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

2 ***anguilla***

3

4 I. Mazzeo*¶, E. Giorgini¶, G. Gioacchini¶, F. Maradonna¶, M.C. Vílchez*, S.

5 Baloche‡, S. Dufour‡, L. Pérez*, O. Carnevali¶, and J.F. Asturiano*∫

6

7 *Grupo de Acuicultura y Biodiversidad. Instituto de Ciencia y Tecnología Animal. Universitat

8 Politècnica de València, 46022 Valencia, Spain,

9 ¶Dipartimento di Scienze della Vita e dell'Ambiente, Università Politecnica delle Marche, via Brecce

10 Bianche, 60131 Ancona, Italy and

11 ‡ Laboratoire de Biologie des Organismes et Ecosystèmes Aquatiques (BOREA), UMR CNRS 7208,

12 IRD 207, UPMC, Muséum d'Histoire Naturelle. Paris, France

13

14

15

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

17

18

19 ∫ Author to whom correspondence should be addressed. Tel.: +34 96 387 93 85; Fax: +34 96 387 74

20 39; email: jfastu@dca.upv.es

21

22

23 **ABSTRACT**

24 A multi-technique approach was used to study the changes occurring in European eel *Anguilla*

25 *anguilla* ovaries during hormonally-induced vitellogenesis. Aside from classic techniques used to

26 monitor the vitellogenic process, such as ovary histology, fat content analysis, sodium dodecyl

27 sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and vitellogenin enzyme linked

28 immunosorbent assay (ELISA), a new technique, Fourier Transform Infrared (FT-IR)

29 Microspectroscopy, was used for the first time to analyze *A. anguilla* ovaries. The results from the

30 different techniques provided different ways of approaching the same process. Although it is

31 considered a time consuming approach, of all the employed techniques, histology provided the

32 most direct evidences about vitellogenesis. SDS-PAGE and ELISA were also useful for studying

33 vitellogenesis, whereas fat analysis cannot be used for this purpose. The FT-IR analysis provided a

34 representative IR spectrum for each ovarian stage (PV, EV, MV and LV), demonstrating that it is a

35 valid method able to illustrate the distribution of the oocytes within the ovary slices. The obtained

36 chemical maps confirmed changes in lipid concentrations, and revealed their distribution within

37 the oocytes at different maturational stages. When the results and the accuracy of the FT-IR

38 analysis were compared to those of the traditional techniques commonly used to establish the

39 vitellogenic stage, it became evident that FT-IR is a useful and reliable tool, with many advantages,

40 including the fact that it requires little biological material, the costs involved are low, analysis times

41 are short, and, last but not least, the fact that it offers the possibility of simultaneously analyzing

42 various biocomponents of the same oocyte.

43

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

45

46

INTRODUCTION

48 The European eel *Anguilla anguilla* L. 1758 has a peculiar life cycle which is not yet fully

49 understood. It is a semelparous species which undergoes a 5-6,000 km migration to reach the

50 Sargasso Sea, the supposed spawning area (van Ginneken & Maes, 2005).

51 The *A. anguilla*, like all Anguillid species, does not mature in captivity unless hormonally

52 stimulated. hCG is commonly used in males – although recently it was demonstrated that

53 recombinant hCG achieves better results – and fish pituitary extract in females (Asturiano *et al*.,

54 2005; Palstra *et al*., 2005; Pérez *et al*., 2008; Peñaranda *et al*., 2010; Gallego *et al*., 2012).

55 Hormonal treatment in males results in good quality sperm (Asturiano *et al*., 2005; Gallego *et al*.,

56 2012), while the current protocol used for females, both in the case of *A. anguilla* and Japanese eel

57 *Anguilla japonica* Temminick and Schlegel 1847, results in low quality eggs and has a negative

58 impact on embryo survival. Possible causes for the low quality eggs might be: inadequate

59 broodstock nutrition leading to altered lipid accumulation (Seoka *et al*., 2003), inappropriate

60 maturation techniques (Pedersen, 2004; Kagawa *et al*., 2005; Horie *et al*., 2008), or the

61 accumulation of pollutants (Palstra *et al*., 2006). Vitellogenesis is a complex process controlled by

62 hormones. It involves the brain, pituitary, ovaries and the liver and is influenced by environmental

63 and internal factors. Vitellogenesis is crucial since egg growth and the uptake of the nutrients

64 which will be used for embryo development are dependent on it (Nagahama, 1994; Brooks *et al*.,

65 1997; Carnevali *et al*., 2001a,b; Polzonetti-Magni *et al*., 2004). Among the different hormones

66 involved, the gonadotropins FSH and LH (produced in the pituitary), as well as E2 (synthesized in

67 the ovary), play important roles since they control the hepatic production of vitellogenin (an

68 important precursor of yolk protein), the plasma levels of which affect the final egg quality

69 (Carnevali *et al*., 2001b; Polzonetti *et al.,* 2002, Lubzens *et al*., 2010).

70 The egg composition of *A. japonica* has been investigated in relation to egg quality (Furuita *et*

71   *al*., 2003, 2006, 2007) however, to date, no studies have focused on *A. anguilla.* Hence, further

72   studies on *A. anguilla* oocytes during vitellogenesis are necessary in order to optimize

73   reproduction in this species.

74   In the last few years, several studies have been carried out in order to gain a better

75   understanding of *A. anguilla* vitellogenesis and zonagenesis (Pérez *et al*., 2011; Mazzeo *et al*., 2012;

76   Peñaranda *et al*., 2013). Due to the complexity of vitellogenesis itself and all the changes that

77   occur during this process, the objective of this study was to compare different techniques which

78   can be employed to study vitellogenesis, in order to evaluate which one is the most suitable in

79   terms of obtained results *vs.* costs and times, and how information provided by each technique

80   can be intercrossed.

81   To achieve this, techniques commonly employed in the study of oocyte growth, such as histology,

82   ELISA, SDS-PAGE, and Folch method for determining fat content were used. In addition, Fourier

83   Transform Infrared (FT-IR) Microspectroscopy was employed for the first time with *A. anguilla*. This

84   technique, previously used in the study of zebrafish *Danio rerio* Hamilton 1882 and mummichog

85   *Fundulus heteroclitus* L. 1766 ovaries and single oocytes, is considered a novel and powerful tool

86   for analyzing the macromolecular composition of ovarian structures (Carnevali *et al*., 2009;

87   Giorgini *et al*., 2010; Lombardo *et al*., 2012). Plasma FT-IR has also been shown to be effective in

88   determining the stage of sexual development in sturgeon *Acipenser transmontanus* Richardson

89   1836 (Lu *et al.*, 2013).

