A comparison of techniques for studying oogenesis in the European eel *Anguilla anguilla*

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Running headline: Techniques to study *A. anguilla* oogenesis

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ABSTRACT

A multi-technique approach was used to study the changes occurring in European eel *Anguilla anguilla* oocytes during hormonally-induced vitellogenesis. Aside from classic techniques used to monitor the vitellogenic process, such as ovary histology, fat content analysis, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and vitellogenin enzyme linked immunosorbent assay (ELISA), a new technique, Fourier Transform Infrared (FT-IR) Microspectroscopy, was used for the first time to analyze *A. anguilla* oocytes. The results from the different techniques provided different ways of approaching the same process. Although it is considered a time consuming approach, of all the employed techniques, histology provided the most direct evidences about vitellogenesis. SDS-PAGE and ELISA were also useful for studying vitellogenesis, whereas fat analysis cannot be used for this purpose. The FT-IR analysis provided a representative IR spectrum for each ovarian stage (PV, EV, MV and LV), demonstrating that it is a valid method able to illustrate the distribution of the oocytes within the ovary slices. The obtained chemical maps confirmed changes in lipid concentrations, and revealed their distribution within the oocytes at different maturational stages. When the results and the accuracy of the FT-IR analysis were compared to those of the traditional techniques commonly used to establish the vitellogenic stage, it became evident that FT-IR is a useful and reliable tool, with many advantages, including the fact that it requires little biological material, the costs involved are low, analysis times are short, and, last but not least, the fact that it offers the possibility of simultaneously analyzing various biocomponents of the same oocyte.

Keywords: ovary, vitellogenesis, SDS-PAGE, Folch, ELISA, FT-IR.
INTRODUCTION

The European eel *Anguilla anguilla* L. 1758 has a peculiar life cycle which is not yet fully understood. It is a semelparous species which undergoes a 5-6,000 km migration to reach the Sargasso Sea, the supposed spawning area (van Ginneken & Maes, 2005).

The *A. anguilla*, like all Anguillid species, does not mature in captivity unless hormonally stimulated. hCG is commonly used in males – although recently it was demonstrated that recombinant hCG achieves better results – and fish pituitary extract in females (Asturiano et al., 2005; Palstra et al., 2005; Pérez et al., 2008; Peñaranda et al., 2010; Gallego et al., 2012). Hormonal treatment in males results in good quality sperm (Asturiano et al., 2005; Gallego et al., 2012), while the current protocol used for females, both in the case of *A. anguilla* and Japanese eel *Anguilla japonica* Temminick and Schlegel 1847, results in low quality eggs and has a negative impact on embryo survival. Possible causes for the low quality eggs might be: inadequate broodstock nutrition leading to altered lipid accumulation (Seoka et al., 2003), inappropriate maturation techniques (Pedersen, 2004; Kagawa et al., 2005; Horie et al., 2008), or the accumulation of pollutants (Palstra et al., 2006). Vitellogenesis is a complex process controlled by hormones. It involves the brain, pituitary, ovaries and the liver and is influenced by environmental and internal factors. Vitellogenesis is crucial since egg growth and the uptake of the nutrients which will be used for embryo development are dependent on it (Nagahama, 1994; Brooks et al., 1997; Carnevali et al., 2001a,b; Polzonetti-Magni et al., 2004). Among the different hormones involved, the gonadotropins FSH and LH (produced in the pituitary), as well as E2 (synthesized in the ovary), play important roles since they control the hepatic production of vitellogenin (an important precursor of yolk protein), the plasma levels of which affect the final egg quality (Carnevali et al., 2001b; Polzonetti et al., 2002, Lubzens et al., 2010).

The egg composition of *A. japonica* has been investigated in relation to egg quality (Furuita et
al., 2003, 2006, 2007) however, to date, no studies have focused on *A. anguilla*. Hence, further studies on *A. anguilla* oocytes during vitellogenesis are necessary in order to optimize reproduction in this species.

