

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

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A multi-technique approach was used to study the changes occurring in European eel *Anguilla anguilla* ovaries during hormonally-induced vitellogenesis. Aside from classic techniques used to monitor the vitellogenic process, such as ovary histology, fat content analysis, sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and vitellogenin enzyme linked immunosorbent assay (ELISA), a new technique, Fourier-transform infrared (FT-IR) microspectroscopy, was used to analyse *A. anguilla* ovaries. 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 (previtellogenic stage, early vitellogenic stage, mid-vitellogenic stage and late vitellogenic stage), demonstrating that it is a valid method able to illustrate the distribution of the oocytes within the ovary slices. The chemical maps obtained 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 with 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 analysing various biocomponents of the same oocyte.

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Key words: ELISA; Folch; FT-IR; ovary; SDS–PAGE; vitellogenesis.

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 5000–6000 km migration to reach the Sargasso Sea, the supposed spawning area (van Ginneken & Maes, 2005).

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Like all anguillid species, *A. anguilla* does not mature in captivity unless hormonally stimulated. Human chorionic gonadotropin (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 & Schlegel 1846, results in low quality eggs and has a negative influence 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 that 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, follicle-stimulating hormone and luteinizing hormone, both produced in the pituitary, as well as oestradiol (E2), synthesized in the ovary, play important roles since they control the hepatic production of vitellogenin (VtG) (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), but as far as is known, no studies previous 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 recent 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 that can be employed to study vitellogenesis, in order to evaluate which one is the most suitable in terms of obtained results *v.* costs and time and how information provided by each technique can be cross validated.

To achieve this, techniques commonly employed in the study of oocyte growth, such as histology, enzyme linked immunosorbent assay (ELISA), sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and Folch method for determining fat content were used. In addition, Fourier transform infrared (FT-IR) microspectroscopy was employed, possibly 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 analysing 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 (mean \pm s.d., 660 ± 162 g whole-body mass) were caught by local fishermen between December and March during their reproductive migration from the Albufera Lagoon, Valencia, Spain ($39^{\circ} 21' N$; $00^{\circ} 21' W$), 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 fresh water and gradually acclimated to seawater salinity (37) and temperature ($18^{\circ} C$, range $\pm 1^{\circ} C$) over the course of 2 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 (E.U., 1986) and under the supervision of the university ethics committee.

HORMONAL TREATMENT

After being anesthetized with 60 mg l^{-1} benzocaine (Sigma-Aldrich; 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 $1260g$ for 10 min. The supernatant was collected and stored at $-20^{\circ} C$ until use.

SAMPLING

Once acclimated to seawater salinity and temperature as previously described, eight fish were sacrificed every 4 weeks. The animals were anaesthetized (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 ($675g$, 15 min) and stored at $-80^{\circ} C$ until further analysis.

The gonads were weighed to calculate the gonad-somatic index ($I_G = M_O M_B^{-1} \times 100$, where M_O is ovary mass and M_B is body mass). 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 $-20^{\circ} C$.

GONAD HISTOLOGY

After dehydration in ethanol, samples were embedded in paraffin wax and $5\text{--}10 \mu\text{m}$ thick sections were cut with a Shandon Hypercut manual microtome (Shandon, Southern Products Ltd; www.southernbiological.com). Slides were stained with haematoxylin 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 (PV) stage, oocyte at perinuclear and oil droplet stages; early vitellogenic (EV) stage, oocytes with small yolk globules at the periphery of the cytoplasm; mid-vitellogenic (MV) stage, oocytes with bigger yolk globules, widely distributed in the cytoplasm but still with a greater abundance of oil droplets; late vitellogenic (LV) stage, oocytes with more yolk globules than oil droplets.

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

ENZYME LINKED IMMUNOSORBENT ASSAY FOR VITELLOGENIN

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⁻¹). After washing, non-specific sites were saturated by the addition of 2% pig serum to the wells and incubated for 2 h 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 (10⁻⁵ 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 immunoglobulin goat antiserum linked to peroxydase was added to each well (4 × 10⁻³) and incubated for 2 h at room temperature. After washing, peroxydase activity was revealed by ortho-diphenylalanine in the presence of H₂O₂, for 15 min in the dark at room temperature. The reaction was stopped by the addition of sulphuric 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 (Burzawa-Gérard *et al.*, 1991) and the intra and inter-assay variation coefficients were 6.2 and 9.1%, respectively.

