary FSH levels only (Berkovich et al., 2013; Heinisch et al., 2014).

As part of the national monitoring program founded by the Italian Ministry of Agricultural, Food and Forestry Policies (MIPAAF), and considering the worldwide importance of ABFT both from scientific and commercial perspective, the present study was conducted to gain further insights regarding the reproductive biology of ABFT. To achieve this aim, ABFT were collected at two different seasons of the reproductive cycle, i.e., at the breeding or spawning season (May-June) and after spawning or non-breeding season (Oct-Nov). At sampling, morphometric data were collected as well as plasma and gonad tissues. Gonad samples were subjected to histological analysis and analyzed according to histological data that are already available. Altogether, our results add to the understanding of the reproductive biology of ABFT, providing further basis for its management and conservation and development of its aquaculture as a valid alternative to the overfishing of this species.

2. Material and methods

2.1. Sampling

Thirty-five (10 male and 25 female) ABFT specimens were collected during the reproductive season (May-June 2017) in Sardinia, Italy, by tuna trap, sacrificed and different tissues were sampled. From tuna trap, some specimens were then transferred to Malta in a tuna farming company, Fish & Fish Co. Ltd, Malta. Here, thirty-three (14 male and 19 female) tunas were sacrificed and blood and tissues sampled during the non-reproductive season in Oct-Nov 2017. The procedures did not include animal experimentation; therefore, ethics approval was not necessary in accordance with the Italian legislation. At sampling, for each specimen, different tissue samples, including gonads, were sampled and stored in RNALater solution (Qiagen, Hilden, Germany) for molecular analysis, and in Bouin's solution for histology. Blood was collected with heparinized needles and syringes and plasma was obtained after centrifugation at 900 \times g for 15 min. Plasma samples were lyophilized and then stored at -20 °C for immunosorbent assays. Total body and gonad weights were recorded to calculate gonadosomatic index (GSI): GSI (%) = (gonad weight * 100)/gutted weight.

2.2. Gonad histology

Gonads fixed in Bouin's solution overnight at 4 °C were washed three times and then stored in 70% ethanol at 4 °C until processing. Several pieces from the same sample were dehydrated through a graded series of ethanol and embedded in paraffin. Consecutive sections were cut at 6–7 μ m of thickness for ovary and 4 μ m for testis using Leica RM2125 RTS microtome and were stained with Mayer's hematoxylin and eosin following Randazzo et al. (2018) and Vargas et al. (2018). The sections were examined by Zeiss Axioscop light microscopy connected to a camera (Canon EOS 6D). The gonadal classification followed the literature so far available on ABFT (Abascal et al., 2016, 2004; Abascal and Medina, 2005; Corriero et al., 2007; Medina et al., 2016, 2002; Sarasquete et al., 2002; Susca et al., 2001a, 2001b; Heinisch et al., 2008).

2.3. RNA extraction and cDNA synthesis

Total RNA was extracted from small pieces (100 mg) of ovary and testis using RNazol solution (Sigma-Aldrich, Milan, Italy) according to the manufacturer's instructions and then eluted with RNase-free water as described in Candemla et al., (2018).

Table 1
Primer list.

| Gene | For Sequence 5'–3' | Rev Sequence 5'–3' | TM | Amplification Efficiency (%) | Source |
|--|-----------------------|-----------------------|----|------------------------------|----------------------|
| Zona pellucida component 1 (<i>zpc1</i>) | CATACCACCCCTTCACCCATC | GCTCCACACTAGCCCATGAT | 57 | 98 | Gardner et al., 2012 |
| Vitelin envelope gamma (<i>Ve γ</i>) | GCTTGCATGTGTCAGGCTTA | GGAGAATGGCTTGACTGCTC | 57 | 91 | Gardner et al., 2012 |
| Alveolin (<i>alveolin</i>) | GCTTCTGCTGCTCTTCGTCT | AACAACACCTGAGGCAGGAC | 55 | 87 | Gardner et al., 2012 |
| Chorogonin L (<i>Chg L</i>) | GAGCAGTCAAGCCATTCTCC | CGTCAATCTCAGTGGCTGAA | 57 | 94 | Gardner et al., 2012 |
| Aquaporin 1 (<i>aquaporin</i>) | CCTGTTTCGCAGTCTTGAT | GGTCGGGTAGGAATCATT | 57 | 89 | Gardner et al., 2012 |
| Transmembrane Channel Like 6 related protein (<i>tmc6</i>) | CCGGTTTCTCCTCACCAATA | TTGTGCGTGACATTCCTGAT | 57 | 87 | Gardner et al., 2012 |
| Fatty acid binding protein (<i>fabp</i>) | ACTGCAATGACCGAAAGACC | CCTCCTTTCCGTAGGTCTCT | 57 | 95 | Gardner et al., 2012 |
| Brain-fatty acid binding protein (<i>bfabp</i>) | CCTACACCTGATGACCGACA | GCTGGGATGATTGCTCATT | 55 | 92 | Gardner et al., 2012 |
| Intestinal-fatty acid binding protein (<i>ifabp</i>) | CGCAGCGAGAATTATGACAA | AGCATGTCACCCCTCCATCTC | 55 | 87 | Gardner et al., 2012 |
| Actin beta (<i>βact</i>) | TATCCTGACCCCTGAAGTA | CATTGTAGAAGGTGTGATG | 60 | 81.3 | Api et al., 2018 |
| Elongation factor 1 alpha (<i>ef1a</i>) | ATGGTCGTCACCTTTGCTCC | CCACGTATCCACGACGGATT | 60 | 81.2 | Api et al., 2018 |