90

91   **MATERIAL AND METHODS**

92   *FISH HANDLING*

93   Thirty-nine silver-stage *A. anguilla* females (660 ± 162 g body mass) were caught by local

94   fishermen between December and March during their reproductive migration from the Albufera

95   lagoon (Valencia, Spain) to the sea, and transported directly to the Universitat Politècnica de

96   València (Spain) aquaculture facilities.

97   The fish were placed in a 1500 l tank with recirculating freshwater and gradually acclimated to

98   seawater salinity (37) and temperature (18 ± 1 °C) over the course of two weeks. The tank was

99   covered to maintain constant darkness, thereby reducing stress. Since *A. anguilla* stop eating at the

100   beginning of their reproductive migration, they were not fed during the whole experiment. The

101   fish were handled in accordance with the European Union regulations concerning the protection of

102   experimental animals (Dir 86/609/EEC) and under the supervision of the University Ethics

103   Committee.

104

105   *HORMONAL TREATMENT*

106   After being anesthetized (benzocaine, 60 mg l^{-1}; www.sigmaaldrich.com/) and weighed to

107   calculate the hormone dosage, the *A. anguilla* females were treated weekly for 12 weeks with

108   intra-peritoneal injections of carp pituitary extract (CPE: Catvis, www.catvis.nl) at a dose of 20 mg

109   kg^{-1}. The CPE was prepared as follows: 1 g of pituitary powder was diluted in 10 ml of NaCl solution

110   (9 g l^{-1}) and centrifuged at 1260 g for 10 min. The supernatant was collected and stored at -20 °C

111   until use.

112

113   *SAMPLING*

114   Once acclimated to seawater salinity and temperature as previously described, eight fish were

115   sacrificed every four weeks. The animals were anesthetized (benzocaine, 60 mg l^{-1}) before being

116   weighed and sacrificed by decapitation. Before sacrificing, blood samples were obtained from the

117   caudal vasculature, and plasma was retrieved by centrifugation (3000 rpm, 15 min) and stored at -

118   80 °C until further analysis.

119   The gonads were weighed to calculate the gonad somatic index (I_G; ovary mass/body mass*100).

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

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

122

123   *GONAD HISTOLOGY*

124   After dehydration in ethanol, samples were embedded in paraffin and 5-10 μm thick sections

125   were cut with a Shandon Hypercut manual microtome (Shandon, Southern Products Ltd,

126   www.southernbiological.com/). Slides were stained with haematoxilin and eosin and observed

127   through a Nikon Eclipse E-400 microscope and pictures were taken with a Nikon DS-5M camera

128   attached to the microscope (www.nikon.com/).

129   The stage of oogenesis was determined following the method described by Pérez *et al*. (2011).

130   The diameters of 100 oocytes from each specimen were measured and the corresponding stage

131   was established on the basis of the most advanced oocyte stage observed in the histological

132   sections. The following stages were observed: Previtellogenic stage (PV): oocyte at perinucleolar

133   and oil droplet stages; Early vitellogenic stage (EV): oocytes with small yolk globules at the

134   periphery of the cytoplasm; Mid-vitellogenic stage (MV): oocytes with bigger yolk globules, widely

135   distributed in the cytoplasm but still with a greater abundance of oil droplets; Late vitellogenic

136   stage (LV): oocytes with more yolk globules than oil droplets.

137   The most advanced stage observed was the nuclear migration stage (NM), characterized by

138   oocyte hydration and the migration of the nucleus towards the animal pole. However, the NM

139   stage was only reached by one animal and it was not considered in the performed analyses due to

140   the poor meaning of comparing with just one specimen.

141

142

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 H_2O_2, 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 Spectrophophotometer (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% 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, 7x10 cm (Selman

167   *et al*., 1993). Molecular weight standards were placed in the well and electrophoresed at a

168   constant current (60 mA). The protein bands were visualized by fixing the gel in 12% trichloroacetic

169   acid for 30 min, then staining for 45 min in 0.2% Coomassie Blue R-350 (Amersham-Pharmacia

170   Biotech, www.gelifesciences.com) in 30% methanol plus 10% acetic acid, and finally de-staining

171   overnight in 25% methanol and 7% acetic acid (Carnevali *et al*., 1992).

172

173   *FAT EXTRACTION*

174   Total fat was extracted according to the method described by Folch *et al*. (1957) with a few

175   modifications. In summary, 1 g of sample was homogenized in 30 ml of Folch solution (2:1, v/v

176   dichloromethane/methanol and 0.75 g l^{-1} butylhydroxytoluene as antioxidant). Homogenization

177   was carried out in a glass tube with an Ultra216 turrax type of homogenizer. The homogenate was

178   filtered through fat-free Whatmann n. 6 paper into a new glass tube. To separate the mixture into

179   two layers - one with lipids and the other with the non-lipid substances - 3.75 ml of potassium

180   chloride were added and the glass tube was kept at 4 °C overnight. Between 8 to 48 h later, once

181   the two layers had formed, the upper layer containing the non-lipid substances was removed with

182   a vacuum pump. Afterwards, the aqueous phase was removed by adding enough sodium sulphate

183   anhydrous. The fat containing phase was filtered again and evaporated using a centrifuge vacuum

184   concentrator (Scan Speed MaxiVac Alpha, www.labogene.com/) at 35 °C. The fat was weighed and

185   its percentage calculated on the basis of the gonad sample mass.