SODIUM DODECYL SULPHATE-POLYACRYLAMIDE GEL ELECTROPHORESIS

Fifty 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% sodium dodecyl sulphate (SDS)] and immediately homogenized. The homogenates were then centrifuged at 14 000g 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% bromophenol 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, 7 × 10 cm (Selman *et al.*, 1993). Molecular mass 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 (IKA; www.ika.com). The homogenate was filtered through fat-free Whatmann no. 6 paper (GE Healthcare Life Sciences; www.gelifesciences.com) 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 KCl were added and the glass tube was kept at 4° C overnight. Between 8 and 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 anhydrous Na₂SO₄. 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 cryo-sectioned 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 (www.perkinelmer.com), equipped with a Perkin Elmer Autoimage microscope and a photoconductive mercury–cadmium–telluride 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 (*c.* 600 \times 500 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 20 \times 20 μm (128 scans), for a total of *c.* 750 spectra. Background scans were acquired and rationed against the sample spectrum. The following software packages were used for data handling: Spectrum Image 1.6 and Spectrum 6.3.1 (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-point baseline fitted in the spectral range 4000–700 cm^{-1} and to normalize the vectors (Wood *et al.*, 2004). Second derivative (nine-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, peak fitting was performed on average spectra (interpolated in the range 1780–1470 cm^{-1} and two-point baseline fitted), to identify the underlying component bands, the number of peaks together with their centre values were carefully identified 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 analysed by a one-way ANOVA followed by a Newman-Keuls *post hoc* test. If normality failed, the data were \log_{10} transformed to perform the ANOVA. All the values are expressed as mean \pm s.d. of mean. 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)–(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 (1) vitellogenesis was preceded by oil droplet accumulation [Fig. 1(a), (b)]; (2) VtG uptake occurred in the EV stage [Fig. 1(c)] and continued until the MV and LV stages [Fig. 1(d), (e)]; (3) 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.



FIG. 1. Ovarian developmental stages in *Anguilla anguilla*. (a) Previtellogenic (PV), perinucleolar stage, (b) PV, lipid-droplet stage, (c) early vitellogenic (EV) stage, (d) mid vitellogenic (MV) stage, (e) late vitellogenic (LV) stage and (f) nuclear migration (NM) stage. † (c)–(e) position of the yolk globules; † (f) the nucleus. Scale bar: (a), (b) and (d): 100 μm ; (c): 50 μm ; (e) and (f): 200 μm .

PLASMA VTG LEVELS

VtG plasma levels (Fig. 2) increased during EV and then remained constant until 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. In spite of the lack of statistical significance, fat levels increased when moving from the PV to the EV stage (Fig. 3).

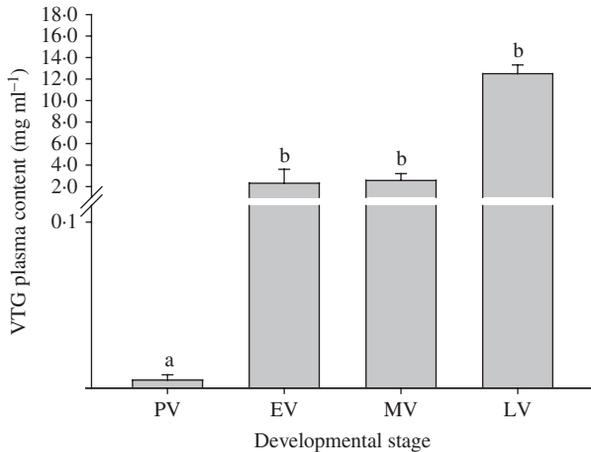


FIG. 2. Changes in mean \pm s.d. VtG plasma levels during vitellogenesis according to *Anguilla anguilla* ovarian developmental stage. VtG levels are measured by homologous ELISA. PV, previtellogenesis ($n = 8$); EV, early vitellogenesis ($n = 9$); MV, mid-vitellogenesis ($n = 2$); LV, late vitellogenesis ($n = 6$). Different lower-case letters indicate significant differences: $P < 0.05$.