2.4. Real-time PCR

Real-time PCRs were performed for target genes with SYBR green method on CFX connect Real time PCR (Bio-Rad) in triplicates as previously described (Carnevali et al., 2019a, 2019b; Maradonna et al., 2015, 2014). The thermal profile for all reactions was 3 min at 95 °C followed by 45 cycles of 20 s at 95 °C, 20 s at 55–60 (see Table 1) and 20 s at 72 °C. Fluorescence was monitored at the end of each cycle. Dissociation curve analysis showed a single peak in all cases. β -actin (*actb*) and elongation factor1a (*ef1a*) were used as reference genes to standardize the results by eliminating variation in mRNA and cDNA quantity and quality. Data were analyzed using Bio-Rad's iQ5 optical system software, version 2.0. Alterations in gene expression were reported with respect to the control sample. Primer sequences are reported in Table 1.

2.5. Determination of plasma FSH

Levels of plasma FSH were determined using a specific competitive ELISA developed for yellowtail kingfish, *Seriola lalandi* (Nocillado et al., 2019). The standard was a recombinant full-length yellowtail kingfish FSH and the antiserum was raised in rabbits against the recombinant β subunit of the hormone. The assay sensitivity was 78 pg/ml. Intra-assay coefficient of variation was 2.2% and inter-assay coefficient of variation was 10.2%. Parallelism was confirmed between the standard curve and the displacement curve of serially diluted ABFT plasma.

2.6. VTG ELISA

Plasma levels of VTG in ABFT were quantified using a sandwich ELISA developed for grouper VTG (Palma et al., 2019) following methods previously described (Takemura et al., 1999). The standard was purified VTG from honeycomb grouper, *Epinephelus merra*, and the antiserum was raised in rabbits (Takemura et al., 1991). Initially, parallelism between the standard curve and displacement curve of serially diluted female ABFT plasma was established. The intra- and inter-assay coefficients of variation were 8.7% and 13.4%, respectively. Assay sensitivity was 5 ng/ml. Plasma samples were diluted 1000-fold with BSA-PBS prior to the assay.

2.7. Steroid assay

Plasma of animals, stored at –20 °C, was thawed and the steroids extracted twice with dichloromethane and the organic phase evaporated. For each sex steroid analysis, 500 μ l plasma/fish was used for extraction. The extracts was re-dissolved in the appropriate buffer for ELISA and used for the assay.

Progesterone (P) levels were measured with a progesterone ELISA Kit (Enzo Life Sciences, Switzerland) using a standard curve in the range of 15.64–1000 pg/ml, according to the kit instructions. The assay sensitivity was 4.8 pg/ml. Inter- and intra-assay coefficients of variation

for the method were 6.5% and 4.9%, respectively. Estradiol (E_2) was measured with a 17 β -estradiol ELISA Kit (Enzo Life Sciences, Switzerland) using a standard curve in the range of 15.64–1000 pg/ml, according to the kit instructions. The assay sensitivity was 5.20 pg/ml and the inter- and intra-assay coefficients of variation were 6.3% and 5.8%, respectively. Testosterone (T) was measured with a Testosterone ELISA Kit (Enzo Life Sciences, Switzerland) using a standard curve in the range of 7.81–2000 pg/ml, according to the kit instructions. The assay sensitivity was 6.2 pg/ml, and the inter- and intra-assay coefficients of variation were 9.7 and 5.8%, respectively. To validate all steroid assays, parallelism between the standard curve and serial dilution of the extracted solution were performed.

2.8. Statistical analysis

Data were presented as mean \pm SD. For sex steroids analysis (E_2 , T and P), significant differences were determined by Student's *t* test comparing mean values between male or female only at the two sampling timepoints (May-June vs Oct-Nov). GSI, FSH, VTG and Real Time PCR data were analyzed by Two-Way ANOVA followed by the Tukey test as a multiple comparisons test to compare experimental groups (male and female during reproductive and non-reproductive season). All statistical analyses were performed using the statistical software package Prism5 (GraphPad Software, Inc. USA) with significance accepted at $P < 0.05$.

