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FEMALES REPRODUCTIVE BIOLOGY OF MEDITERRANEAN SWORDFISH (*XIPHIAS GLADIUS* L.): NEW INSIGHTS FROM A MULTIDISCIPLINARY STUDY

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ABSTRACT

A multidisciplinary approach which include histological, macromolecular and molecular assays, is of great importance to fully understand a complex process such as the reproductive biology of swordfish (XIPHIAS GLADIUS L.). Accordingly, the optimization of reliable protocols for the collection of biological samples, intended for the different analytical tools, is a mandatory step considering the logistic constraints associated with on board sampling procedures.

In this study have been optimized three analytical tools to assess reproductive status of Mediterranean swordfish: histological assay; FTIR microspectroscopy and transcriptomic analysis. The histological approach, based on the presence of specific characteristic structures, let us classify the ovary maturation in the five following developmental stages: "immature"; "developing"; "spawning"; "regressing" and "regenerating". The use of the FTIR microspectroscopy provided information about the macromolecular composition of the oocytes at different developmental stages, providing specific chemical map for each class of oocyte. Finally, by the de novo transcriptome assembly approach, the molecular dynamics governing ovarian maturation were elucidated and molecular biomarkers of swordfish reproduction were identified. For each analytical tool, the protocol for samples collection was optimized and adapted to difficulties of on-board sampling procedures.

KEYWORDS: Reproductive cycle, Sex determination, Animal reproductive

Organs, Biochemical analysis, Sexual maturity,

Spawning, Biological sampling, Mediterranean Sea, longline.

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1. Introduction

The swordfish (*Xiphias gladius*) is a highly valuable commercial target specie with extensive seasonal migrations and a global distribution. This migratory behaviour in addition to a challenging management led to severe overfishing of the Mediterranean stock (ICCAT, 2016a). Indeed, recent estimations of the swordfish stock status in this area have indicated a 40% reduction in spawning stock mass over the past 20 years. This reduction, combined with high fishing mortality rates, gave rise to "non-negligible" risks of rapid future declines in stock. In order to recover the Mediterranean stock, in 2016, the ICCAT established a multi-annual plan containing measures such as: Total Allowable Catches (TAC); fishing fleet reduction; closed fishing season and a minimum landing size (ICCAT, 2016b).

To face the decline of swordfish stock and properly support the ICCAT recovery plan, based on scientific evidences, deeper insights on swordfish reproductive biology in the Mediterranean Sea are urgently needed. The importance of biological data was successfully demonstrated in the last years in the rebuilding of the Atlantic swordfish stock (Neilson et al., 2013), achieved by the integration of physiological responses (i.e. reproduction) with stock assessment modelling.

The swordfish is a gonochoristic species with a limited spawning season and, in the Mediterranean Sea, shows one of the highest estimated batch fecundity compared with other areas (Young et al., 2003; Poisson and Fauvel, 2009a). Ovaries exhibited ovigerous lamellae, asynchronous oocyte development and the spawning occurred in multiple batches with a spawning frequency which ranged 2.6-3.0 days (Arocha, 1997; Young et al., 2003; Poisson and Fauvel, 2009a). Despite previous studies investigated the swordfish reproduction in the Mediterranean Sea (Macías et al., 2005; Corriero et al., 2007; Alıçlı et al., 2012), comprehensive information on reproduction strategies, gonadal development and spawning patterns are far from being firmly established. To fill such a gap of knowledge, we optimized protocols to study spawning patterns and gonadal development of Mediterranean swordfish through a multidisciplinary approach. Microscopic approaches such as histological analysis, FTIR microspectroscopy in addition to transcriptomic assays have been optimized. The analysis of Gonadal Index (GI) of immature, developing and spawning females were also performed. Furthermore, we depicted and optimized an *ad-hoc* samplings method for each analytical tool adapted to constraints typically encountered during on board sampling procedures.

2. Methodology

Biological sampling was carried out by the scientific staff of the Department of Life and Environmental Sciences (DiSVA) - Università Politecnica delle Marche, Ancona (Italy) with the support of OCEANIS Srl for the years 2016, 2017 and 2018 within the scientific monitoring program funded by the Italian Ministry of Agricultural, Food and Forestry Policies (MiPAAF), General Directorate of Fisheries and Aquaculture. The area of study covers the longline fisheries targeting SWO in Mediterranean waters around Sicily, Sardinia and the Adriatic Sea.

Ovaries were retrieved from 126 swordfish with a Lower Jaw-Fork Length (LJFL) > 100 cm, according to Italian legislatio. Soon after capture, LJFLs were measured to the nearest cm using a tape measure; ovaries were removed and the gonads' weight (WG) measured. Small gonad portions (2 cm^3) were taken from the middle part of the largest ovary of all sampled specimens and fixed in a solution of Formaldehyde 36.5% and glutaraldehyde 25%, and stored at 4°C until histological and FTIRM analyses. At the same time additional small gonad portions were placed in RNAlater solution (Ambion, Austin, TX, USA), stored at 4°C till boarding and once landed transferred to -20°C. At landing, the eviscerated body weight (WB) was also measured.

