Authentication of farmed and wild european eel (*Anguilla anguilla*) by fatty acid profile and carbon and nitrogen isotopic analyses

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Abstract

We determined fatty acid profile and protein carbon and nitrogen isotopic composition in muscle of wild and farmed eels, in order to discriminate the geographical origin of fish and their dietary habits. The samples were divided into groups according to the farm origin (Italy, Denmark and Netherland), the market size (capitoni and buratelli) and the farming system (pond, RAS, lagoon and wild). Biometric measurements, proximate composition analysis (moisture, ash, lipid and protein content), fatty acids profile and carbon and nitrogen stable isotopes measurements were performed. Statistical analysis was fulfilled to investigate the homogeneity of variance and the means distribution, including Principal Component Analysis and Partial Least Squares Discriminant Analysis. The results suggested that the different farming conditions represented the main factor affecting fish muscle composition. In this scenario, fatty acid profile and $\delta^{13}C$ and $\delta^{15}N$ values of the defatted muscle represented useful tools to distinguish between eels reared under different feeding regimes (wild vs captive-bred). No differences were found between eels reared in different farms.

Keywords: Food analysis; Food composition; Isotopic Composition; Fatty Acids Profile; Aquaculture; Eels; Farming system; Food of animal origin; Isotopes; Anguilla anguilla

1. Introduction

European eel *(Anguilla anguilla*) is an important commercial fish species in Europe. The Italian production of eels, based on capture from wild population and extensive/intensive farming, cannot cover the request of this product during the peak of demand, concentrated around the Christmas period, when occurs a large importation of eels from other European producer countries, where the

farming methods are different. Italian eel production reached an amount of 1250 tons (API, 2016) in 2016. Traditionally, the main European producers of farmed eels are Denmark (789 tons) and The Netherlands (2885 tons). Recently, Germany started an intensive eel farming, with a production that has rapidly increased, reaching 927 tons in 2016 (FEAP 2017). In 2013, the whole farming system of European eel lost more than half of its production compared with 2003 (FAO), when it reached its peak.

Farming of European eel is based on the fattening of juvenile eels collected from wild population, since the artificial reproduction and larval weaning are currently limited to research experiences (O. Mordenti, Di Biase, Sirri, S., & A. 2012). The lack of availability of glass eels resulted in an increase of the product price and in a strong reduction of European production. The wild stock of European eel is critically endangered: the availability of glass eel is estimated to be only the 5% of the level recorded in the early 1980s (S. Bonhommeau et al. 2008; Bonhommeau, Chassot, & Rivot 2008). This aspect is due to many factors: first of all, eel is a vulnerable species that reproduces only once during its life; furthermore, eel overfishing is practiced in all phases of its life, from the juvenile phase to the adult (Støttrup et al., 2013). In addition, barriers to migration (e.g. dams, weirs) and parasites represent an obstacle to their life cycle (Dekker 2003). Due to the intensive depletion of natural stocks, the European eel (*Anguilla anguilla)* is inserted in the Appendix II of the Convention on International Trade in Endangered Species of wild Flora and Fauna (CITES), being Critically Endangered according to IUCN Criteria (Jacoby et al. 2015). For all these reasons, with the Reg. (EC) 1100/07, European legislator has established a number of measures aiming to protect and to make a sustainable use of this endangered species in communitarian waters. Moreover, each Member State has the commitment to define national management plans to avoid an additional depletion of juvenile samples in its waters.

Mediterranean production of eel involves a different farming method, if compared to Northern Europe countries, especially Netherland and Denmark. In Spain, Greece and Italy eels are farmed

mainly in extensive conditions, named *vallicoltura*, that are limited portion of lagoons of brackish waters, where juvenile of various fish species enter and grow using natural food. After that they are collected when commercial size is reached. Farmers, acting with the sea water incoming in the *Valle*, attract eels in the fish trap, called *lavoriero*, during the autumnal migration of eels from freshwater to the open sea. This rearing system uses lower densities than that practiced by intensive farming of Netherland and Denmark, which can reach over than 100 kg/ m^3. In Italy eels are farmed also in pond systems, fed with a moist paste, directly prepared by the farmers with commercial raw materials.