3. Results

3.1. Gonadal histology

In female fish caught in May-June, two different ovarian developmental stages can be identified: active non-spawning (ANS) (Fig. 1A) and active spawning (AS) (Fig. 1B). ANS is characterized by the presence of oocytes at advanced stage of vitellogenesis together with previtellogenic (PV) and early vitellogenic (EV) oocytes. In AS stage, early (EV) and late vitellogenic (LV) oocytes, post ovulatory follicles (POF) and few α -atretic follicles were found. In males caught in May-June, testis sections showed gonads either at early spermatogenesis predominantly containing spermatocytes (Sc) and spermatids (Sd) with few cysts releasing spermatozoa (Sz) and small clusters of spermatozoa in lobules can be observed (Fig. 2A), or late spermatogenesis with the testis exhibiting predominantly spermatozoa (Sz) (Fig. 2B). In Oct-Nov, majority of ovaries were at inactive stage, mainly containing previtellogenic oocytes and α -atretic follicles and β -atretic follicles (Fig. 3A). During this time, testis were spent. Spermatogonia (Sg) were predominant in the germinal epithelium (Fig. 3B). Some residual spermatozoa in the lumen and few spermatocytes were detected, but their number is very low if compared to that of spermatogonia.

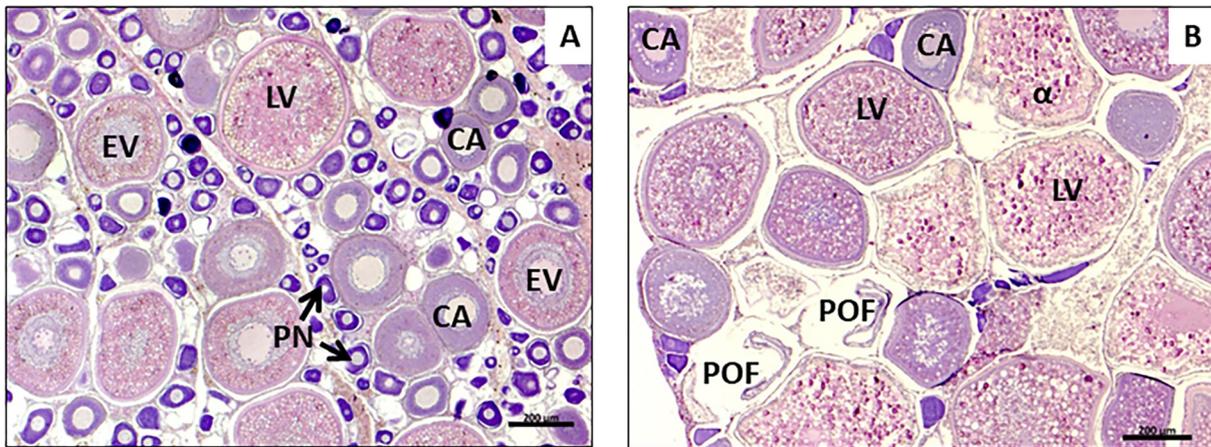


Fig. 1. Tissue sections of mature ovary during the reproductive season (May-June). (A) Mature ovary in active non-spawning stage (ANS). (B) Mature ovary in active spawning stage (AS); PN = perinuclear oocytes; CA = cortical alveolar oocytes; EV = early-vitellogenic oocytes; LV = late-vitellogenic oocytes; POF = post-ovulatory follicle; α = α -atretic follicle. Scale bar is 200 μm .

3.2. Gonadosomatic index (GSI)

GSI varied with the developmental stage of the gonads. In ANS females, the GSI was 3.88 ± 1.12 , which was significantly lower than the GSI (6.66 ± 1.99) in AS fish ($P < 0.05$) (Fig. 4a). The lowest GSI (0.71 ± 0.21) values in females were observed in IM specimens caught in Oct-Nov. In contrast to females, the GSI in males caught in May-June did not differ significantly between the ES and LS developmental stages (4.81 ± 0.45 and 6.78 ± 3.6 , respectively) (Fig. 4b). In Oct-Nov, the GSI in males significantly decreased (0.31 ± 0.09 ; $P < 0.05$). Supplementary Table 1A and B show the curved fork length (CFL in cm), total weight (W in Kg), gonad weight, (GW in Kg), GSI and the gonadal developmental stages (according to Medina et al., 2002; Schaefer, 2001) in male and female fish sampled in May-June and in Oct-Nov.

3.3. Plasma FSH levels in relation to sex and spawning season

Circulating levels of FSH were detected in ABFT both during the spawning and non-spawning seasons, with the levels being significantly higher ($P < 0.05$) in male and female fish sampled during the non-breeding period (Fig. 4c).

3.4. Sex steroid levels

In females, plasma E_2 was significantly higher in May-June than in Oct-Nov while plasma T levels did not differ between the two sampling times (Fig. 4d). In males, plasma levels of E_2 and T were significantly higher ($P < 0.05$) in May-June with respect to Oct-Nov samples (Fig. 4e). Plasma progesterone levels did not vary with season in either males or females (Fig. 4d-e).