Gonadal Index: the gonadal index (GI) was assessed following Hinton et al. (1997): GI=ln(GW)/ln (a LJFL – b) where GW indicates the weight of the gonad (expressed in g) and a, b parameters have been *ad hoc* calculated.

Histological assay: Ovary samples were processed as described in (Forner-Piquer et al, 2018). Briefly, fixed samples were dehydrated in a series of alcohol baths, cleared in Xylene and finally embedded in paraffin. Sections 5 µm thick were cut with a microtome and stained with Mayer's haematoxylin – eosin. The histological slides were observed under a Zeiss Aixio Imager M2 microscope and microphotographed with a high-resolution camera (Zeiss Axiocam 105 color). Female samples were classified on five developmental stages, such as

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"immature", "developing", "spawning", "regressing" and "regenerating". Based on the FTIR microspectroscopy assay, ovaries were processed as described in (Carnevali et al 2009, Santangeli et al, 2017). Briefly, from each fixed ovarian sample, three thin sections (10 µm thickness) were cut by using a cryomicrotome. Sample sections were then deposited, without any fixation process, onto CaF2 optical windows and then analyzed with a Bruker VERTEX 70 interferometer coupled with a Hyperion 3000 Vis-IR microscope equipped with bidimensional Focal Plane Array (FPA). The visible image of each ovarian section was obtained with a 15X condenser/objective and used to select areas containing oocytes at different development stages (previtellogenic, vitellogenic, mature, and atretic). On these selected areas, IR maps were collected in transmission mode in the 4000-900 cm-1 MIR range with a spatial resolution of ~2.56 µm. These preprocessed IR maps were integrated under the following spectral regions, to obtain false colour images representing the topographical distribution and relative amount of the most relevant biochemical features27: 3034-2995 cm-1 (containing the vibrational modes of unsaturated groups in lipid alkyl chains, named CH); 2995–2825 cm-1 (containing the vibrational modes of lipids, named LIP); 1754-1718 cm-1 (containing the vibrational modes of fatty acids, named FA); 1718–1481 cm-1 (containing the vibrational modes of proteins, named PRT); 1427-1360 cm-1 (containing the vibrational modes of COO- groups in glutamate and aspartate amino acids, named COO); 1274–1181 cm-1 (containing the vibrational modes of phosphates groups inside nucleic acids, named PHOSPHO), and 1130–1013 cm-1 (containing above all the vibrational modes of carbohydrates, named CARBO). An arbitrary colour scale was used, white colour indicating areas with the highest absorbance values and blue colour areas with the lowest ones.

Transcriptomic analysis: total RNA was isolated from ovaries of 3 immature females and 3 mature females. Libraries were created with the Illumina TruSeq Stranded mRNA Library Prep Kit and then sequenced with an HiSeq2500. Illumina paired-end 150 bp reads from X. gladius samples were processed to produce the transcriptome assembly. Functional annotation of the transcriptome was performed following the AHRD pipeline. A Gene Ontology Enrichment Analysis (GOEA) and a KEGG enrichment analysis were performed to evaluate differentially expressed transcripts. Finally, we set up a dedicated database (www.swordfishomics.com) that is publicly available where all the information about the swordfish transcriptome can be directly accessed by the scientific community, offering a powerful tool for the integration of our findings with other approaches for stock assessment analysis.

3. Results

Since the difficulties in macroscopically recognizing of swordfish gonads during non-reproductive period, a microscopically investigation and classification of ovary becomes necessary.

Figure 1 shows the histological classification of follicles at different developmental stages: the earlier developmental stages (Figure 1A) are characterized by the presence of lipid droplet and cortical alveoli beside to the absence of yolk vesicle in the cytoplasm. Figure 1B represents a vitellogenic follicle in which is evident the yolk deposition within the cytoplasm into yolk vesicles. Figure 1C represents a follicle starting maturation where is evident the migration of nucleus towards the animal pole. When follicles reach maturation (figure 1D) yolk deposition stops, ooplasm hydration becomes evident, yolk globules start to coalesce, and lipid droplets fuse in a single oil droplet. Figures 1E and 1F represent the final destiny of follicles within the ovary: the Post Ovulatory Follicle (POF), which is constituted by follicular cells left by the ovulated oocyte or the Atretic follicle where the oocyte undergoes cell death: it loses its spherical shape, due to the degradation of the envelope and its cytoplasm becomes full of vacuoles.

Ovaries were histologically classified in 5 developmental stages: Immature; Developing; Spawning; Regressing and Regenerating (Figure 2A – E).