186

187   *FT-IR MEASUREMENTS AND DATA ANALYSIS*

188   Ovaries from five different specimens were cryosectioned in thin slices at a predefined thickness

189   of 5 μm. Two adjacent slices were obtained from each sample: one of which was placed on silicon

190   supports for the vibrational analysis and the other on conventional glass slides for morphological

191 examination (haematoxylin and eosin stained). Spectral data were achieved at room temperature

192 by using a Perkin Elmer Spectrum GXI Spectrometer, equipped with a Perkin Elmer Autoimage

193 microscope and a photoconductive HgCdTe, MCT, array detector, operating at liquid nitrogen

194 temperature and covering the entire IR spectral range from 4000 to 700 cm^{-1}. Using the

195 microscope television camera, specific areas of each sample where the tissue distribution

196 appeared homogeneous were selected. In these zones (ca. 600x500 μm), the chemical maps,

197 which represent the total intensity of the infrared absorption with each pixel corresponding to a

198 single spectrum, were acquired in transmission mode, with a spectral resolution of 4 cm^{-1} and a

199 spatial resolution of 20x20 μm (128 scans), for a total of ca. 750 spectra. Background scans were

200 acquired and rationed against the sample spectrum. For data handling, the following software

201 packages were used: Spectrum Image 1.6 and Spectrum 6.3.1 (Perkin Elmer,

202 www.perkinelmer.com/lab-solutions/default.xhtml), and Grams AI 7.02 (Galactic Industries,

203 www.spectra.co.jp/pdf/grams.pdf). The spectra obtained from each sample were used to build a

204 two points baseline fitted in the spectral range 4000-700 cm^{-1} and to normalize the vectors (Wood

205 *et al*., 2004). Second Derivative (9-point smoothing) and Peak Fitting (Gaussian algorithm)

206 procedures were adopted to determine the correct position and absorbance intensity of bands. By

207 using GRAMS/AI 7.02 (Galactic Industries, www.spectra.co.jp/pdf/grams.pdf), peak fitting was

208 performed on average spectra (interpolated in the range 1780-1470 cm^{-1} and two points baseline

209 fitted), to identify the underlying component bands, the number of peaks together with their

210 center values were carefully individuated according to the second derivative results and fixed

211 before running the iterative process, to obtain the best reconstructed curve (residual close to zero).

212 Correlation maps were obtained by loading second derivative representative spectra onto the

213 chemical maps (Wood *et al*., 2004). This procedure, which enables the localization of biological

214 components in the sample, correlates a selected spectrum with all the spectra in the map,

215 affording a colorimetric and numeric scale of correlation percentage. Attribution of the bands was

216 carried out according to literature (Jackson & Mantsch, 1993, 2002; Pacifico *et al.*, 2003).

217

218 *STATISTICAL ANALYSIS*

219 After establishing data normality, the data were analyzed by a one way analysis of variance

220 (ANOVA) followed by a Newman-Keuls post-hoc test. If normality failed, the data were Log

221 transformed to perform the ANOVA. All the values are expressed as mean ± standard error of

222 mean (SEM). Differences were considered significant at $p < 0.05$. All statistical procedures were run

223 using Statgraphics Plus 5.1 (www.statgraphics.com/).

224

225 **RESULTS**

226 *HISTOLOGY*

227 Five different vitellogenic stages during vitellogenesis were observed using histology [Fig. 1]. At

228 the beginning of the treatment, all the specimens were in the PV stage (I_G: 0.83), which includes

229 the perinucleolar and oil droplet stages [Fig. 1(a,b)]. The stages progressed in line with the

230 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

231 reached. The most advanced stage reached was NM (I_G: 39.26) [Fig. 1(f)], observed in just one

232 female. Histological observation clearly demonstrated that (i) vitellogenesis was preceded by oil

233 droplet accumulation [Fig. 1(a,b)]; (ii) VTG uptake occurred in the EV stage [Fig. 1(c)] and continued

234 until the MV and LV stages [Fig. 1(d,e)]; (iii) in the NM stage, nucleus migration and coalescence of

235 yolk granules were evident [Fig. 1(f)].

236 For the remainder of the analyses, specimens were grouped by developmental stage in order to

237 identify differences relating to the progression of vitellogenesis rather than to the week of

238 treatment.

10

240 *PLASMA VTG LEVELS*

241   VTG plasma levels [Fig. 2] increased during early vitellogenesis (EV) and then remained constant

242 until late vitellogenesis (LV). The plasma VTG increase was accompanied by the appearance of yolk

243 vesicles in the oocytes in the EV stage [Fig. 1(c)].

244

245 *FAT CONTENT*

246   In the ovaries, no differences were found in any of the different phases of vitellogenesis with

247 respect to fat content. However, in spite of the lack of statistical significance, fat levels increased

248 when moving from the PV to the EV stage [Fig. 3].

249

250 *SDS-PAGE*

251   The presence of yolk proteins in the growing oocyte was confirmed by the appearance of four

252 distinct components with an apparent molecular weight of 100, 60, 30 and 26 kDa in the EV, MV

253 and LV stages, as shown by SDS-PAGE. An additional band with an apparent molecular weight of 15

254 kDa was observed in the LV stage [Fig. 4].

255

256 *FT-IR*

257   The comparative analysis of representative spectra allowed the visualization of the changes that

258 occurred in the biochemical composition of the different samples.

259   With regards to the ovary, when moving from the PV to the LV stage, the uptake of VTG was

260 substantiated by an increase in lipids, proteins, carbohydrates and phosphates. In the region 3100–

261 2800 cm^{-1}, the convoluted band with a maximum at 2926 cm^{-1} increased between the PV and the

262 LV stage: in particular, the analysis of the 2926/2954 cm^{-1} (ν_{asym} CH_2/CH_3) and 2854/2873 cm^{-1} (ν_{sym}

263   CH_2/CH_3) absorbance band ratios pin-pointed an increase in CH_2 moiety [Fig. 5]. According to the

264   vibrational analysis in the spectral region 1800–1900 cm^{-1}, between the PV and the LV stages in the

265   ovary, some conclusions can be drawn: (i) the vibrational mode at 1738 cm^{-1} increased; (ii) changes

266   in protein content and secondary structure were observed by analyzing Amide I and Amide II

267   bands; in particular, the bands at 1695 cm^{-1} (β-turn) and at 1631 cm^{-1} (β-sheet) increased, while

268   those at 1640 cm^{-1} (random coil) disappeared; (iii) the increase in the lipidic chain length was

269   confirmed by the increase of the band at 1458 cm^{-1} ($\delta CH_2/_3$) ; (iv) the increase in the phosphate

270   groups was demonstrated by the bands at 1239 cm^{-1} ($\nu_{asym}\ PO_2^-$) and 1081 ($\nu_{sym}\ PO_2^-$); (v) there

271   was a rise in the glucidic component, confirmed by the band at 1060 cm^{-1} (ν_{sym} CO-O-C) [Fig. 5]. In

272   addition, correlation maps guaranteed the correspondence between the analyzed samples and an

273   average representative spectrum for each ovarian stage (PV, EV, MV and LV), validating the method

274   and illustrating the distribution of the oocytes within the ovary slices [Fig. 6]. At the same time,

275   chemical maps obtained by correlating the region 3100-2800 cm^{-1} (corresponding to CH_2 and CH_3

276   moieties) confirmed changes in the concentration of the analyzed components and showed the

277   distribution of the lipids within the ovaries in the different maturational stages [Fig. 6].