In the last few years, several studies have been carried out in order to gain a better understanding of *A. anguilla* vitellogenesis and zonagenesis (Pérez *et al*., 2011; Mazzeo *et al*., 2012; Peñaranda *et al*., 2013). Due to the complexity of vitellogenesis itself and all the changes that occur during this process, the objective of this study was to compare different techniques which can be employed to study vitellogenesis, in order to evaluate which one is the most suitable in terms of obtained results vs. costs and times, and how information provided by each technique can be intercrossed.

To achieve this, techniques commonly employed in the study of oocyte growth, such as histology, ELISA, SDS-PAGE, and Folch method for determining fat content were used. In addition, Fourier Transform Infrared (FT-IR) Microspectroscopy was employed for the first time with *A. anguilla*. This technique, previously used in the study of zebrafish *Danio rerio* Hamilton 1882 and mummichog *Fundulus heteroclitus* L. 1766 ovaries and single oocytes, is considered a novel and powerful tool for analyzing the macromolecular composition of ovarian structures (Carnevali *et al*., 2009; Giorgini *et al*., 2010; Lombardo *et al*., 2012). Plasma FT-IR has also been shown to be effective in determining the stage of sexual development in sturgeon *Acipenser transmontanus* Richardson 1836 (Lu *et al*., 2013).

**MATERIAL AND METHODS**

**FISH HANDLING**

Thirty-nine silver-stage *A. anguilla* females (660 ± 162 g body mass) were caught by local fishermen between December and March during their reproductive migration from the Albufera
lagoon (Valencia, Spain) to the sea, and transported directly to the Universitat Politècnica de
València (Spain) aquaculture facilities.

The fish were placed in a 1500 l tank with recirculating freshwater and gradually acclimated to
seawater salinity (37) and temperature (18 ± 1 °C) over the course of two weeks. The tank was
covered to maintain constant darkness, thereby reducing stress. Since A. anguilla stop eating at the
beginning of their reproductive migration, they were not fed during the whole experiment. The
fish were handled in accordance with the European Union regulations concerning the protection of
experimental animals (Dir 86/609/EEC) and under the supervision of the University Ethics
Committee.

HORMONAL TREATMENT

After being anesthetized (benzocaine, 60 mg l⁻¹; www.sigmaaldrich.com/) and weighed to
calculate the hormone dosage, the A. anguilla females were treated weekly for 12 weeks with
intra-peritoneal injections of carp pituitary extract (CPE: Catvis, www.catvis.nl) at a dose of 20 mg
kg⁻¹. The CPE was prepared as follows: 1 g of pituitary powder was diluted in 10 ml of NaCl solution
(9 g l⁻¹) and centrifuged at 1260 g for 10 min. The supernatant was collected and stored at -20 °C
until use.

SAMPLING

Once acclimated to seawater salinity and temperature as previously described, eight fish were
sacrificed every four weeks. The animals were anesthetized (benzocaine, 60 mg l⁻¹) before being
weighed and sacrificed by decapitation. Before sacrificing, blood samples were obtained from the
caudal vasculature, and plasma was retrieved by centrifugation (3000 rpm, 15 min) and stored at -
80 °C until further analysis.
The gonads were weighed to calculate the gonad somatic index ($I_G$, ovary mass/body mass*100).

For the histological analysis, gonad samples were preserved in 10% buffered formalin (pH 7.4).

Gonad samples for fat, FT-IR and SDS-PAGE analyses were frozen at -20 °C.

GONAD HISTOLOGY

After dehydration in ethanol, samples were embedded in paraffin and 5-10 µm thick sections were cut with a Shandon Hypercut manual microtome (Shandon, Southern Products Ltd, www.southernbiological.com/). Slides were stained with haematoxilin and eosin and observed through a Nikon Eclipse E-400 microscope and pictures were taken with a Nikon DS-5M camera attached to the microscope (www.nikon.com/).