Ovaries from immature females (Figure 2A) show perinucleolar oocytes (PG) and oogonial nests, there are no signs of atresia, the ovarian wall is thin, and oocyte are homogenously distributed. Specimens in the developing phase (Figure 2B) show early (Es) and middle (Ms) vitellogenic oocytes and some atretic ones. If mature oocytes (mt) and POFs are present in the ovarian tissue, the specimen is in the actively spawning phase (Figure 2C). The regressing phase (Figure 2D) follows the spawning process. In this phase the female has completed its breeding period. POFs are still visible. The remaining oocytes are in lipid, early or middle vitellogenic phase an many of them will undergo reabsorption by atresia. Finally, the regenerating ovary (Figure 2E) is characterized by the only presence of previtellogenic oocytes but differs from the immature one for the presence of lipids droplets and cortical alveoli stages; it is representative of the pre-reproductive period.

Figure 3 represents the GI mean of immature, developing and spawning stages females determined by histological analysis.

Figure 4A shows the morphological features of a section of an immature ovary, containing oogonia (O) and perinucleolar oocytes (PN. The vibrational imaging analysis showed both in O and PN oocytes a homogeneous distribution of proteins, which appeared more abundant than the other molecules, (Figure 4B) and lipids, (Figure

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4C). Conversely, phosphate groups and carbohydrates were more concentrated in O with respect to PN (Figure 4D and 4E).
Due to the bigger size, only a portion of an oocyte in vitellogenesis stage was shown in Figure 5A. The topographic distribution of proteins (Figure 5B) allowed to highlight not only the presence of Zona Radiata surrounding the oolemma but its molecular structure since it appears composed by different layers of proteins, lipids, carbohydrate and phosphates groups (Figure 5C,E,D). The topographic distribution of proteins, lipids, fatty acids, carbohydrates and phosphates let identify vitellogenin vesicles, oil droplets and cortical alveoli in the inner part of the oocyte.
Figure 6 shows the Volcano plot representative of differential expressed genes between immature and mature ovaries. The differential expression analysis revealed a total of 6.501 transcripts differentially expressed, with 4007 predicted to be up-regulated in mature ovaries.
Figure 7 shows the results obtained by Gene Ontology Enrichment Analysis (GOEA). This analysis highlighted Biological Processes (BP), Cellular Component (CC) and Molecular Function (MF) significantly modulated during ovarian maturation. In particular, among BP we found translation, transport, transmembrane receptor proteins and lipid transport. Among (CC) we found Nucleus, Cytoplasm, Membrane and mitochondrion. Finally, among MF we found nucleotide and RNA binding, transporter activity and lipid transport.

4. Discussions and Conclusions

In conclusion, according to the ICCAT plan, recovery of the Mediterranean swordfish stock has at least a 60% probability of success. Despite this percentage represents an optimistic goal, filling the gap of knowledge about swordfish biology of reproduction has the potential to significantly improve the predicted scenario. In this context, this multidisciplinary study provides comprehensive step towards the complete understanding of Mediterranean swordfish reproductive biology. Macroscopic classification of gonads is recognized as not valid for the determination of sex and gonadal developmental stages. On the basis of an optimized histological protocol for gonadal analysis it was possible to classify follicles and ovaries into distinct developmental stages.
The histological classification was the basis for the analysis of GI evaluation in immature, developing and spawning females and in this light the GI should be assumed and validated as a solid indicator of gonadal developmental stages.
Females reproductive performances rely also with egg quality which in turn depends on `building blocks' such as amino acids, lipids, carbohydrates and maternal determinants accumulated in the egg. When eggs lack specific compounds, or contain inappropriate amounts of them, they will be not able to sustain fertilization or the development of a viable embryo. In this light, the macromolecular analysis of swordfish oocyte made by FTIR spectroscopy represents a deeper understanding of reproductive biology of this species. Furthermore, by applying transcriptomic analysis we clarified the molecular dynamics governing ovarian maturation. Finally, the establishment of a publicly available database containing information on the swordfish transcriptome aims to boost research on this species with the long-term of developing more comprehensive and successful stock management plans. Concluding, all protocols for the different tools were optimized including those for sample collection considering constraints usually encountered during on board sampling procedures.

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6. References

Alıçlı, T. Z., Oray, I. K., Karakulak, F. S., and Kahraman, A. E. 2012. Age, sex ratio, length-weight relationships and reproductive biology of Mediterranean swordfish, Xiphias gladius L., 1758, in the eastern Mediterranean. African Journal of Biotechnology, 11: 3673–3680. http://www.academicjournals.org/ajb/abstracts/abs2012/21Feb/Alicli et al.htm.

Arocha, F. 1997. The reproductive dynamics of swordfish Xiphias gladius L and management implications in the northwestern Atlantic. University of Miami. https://scholarlyrepository.miami.edu/dissertations/3432.