3.5. Plasma VTG

VTG was detected in ABFT plasma samples collected during the spawning period, both in males and females, although a significantly higher VTG level was found in females than in males (Fig. 4f). The relatively low overall values of plasma VTG detected in this study could be attributed to the use of a heterologous ELISA. Plasma of post-spawning specimens had undetectable levels of VTG both in males and in females.

3.6. Expression level of genes involved in gametogenesis

Fig. 5 shows the mRNA levels of key genes involved in gametogenesis in the ovary and the testis of fish caught in May-June and Oct-Nov. *zpc1* mRNA levels did not vary in the ovaries of tuna between May-June and Oct-Nov samplings (Fig. 5a). In contrast, *ve γ* , *Chg L* and

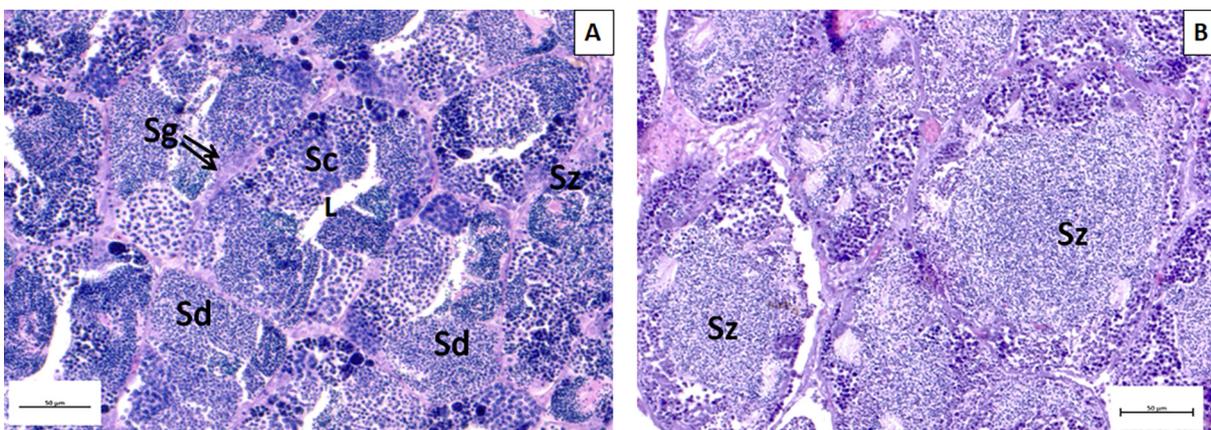


Fig. 2. Tissue sections of ABFT testis at different developmental stages of during the reproductive season (May-June). (A) Early spermatogenesis, dominance of spermatocytes and spermatids, small clusters of spermatozoa in lobules, and few spermatocysts that release spermatozoa; (B) Spermiation (late spermatogenesis), high number of spermatozoa in the lumen. Sg = spermatogonia; Sc = spermatocytes; Sd = spermatids; Sz = spermatozoa; L = lumen. The scale bar is 50 μm .

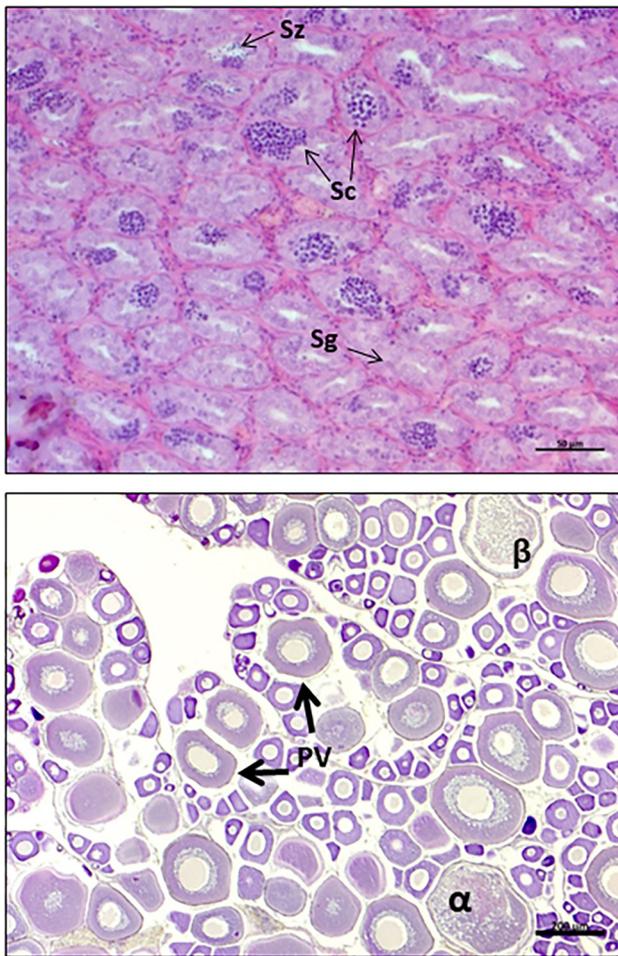


Fig. 3. Tissue sections of the gonads of ABFT during the non-reproductive season (Oct-Nov). (A) Ovary; (B) Testis. PV = pre-vitellogenic oocytes; α = α -atretic follicle; β = β -atretic follicle; Sg = spermatogonia; Sc = spermatocytes; Sz = spermatozoa. Scale bar: A = 200 μ m; B = 50 μ m.