SODIUM DODECYL SULPHATE–POLYACRYLAMIDE GEL ELECTROPHORESIS

The presence of yolk proteins in the growing oocyte was confirmed by the appearance of four distinct components with an apparent molecular mass 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 mass of 15 kDa was observed in the LV stage (Fig. 4).

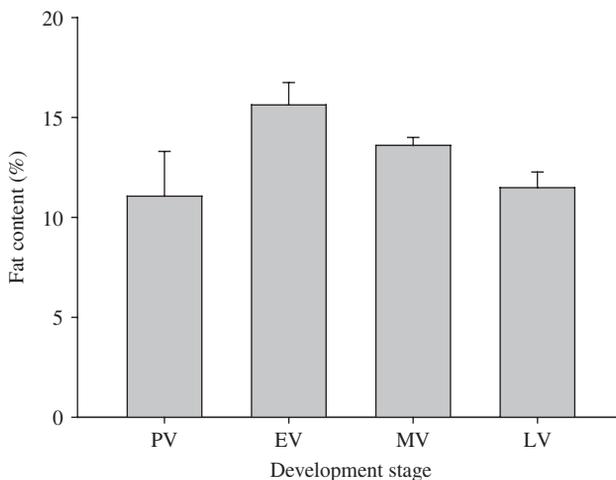


FIG. 3. Changes in mean \pm s.d. *Anguilla anguilla* ovarian fat content during ovarian development according to developmental stage. Fat content was measured by Folch method. PV, pre-vitellogenesis ($n = 3$); EV, early vitellogenesis ($n = 10$); MV, mid-vitellogenesis ($n = 2$); LV, late vitellogenesis ($n = 6$).

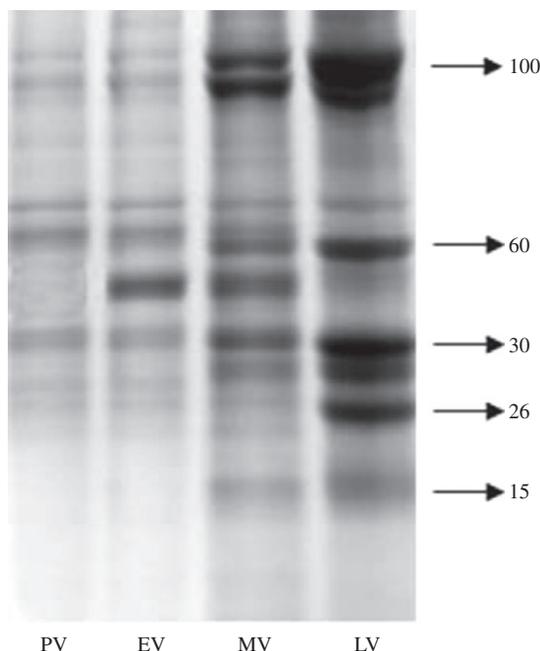


FIG. 4. SDS-PAGE showing changes in protein during *Anguilla anguilla* oocyte development. PV, pre-vitellogenesis; EV, early vitellogenesis; MV, mid-vitellogenesis; LV, late vitellogenesis; NM, nuclear migration.

FOURIER TRANSFORM INFRARED

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 $3100\text{--}2800\text{ cm}^{-1}$, the following absorption bands were found: 2954 and 2926 cm^{-1} (asymmetric stretching modes of CH_3 and CH_2 moieties, named respectively $n_{\text{asym}}\text{CH}_3$ and $n_{\text{asym}}\text{CH}_2$); 2873 and 2854 cm^{-1} (symmetric stretching modes of CH_3 and CH_2 moieties, named respectively $n_{\text{sym}}\text{CH}_3$ and $n_{\text{sym}}\text{CH}_2$). The analysis of the $2926\text{--}2954\text{ cm}^{-1}$ ($n_{\text{asym}}\text{CH}_2\text{--}n_{\text{asym}}\text{CH}_3$) and $2854\text{--}2873\text{ cm}^{-1}$ ($n_{\text{sym}}\text{CH}_2\text{--}n_{\text{sym}}\text{CH}_3$) absorbance-band ratios pin-pointed an increase in CH_2 moiety in PV stage with respect to LV one (Fig. 5). According to the vibrational analysis in the spectral region $1800\text{--}1900\text{ cm}^{-1}$, between the PV and the LV stages in the ovary, some conclusions can be drawn: (1) the vibrational mode at 1738 cm^{-1} (stretching vibration of $\text{C}=\text{O}$ ester in lipids) increased; (2) changes in protein content and secondary structure were observed by analysing amide I and amide II bands; in particular, the bands at 1695 cm^{-1} (due to β -turn structures) and at 1631 cm^{-1} (due to β -sheet structures) increased, while that at 1640 cm^{-1} (representative of random coil components) disappeared; (3) the increase in the lipid-chain length was confirmed by the increase of the band at 1458 cm^{-1} (bending vibrations of CH_2 and CH_3 moieties, named $d\text{CH}_{2/3}$); (4) the increase in the phosphate groups was demonstrated by the bands at 1239 cm^{-1} (asymmetric stretching mode of phosphate groups, named $n_{\text{asym}}\text{PO}_2^-$) and 1081 (symmetric stretching mode