The stage of oogenesis was determined following the method described by Pérez et al. (2011). The diameters of 100 oocytes from each specimen were measured and the corresponding stage was established on the basis of the most advanced oocyte stage observed in the histological sections. The following stages were observed: Previtellogenic stage (PV): oocyte at perinucleolar and oil droplet stages; Early vitellogenic stage (EV): oocytes with small yolk globules at the periphery of the cytoplasm; Mid-vitellogenic stage (MV): oocytes with bigger yolk globules, widely distributed in the cytoplasm but still with a greater abundance of oil droplets; Late vitellogenic stage (LV): oocytes with more yolk globules than oil droplets.

The most advanced stage observed was the nuclear migration stage (NM), characterized by oocyte hydration and the migration of the nucleus towards the animal pole. However, the NM stage was only reached by one animal and it was not considered in the performed analyses due to the poor meaning of comparing with just one specimen.
ENZYME LINKED IMMUNOSORBENT ASSAY FOR VTG

VTG plasma levels were assayed using a homologous ELISA previously developed for *A. anguilla* (Burzawa Gérard *et al.*, 1991). In summary, purified *A. anguilla* VTG was fixed on 96-well plates, by 24 h incubation at 4 °C (200 ng/well). After washing, non-specific sites were saturated by the addition of 2% pig serum to the wells and incubated for 2 hours at room temperature, and being washed again. Serial dilutions of *A. anguilla* VTG standard, or of *A. anguilla* plasma samples, were pre-incubated with anti-*A. anguilla* Vtg rabbit antiserum (1/100 000 final dilution) for 24 h at 4 °C. The mixtures were added to the wells in duplicate and incubated for 24 h at 4 °C. After washing, anti-rabbit IgG goat antiserum linked to peroxydase was added to each well (1/4000) and incubated for 2 h at room temperature. After washing, peroxydase activity was revealed by ortho-diphenylalanine (ODP) in the presence of H₂O₂, for 15 minutes in the dark at room temperature. The reaction was stopped by the addition of sulfuric acid, and the Optic Density measured using a Appolo LB 913 Spectrophotometer (Berthold Biotechnologies, www.bionity.com). The sensitivity of the ELISA was 1.7 ng ml⁻¹ and the intra and inter-assay variation coefficients were 6.2% and 9.1%, respectively. The sensitivity of the ELISA was 1.7 ng ml⁻¹ (Burzawa Gérard *et al.*, 1991).

SODIUM DODECYL SULFATE–POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS–PAGE)

50 mg of ovary from different stages (PV, EV, MV and LV) were placed in 500 µL Eppendorf tubes containing 10 µL of lysis buffer (10 mM TRIS-HCl, pH 6.8, 1% SDS) and immediately homogenized. The homogenates were then centrifuged at 14000 g for 15 min at 4 °C to separate the dissolved yolk from the insoluble cellular debris. Protein concentration was determined by a Bradford assay (Bradford, 1976). The supernatant was added to the sample buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromphenol blue, 0.125 M TRIS-HCl, pH 6.8) in the proportion of 1:1 and run on SDS-PAGE in stacking (4%) and resolving (10%) acrylamide mini-gels, 7x10 cm (Selman
et al., 1993). Molecular weight standards were placed in the well and electrophoresed at a constant current (60 mA). The protein bands were visualized by fixing the gel in 12% trichloroacetic acid for 30 min, then staining for 45 min in 0.2% Coomassie Blue R-350 (Amersham-Pharmacia Biotech, www.gelifesciences.com) in 30% methanol plus 10% acetic acid, and finally de-staining overnight in 25% methanol and 7% acetic acid (Carnevali et al., 1992).

**FAT EXTRACTION**

Total fat was extracted according to the method described by Folch et al. (1957) with a few modifications. In summary, 1 g of sample was homogenized in 30 ml of Folch solution (2:1, v/v dichloromethane/methanol and 0.75 g l⁻¹ butylhydroxytoluene as antioxidant). Homogenization was carried out in a glass tube with an Ultra216 turrax type of homogenizer. The homogenate was filtered through fat-free Whatmann n. 6 paper into a new glass tube. To separate the mixture into two layers - one with lipids and the other with the non-lipid substances - 3.75 ml of potassium chloride were added and the glass tube was kept at 4 °C overnight. Between 8 to 48 h later, once the two layers had formed, the upper layer containing the non-lipid substances was removed with a vacuum pump. Afterwards, the aqueous phase was removed by adding enough sodium sulphate anhydrous. The fat containing phase was filtered again and evaporated using a centrifuge vacuum concentrator (Scan Speed MaxiVac Alpha, www.labogene.com/) at 35 °C. The fat was weighed and its percentage calculated on the basis of the gonad sample mass.