Carnevali O., Conti C., Ferraris P., Garavaglia M.G., Gioacchini G., Giorgini E., Rubini C., Sabbatini S., Tosi G. (2009). FT-IR Microspectroscopy on molecular building of Zebrafish oocytes. J. Mol. Structure 938, 207-213.

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Corriero, A., Desantis, S., Bridges, C. R., Kime, D. E., Megalofonou, P., Santamaria, N., Cirillo, F., et al. 2007. Germ cell proliferation and apoptosis during different phases of swordfish (Xiphias gladius L.) spermatogenetic cycle. Journal of Fish Biology, 70: 83–99.

de la Serna, J. M., Ortiz de Urbina, J. M., and Macias, D. 1996. Observations on sex-ratio, maturity and fecundity by length-class for swordfish (Xiphias gladius) captured with surface longline in the western Mediterranean. 115-139 pp.

Forner-Piquer, I. et al. Endocrine disruptors in the diet of male Sparus aurata: Modulation of the endocannabinoid system at the hepatic and central level by Di-isononyl phthalate and Bisphenol A. Environ. Int. 119, 54–65 (2018).

ICCAT. 2016a. Report of the 2016 Mediterranean swordfish stock assessment meeting. Casablanca.

ICCAT. 2016b. Recommendation by ICCAT replacing the recommendation [13-04] and establishing a multi-annual recovery plan for Mediterranean swordfish.

Neilson, J., Arocha, F., Cass-Calay, S., Mejuto, J., Ortiz, M., Scott, G., Smith, C., et al. 2013. The Recovery of Atlantic Swordfish: The Comparative Roles of the Regional Fisheries Management Organization and Species Biology. Reviews in Fisheries Science, 21: 59–97.

Poisson, F., and Fauvel, C. 2009a. Reproductive dynamics of swordfish (Xiphias gladius) in the southwestern Indian Ocean (Reunion Island). Part 2: fecundity and spawning pattern. Aquatic Living Resources, 22: 59–68.

Poisson, F., and Fauvel, C. 2009b. Reproductive dynamics of swordfish (Xiphias gladius) in the southwestern Indian Ocean (Reunion Island). Part 1: oocyte development, sexual maturity and spawning. Aquatic Living Resources, 22: 45–58. http://archimer.ifremer.fr/doc/00000/6353/.

Santangeli S., Maradonna F., Zanardini M., Notarstefano, V., Gioacchini, G., Forner-Piquer, I., Habibi, H., Carnevali, O. 2017 Effects of diisononyl phthalate on Danio rerio reproduction. Environmental Pollution 23: 1051e1062

Young, J. L., Young, J. L., Bornik, Z. B., Bornik, Z. B., Marcotte, M. L., Marcotte, M. L., Charlie, K. N., et al. 2006. Integrating physiology and life history to improve fisheries management and conservation. Fish and Fisheries, 7: 262–283.

Young, J., Drake, A., Brickhill, M., Farley, J., and Carter, T. 2003. Reproductive dynamics of broadbill swordfish, Xiphias gladius, in the domestic longline fishery off eastern Australia. Marine and Freshwater Research, 54: 315–332.

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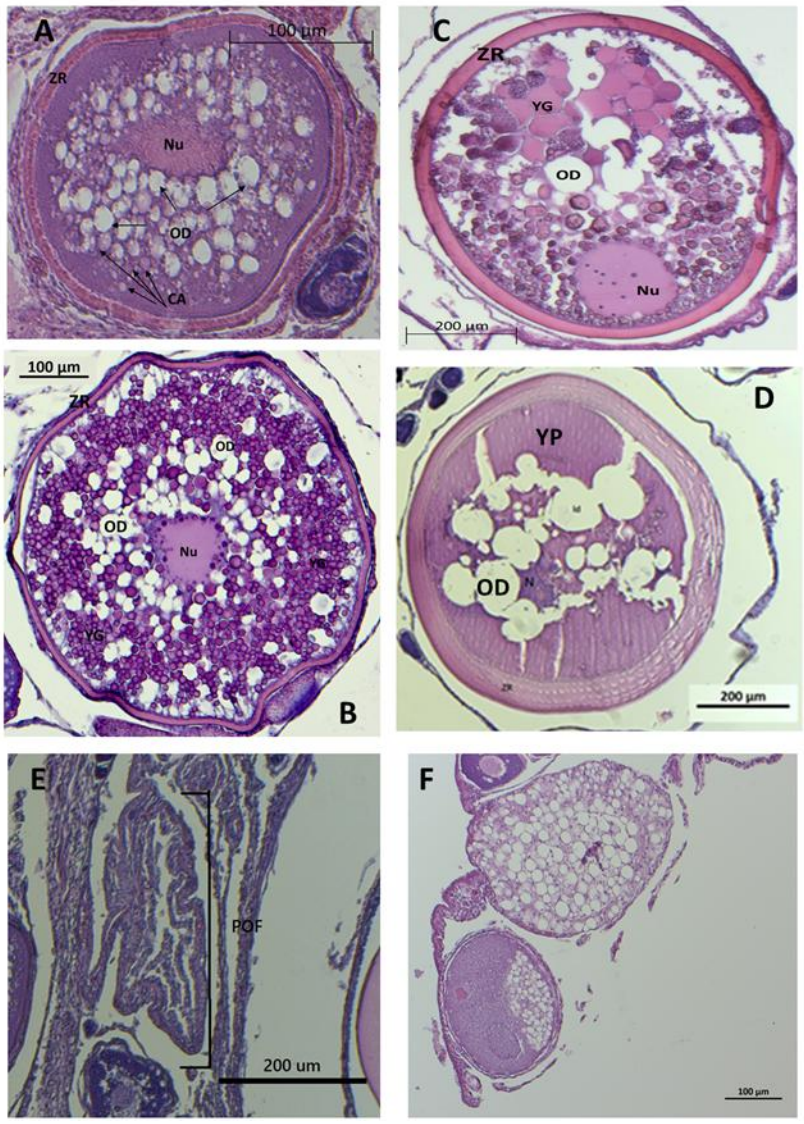