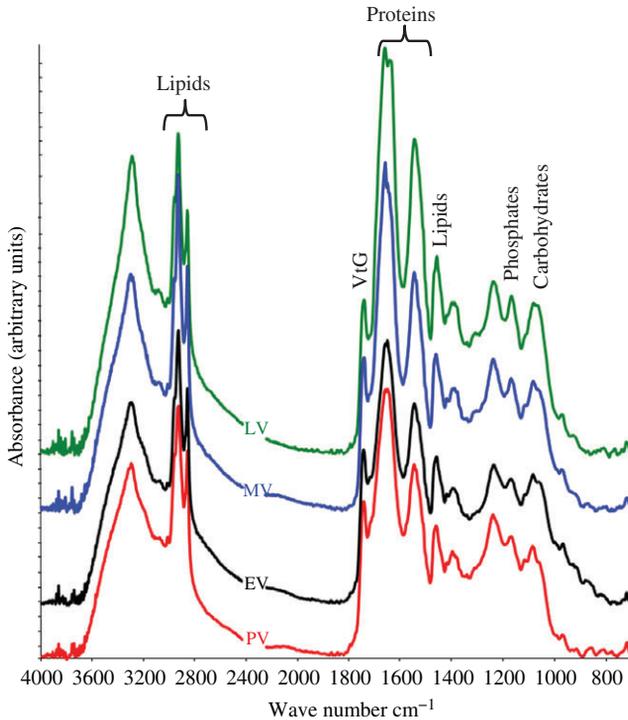


FIG. 5. Representative spectra of the different stages of *Anguilla anguilla* oocyte development analysed by FT-IR analysis. LV, pre-vitellogenesis; EV, early vitellogenesis; MV, mid-vitellogenesis; PV, late vitellogenesis. The meaningful peaks are labelled.

of phosphate groups, named $n_{\text{sym}} \text{PO}_2^-$); (5) there was a rise in the glucidic component, confirmed by the band at 1060 cm^{-1} (symmetric stretching mode of C–O bond in sugars, named $n_{\text{sym}} \text{CO–O–C}$) (Fig. 5). In addition, correlation maps guaranteed the correspondence between the analysed 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 $3100\text{--}2800 \text{ cm}^{-1}$ (corresponding to CH_2 and CH_3 moieties) confirmed changes in the concentration of the analysed 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 VtG is synthesized, transported and accumulated. The reason for this could be that lipids and