278

279   **DISCUSSION**

280   The aim of this study was to integrate information from different techniques to gain a wider and

281   more complete understanding of vitellogenesis in *A. anguilla* and to identify the most exhaustive

282   and convenient technique.

283   According to the histological analysis of the ovaries, VTG plasma content and fat content follow

284   a similar pattern, with both showing a first phase of lipid accumulation followed by a phase in

285   which vitellogenin is synthesized, transported and accumulated. The reason for this could be that

286   lipids and yolk proteins are the most important macromolecules for the developing future embryo

287   (Brooks *et al*., 1997; Carnevali *et al.,* 2001a,b).

288   In particular, fat accumulation in the ovary occurs between the PV and the EV stages, as

289   illustrated in particular by histology and fat analysis (in spite of a lack of statistical significance, an

290   increase in ovarian fat content was appreciated). This first stage of vitellogenesis, characterized by

291   the presence of fat, indicates that lipid composition, storage and mobilization are important for

292   successful vitellogenesis and probably deserve more attention in the attempt to optimize breeding.

293   The progression of vitellogenesis is supported by histology, the increase of VTG plasma content

294   and the SDS-PAGE. Vitellogenin is synthesized by the liver in response to 17β-estradiol produced by

295   the ovary, and is carried in the blood to the ovary (Nagahama, 1994). Hence, VTG plasma content

296   can be an indicator of the progression of vitellogenesis. These results were validated by FT-IR

297   analysis, which showed an increase in vitellogenin during oocyte maturation. In fact, the increase

298   in CH_2 moiety and in the vibrational mode at 1738 cm^{-1} were attributable to changes in lipidic

299   backbone as well as amino acid side chains (Wood *et al*., 2008), and to VTG (Carnevali *et al*., 2009),

300   respectively.

301   In *A. anguilla,* in order to classify animals according to their developmental stage, eye index

302   together with skin and fin coloration can also be used, but these are indirect evidences that may

303   help to distinguish migrating from non-migrating individuals (Durif *et al*., 2005). So, it is important

304   to find a technique which can give a reliable and more direct indication of the developmental stage

305   reached by a specimen.

306   The advantages and disadvantages of each technique are summarized in Table I. Histology has

307   traditionally been the most employed technique for establishing the developmental stage of fish,

308   since it allows the direct observation of the progression of vitellogenesis through the analysis of

309   the ovaries. Unfortunately, this technique is very time consuming (Brewer *et al*., 2008) and

310   generates of a lot of waste because of the use of alcohols and chemical reagents. Waste

311 production is also one of the disadvantages of fat content analysis by Folch method. However,

312 unlike histology, ovarian fat content is not a very good technique for studying the progression of

313 vitellogenesis. In fact, in addition to it also being time consuming, it does not give an immediate

314 result and, above all, fat content cannot be linked exactly to any vitellogenic stage. However, since

315 it has been demonstrated that dietary lipid content affects fatty acid composition and egg quality,

316 studies on fat composition and mobilization – fat content was also studied in other body tissues,

317 including the muscle and the liver – should be performed to establish the energetic needs of

318 maturing *A. anguilla* and to define specific breeder diets (García-Gallego & Akharbach, 1998;

319 Furuita *et al.*, 2007).

320 In addition, VTG plasma content and SDS-PAGE only give partial indications on the progression

321 of vitellogenesis. According to our results, VTG plasma content analysis, which has the advantage

322 of not needing to sacrifice the fish as blood can be retrieved by anesthetized specimens, could be

323 used to distinguish the PV stage from more advanced stages. This is because a significant increase

324 in the levels is only observed in the PV and EV stages, with no differences found in the other stages.

325 When using SDS-PAGE, real differences were found between animals at the very beginning of

326 vitellogenesis and others at more advanced stages, but this technique cannot be used to find out

327 exactly in which stage a specimen is. However, it would be interesting to carry out a more in depth

328 analysis in order to understand which yolk proteins appear during the progression of vitellogenesis.

329 In addition, SDS-PAGE is a fast method, which generates little waste and requires small amounts of

330 tissue.

331 FT-IR analysis is a novel technique, which was successfully applied to determine the

332 developmental stage in maturing *A. anguilla*. Although specific instruments and trained staff are

333 needed, this technique has the advantage of short analysis times and the possibility of analyzing

334 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.

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-

359   assay) for vitellogenin and vitellus in the eel (*Anguilla anguilla* L.) and in the Indian major

360   carp (*Labeo rohita*). In *Proc. 4th Int. Symp. Reproductive Physiology of Fish, FishSymp91*

361   (Scott, A. P., Sumpter, J. P., Kime, D. E. & Rolfe, M. S. eds), pp 319-320. Sheffield.

362   Carnevali, O., Mosconi, G., Roncarati, A., Belvedere, P., Romano, M. & Limatola, E. (1992) Changes

363   in the electrophoretic pattern of yolk proteins during vitellogenesis in the gilthead seabream

364   *Sparus aurata* L. *Comparative Biochemistry and Physiology* **103B,** 955–962. doi:

365   10.1016/0305-0491(92)90222-D

366   Carnevali, O., Mosconi, G., Cardinali, M., Meiri, I. & Polzonetti Magni, A. M. (2001a) Molecular

367   components related with egg viability in the sea bream *Sparus aurata*. *Molecular*

368   *Reproduction and Development* **58**, 330-335. doi: 10.1002/1098-2795(200103)58:3<330

369   Carnevali, O., Mosconi, G., Cambi, A., Ridolfi, S., Zanuy, S. & Polzonetti-Magni, A. M. (2001b)

370   Changes of lysosomal enzyme activities in sea bass *Dicentrachus labrax* egg and developing

371   embryo. *Aquaculture* **202,** 249-256. doi: 10.1016/S0044-8486(01)00775-X

372   Carnevali, O., Conti, C., Ferraris, P., Garavaglia, M. G., Gioacchini, G., Giorgini, E., Rubini, C.,

373   Sabbatini, S. & Tosi, G. (2009) FT-IR Microspectroscopy on molecular building of Zebrafish

374   oocytes. *Journal of Molecular Structure* **938**, 207–213. doi: 10.1016/j.molstruc.2009.09.029

375   Durif, C., Dufour, S. & Elie, P. (2005) The silvering process of *Anguilla anguilla*: a new classification

376   from the yellow resident to the silver migrating stage. *Journal of Fish Biology* **66,** 1025–1043.