Figure 1: Mediterranean swordfish follicles at different developmental stages: A) Cortical Alveoli/lipid droplets stage follicle, B) Vitellogenic stage follicle, C) in maturation stage follicle, D) Mature/hydrated stage follicle, E) Post Ovulatory follicle, F) atretic follicle; Nu: nucleus, OD oil droplet, CA: cortical alveoli, YG: yolk globules ZR: Zona Radiata (E:esterna, I:interna), YC yolk coalescence

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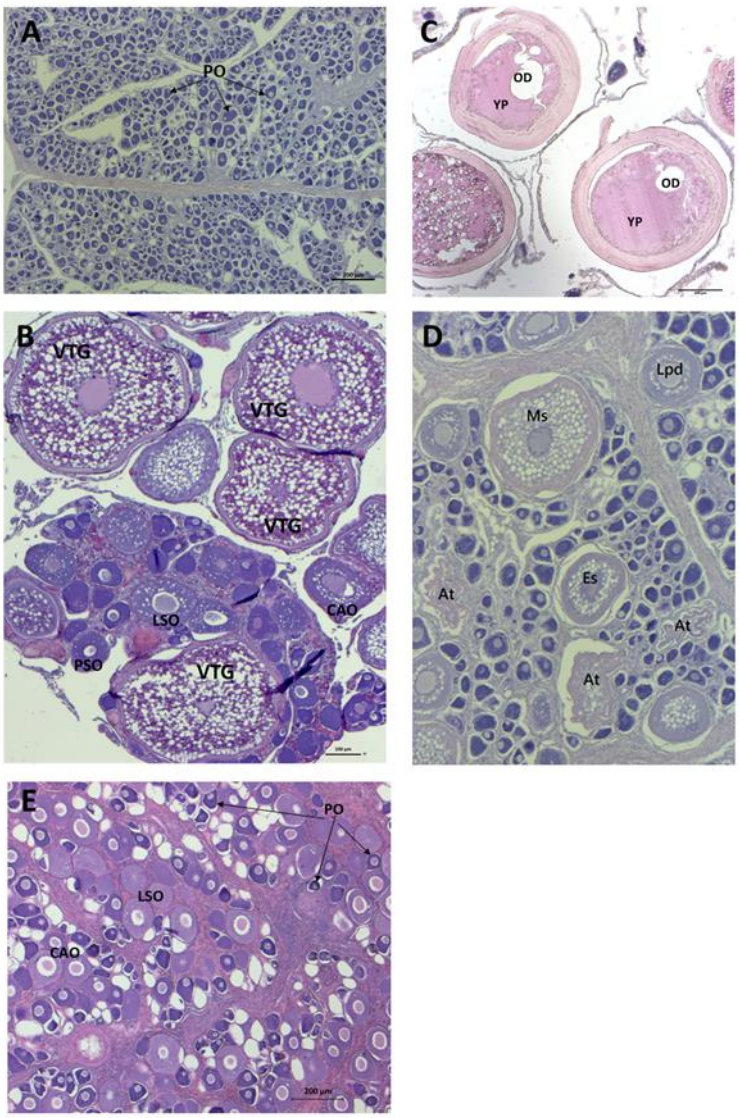

Figure 2: Mediterranean swordfish ovaries at different developmental stages A) immature, B) developing, C) spawning, D) regressing and E) regenerating. PO = primary oocyte, Lpd and LSO = lipid stage, CAO= cortical alveoli stage, Es = early vtg stage, Ms = middle vtg stage, Ls = late vtg stage, mt = mature, At = atretic, PSO: perinucleolar stage follicle YP= yolk platelet, OD = oil droplet