alveolin mRNA expression was significantly higher ($P < 0.05$) in May-June females than in Oct-Nov ones (Fig. 5b–d). The transcripts of *zpc1*, *vey*, *Chg L* and *alveolin* genes were undetected in males during both sampling periods. Transcript of genes encoding for proteins involved in gamete hydration, *tmc6* related protein 1 (*tmc6*) and aquaporin 1, were also measured in both ovary and testis. *Tmc6* mRNA expression was higher ($P < 0.05$) in females caught in May-June, with levels decreasing in Oct-Nov catches (Fig. 5e). In males, a significantly higher ($P < 0.05$) *Tmc6* mRNA expression was observed in May-June with respect to Oct-Nov, with a similar trend in female (Fig. 5e). *Aquaporin* mRNA levels were significantly higher ($P < 0.05$) in May-June females than in Oct-Nov samples. On the contrary, in the testis, *aquaporin* mRNA was higher ($P < 0.05$) in Oct-Nov males than in May-June males (Fig. 5f).

The levels of a set of fatty acid binding protein mRNAs were also measured. Fatty acid binding protein (*fabp*) mRNA expression levels were significantly higher ($P < 0.05$) in Oct-Nov females than in females caught in May-June. In males, *fabp* expression was not significantly different between the two sampling times but the levels were significantly higher ($P < 0.05$) when compared to females (Fig. 5g). Intestinal Fatty acid binding protein (*ifabp*) transcripts were similar in males during the May-June and Oct-Nov sampling. In females, *ifabp* expression was significantly higher ($P < 0.05$) in May-June specimens than in Oct-Nov caught individuals, however, the relative abundance value was lower than in males (Fig. 5h). Brain-type Fatty acid binding protein (*bfabp*) mRNA expression was significantly higher ($P < 0.05$)

in Oct-Nov females than in May-June ones. Similarly, in males, the *bfabp* mRNA expression was 4-fold higher in Oct-Nov fish than in May-June (Fig. 5i).

4. Discussion

Results of the present study provide new insights regarding the reproductive biology of ABFT caught in the Mediterranean Sea at two different time points. During the reproductive season (May-June), the histological characteristics and GSI levels of fish were similar to those of wild specimens previously captured around the Balearic Island, which has been previously indicated as another main spawning ground (Corriero et al., 2007; Rosenfeld et al., 2012). The variability of GSI among the fish reported in this study reflected the two distinct reproductive stages, ANS and AS for females, ES and LS for males. GSI values were significantly lower in Oct-Nov, when mature ovaries were inactive, and the testes spent. In contrast from previous observations by other authors (Medina et al., 2016), atretic oocytes were still detectable in Oct-Nov in the present study, showing a prolonged period for their resorption (IM reproductive stage). In May-June females, the higher levels of GSI is also accompanied by higher level of plasma VTG. This trend reflects the stage of maturity of the ovary, as VTG is synthesized in the liver under E_2 stimulation (Babin et al., 2007; Carbonara et al., 2015; Mosconi et al., 1998) and is immediately transported to the developing oocytes. These observations are supported by the increase of plasma E_2 which was elevated in female ABFT in May-June but decreased in Oct-Nov. In most species, both T and E_2 increase concomitantly during the pre-spawning period, and peak at spawning (Guzmán et al., 2008). In male tuna, in May-June, E_2 and T reached similar levels, significantly higher than in Oct-Nov, possibly because of its role in the renewal of spermatogonia, as demonstrated in three-spot wrasse, Japanese eel and other vertebrate species (Miura et al., 1999; Schulz et al., 2010). In contrast to males, in May-June females, T levels were similar to those measured in Oct-Nov, possibly because T is being actively converted to E_2 , steering the vitellogenic process. P levels were not affected by seasonality either in male nor in females, despite its role as a key intermediate in the biosynthesis of several active steroids, including androgens (T and 11-KT), cortisol, E_2 , or 17 α , 20 β -P.