377   doi: 10.1111/j.1095-8649.2005.00662.x

378   Folch, J., Lees, M. & Sloane Stanley, G. H. (1957) A simple method for the isolation and purification

379   of total lipides from animal tissues. *The Journal of Biological Chemistry* **226(1),** 497-509.

380   Furuita, H., Ohta, H., Unuma, T., Tanaka, H., Kagawa, H., Suzuki, N. & Yamamoto, T. (2003)

381   Biochemical composition of eggs in relation to egg quality in the Japanese eel, *Anguilla*

382   *japonica*. *Fish Physiology and Biochemistry* **29,** 37-46.

383   Furuita, H., Unuma, T., Nomura, K., Tanaka, H., Okuzawa, K., Sugita, T. & Yamamoto, Y. (2006) Lipid

384   and fatty acid composition of eggs producing larvae with high survival rate in the Japanese

385   eel. *Journal of Fish Biology* **69,** 1178-1189. doi: 10.1111/j.1095-8649.2006.01196.x

386   Furuita, H., Hori, K., Suzuki, N., Sugita, T. & Yamamoto, T. (2007) Effect of n-3 and n-6 fatty acids in

387   broodstock diet on reproduction and fatty acid composition of broodstock and eggs in the

388   Japanese eel *Anguilla japonica*. *Aquaculture* **267,** 55-61. doi:

389   10.1016/j.aquaculture.2007.01.039

390   Gallego, V., Mazzeo, I., Vílchez, M. C., Peñaranda, D. S., Carneiro, P. C. F., Pérez, L. & Asturiano, J. F.

391   (2012) Study of the effects of thermal regime and alternative hormonal treatments on the

392   reproductive performance of European eel males (*Anguilla anguilla*) during induced sexual

393   maturation. *Aquaculture* **354-355,** 7-16. doi: 10.1016/j.aquaculture.2012.04.041

394   García-Gallego, M. & Akharbach, H. (1998) Evolution of body composition of European eels during

395   their growth phase in a fish farm, with special emphasis on the lipid component.

396   *Aquaculture International* **6,** 345–356.

397   Giorgini, E., Conti, C., Ferraris, P., Sabbatini, S., Tosi, G., Rubini, C., Vaccari, L., Gioacchini, G. &

398   Carnevali, O. (2010) Effects of *Lactobacillus rhamnosus* on zebrafish oocyte maturation: an

399   FTIR imaging and biochemical analysis. *Analytical and Bioanalytical Chemistry* **398(7-8)**,

400   3063-72. doi: 10.1007/s00216-010-4234-2

401   Heinsbroek, L. T. N., Støttrup, J. G., Jacobsen, C., Corraze, G., Kraiem, M. M., Holst, L. K., Tomkievicz,

402   J. & Kaushik, S. J. (2013) A review on broodstock nutrition of marine pelagic spawners: the

403   curious case of the freshwater eels. *Aquaculture Nutrition* **19,** 1-24. doi: 10.1111/anu.12091

404   Horie, N., Utoh, T., Mikawa, N., Yamada, Y., Okamura, A., Tanaka, S. & Tsukamoto, K. (2008) Influence of

405   artificial fertilization methods of the hormone-treated Japanese eel *Anguilla japonica* upon

406   the quality of eggs and larvae (Comparison between stripping-insemination and spontaneous

407   spawning methods). *Nippon Suisan Gakkaishi* **74,** 26-35.

408   Jackson, M. & Mantsch, H. H. (1993) Biomembrane structure from FT-IR spectroscopy.

409   *Spectrochimica Acta Reviews* **15,** 53-69.

410   Jackson, M. & Mantsch, H. H. (2002) Pathology by Infrared and Raman Spectroscopy. In *Handbook*

411   *of Vibrational Spectroscopy*, Vol. 5 (Chalmers, J. M. & Griffiths, P. R. eds.), pp. 3227–3245.

412   Chichester, Wiley.

413   Kagawa, H., Tanaka, H., Ohta, H., Unuma, T. & Nomura, K. (2005) The first success of glass eel

414   production in the World: basic biology on fish reproduction advances new applied

415   technology in aquaculture. *Fish Physiology and Biochemistry* **31,** 193-199. doi:

416   10.1007/s10695-006-0024-3

417   Lombardo, F., Giorgini, E., Gioacchini, G., Maradonna, F., Ferraris, P. & Carnevali, O. (2012)

418   Melatonin effects on *Fundulus heteroclitus* reproduction. *Reproduction, Fertility and*

419   *Development* **24(6),** 794-803. doi: 10.1071/RD11267.

420   Lu, X., Talbott, M. J., Eenennaam, J. P. V., Webb, M. A. H., Doroshov, S. I., Ovissipour, M. & Rasco, B.

421   (2013) Determining Ovarian Maturity in Farmed Sturgeon (*Acipenser transmontanus*) for

422   Caviar Production Using Fourier Transform Infrared Spectroscopy (FT-IR). *Journal of*

423   *Aquaculture Research and Development* **5,** 1. doi: 10.4172/2155-9546.1000202.

424   Lubzens, E., Young, G., Bobe, J. & Cerdà, J. (2010) Oogenesis in teleosts: How fish eggs are formed.

425   *General and Comparative Endocrinology* **165,** 367–389. doi: 10.1016/j.ygcen.2009.05.022

426   Mazzeo, I., Peñaranda, D. S., Gallego, V., Hildahl, J., Nourizadeh-Lillabadi, R., Asturiano, J. F., Pérez,

427   L. & Weltzien, F-A. (2012) Variations in the gene expression of zona pellucida proteins, zpb

428   and zpc, in female European eel (*Anguilla anguilla*) during induced sexual maturation.

429   *General and Comparative Endocrinology* **178,** 338–346. doi: 10.1016/j.ygcen.2012.06.003

430   Nagahama, Y. (1994) Endocrine regulation of gametogenesis in fish. *The International Journal of*

431   *Development Biology* **38,** 217-229.