Central and Northern Europe countries use an intensive production system, taking advantage of recirculating aquaculture system (RAS), in which eels are kept at their optimum temperature and automatically fed with extruded dry feed (1.5 - 3 mm), several times a day. This system is based on water recycling; tanks' water is moved by a circulating pump and depurated by mechanical and biological filters and sterilized by UV irradiation or ozonization (Mordenti et al., 2014). Thanks to RAS system, farmers have the advantage of controlling water temperatures; in this case, the duration of farming cycle is shorter if compared with the Italian pond system and this is due to the use of an optimum water temperature range for eels.

As mentioned above, the common aspect of all these systems is that every farming cycle starts from the collection of juvenile animals, since reproduction is actually possible only in nature, precisely in Sargasso Sea. On the other hand, different densities and feeding techniques influence the growing time and the muscle composition of eels.

The intensive production leads to the availability of two market size of eels. Males, named "*buratelli*" in Italy, are sold when they reach 120-150 g of weight, obtained in 8–12 months in plants working with water temperatures above 18°C for at least 7 months; females, named "*capitoni*", reach 400-600 g of weight in a farming cycle 4-7 months longer (Parisi et al., 2014).

The isotope ratio mass spectrometry (IRMS) represents an innovative technique that could be applied in order to discriminate different samples of eels. The aim to investigate the stable isotope ratio, especially of light elements, in food traceability and authenticity issues is supported by the fact that their fractionation is significantly influenced by geographical origin, climate conditions, soil geology and diet composition. All these factors also influence the isotopic ratio of elements that are captured in animal tissues through feeding, drinking, breathing and all the existing interactions between the animal organisms and the environment (van Leeuwen et al., 2014).

It is demonstrated that the isotopic composition of $\delta^{13}C$ and $\delta^{15}N$ of terrestrial or aquatic organisms reflects the isotopic composition of their diet, with a nitrogen enrichment much higher than for carbon at each trophic transfer, providing a valuable measure of the position of an organism within the food web (Michener & Kaufman, 2007). The carbon isotopic composition in animal tissues is useful in tracing food webs in systems where food sources show large differences in $\delta^{13}C$ values, such as C3 vs. C4 plants, marine vs. terrestrial systems, or nearshore vs. offshore systems (Koch, 2007). Consequently, the isotopic composition in $^{13}C/^{12}C$ changes if food furnished to farmed fish has a mainly vegetal or animal origin (Moreno-Rojas et al., 2008). In a similar way, the $^{15}N/^{14}N$ ratio is considered the signature of agricultural practices. Organic fertilizers and intensive farming methods increase the level of ^{15}N in the soil. Other factors such as water stress and climatic conditions affect the biological turnover of the nitrogen (mineralization, nitrification, nitrogen denitrification and leaching) and they are accompanied by significant isotopic fractionation.

IRMS has been demonstrated to be a technique capable of revealing the origin of fish in terms of dietary habits, rearing system, geographical area of capture (Busetto et al., 2008). This analytical approach is able to confirm traceability issues established by EU law (EC Reg. 2065/2001): production method (wild or farmed, sea or freshwater), geographical origin and biological species. Differentiation between wild and farmed fish was the first approach introducing analytical determination of stable isotope ratios, mainly C, N and H, in different tissues or lipid fraction. The

120 relative enrichment or depletion of $\delta^{15}N$ and $\delta^{13}C$ in farmed and wild fish resulted influenced by
121 diet behaviors of the species examined (such as origin and type of protein consumed) and metabolic
122 turnover related to the scarcity or abundance of food.
123 Studies of changes in isotopic ratios based on either biomass gain or metabolic tissue turnover rates
124 have been mostly evidenced on larvae or juvenile organisms. In juvenile rainbow trout stable
125 isotopic values in white muscle resulted less variable than in other tissues. Juvenile animals that
126 migrate offshore from estuaries will tend to change their isotopic composition as they incorporate a
127 new diet.
128 Fatty acid and chemical composition were coupled with C and N isotope ratio analysis to
129 differentiate between different wild and farmed sea and freshwater fish species: turbot (Busetto el
130 al., 2008), sea bass (Fasolato et al., 2010), trout (Moreno-Rojas et al., 2008), mackerel, yellow
131 croaker and pollock (Kim et al., 2015; Thomas et al., 2008; Moreno-Rojas et al., 2008; Moreno-
132 Rojas et al., 2007).
133 The aim of the present study was to examine factors affecting carbon and nitrogen isotopic
134 composition and fatty acid profile in farmed and wild eels, in order to identify appropriate
135 biomarkers for discrimination of eels of different origin, farming system and dietary habits.
136

137 ## 2. Materials and methods

138 ### 2.1 Fish sampling and biometric measurements

139 Thirty-seven eels were sampled during winter across year 2014 and 2015. Eighteen farmed eels, 6
140 originating from Italy, 6 from Netherlands and 6 from Denmark, were purchased on Italian retail
141 market; other 8 eels were collected directly from an Italian farmer. Five wild eels were sampled
142 from the brackish lagoon of Bonello (Northern Adriatic sea) and 6 wild eels were purchased from a
143 local fisherman who operated in the Sacca of Scardovari (Northern Adriatic sea).