In this study, we detected FSH in the plasma in Oct-Nov and in May-June, both in male and female fish, with higher levels observed during the non-reproductive season in both sexes. It is possible that the high levels of FSH in mature but inactive ABFT is necessary in preparation for the next cycle of oogenesis and spermatogenesis, as reported in trout (Breton et al., 1998). In immature ABFT, the pituitary content of FSH was always higher than luteinizing hormone (LH) (Berkovich et al., 2013; Heinisch et al., 2014), indicating the role of FSH in preparing the gonads for gametogenesis. In general, FSH plays a stimulatory role at the onset of puberty and at early stages of reproductive development while LH plays a role at advanced stages of maturation and spawning (Yaron et al., 2003). The histological analyses here clearly demonstrated that all the analyzed specimens were sexually mature. Considering that tuna is a multiple spawning species, it is likely that FSH still has a role in promoting the development of early stage oocytes recruited for the season. In species that are capable of spawning multiple times within a season, circulating FSH persists at concentrations like those observed at the beginning of vitellogenesis, even at the point when the fish are already spawning. This has been observed in European seabass, *Dicentrarchus labrax* (Molés et al., 2012), greater amberjack, *Seriola dumerili* (Nyuji et al., 2016) and yellowtail kingfish, *Seriola lalandi* (Nocillado et al., 2019). In this study, the significantly higher T in males and E_2 in females during the spawning season suggest that the gonads were actively undergoing steroidogenesis. This process is known to be regulated both by FSH and LH in several species of fish (Yaron et al., 2003). Future studies including determination of circulating LH levels are needed to verify the role of LH in this process as pituitary content of LH did not vary in ABFT of different size classes,

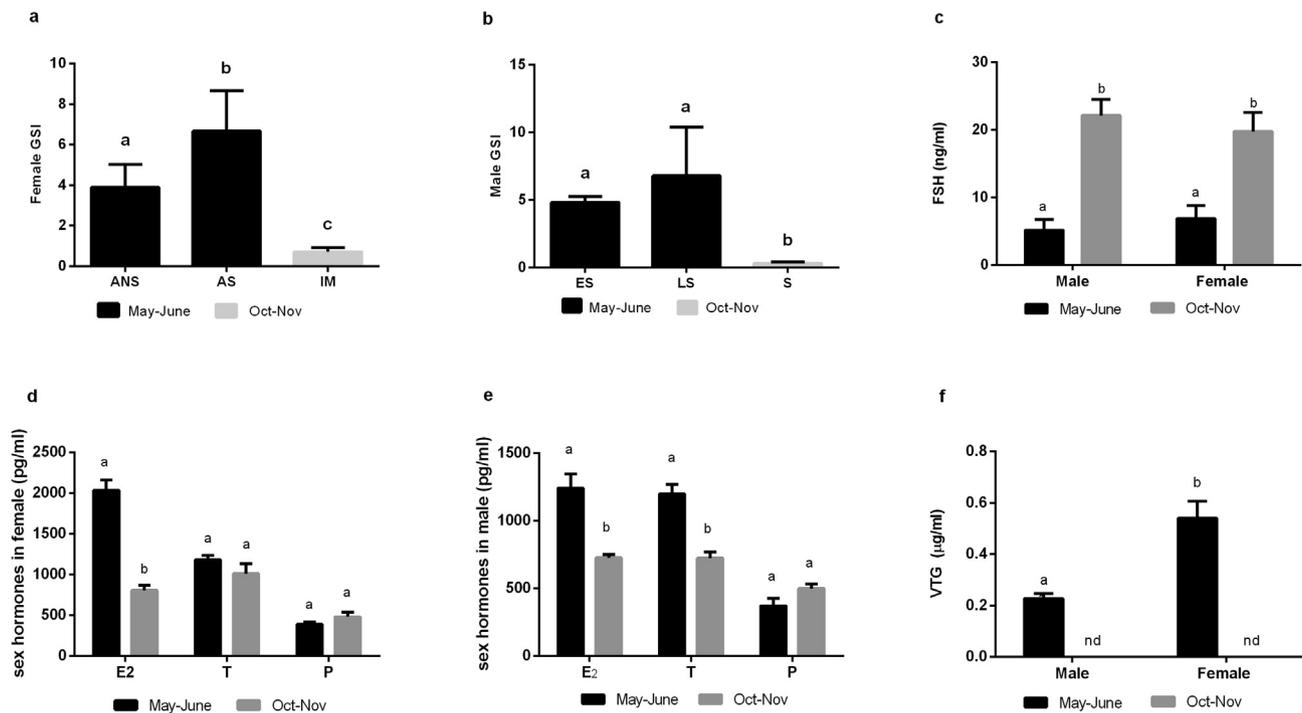


Fig. 4. Gonadosomatic index (GSI) in female (a) and male (b) ABFT during the reproductive (May-June) and non-reproductive season (Oct-Nov) according to the oocyte and spermatogenic stages, respectively, and the corresponding plasma levels of FSH (c), sex steroids in female (d) and male (e) fish, and VTG (f) in male and female fish. Data are shown as means \pm S.D. Different letters in graphs denote significant differences among the experimental groups ($P < 0.05$). LS: late spermatogenesis, ES: early spermatogenesis, S: spent, ANS: active non-spawning, AS: active spawning, IM: inactive mature.

whose ovaries contained oocytes at various stages, during the spawning season (Heinisch et al., 2014). Furthermore, future studies should also determine whether the ratio of plasma FSH and LH levels can be used as an indicator of gonadal maturation as previously shown for the ratio of the FSH and LH content in the pituitary (Heinisch et al., 2014).