432 Pacifico, A., Chiriboga, L., Lasch, P. & Diem, M. (2003) Infrared spectroscopy of cultured cells. II.

433   Spectra of exponentially growing, serum-deprived and confluent cells. *Vibrational*

434   *Spectroscopy* **32,** 107–115. doi: 10.1016/S0924-2031(03)00051-1

435 Palstra, A. P., Cohen, E. G. H., Niemantsverdriet, P. R. W., van Ginneken, V. J. T. & van den Thillart, G.

436   E. E. J. M. (2005) Artificial maturation and reproduction of European silver eel: Development

437   of oocytes during final maturation. *Aquaculture* **249,** 533-547. doi:

438   10.1016/j.aquaculture.2005.04.031

439 Palstra, A. P., van Ginneken, V. J. T., Murk, A. J. & van den Thillart, G. E. E. J. M. (2006) Are dioxin-

440   like contaminants responsible for the eel (*Anguilla anguilla*) drama?. *Naturwissenschaften*

441   **93,** 145–148. doi: 10.1007/s00114-005-0080-z

442 Pedersen, B. H. (2004) Fertilisation of eggs, rate of embryonic development and hatching following

443   induced maturation of the European eel *Anguilla anguilla*. *Aquaculture* **237,** 461-473. doi:

444   10.1016/j.aquaculture.2004.04.019

445 Peñaranda, D. S., Pérez, L., Gallego, V., Jover, M., Baloche, S., Dufour, S. & Asturiano, J. F. (2010)

446   Molecular and physiological study of the artificial maturation process in the European eel

447   males: from brain to testis. *General and Comparative Endocrinology* **166,** 160-171. doi:

448   10.1016/j.ygcen.2009.08.006

449 Peñaranda, D. S., Mazzeo, I., Hildahl, J., Gallego, V., Nourizadeh-Lillabadi, R., Pérez, L., Asturiano, J.

450   F. & Weltzien, F-A. (2013) Molecular characterization of three GnRH receptor paralogs in the

451   European eel, *Anguilla anguilla*: tissue-distribution and changes in transcript abundance

452   during artificially induced sexual development. *Molecular and Cellular Endocrinology* **369(1-**

453   **2),** 1-14. doi: 10.1016/j.mce.2013.01.025

454 Pérez, L., Peñaranda, D. S., Jover, M. & Asturiano, J. F. (2008) Results of maturation and ovulation in

455   European eel females. *Cybium* **32 (2),** 320.

456   Pérez, L., Peñaranda, D. S., Dufour, S., Baloche, S., Palstra, A. P., van den Thillart, G. E. E. J. M. &

457   Asturiano, J. F. (2011) Influence of temperature regime on endocrine parameters and

458   vitellogenesis during experimental maturation of European eel (*Anguilla anguilla*) females.

459   *General and Comparative Endocrinology* **17,** 51-59. doi: 10.1016/j.ygcen.2011.08.009

460   Polzonetti, V., Cardinali, M., Mosconi, G., Natalini, P., Meiri, I. & Carnevali, O. (2002) Cyclic ADPR

461   and calcium signaling in seabream (*Sparus aurata*) egg fertilization. *Molecular Reproduction*

462   *and Development* **61,** 213-217. doi: 10.1002/mrd.1150

463   Polzonetti-Magni, A. M., Mosconi, G., Soverchia, L., Kikuyama, S. & Carnevali, O. (2004)

464   Multihormonal control of vitellogenesis in lower vertebrates. *International Review of*

465   *Cytology* **239,** 1–46. doi: 10.1016/S0074-7696(04)39001-7

466   Selman, K., Wallace, R. A., Sarka, A. & Xiaoping, Q. (1993) Stages of oocyte development in the

467   zebrafish, *Brachydanio rerio*. *Journal of Morphology* **218,** 203–224. doi:

468   10.1002/jmor.1052180209

469   Seoka, M., Yamada, S., Iwata, Y., Yanagisawa, T., Nakagawa, T. & Kumai, H. (2003) Differences in the

470   biochemical content of buoyant and non-buoyant eggs of the Japanese eel, *Anguilla japonica*.

471   *Aquaculture* **216,** 355–362.

472   Strøttrup, J. C., Jacobsen, C., Tomkievicz, J. & Jarlbǽk, H. (2013) Modifications of essential fatty

473   acids composition in broodstock of cultured European eel *Anguilla anguilla* L. *Aquaculture*

474   *Nutrition* **19,** 172-185. doi: 10.1111/j.1365-2095.2012.00967.x

475   van Ginneken, V. & Maes, G. (2005) The European eel (*Anguilla anguilla*, Linnaeus), its lifecycle,

476   evolution and reproduction: a literature review. *Reviews in Fish Biology and Fisheries* **15,** 367-

477   398. doi: 10.1007/s11160-006-0005-8

478   Wood, B. R., Chiriboga, L., Yee, H., Quinn, M. A., McNaughton, D. & Diem, M. (2004) Fourier

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

480   dysplastic squamous epithelium. *Gynecologic Oncology* **93,** 59-68. doi:

481   10.1016/j.ygyno.2003.12.028

482 Wood, B. R., Chernenko, T., Matthäus, C., Diem, M., Chong, C., Bernhard, U., Jene, C., Brandli, A. A.,

483   McNaughton, D., Tobin, M. J., Trounson, A. & Lacham-Kaplan, O. (2008) Shedding new light

484   on the molecular architecture of oocytes using a combination of synchrotron Fourier

485   transform-infrared and Raman spectroscopic mapping. *Analytical Chemistry* **80**, 9065–9072.

486   doi: 10.1021/ac8015483.

Table

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

Technique	Advantages	Diasadvantages
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 *vs* other stages	- No direct information on developmental stage
SDS-PAGE	- Screening of specimens in PV stage *vs* other stages - Fast - Small amount of tissue needed	- No direct information on developmental stage
FT-IR	- Once spectra are acquired, information on developmental stage - Fast - Study of macromolecules	- Expensive equipment - Trained personnel needed

1 **Figures captions**

2

3 **Fig. 1.** Ovarian developmental stages in *Anguilla anguilla*. A) Previtellogenic

4 (PV), perinucleolar stage; B) Previtellogenic, lipid droplet stage; C) Early

5 vitellogenic (EV) stage; D) Mid vitellogenic (MV) stage; E) Late vitellogenic

6 (LV) stage; F) Nuclear migration (NM) stage. Arrows in C, D, E show the

7 position of the yolk globules, arrow in F points to the nucleus. Scale Bar: A, B,

8 D: 100 µm; C: 50 µm; E, F: 200 µm.

9

10 **Fig 2.** Changes in VTG plasma levels (mg ml^{-1}) during vitellogenesis according

11 to *Anguilla anguilla* ovarian developmental stage. VTG levels are measured by

12 homologous ELISA. PV: pre-vitellogenesis ($n = 8$); EV: early vitellogenesis (n

13 $= 9$); MV: mid-vitellogenesis ($n = 2$); LV: late vitellogenesis ($n = 6$). $P < 0.05$.