**FT-IR MEASUREMENTS AND DATA ANALYSIS**

Ovaries from five different specimens were cryosectioned in thin slices at a predefined thickness of 5 μm. Two adjacent slices were obtained from each sample: one of which was placed on silicon supports for the vibrational analysis and the other on conventional glass slides for morphological
examination (haematoxylin and eosin stained). Spectral data were achieved at room temperature by using a Perkin Elmer Spectrum GXI Spectrometer, equipped with a Perkin Elmer Autoimage microscope and a photoconductive HgCdTe, MCT, array detector, operating at liquid nitrogen temperature and covering the entire IR spectral range from 4000 to 700 cm\(^{-1}\). Using the microscope television camera, specific areas of each sample where the tissue distribution appeared homogeneous were selected. In these zones (ca. 600x500 μm), the chemical maps, which represent the total intensity of the infrared absorption with each pixel corresponding to a single spectrum, were acquired in transmission mode, with a spectral resolution of 4 cm\(^{-1}\) and a spatial resolution of 20x20 μm (128 scans), for a total of ca. 750 spectra. Background scans were acquired and rationed against the sample spectrum. For data handling, the following software packages were used: Spectrum Image 1.6 and Spectrum 6.3.1 (Perkin Elmer, www.perkinelmer.com/lab-solutions/default.xhtml), and Grams AI 7.02 (Galactic Industries, www.spectra.co.jp/pdf/grams.pdf). The spectra obtained from each sample were used to build a two points baseline fitted in the spectral range 4000-700 cm\(^{-1}\) and to normalize the vectors (Wood et al., 2004). Second Derivative (9-point smoothing) and Peak Fitting (Gaussian algorithm) procedures were adopted to determine the correct position and absorbance intensity of bands. By using GRAMS/AI 7.02 (Galactic Industries, www.spectra.co.jp/pdf/grams.pdf), peak fitting was performed on average spectra (interpolated in the range 1780-1470 cm\(^{-1}\) and two points baseline fitted), to identify the underlying component bands, the number of peaks together with their center values were carefully individuated according to the second derivative results and fixed before running the iterative process, to obtain the best reconstructed curve (residual close to zero). Correlation maps were obtained by loading second derivative representative spectra onto the chemical maps (Wood et al., 2004). This procedure, which enables the localization of biological components in the sample, correlates a selected spectrum with all the spectra in the map,
affording a colorimetric and numeric scale of correlation percentage. Attribution of the bands was
carried out according to literature (Jackson & Mantsch, 1993, 2002; Pacifico et al., 2003).

STATISTICAL ANALYSIS

After establishing data normality, the data were analyzed by a one way analysis of variance
(ANOVA) followed by a Newman-Keuls post-hoc test. If normality failed, the data were Log
transformed to perform the ANOVA. All the values are expressed as mean ± standard error of
mean (SEM). Differences were considered significant at p<0.05. All statistical procedures were run
using Statgraphics Plus 5.1 (www.statgraphics.com/).

RESULTS

HISTOLOGY

Five different vitellogenic stages during vitellogenesis were observed using histology [Fig. 1]. At
the beginning of the treatment, all the specimens were in the PV stage (I_G: 0.83), which includes
the perinucleolar and oil droplet stages [Fig. 1(a,b)]. The stages progressed in line with the
hormonal treatment, with stages EV (I_G: 2.83), MV (I_G: 5.32) and LV (I_G: 17.26) [Fig. 1(c,d,e)] being
reached. The most advanced stage reached was NM (I_G: 39.26) [Fig. 1(f)], observed in just one
female. Histological observation clearly demonstrated that (i) vitellogenesis was preceded by oil
droplet accumulation [Fig. 1(a,b)]; (ii) VTG uptake occurred in the EV stage [Fig. 1(c)] and continued
until the MV and LV stages [Fig. 1(d,e)]; (iii) in the NM stage, nucleus migration and coalescence of
yolk granules were evident [Fig. 1(f)].