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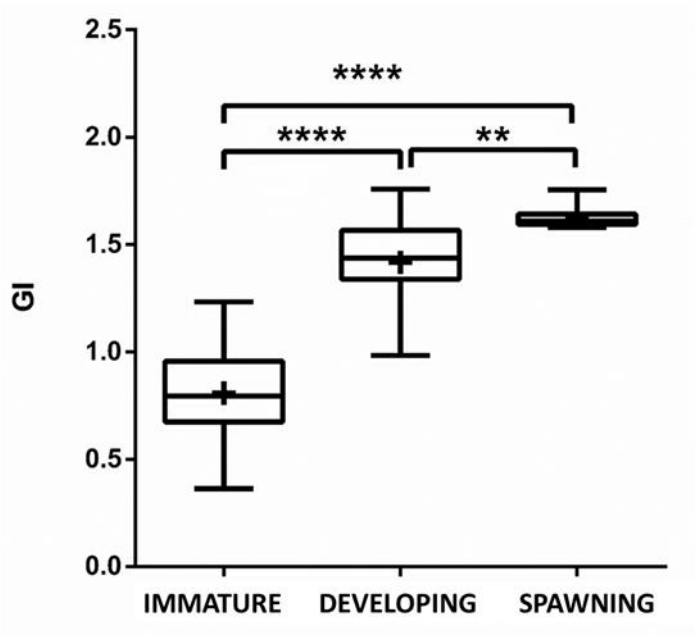

Figure 3: Gonadal Index (GI) trend in females at different ovarian developmental stage. **P<0.01; ****P<0.001

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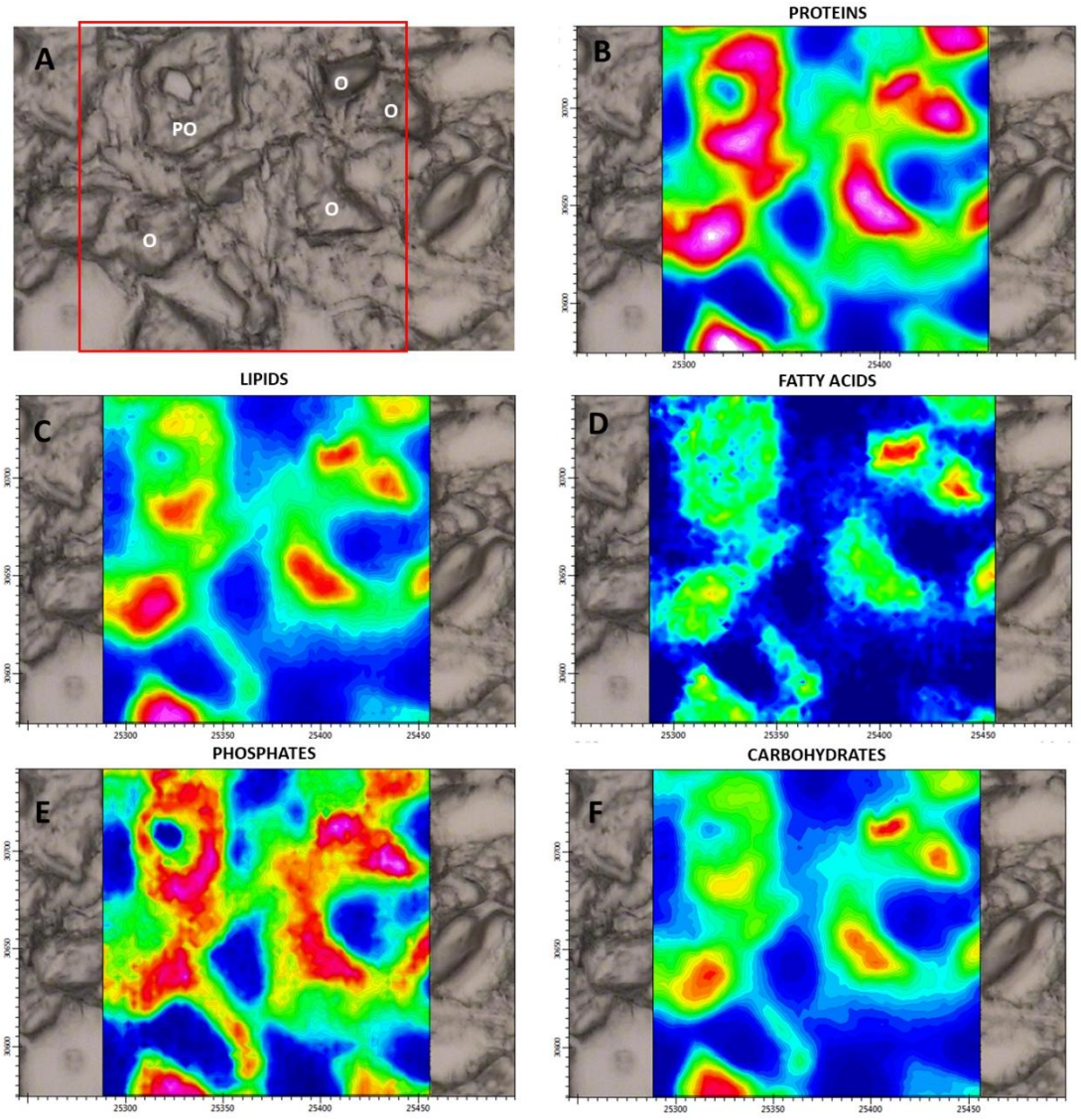