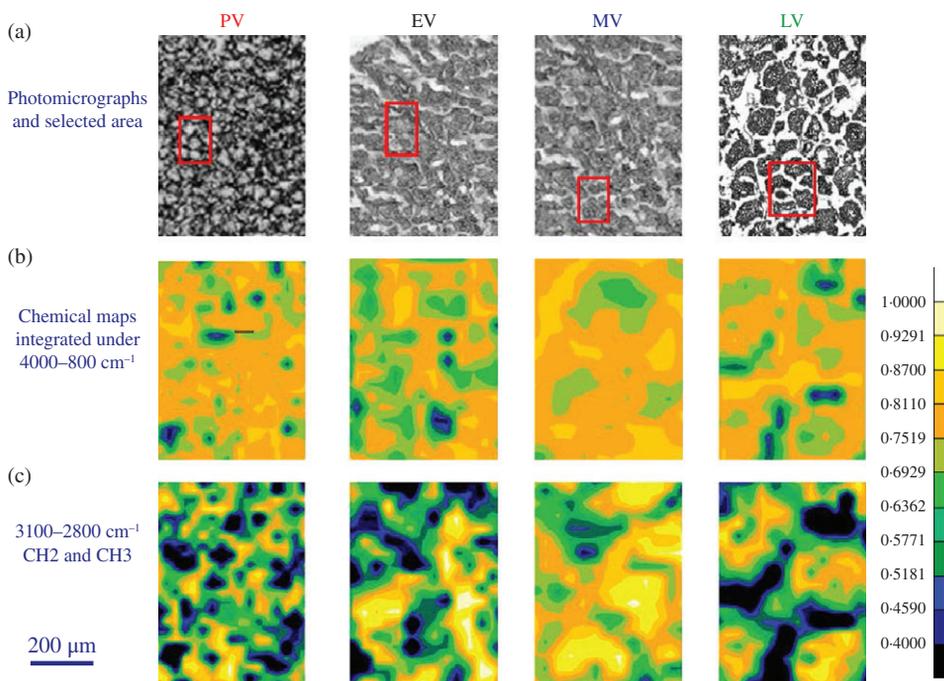


FIG. 6. (a) Photomicrographs of *Anguilla anguilla* ovary sections from fish at different vitellogenic stages: PV, pre-vitellogenesis; EV, early vitellogenesis; MV, mid-vitellogenesis; LV, late vitellogenesis. (b) Correlation maps of different vitellogenic stages (PV, EV, MV and LV) integrated under the corresponding representative spectrum. (c) Chemical maps integrated under the 3100–2800 cm^{-1} region corresponding to lipids. The colour scale indicates the intensity of the signal detected.

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 apparent). 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. VtG is synthesized by the liver in response to 17β -oestradiol 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 VtG 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 colouration can also be used, but these are indirect

TABLE I. Comparison of the advantages and disadvantages of the various techniques used to study vitellogenesis in *Anguilla anguilla*

Technique	Advantages	Disadvantages
Histology	Direct observation of the ovary and determination of the developmental stage	Time consuming Waste production
Folch method	Basis to study mobilization and nutrient requests	No direct information on developmental stage Time consuming Waste production
VtG content	No sacrifice needed Screening of specimens in PV stage v. other stages	No direct information on developmental stage
SDS–PAGE	Fast Small amount of tissue needed Screening of specimens in PV stage v. other stages	No direct information on developmental stage
FT-IR	Fast Study of macromolecules Once spectra are acquired, information on developmental stage	Expensive equipment Trained personnel needed

VtG, vitellogenin; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; FT-IR, Fourier-transform infrared microspectroscopy; PV, previtellogenic.

evidences that may help to distinguish migrating from non-migrating individuals (Durif *et al.*, 2005). So, it is important to find a technique that 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 fishes, 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 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. Unlike histology, however, ovarian-fat content is not a very good technique for studying the progression of vitellogenesis. 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. Since it has been demonstrated, however, 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 the results presented here, VtG plasma content analysis, which has the advantage of not needing to sacrifice the fish as blood can be retrieved from anaesthetized 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. It would be interesting, however, 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 analysing various molecules of interest at the same time on the same oocyte. 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; Strøttrup *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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References