For the remainder of the analyses, specimens were grouped by developmental stage in order to
identify differences relating to the progression of vitellogenesis rather than to the week of
treatment.
PLASMA VTG LEVELS

VTG plasma levels [Fig. 2] increased during early vitellogenesis (EV) and then remained constant until late vitellogenesis (LV). The plasma VTG increase was accompanied by the appearance of yolk vesicles in the oocytes in the EV stage [Fig. 1(c)].

FAT CONTENT

In the ovaries, no differences were found in any of the different phases of vitellogenesis with respect to fat content. However, in spite of the lack of statistical significance, fat levels increased when moving from the PV to the EV stage [Fig. 3].

SDS-PAGE

The presence of yolk proteins in the growing oocyte was confirmed by the appearance of four distinct components with an apparent molecular weight of 100, 60, 30 and 26 kDa in the EV, MV and LV stages, as shown by SDS-PAGE. An additional band with an apparent molecular weight of 15 kDa was observed in the LV stage [Fig. 4].

FT-IR

The comparative analysis of representative spectra allowed the visualization of the changes that occurred in the biochemical composition of the different samples.

With regards to the ovary, when moving from the PV to the LV stage, the uptake of VTG was substantiated by an increase in lipids, proteins, carbohydrates and phosphates. In the region 3100–2800 cm\(^{-1}\), the convoluted band with a maximum at 2926 cm\(^{-1}\) increased between the PV and the LV stage: in particular, the analysis of the 2926/2954 cm\(^{-1}\) (\(\nu_{\text{asym}}\ CH_2/CH_3\)) and 2854/2873 cm\(^{-1}\) (\(\nu_{\text{sym}}\)
CH$_2$/CH$_3$) absorbance band ratios pointed an increase in CH$_2$ moiety [Fig. 5]. According to the vibrational analysis in the spectral region 1800–1900 cm$^{-1}$, between the PV and the LV stages in the ovary, some conclusions can be drawn: (i) the vibrational mode at 1738 cm$^{-1}$ increased; (ii) changes in protein content and secondary structure were observed by analyzing Amide I and Amide II bands; in particular, the bands at 1695 cm$^{-1}$ (β-turn) and at 1631 cm$^{-1}$ (β-sheet) increased, while those at 1640 cm$^{-1}$ (random coil) disappeared; (iii) the increase in the lipidic chain length was confirmed by the increase of the band at 1458 cm$^{-1}$ ($\delta$CH$_2$/3); (iv) the increase in the phosphate groups was demonstrated by the bands at 1239 cm$^{-1}$ ($\nu_{\text{asym}}$ PO$_2^-$) and 1081 ($\nu_{\text{sym}}$ PO$_2^-$); (v) there was a rise in the glucic component, confirmed by the band at 1060 cm$^{-1}$ ($\nu_{\text{sym}}$ CO-O-C) [Fig. 5]. In addition, correlation maps guaranteed the correspondence between the analyzed samples and an average representative spectrum for each ovarian stage (PV, EV, MV and LV), validating the method and illustrating the distribution of the oocytes within the ovary slices [Fig. 6]. At the same time, chemical maps obtained by correlating the region 3100-2800 cm$^{-1}$ (corresponding to CH$_2$ and CH$_3$ moieties) confirmed changes in the concentration of the analyzed components and showed the distribution of the lipids within the ovaries in the different maturational stages [Fig. 6].

**DISCUSSION**

The aim of this study was to integrate information from different techniques to gain a wider and more complete understanding of vitellogenesis in *A. anguilla* and to identify the most exhaustive and convenient technique.