Figure 4: FTIR analysis of a representative Swordfish immature gonad containing oogonia (O) and perinucleolar oocytes (PO). (A) Microphotograph. IR maps representing the topographical distribution of: (B) proteins, (C) lipids, (D) fatty acids, (E) phosphate groups, and (F) carbohydrates. Due to different molar extinction coefficients of the analysed peaks, different scales were used for each IR map (blue colour indicating the areas with the lowest absorption values, while white colour the highest ones).

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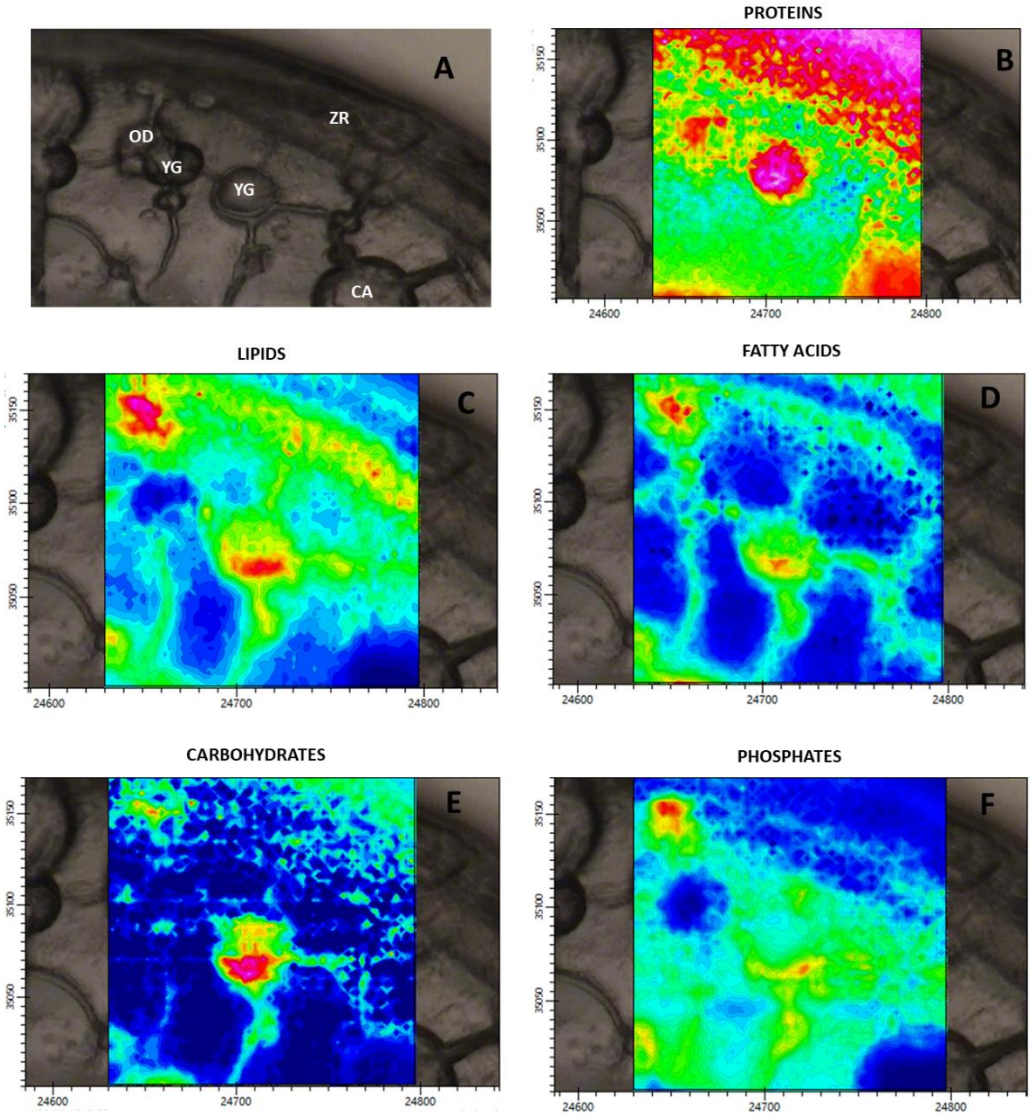

Figure 5: FTIR analysis of a representative Swordfish vitellogenic oocyte containing the Zona Radiata (ZR), oil droplets (OD), yolk vesicles (YG) and Cortical alveoli (CA). (A) Microphotograph. IR maps representing the topographical distribution of: (B) proteins, (C) lipids, (D) fatty acids, (E) phosphate groups and (F) carbohydrates. Due to different molar extinction coefficients of the analysed peaks, different scales were used for each IR map (blue colour indicating the areas with the lowest absorption values, while white colour the highest ones).