144 The fish were immediately stored under ice in polystyrene boxes until their arrival in the laboratory,
145 where fish were measured for total length and weighed for total mass; then fish were eviscerated
146 and stored in vacuum bags at -25°C until analysis.
147
148 **2.2 Proximate Composition and Fatty Acid Analysis**
149 All assays for proximate composition analysis were performed using official methods of analysis of
150 the Association of Official Analytical Chemists (AOAC, 1996). Moisture content of fillets was
151 determined by drying samples in an oven at 105 °C for 16–18 hours, according to AOAC method
152 950.46. Total protein was determined by wet digestion of the sample and subsequent distillation and
153 titration of ammonia content. Nitrogen concentration was converted to protein using a conversion
154 ratio of 6.25, according to AOAC method 940.25. For the analysis, an automated distillation unit
155 (Büchi 339, Switzerland) was used. Ash was determined by heating the dried sample in a muffle
156 furnace for about 10-12 hours at 650°C and weighting it after cooling, according to AOAC method
157 938.08.
158 The extraction and determination of total lipids was performed according to Folch et al. (1957) with
159 chloroform/methanol (2/1, v/v) on 500 mg of eel muscle tissue collected from dorsal, ventral and
160 caudal regions of fillets. The preparation of fatty acid methyl esters was performed according to
161 Christie (2003). Briefly, the lipid sample (20 mg) was dissolved in 10% methanolic hydrogen
162 chloride (2 mL). A 1 mL solution of tricosanoic acid (1 mg mL^{-1}) in toluene was added as internal
163 standard. The sample was sealed and heated at 50 °C overnight; then, 2 mL of a 1M potassium
164 carbonate solution and 5 mL of 5% NaCl solution were added to each sample. The FAMEs were
165 extracted with 2×2 mL of hexane and the mixture was evaporated to dryness under a stream of
166 nitrogen. The sample was dissolved in 1 mL hexane and 1 µL was injected into the gas-
167 chromatograph, in split mode (split ratio 1:100). Fatty acid analysis was carried out on an Agilent
168 gas-chromatograph (Model 6890 Series GC) fitted with an automatic sampler (Model 7683) and

169 FID detector. The carrier gas was helium with a flow rate of 1.0 mL min^{-1} at an i pressure of 16.9
170 psi. A HP-Innowax fused silica capillary column (30m×0.25mm I.D., 0.25 µm film thickness;
171 Agilent Technologies) was used to separate fatty acid methyl esters. The oven temperature program
172 was from 100 to 180 °C at 3 °C min^{-1}, then from 180 to 250 °C at 2.5 °C min^{-1} and held for 10 min.
173 Fatty acids were identified relative to known external standards.
174
175 **2.3 Isotope measurements**
176 For the determination of $\delta^{13}C$ and $\delta^{15}N$ 3 g of eel muscle were defatted using a non-chlorinated
177 solvents method (Smedes 1999). Briefly, 3 g of sample was added with 15 ml of cyclohexane and
178 12 ml of 2-propanol and homogenized with an Ultra-Turrax (IKA), then 15 ml of demineralized
179 water were added. Successively hydrophilic and hydrophobic phases were separated by
180 centrifugation and hydrophobic phase was discarded. A second extraction was performed by adding
181 to the mixture 20 ml of cyclohexane containing 13% 2-propanol (v/v). Before stable isotope
182 analysis, samples were dried under vacuum for 24 hours and then freeze-dried to obtain the defatted
183 dry samples.