According to the histological analysis of the ovaries, VTG plasma content and fat content follow a similar pattern, with both showing a first phase of lipid accumulation followed by a phase in which vitellogenin is synthesized, transported and accumulated. The reason for this could be that lipids and yolk proteins are the most important macromolecules for the developing future embryo.
(Brooks et al., 1997; Carnevali et al., 2001a,b).

In particular, fat accumulation in the ovary occurs between the PV and the EV stages, as illustrated in particular by histology and fat analysis (in spite of a lack of statistical significance, an increase in ovarian fat content was appreciated). This first stage of vitellogenesis, characterized by the presence of fat, indicates that lipid composition, storage and mobilization are important for successful vitellogenesis and probably deserve more attention in the attempt to optimize breeding.

The progression of vitellogenesis is supported by histology, the increase of VTG plasma content and the SDS-PAGE. Vitellogenin is synthesized by the liver in response to 17β-estradiol produced by the ovary, and is carried in the blood to the ovary (Nagahama, 1994). Hence, VTG plasma content can be an indicator of the progression of vitellogenesis. These results were validated by FT-IR analysis, which showed an increase in vitellogenin during oocyte maturation. In fact, the increase in CH$_2$ moiety and in the vibrational mode at 1738 cm$^{-1}$ were attributable to changes in lipidic backbone as well as amino acid side chains (Wood et al., 2008), and to VTG (Carnevali et al., 2009), respectively.

In _A. anguilla_, in order to classify animals according to their developmental stage, eye index together with skin and fin coloration can also be used, but these are indirect evidences that may help to distinguish migrating from non-migrating individuals (Durif et al., 2005). So, it is important to find a technique which can give a reliable and more direct indication of the developmental stage reached by a specimen.

The advantages and disadvantages of each technique are summarized in Table I. Histology has traditionally been the most employed technique for establishing the developmental stage of fish, since it allows the direct observation of the progression of vitellogenesis through the analysis of the ovaries. Unfortunately, this technique is very time consuming (Brewer et al., 2008) and generates of a lot of waste because of the use of alcohols and chemical reagents. Waste
production is also one of the disadvantages of fat content analysis by Folch method. However, unlike histology, ovarian fat content is not a very good technique for studying the progression of vitellogenesis. In fact, in addition to it also being time consuming, it does not give an immediate result and, above all, fat content cannot be linked exactly to any vitellogenic stage. However, since it has been demonstrated that dietary lipid content affects fatty acid composition and egg quality, studies on fat composition and mobilization – fat content was also studied in other body tissues, including the muscle and the liver – should be performed to establish the energetic needs of maturing *A. anguilla* and to define specific breeder diets (García-Gallego & Akharbach, 1998; Furuita *et al*., 2007).

In addition, VTG plasma content and SDS-PAGE only give partial indications on the progression of vitellogenesis. According to our results, VTG plasma content analysis, which has the advantage of not needing to sacrifice the fish as blood can be retrieved by anesthetized specimens, could be used to distinguish the PV stage from more advanced stages. This is because a significant increase in the levels is only observed in the PV and EV stages, with no differences found in the other stages. When using SDS-PAGE, real differences were found between animals at the very beginning of vitellogenesis and others at more advanced stages, but this technique cannot be used to find out exactly in which stage a specimen is. However, it would be interesting to carry out a more in depth analysis in order to understand which yolk proteins appear during the progression of vitellogenesis. In addition, SDS-PAGE is a fast method, which generates little waste and requires small amounts of tissue.

FT-IR analysis is a novel technique, which was successfully applied to determine the developmental stage in maturing *A. anguilla*. Although specific instruments and trained staff are needed, this technique has the advantage of short analysis times and the possibility of analyzing various molecules of interest at the same time on the same oocyte. In fact, FT-IR also provides
information about the composition of the ovary. Recently, studies on A. anguilla reproduction have been focusing on the effect of nutrition and broodstock diets on egg quality (Heinsbroek et al., 2013; Støttrup et al., 2013). FT-IR could prove a useful tool in this respect, since it allows the study how different diets affect ovarian macromolecular composition and oocyte quality.