Plasma VTG was detected during the reproductive season not only in females but also in males. The presence of vitellogenin in male ABFT has been reported previously, both at gene expression and protein levels, and has been proposed, in many teleost species, to be a result of exposure to estrogenic pollutants (Maradonna et al., 2014; Maradonna et al., 2013; Maradonna et al., 2004), particularly of large fish (> 100 kg) (Barucca et al., 2006; Fossi et al., 2002). The VTG concentrations detected in the males in this study were much lower compared with circulating levels sampled in May-June female ABFT (Susca et al., 2001a), however only two of the sampled males were more than 100 kg. Nevertheless, our results support previous warning of the potential environmental hazard caused by endocrine pollutants on top predators in the Mediterranean Sea (Fossi et al., 2006).

Major gaps exist about the molecular and biochemical mechanisms that lead to the production of good quality gametes. Gametogenesis is a very complex process and consists of gamete growth and differentiation. During oogenesis, a pivotal event is the formation of vitelline envelop (VE) and on this regard, the expression of three different genes, namely *zpc1*, *vey* and *Chg L*, encoding for vitelline envelope proteins was analyzed. Their mRNA levels increased in May-June females and was followed by a significant decline in Oct-Nov fish, confirming their vital role during oogenesis and suggesting their role as a sperm-binding protein, sperm receptor and acrosome reaction inducer, as previously described in *Cynoglossus semilaevis* and in medaka (*Oryzias latipes*) (Kanamori et al., 2003; Sun et al., 2010a). The mRNA detection in the ovary indicate an extrahepatic synthesis, as previously described in other species (Mold et al., 2009; Murata et al., 2014).

At fertilization, the trigger of egg envelope hardening is alveolin, as reported in medaka (Shibata et al., 2012). Its mRNA expression revealed a marked seasonality, increasing significantly in May-June

ovaries compared to Oct-Nov samples, suggesting its main role at advanced stage of oogenesis. One of the factors that regulate hydration process in eggs is *tmc6*. The higher *tmc6* mRNA level detected in May-June samples compared to those collected in Oct-Nov suggests a role in increasing the internal osmolality and driving the influx of water as previously reported (Gardner et al., 2012), a key step to gain buoyancy in pelagic eggs. The detection of this mRNA in the testis suggests its possible involvement in sperm maturation.

As an adaptation to the hyperosmotic condition of seawater, the oocytes of marine teleost hydrate during meiotic resumption (oocyte maturation) and aquaporins play a pivotal role in the adaptation of gametes and early embryos to the external spawning environment (Cerdà et al., 2017; Martos-Sitcha et al., 2015). This mechanism provides a water reservoir in the embryo to compensate for the passive water efflux until osmoregulatory organs develop and improves oxygen exchange and egg dispersal in the ocean. In seabream, aquaporin 1b-containing vesicles are transported toward the oocyte cortex and during meiotic maturation and hydration they are temporarily inserted into the oocyte plasma membrane (Fabra et al., 2006). Considering this evidence, it is acceptable that its levels in the ovary were higher during the reproductive phase, as observed in May-June fish, than after spawning (Oct-Nov). In males, aquaporin mRNA level in Oct-Nov were higher than in May-June. Since aquaporin channels are involved in the hydration of the seminal fluid in the acquisition of sperm motility (Boj et al., 2015), we could hypothesize that in spent testis, the aquaporin mRNA is not immediately translated into protein and could be stored until the next reproductive season as occurs in the gonad of several teleost species (Kagawa et al., 2011; Ozgyin et al., 2015; Sun et al., 2010b; Tingaud-Sequeira et al., 2010).

Lipids accumulated within gametes are an important source of energy and therefore an important factor for good gamete quality (Eslami et al., 2018; Yuan et al., 2019). Lipids are transported by fatty acid binding proteins, the most expressed (*fabp*) has a higher homology with *fabp11*, whose expression in flatfish ovarian follicle cells directly correlated with follicular atresia. The increase of *fabp* levels in tunas