184 $\delta^{13}C$ and $\delta^{15}N$ values were measured by continuous flow IRMS (Conflo IV Universal Interface)
185 using a Flash 2000 HT elemental analyzer coupled to a Delta V Advantage Isotope Ratio Mass
186 Spectrometer from Thermo Fisher Scientific (Bremen, Germany). This system was equipped with
187 the ISODAT Thermo Scientific software v 3.0 (Bremen, Germany) that was used to acquire and
188 process the signal obtained by IRMS instrument. Since fish muscle has a C:N ratio less than 5:1, the
189 CF-EA-IRMS system was operated in the dual isotope mode, allowing $\delta^{15}N$ and $\delta^{13}C$ to be
190 measured on the same sample. Amounts of 0.45–0.55 mg of defatted dry muscle sample were
191 weighed into tin capsules for measurements. The results of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$)
192 isotope ratio analyses were reported in per mil (‰) on the relative δ-scale and referred to the
193 international standards V-PDB (Vienna Pee Dee Belemnite) and Atmospheric Air for carbon and

194 nitrogen isotope ratios, respectively. All results were calculated according to the following
195 equation:
196 X (‰) = [(R_{sample}/$R_{reference}$) - 1] x 1000

197 where X is the ratio of the heavy to the light stable isotope (e.g. $^{13}C/^{12}C$) in the sample (R_{sample}) and
198 in the standard ($R_{reference}$). The calibration of the control gases (CO_2, N_2) was performed using the
199 following reference materials:

200 IAEA-CH7-Polyethylene ($\delta^{13}C$ = –32.15 ‰) and IAEA-CH6-sucrose ($\delta^{13}C$ = –10.4 ‰) for CO_2 gas
201 cylinder calibration (used for $\delta\ ^{13}C$ measurements).
202 IAEA-N1-Ammonium sulfate ($\delta^{15}N$ = 0.4 ‰) and IAEA-N2-ammonium sulfate ($\delta^{15}N$ = 20.3 ‰)
203 for N_2 gas cylinder calibration (used for $\delta\ ^{15}N$ measurements).

204 An internal standard was analyzed at regular intervals to control that the repeatability of the
205 measurements were acceptable, and to correct for possible drifts in the measurements. The relative
206 standard deviation (n = 10) determined using the corresponding reference gas was 0.05 % for $\delta^{13}C$
207 and 0.08 for $\delta^{15}N$. Each sample was analyzed in duplicate with variability less than 0.2 ‰.
208 One muscle sample was calibrated with the international reference materials mentioned before and
209 used as a working standard. The working standard was analyzed at regular intervals to control the
210 repeatability of the measurements and to correct possible deviations in the measurements.
211

212 **2.4 Statistical analysis**

213 Normal distribution and homogeneity of variance was confirmed using the Kolmogorov-Smirnov
214 and the Hartley test, respectively. Then, comparison between means was performed by analysis of
215 variance. The Student-Newman-Keuls was used as post-Hoc test for comparison of the means
216 among different eel origins. Significance was accepted at probabilities of 0.05 or less. These
217 statistical analyses were performed by SPSS version 25.0 (SPSS Inc. Chicago, Illinois).

Principal component analysis was performed in order to compare different samples and to detect the most important factors affecting the distribution of eel samples, using The Unscrambler version 10.4 (Camo, Norway). Afterwards, sparse partial least squares discriminant analysis (sPLS-DA) was performed on the data with R by means of the package mixOmics (Rohart et al., 2017) in order to point out the effect of the farming system, market size and farm origin. Autoscaling was used as pre-treatment of the data. Leave one out cross validation (LOOCV) was used as resampling method. The balanced error rate (BER) measured at the Mahalanobis distance was used to test the prediction ability of each model.

3. Results

3.1 Biometric Measurements and Proximate Composition

Table 1 shows eel used in our study according to their provenience (Italy, Netherland, Denmark, Lagoon or Sea), rearing system (pond, RAS, lagoon or wild) and commercial category (buratelli and capitoni). Capitoni average weight is above 500 g, with a length of more than 60 cm, while buratelli presented a mean weight less than 170 g and a length less than 50 cm. Lagoon eels were comparable to capitoni category, according with their dimension, while wild eels were in an intermediate size between capitoni and buratelli.