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transform infrared (FTIR) spectral mapping of the cervical transformation zone, and

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on the molecular architecture of oocytes using a combination of synchrotron Fourier
Table I. Comparison of the advantages and disadvantages of the various techniques used to study vitellogenesis in *Anguilla anguilla*.

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<th>Technique</th>
<th>Advantages</th>
<th>Disadvantages</th>
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<td>Histology</td>
<td>- Direct observation of the ovary and determination of the developmental stage</td>
<td>- Time consuming</td>
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<td>- Waste production</td>
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<td>Folch method</td>
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<td>- Waste production</td>
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<td>VTG content</td>
<td>- No sacrifice needed</td>
<td>- No direct information on developmental stage</td>
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<td></td>
<td>- Screening of specimens in PV stage vs other stages</td>
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<tr>
<td>SDS-PAGE</td>
<td>- Screening of specimens in PV stage vs other stages</td>
<td>- No direct information on developmental stage</td>
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<td>- Fast</td>
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<td></td>
<td>- Small amount of tissue needed</td>
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<tr>
<td>FT-IR</td>
<td>- Once spectra are acquired, information on developmental stage</td>
<td>- Expensive equipment</td>
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<td>- Fast</td>
<td>- Trained personnel needed</td>
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<td>- Study of macromolecules</td>
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Figures captions

**Fig. 1.** Ovarian developmental stages in *Anguilla anguilla*. A) Previtellogenic (PV), perinucleolar stage; B) Previtellogenic, lipid droplet stage; C) Early vitellogenic (EV) stage; D) Mid vitellogenic (MV) stage; E) Late vitellogenic (LV) stage; F) Nuclear migration (NM) stage. Arrows in C, D, E show the position of the yolk globules, arrow in F points to the nucleus. Scale Bar: A, B, D: 100 µm; C: 50 µm; E, F: 200 µm.

**Fig. 2.** Changes in VTG plasma levels (mg ml\(^{-1}\)) during vitellogenesis according to *Anguilla anguilla* ovarian developmental stage. VTG levels are measured by homologous ELISA. PV: pre-vitellogenesis \((n = 8)\); EV: early vitellogenesis \((n = 9)\); MV: mid-vitellogenesis \((n = 2)\); LV: late vitellogenesis \((n = 6)\). \(P < 0.05\).

**Fig. 3.** Changes in *Anguilla anguilla* ovarian fat content during ovarian development according to developmental stage. Fat content was measured by Folch method. PV: pre-vitellogenesis \((n = 3)\); EV: early vitellogenesis \((n = 10)\); MV: mid-vitellogenesis \((n = 2)\); LV: late vitellogenesis \((n = 6)\). \(P < 0.05\).

**Fig. 4.** SDS-PAGE showing changes in protein during *Anguilla anguilla* oocyte development. PV: pre-vitellogenesis; EV: early vitellogenesis; MV: mid-vitellogenesis; LV: late vitellogenesis; NM: nuclear migration.
**Fig. 5.** Representative spectra of the different stages of *Anguilla anguilla* oocyte development analyzed by FT-IR analysis. PV: pre-vitellogenesis; EV: early vitellogenesis; MV: mid-vitellogenesis; LV: late vitellogenesis. The meaningful peaks are labelled.

**Fig. 6.** A: Photomicrographs of *Anguilla anguilla* ovary sections from fish at different vitellogenic stages (PV: pre-vitellogenesis; EV: early vitellogenesis; MV: mid-vitellogenesis; LV: late vitellogenesis). B: Correlation maps of different vitellogenic stages (PV, EV, MV and LV) integrated under the corresponding representative spectrum. C: Chemical maps integrated under the 3010-2800 cm\(^{-1}\) region corresponding to lipids. The color scale indicates the intensity of the signal detected.
Fig. 1
Fig. 2

(SigmaPlot)
Fig. 3.
(SigmaPlot)
Fig. 5
B
Chemical maps integrated under 4000-800 cm\(^{-1}\)

C
3100-2800 cm\(^{-1}\)
CH2 and CH3
200 μm