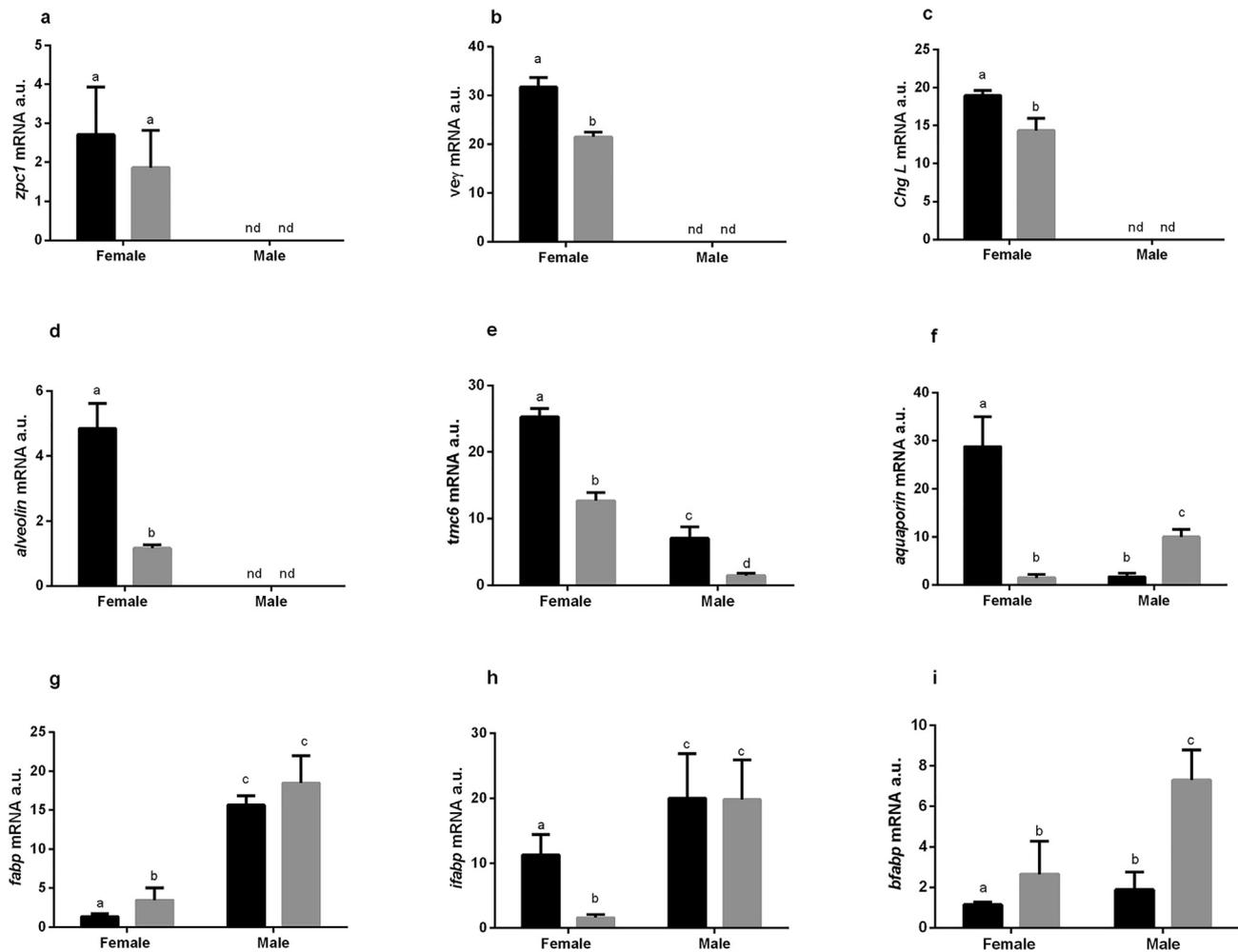


Fig. 5. Relative expression level of *zpc1*, *Chg L*, *vex*, *alveolin*, *tmc6*, *aquaporin*, *fabp*, *ifabp*, *bfabp* in the gonads of male and female ABFT during the reproductive (May-June, black bars) and non-reproductive (Oct-Nov, grey bars) season. Values are presented as arbitrary units (a.u.) and are expressed as mean \pm S.D. Different letters denote significant differences among experimental groups ($P < 0.05$).

caught in Oct-Nov correlated well with the increase of follicular atresia (α, β) observed in Oct-Nov ovaries. The specific function of *fabp* in testis is still unclear, where two additional *fabp* forms, *ifabp* and *bfabp*, are expressed. These two isoforms are cytosolic transporters of long-chain fatty acids and other hydrophobic ligands. *bfabp* has been shown to have the highest affinity for docosahexaenoic acid (DHA), which contributes to membrane fluidity, necessary for the motility of the axoneme (Gardner et al., 2012). As previously observed, and also in this study, the results demonstrated a large difference in the relative expression of *bfabp* between the reproductive and the post-reproductive ovaries, possibly due to a different DHA requirement: oocyte needs of DHA may be considerably less than those of sperm, or it can be speculated that DHA uptake within oocytes could be triggered by other mechanisms. As well as *bfabp*, this study focused also on *ifabp*, the most abundant isoform in the intestine, detectable in many other vertebrate tissues, where expression correlates with obesity and insulin resistance highlighting its role in the trans-membrane uptake of dietary fatty acids. These expression levels can be translated in a gonadal context and the higher level detected in the testis with respect to the ovary suggests its involvement in the sex-specific energy budget maintenance and energy homeostasis.

5. Conclusion

In conclusion, this study provides additional molecular information on the reproductive biology of ABFT (*Thunnus thynnus*). The

comparison at morphological, hormonal and gonadal gene expression levels during the two reproductive periods is an important step towards the comprehension of the reproductive process of this considerably important species. Integration of these results with recently published data regarding the molecular features of ABFT body growth (Api et al., 2018) and Southern bluefin tuna reproduction (Bar et al., 2016) can contribute to the endeavors of successfully farming this species as well as conserving natural stocks.

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Declaration of Competing Interest

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ygcen.2019.113216>.

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