Moisture is higher in eels from lagoon (65.36%) and from the sea (64.54%) if compared with eels from intensive farming (ranging from 50.07% to 58.66%) with a statistical significance ($p<0.05$). In contrast, lipid has the opposite tendency, being higher in eels from intensive farming systems (from 24.96% to 34.89%) than in eels from the extensive farm (16.69% in lagoon and 15.97% in wild eels) with a statistical significance ($p<0.05$). Protein is higher ($p<0.05$) in wild eels (18.46%) and in eels from lagoon (16.74%) if compared with eels from intensive farming (ranging from 13.10% and 15.29%). Ash does not present any statistically significant difference between groups. No

differences are notable in proximate composition of eels farmed in Italy or abroad. If we consider only intensive eels, or rather eels that received artificial feed, therefore excluding lagoon and wild eels, we can observe differences in their proximate composition between commercial sizes (Table 2). Buratelli present a higher lipid content (33.86%) if compared with capitoni (26.87%) with a statistical significance ($p<0.05$). Consequently, capitoni have a higher moisture content and also a higher protein content, if compared to buratelli ($p<0.05$).

3.2 Fatty Acid Composition

Fatty acid composition of the thirty-seven eels analysed in our study are shown in Table 3. Monounsaturated fatty acids (MUFA) are dominants in all samples, followed by saturated fatty acids (SFA) and polyunsaturated fatty acids (PUFA). Palmitic acid (16:0, PA) is the dominant SFA in all eels, even if some differences are present among groups. Its value is statistically lower ($p<0.05$) in Danish and Dutch buratelli (14%) compared with all the other eels (19%). A similar trend is notable in stearic acid (18:0, SA), conversely myristic acid (14:0, MA) presents a higher value in buratelli farmed in Northern Europe. Oleic acid (18:1 n-9, OA) is the more abundant fatty acid among MUFA. Its presence follows the same trend of PA and SA. Cis-vaccenic acid (18:1 n-7) characterizes non-fed eels (lagoon and wild), in which it reaches a value of 5%, compared with the 3% of intensive farmed eels. Gondoic (20:1 n-9, GA), gadoleic (20:1 n-11) and cetoleic acid (22:1 n-11, CA) are MUFA that abounds in Dutch and Danish buratelli; gondoic acid in these fish reaches a value superior to 10% of total fatty acid while in all other eels its value is lower than 2.2 %. Similarly, cetoleic acid in Norther Europe buratelli reaches the notable value of 4.5 % and 5.4 %, while in all other eels its value is below 0.5 %, and not detected in eel caught in Adriatic Sea. PUFA are not particularly abundant in eels. Linoleic acid (18:2 n-6, LA) characterizes fed eels, particularly all capitoni. A difference is also notable in non-fed eels, where LA in lagoon eels (3.6%) is more than double of the value of sea eels (1.3%). Alfa linolenic acid (18:3 n-3, ALA) and gamma

linolenic acid (18:4 n-3) are more abundant in Northern Europe buratelli. Arachidonic acid (20:4 n-6, ARA) highest value is reached in lagoon eels (2.2%), followed by sea eels (1.3 %), whereas in all others eels it does not reach the 1%. Eicosapentaenoic acid (20:5 n-3, EPA) and docosapentaenoic acid (22:5 n-3, DPA) have the same trend, being more abundant in non-fed eels. Northern Europe buratelli present an EPA content similar to lagoon eels; EPA highest content is recorded in sea eels (4.5 %). Conversely, docosahexaenoic acid (22:6 n-3, DHA) is more abundant in fed eels, in particular in Danish buratelli, in which it reaches the highest value (6.5 %). DHA content differs in non-fed eels: it is more abundant in lagoon eels (4.3 %) compared with sea eels (2.5 %). The n-3/n-6 ratio is superior to 1 in all eels, with some statistical significative differences ($p<0.05$). Highest value is achieved in sea eels, mainly due to the lowest n-6 value than to a high n-3 presence. Similarly, Northern Europe capitoni have an n-3/n-6 ratio higher than 2, due to their high value of n-3 and low n-6. Lagoon eels present an n-3/n-6 ratio lower than 2, even if they have a high n-3 content, because of a relevant content of n-6 fatty acids.

3.3 Stable isotopes

Table 1 presents the trend of the isotopic values of the eels analysed. $\delta^{13}C$ values ranged between -23.13±1.57‰ and -18.1±0.50‰, while $\delta^{15}N$ values between 11.68±1.15‰ and 15.28±1.1‰. Carbon isotopic ratios were statistically different between sea eels (-23.13±1.57‰), Danish and Dutch buratelli (respectively, -20.27±0.22‰ and -20.37±0.48‰) and all the other samples (Italian pond fish, lagoon eels and Danish and Dutch capitoni) ($p < 0.01$). Nitrogen isotopic ratios were statistically different between lagoon eels (11.68±1.15‰), Danish and Dutch buratelli (respectively, 12.90±0.17‰ and 12.62±0.21‰) and all the other samples (wild eels, Italian pond eels and Danish and Dutch capitoni) ($p < 0.01$).