- Asturiano, J. F., Pérez, L., Garzón, D. L., Peñaranda, D. S., Marco-Jiménez, F., Martínez-Llorens, S., Tomás, A. & Jover, M. (2005). Effect of different methods for the induction of spermiation on semen quality in European eel. *Aquaculture Research* **36**, 1480–1487. doi: 10.1111/j.1365-2109.2005.01366.x
- Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**, 248–254.
- Brewer, S. K., Rabeni, C. F. & Papoulias, D. M. (2008). Comparing histology and gonadosomatic index for determining spawning condition of small-bodied riverine fishes. *Ecology of Freshwater Fish* **17**, 54–58. doi: 10.1111/j.1600-0633.2007.00256.x
- Brooks, S., Tyler, C. R. & Sumpter, J. P. (1997). Egg quality in fish: what makes a good egg? *Reviews in Fish Biology and Fisheries* **7**, 387–416. doi: 10.1023/A:1018400130692
- Burzawa-Gérard, E., Nath, P., Baloche, S. & Peyon, P. (1991). ELISA (enzyme-linked-immunosorbent-assay) for vitellogenin and vitellus in the eel (*Anguilla anguilla* L.) and in the Indian major carp (*Labeo rohita*). In *Reproductive Physiology of Fish* (Scott, A. P., Sumpter, J. P., Kime, D. E. & Rolfe, M. S., eds), pp. 319–320. Sheffield: Fish Symposium 91.
- Carnevali, O., Mosconi, G., Roncarati, A., Belvedere, P., Romano, M. & Limatola, E. (1992). Changes in the electrophoretic pattern of yolk proteins during vitellogenesis in the gilthead seabream *Sparus aurata* L. *Comparative Biochemistry and Physiology. B* **103**, 955–962. doi: 10.1016/0305-0491(92)90222-D
- Carnevali, O., Mosconi, G., Cardinali, M., Meiri, I. & Polzonetti Magni, A. M. (2001a). Molecular components related with egg viability in the sea bream *Sparus aurata*. *Molecular Reproduction and Development* **58**, 330–335. doi: 10.1002/1098-2795(200103)58:3<330
- Carnevali, O., Mosconi, G., Cambi, A., Ridolfi, S., Zanuy, S. & Polzonetti-Magni, A. M. (2001b). Changes of lysosomal enzyme activities in sea bass *Dicentrarchus labrax* egg and developing embryo. *Aquaculture* **202**, 249–256. doi: 10.1016/S0044-8486(01)00775-X
- 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. *Journal of Molecular Structure* **938**, 207–213. doi: 10.1016/j.molstruc.2009.09.029
- Durif, C., Dufour, S. & Elie, P. (2005). The silvering process of *Anguilla anguilla*: a new classification from the yellow resident to the silver migrating stage. *Journal of Fish Biology* **66**, 1025–1043. doi: 10.1111/j.1095-8649.2005.00662.x
- Folch, J., Lees, M. & Sloane Stanley, G. H. (1957). A simple method for the isolation and purification of total lipides from animal tissues. *The Journal of Biological Chemistry* **226**, 497–509.
- Furuita, H., Ohta, H., Unuma, T., Tanaka, H., Kagawa, H., Suzuki, N. & Yamamoto, T. (2003). Biochemical composition of eggs in relation to egg quality in the Japanese eel, *Anguilla japonica*. *Fish Physiology and Biochemistry* **29**, 37–46.
- Furuita, H., Unuma, T., Nomura, K., Tanaka, H., Okuzawa, K., Sugita, T. & Yamamoto, Y. (2006). Lipid and fatty acid composition of eggs producing larvae with high survival rate in the Japanese eel. *Journal of Fish Biology* **69**, 1178–1189. doi: 10.1111/j.1095-8649.2006.01196.x