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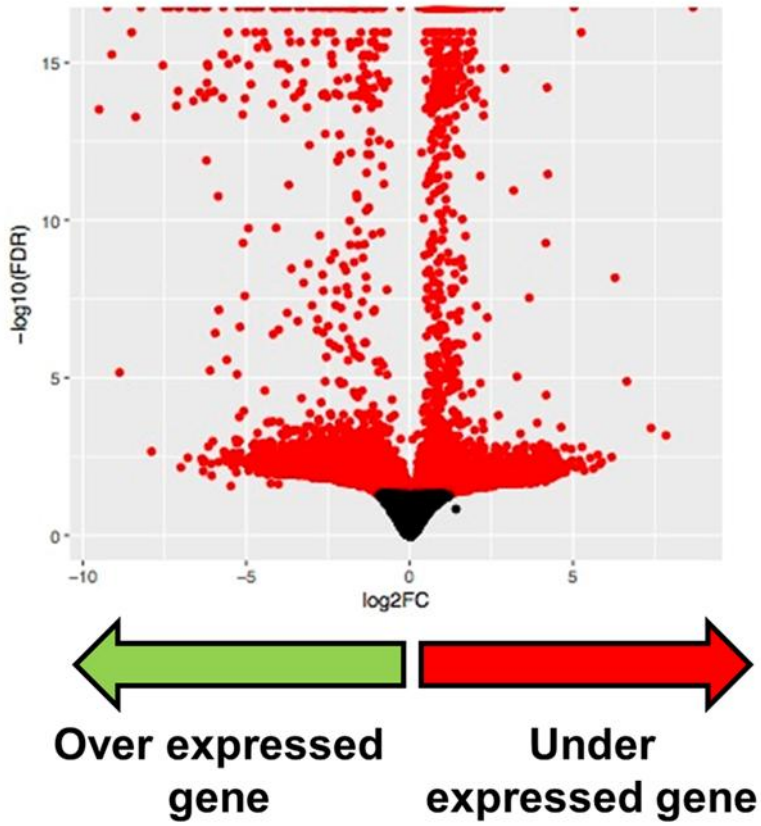

Figure 6: Volcano plot representative of 6.501 differential expressed genes between immature and mature ovaries with 4007 be up-regulated and 2494 down-regulated in mature ovaries.

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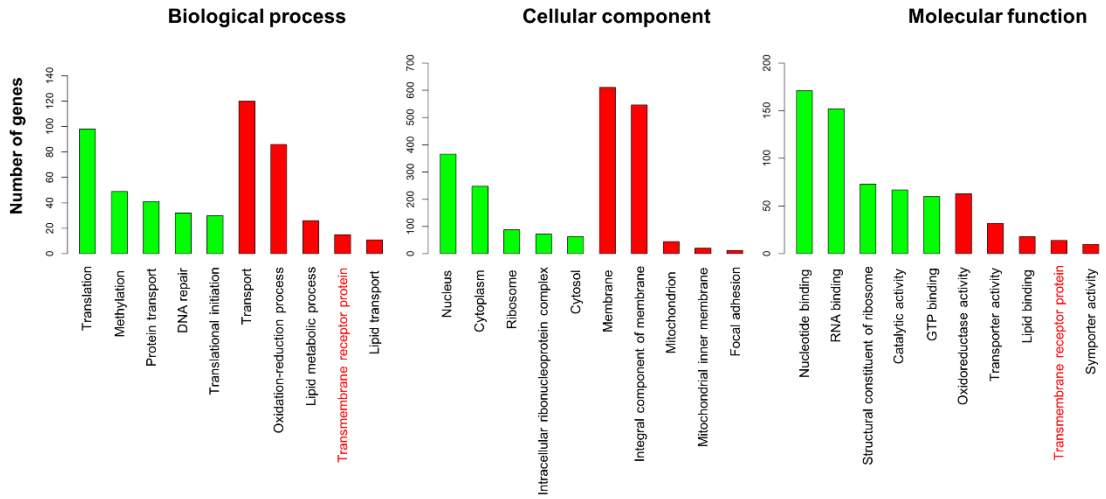

Figure 7: Gene Ontology differences underlying immature and mature ovaries. The bar plot shows the Gene Ontology terms enriched of genes differentially expressed between mature and immature ovaries. The y-axis represents the N° of genes and gene ontology terms have been organized according to the three main categories of Biological Process, Molecular Functions and Cellular Components. Red and green bars stand for terms up-regulated and down-regulated in the mature ovaries compared with the immature ones, respectively.

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