Both $\delta^{13}C$ and $\delta^{15}N$ allow discriminating eels coming from sea and lagoon and buratelli from capitoni in RAS farmed eels. Pond Italian eels, both buratelli and capitoni, showed $\delta^{13}C$ values

292 (respectively, -18.14±0.50‰ and -18.50±0.48‰) closer to lagoon ones (-18.12±0.34‰), whereas
293 their $\delta^{15}N$ values (respectively, 14.65±0.1‰ and 14.42±0.40‰) are statistically comparable with
294 sea eels values (15.28±1.10‰).

295 Capitoni and buratelli coming from Netherland or Denmark cannot be clearly distinct by stable
296 isotopes analysis.

297

298 **3.4 Multivariate data analysis**

299 PCA was used to provide an overview of the capacity of the variables (chromatographic and
300 isotopic measurements) to discriminate eels samples and to find the discriminating power of the
301 variables**.** PCA scores and loadings plots, obtained setting PC-1 and PC-2 values on the axes, are
302 presented in Figure 1; in this way, PCA explained the 65% of the variance, with PC-1 accounting
303 for 36 % and PC-2 for 29 % of the total variance. In addition, PC-3 accounted for the 11%, so that
304 the sum of the first three principal component explains the 76% of the total variance.
305 Eels sampled in this study could be separated by PCA model into 4 groups: the group containing
306 eels coming from the sea, the one containing eels coming from lagoon, one group containing
307 buratelli farmed in RAS in Netherlands and Denmark and another group that contains all the eels
308 farmed in pond system and capitoni farmed in RAS. Among variables, fatty acids are the most
309 contributing descriptors that influence the groups' separation. For instance, ARA and DPA were
310 potential markers for sea and lagoon eels, LA for pond eels and CA with GA for RAS buratelli. To
311 deepen knowledge on the differences among samples in regard to the three factors into which data
312 was divided (farming system, market size and farm origin), we carried out a series of discriminant
313 analyses. The sparse version of the partial least squares discriminant analysis (sPLS-DA) enabled us
314 to select the most predictive variables (potential markers) as well as classify the samples in one step
315 procedure. In addition, equal or better discrimination abilities were obtained in comparison to the

316 PLS-DA model when applied into the same data. Performance assessment was based on the
317 balanced error rate (BER), since it is appropriate when the number of samples per class is
318 unbalanced. It calculates the average proportion of samples that are wrongly classified, taken into
319 account the number of samples in each class, thus avoiding bias towards the majority classes.
320 Related to the farming system, a good classification performance was obtained, with an overall
321 BER of 0.053 in the cross-validation. The model was optimized for 3 components and the overall
322 BER was due to misleading classification of pond eels (with a class BER of 0.214), whereas RAS,
323 lagoon and sea eels were correctly classified in all cases, displaying a class BER of 0. The
324 component 1 and 3 (represented on the X-variate 1 and X-variate 3 respectively) showed the most
325 interesting differences among samples in regard to the farming system (Figure 2). Farmed eels
326 (POND and RAS systems) were clearly differentiated from the wild eels (lagoon and sea) in the
327 X-variate 1, which accounted for the 33 % of the total variance found in samples. The most
328 predictive compounds selected in the X-variate 1, and hence labelled as potential markers, were
329 cis-vaccenic acid, eicosatrienoic acid (20:3 n-3), DPA and adrenic acid (22:4 n-6), which displayed
330 overall higher values in sea and lagoon eels. Meanwhile, LA reached the highest contents in farmed
331 eel samples, especially in POND eels. Additionally, the X-variate 3, that explained 10 % of the
332 variance found in samples, allowed us to differentiate lagoon from sea eels. Variables driving these
333 differences were $\delta\ ^{15}N$, palmitoleic acid (16:1 n-7), 8,11,14-octadecatrienoic acid (18:3 n-4) and
334 paullinic acid (20:1 n-7), showing higher levels in sea eels as well as $\delta\ ^{13}C$, length, weight, ARA,
335 eicosadienoic acid (20:2 n-6) and ash, which in contrary displayed maximal mean values in lagoon
336 eel samples. In order to construct the market size model, length and weight variables were kept out
337 of the model to focus on the chemical differences between both groups. The model was optimized
338 for 3 components and displayed an overall BER of 0.115 in the leave one out cross-validation,
339 corresponding to a class BER of 0.154 for buratelli and 0.077 for capitoni eel groups. Differences
340 between both classes and the most predictive variables selected in the sPLS-DA model can be