- Furuita, H., Hori, K., Suzuki, N., Sugita, T. & Yamamoto, T. (2007). Effect of n-3 and n-6 fatty acids in broodstock diet on reproduction and fatty acid composition of broodstock and eggs in the Japanese eel *Anguilla japonica*. *Aquaculture* **267**, 55–61. doi: 10.1016/j.aquaculture.2007.01.039
- Gallego, V., Mazzeo, I., Vílchez, M. C., Peñaranda, D. S., Carneiro, P. C. F., Pérez, L. & Asturiano, J. F. (2012). Study of the effects of thermal regime and alternative hormonal treatments on the reproductive performance of European eel males (*Anguilla anguilla*) during induced sexual maturation. *Aquaculture* **354–355**, 7–16. doi: 10.1016/j.aquaculture.2012.04.041
- García-Gallego, M. & Akhbarach, H. (1998). Evolution of body composition of European eels during their growth phase in a fish farm, with special emphasis on the lipid component. *Aquaculture International* **6**, 345–356.
- van Ginneken, V. & Maes, G. (2005). The European eel (*Anguilla anguilla*, Linnaeus), its lifecycle, evolution and reproduction: a literature review. *Reviews in Fish Biology and Fisheries* **15**, 367–398. doi: 10.1007/s11160-006-0005-8
- Giorgini, E., Conti, C., Ferraris, P., Sabbatini, S., Tosi, G., Rubini, C., Vaccari, L., Gioacchini, G. & Carnevali, O. (2010). Effects of *Lactobacillus rhamnosus* on zebrafish oocyte maturation: an FT-IR imaging and biochemical analysis. *Analytical and Bioanalytical Chemistry* **398**, 3063–3072. doi: 10.1007/s00216-010-4234-2
- Heinsbroek, L. T. N., Støttrup, J. G., Jacobsen, C., Corraze, G., Kraiem, M. M., Holst, L. K., Tomkiewicz, J. & Kaushik, S. J. (2013). A review on broodstock nutrition of marine pelagic spawners: the curious case of the freshwater eels. *Aquaculture Nutrition* **19**, 1–24. doi: 10.1111/anu.12091
- Horie, N., Utoh, T., Mikawa, N., Yamada, Y., Okamura, A., Tanaka, S. & Tsukamoto, K. (2008). Influence of artificial fertilization methods of the hormone-treated Japanese eel *Anguilla japonica* upon the quality of eggs and larvae (comparison between stripping-insemination and spontaneous spawning methods). *Nippon Suisan Gakkaishi* **74**, 26–35.
- Jackson, M. & Mantsch, H. H. (1993). Biomembrane structure from FT-IR spectroscopy. *Spectrochimica Acta Reviews* **15**, 53–69.
- Jackson, M. & Mantsch, H. H. (2002). Pathology by infrared and Raman spectroscopy. In *Handbook of Vibrational Spectroscopy*, Vol. 5 (Chalmers, J. M. & Griffiths, P. R., eds), pp. 3227–3245. Chichester: Wiley.
- Kagawa, H., Tanaka, H., Ohta, H., Unuma, T. & Nomura, K. (2005). The first success of glass eel production in the world: basic biology on fish reproduction advances new applied technology in aquaculture. *Fish Physiology and Biochemistry* **31**, 193–199. doi: 10.1007/s10695-006-0024-3
- Lombardo, F., Giorgini, E., Gioacchini, G., Maradonna, F., Ferraris, P. & Carnevali, O. (2012). Melatonin effects on *Fundulus heteroclitus* reproduction. *Reproduction, Fertility and Development* **24**, 794–803. doi: 10.1071/RD11267
- Lu, X., Talbott, M. J., Eenennaam, J. P. V., Webb, M. A. H., Doroshov, S. I., Ovissipour, M. & Rasco, B. (2013). Determining ovarian maturity in farmed sturgeon (*Acipenser transmontanus*) for caviar production using Fourier transform infrared spectroscopy (FT-IR). *Journal of Aquaculture Research & Development* **5**, 1. doi: 10.4172/2155-9546.1000202