14

15 **Fig. 3.** Changes in *Anguilla anguilla* ovarian fat content during ovarian

16 development according to developmental stage. Fat content was measured by

17 Folch method. PV: pre-vitellogenesis ($n = 3$); EV: early vitellogenesis ($n =$

18 10); MV: mid-vitellogenesis ($n = 2$); LV: late vitellogenesis ($n = 6$). $P < 0.05$.

19

20 **Fig. 4.** SDS-PAGE showing changes in protein during *Anguilla anguilla* oocyte

21 development. PV: pre-vitellogenesis; EV: early vitellogenesis; MV: mid-

22 vitellogenesis; LV: late vitellogenesis; NM: nuclear migration.

23

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.
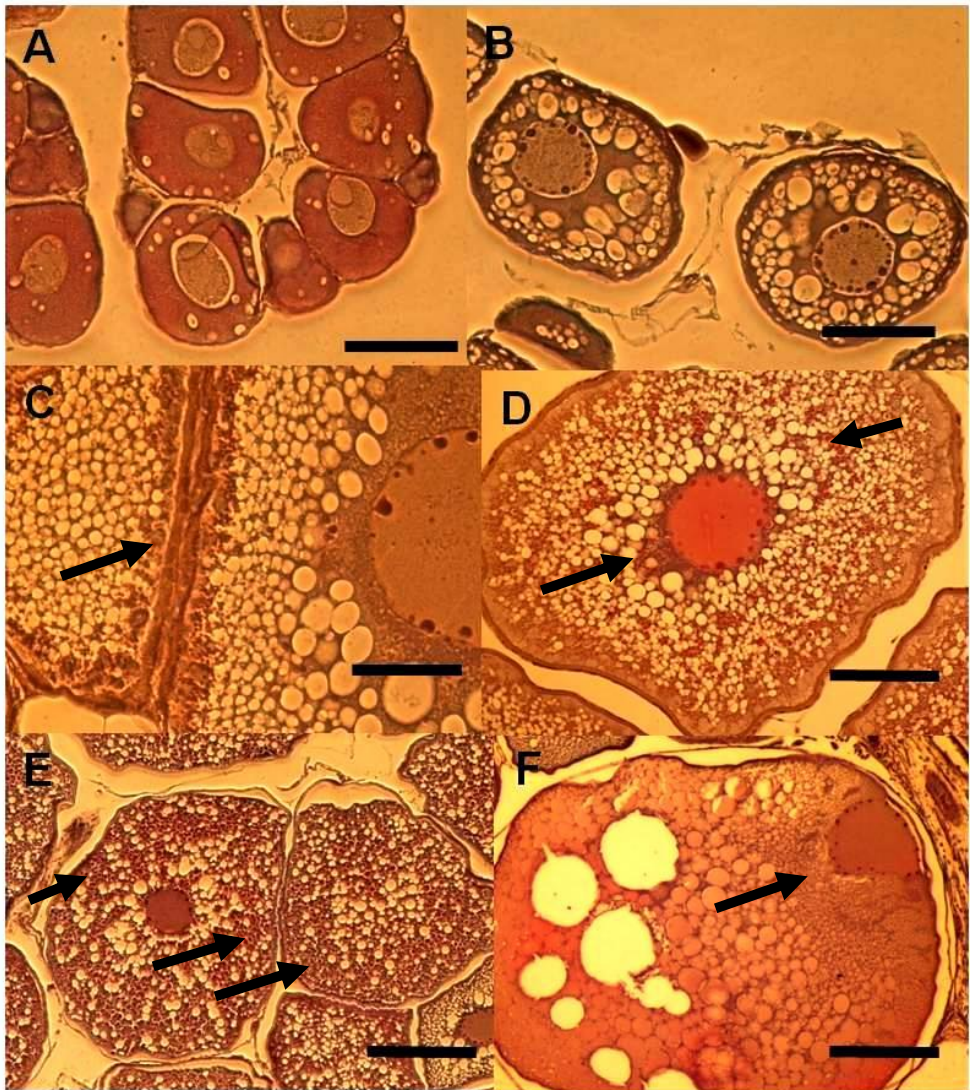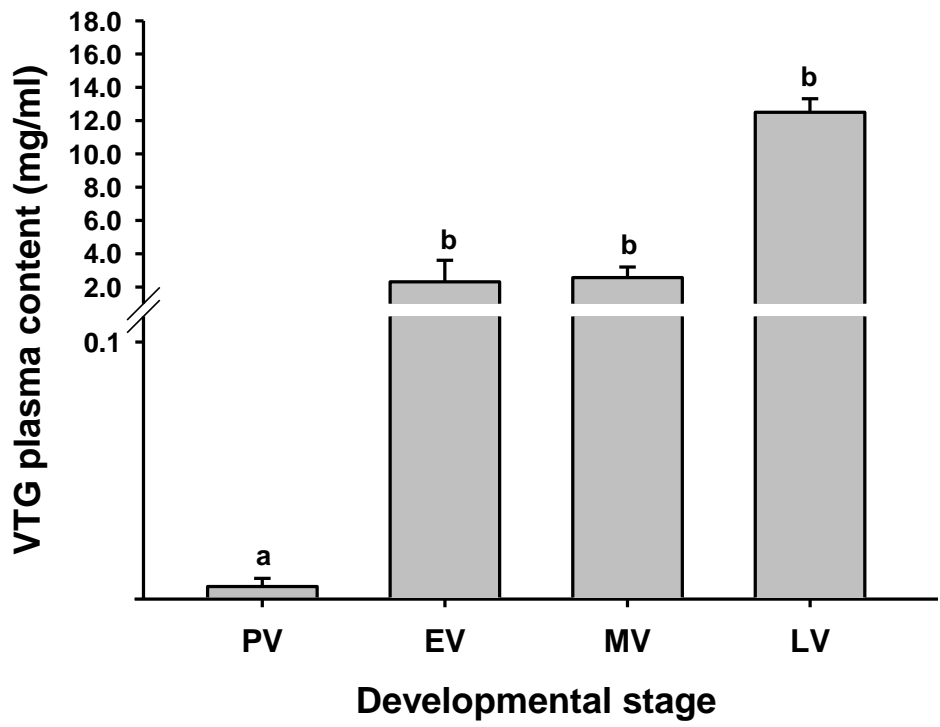
Figure
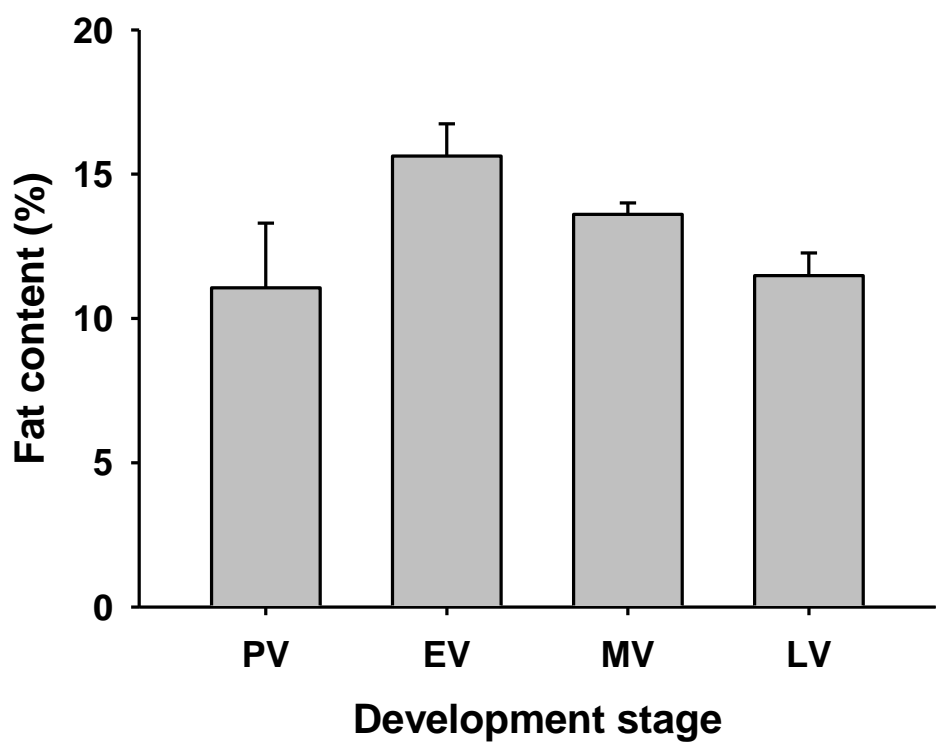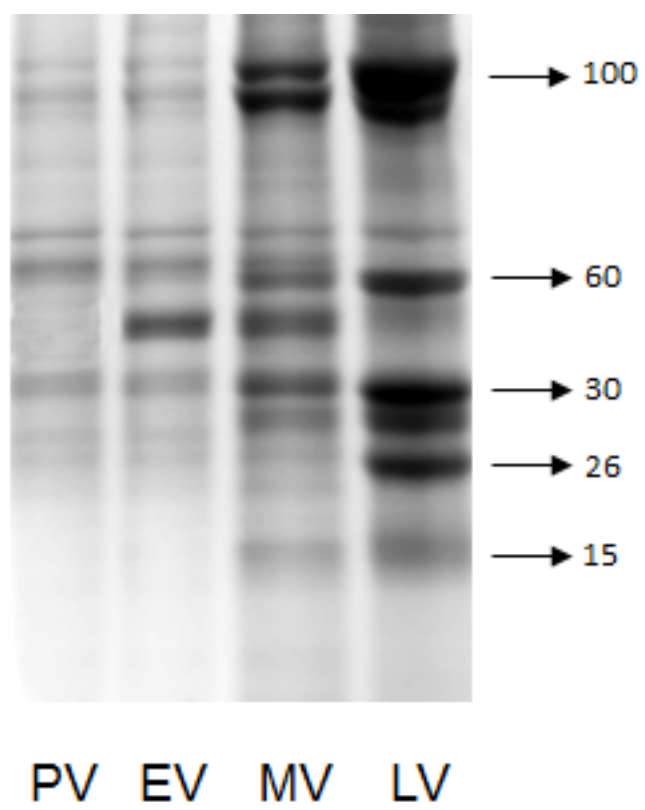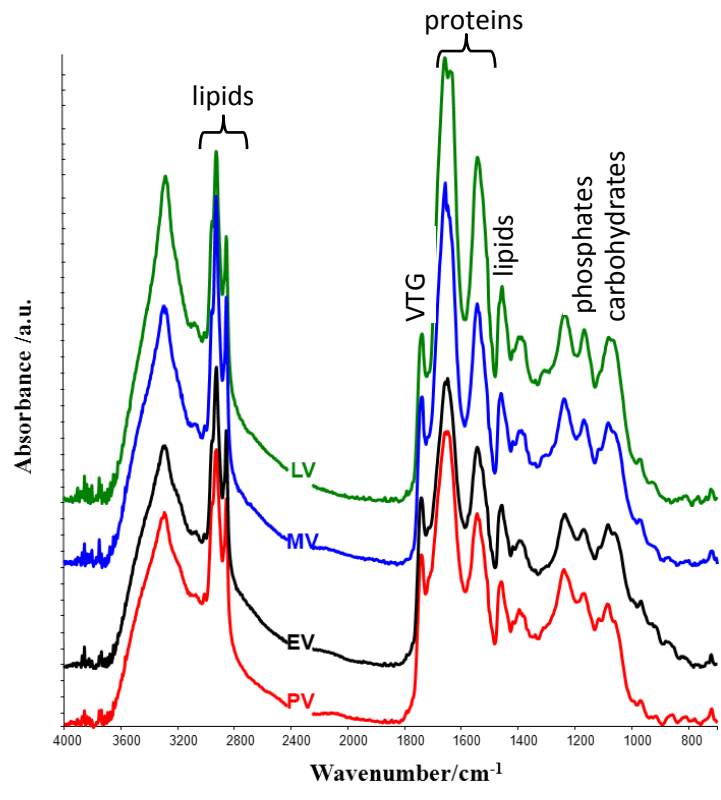

Fig. 1

Figure

Fig. 2

(SigmaPlot)

Figure

Fig. 3.

(SigmaPlot)

Figure

Figure

Fig. 5

Figure

	PV	EV	MV	LV
A Photomicrographs and selected area				
B Chemical maps integrated under 4000-800 cm^{-1}				
C 3100-2800 cm^{-1} CH2 and CH3				

200 μm

1.0000
0.9291
0.8700
0.8110
0.7519
0.6929
0.6362
0.5771
0.5181
0.4590
0.4000