- Lubzens, E., Young, G., Bobe, J. & Cerdà, J. (2010). Oogenesis in teleosts: how fish eggs are formed. *General and Comparative Endocrinology* **165**, 367–389. doi: 10.1016/j.ygcen.2009.05.022
- Mazzeo, I., Peñaranda, D. S., Gallego, V., Hildahl, J., Nourizadeh-Lillabadi, R., Asturiano, J. F., Pérez, L. & Weltzien, F.-A. (2012). Variations in the gene expression of zona pellucida proteins, *zpb* and *zpc*, in female European eel (*Anguilla anguilla*) during induced sexual maturation. *General and Comparative Endocrinology* **178**, 338–346. doi: 10.1016/j.ygcen.2012.06.003
- Nagahama, Y. (1994). Endocrine regulation of gametogenesis in fish. *The International Journal of Developmental Biology* **38**, 217–229.
- Pacifico, A., Chiriboga, L., Lasch, P. & Diem, M. (2003). Infrared spectroscopy of cultured cells. II. Spectra of exponentially growing, serum-deprived and confluent cells. *Vibrational Spectroscopy* **32**, 107–115. doi: 10.1016/S0924-2031(03)00051-1
- Palstra, A. P., Cohen, E. G. H., Niemantsverdriet, P. R. W., van Ginneken, V. J. T. & van den Thillart, G. E. E. J. M. (2005). Artificial maturation and reproduction of European silver eel: development of oocytes during final maturation. *Aquaculture* **249**, 533–547. doi: 10.1016/j.aquaculture.2005.04.031
- Palstra, A. P., van Ginneken, V. J. T., Murk, A. J. & van den Thillart, G. E. E. J. M. (2006). Are dioxin-like contaminants responsible for the eel (*Anguilla anguilla*) drama? *Naturwissenschaften* **93**, 145–148. doi: 10.1007/s00114-005-0080-z
- Pedersen, B. H. (2004). Fertilisation of eggs, rate of embryonic development and hatching following induced maturation of the European eel *Anguilla anguilla*. *Aquaculture* **237**, 461–473. doi: 10.1016/j.aquaculture.2004.04.019
- Peñaranda, D. S., Pérez, L., Gallego, V., Jover, M., Baloché, S., Dufour, S. & Asturiano, J. F. (2010). Molecular and physiological study of the artificial maturation process in the European eel males: from brain to testis. *General and Comparative Endocrinology* **166**, 160–171. doi: 10.1016/j.ygcen.2009.08.006
- Peñaranda, D. S., Mazzeo, I., Hildahl, J., Gallego, V., Nourizadeh-Lillabadi, R., Pérez, L., Asturiano, J. F. & Weltzien, F.-A. (2013). Molecular characterization of three GnRH receptor paralogs in the European eel, *Anguilla anguilla*: tissue-distribution and changes in transcript abundance during artificially induced sexual development. *Molecular and Cellular Endocrinology* **369**, 1–14. doi: 10.1016/j.mce.2013.01.025
- Pérez, L., Peñaranda, D. S., Jover, M. & Asturiano, J. F. (2008). Results of maturation and ovulation in European eel females. *Cybius* **32**, 320.
- Pérez, L., Peñaranda, D. S., Dufour, S., Baloché, S., Palstra, A. P., van den Thillart, G. E. E. J. M. & Asturiano, J. F. (2011). Influence of temperature regime on endocrine parameters and vitellogenesis during experimental maturation of European eel (*Anguilla anguilla*) females. *General and Comparative Endocrinology* **17**, 51–59. doi: 10.1016/j.ygcen.2011.08.009
- Polzonetti, V., Cardinali, M., Mosconi, G., Natalini, P., Meiri, I. & Carnevali, O. (2002). Cyclic ADPR and calcium signaling in seabream (*Sparus aurata*) egg fertilization. *Molecular Reproduction and Development* **61**, 213–217. doi: 10.1002/mrd.1150
- Polzonetti-Magni, A. M., Mosconi, G., Soverchia, L., Kikuyama, S. & Carnevali, O. (2004). Multihormonal control of vitellogenesis in lower vertebrates. *International Review of Cytology* **239**, 1–46. doi: 10.1016/S0074-7696(04)39001-7
- Selman, K., Wallace, R. A., Sarka, A. & Xiaoping, Q. (1993). Stages of oocyte development in the zebrafish, *Brachydanio rerio*. *Journal of Morphology* **218**, 203–224. doi: 10.1002/jmor.1052180209
- Seoka, M., Yamada, S., Iwata, Y., Yanagisawa, T., Nakagawa, T. & Kumai, H. (2003). Differences in the biochemical content of buoyant and non-buoyant eggs of the Japanese eel, *Anguilla japonica*. *Aquaculture* **216**, 355–362.
- Strøttrup, J. C., Jacobsen, C., Tomkiewicz, J. & Jarlbæk, H. (2013). Modifications of essential fatty acids composition in broodstock of cultured European eel *Anguilla anguilla* L. *Aquaculture Nutrition* **19**, 172–185. doi: 10.1111/j.1365-2095.2012.00967.x
- Wood, B. R., Chiriboga, L., Yee, H., Quinn, M. A., McNaughton, D. & Diem, M. (2004). Fourier transform infrared (FT-IR) spectral mapping of the cervical transformation zone and dysplastic squamous epithelium. *Gynecologic Oncology* **93**, 59–68. doi: 10.1016/j.ygyno.2003.12.028

- Wood, B. R., Chernenko, T., Matthäus, C., Diem, M., Chong, C., Bernhard, U., Jene, C., Brandli, A. A., McNaughton, D., Tobin, M. J., Trounson, A. & Lacham-Kaplan, O. (2008). Shedding new light on the molecular architecture of oocytes using a combination of synchrotron Fourier transform-infrared and Raman spectroscopic mapping. *Analytical Chemistry* **80**, 9065–9072. doi: 10.1021/ac8015483

Electronic Reference

- E.U (1986). Council Directive 86/609/EEC on the approximation of laws, regulations and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes. *Official Journal of the European Communities* **L 358**, 1–28. Available at <http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:31986L0609&from=EN/>.