observed in Figure 3. The main variation between classes was explained in the component 1 (X-variate 1), which accounted for the 52 % of the total variance found in samples. Variables selected in this X-variate were 9,12-hexadecadienoic acid (16:2 n-4), EPA, lipid content, MA and Eicosatetraenoic acid (20:4 n-3, ETA) which showed the overall maximal values in buratelli eels, while larger levels of LA, SA, Eicosadienoic acid and moisture content were observed in capitoni eel samples. Meanwhile, the overall BER obtained in the sPLS-DA performed on the farm origin factor was 0.624. Thus, the classification of the samples based on this factor was rejected.

4. Discussion

Eel is one the fattest fish species. Lipid content of eel muscle analysed in our study overcomes 33% in intensively farmed buratelli and 25% in intensively farmed capitoni. Non-fed eels definitely present the lower lipid content (16%), showing a statistically significative difference ($p<0.05$) between farmed and wild fish. Our results confirm the knowledge that, generally, farmed eels are fatter than wild caught eels (Lie et al, 1990; Abrami et al, 1992). In our work, it has been possible to compare eels of different size obtained with the same farming system; buratelli presents a statistically significant higher lipid content respect to capitoni ($p<0.05$), even if they have been farmed in both pond and RAS system. Amerio et al. (1996) found that farmed eels present a different lipid content according to their size, 27.19% and 30.57% for eels of 700g and 200g, respectively. An explication of the higher lipid content of buratelli, which are mature males, respect to capitoni, mature females, could be linked to the energy required by eels during their migration. Females, that are longer than male, have a better swimming performances and they would arrive earlier than male in the site of reproduction. Even if males start their migration earlier than females (Trischitta et al., 2014), they have to swim faster to arrive at the same time of female (Burgerhout et

al., 2013). The swimming cost for male eels would be much higher than for females because they are smaller and have to swim at higher speed (Van den Thillart et al. 2009).

Boëtius I. and Boëttius J. (1985) analysed eel samples caught from the Baltic Sea and at various time of starvation. They found silver eels (sexual mature eels that are starting their migration) fatter than yellow eels (immature on growing eels). Our findings confirm these results, since in our work intensive farmed eels were all classifiable as silver eels, while only two eels of wild and lagoon eels were silver. The onset of silvering is not completely clear. Durif et al. (2009) carried out a study collecting European silver eels from 5 different locations in France, whose ages ranged from 5 to 24 years. Initially, it was hypothesized that eels start the silvering when they reach a determinate muscle fat content, being the high rate of lipid in silver eel muscles an energy reserve for their long-range trans-oceanic migration, during which eels do not eat (Larson et al. 1990). Later, Svedäng and Wickström (1996) found that some female silver European eels presented muscle fat content lower than 10%. They suggested that the maturation process in eels may be more flexible than it was thought before, and that a possible temporary interruption of the migration occurs to resume feeding, with the purpose of accumulating enough fat reserves before migration starts again. A similar result has been achieved in two population of wild silver eels collected from Italian lagoon (*Comacchio* and *Marano/Grado*). The highest lipid storage in *Comacchio* eels did not lead to a real advantage in terms of reproduction (Mordenti et al, 2014). In general, a minimum, critical amount of fat is needed to start the metamorphosis but it is not the only factor that affects silvering.

Generally, fish muscle presents an inverse correlation between lipid and moisture content, while the protein content is constant and genetically determined (Shearer, 1994). In our study, the muscle protein content is inversely related with lipid content. It decreases when the percentage of lipid in muscle increases. A possible explanation for this phenomenon is the presence of adipose tissue intermixed in flesh of extreme fat eels that could have been analysed together with white and red muscle tissues.

389 Natural feeding habits of eels are various: adults feed on other fishes and benthic organisms, like
390 crustaceans and molluscs (Aida et al, 2003). Clearly, intensive farmed eels receive an artificially
391 diet, established to improve growth and to reach in the shortest time the commercial size.
392 Traditionally, eel feed is formulated using fish meal and oil as principal ingredients (Arai, 1989);
393 afterwards these two ingredients has been replaced with plant derived raw materials, in order to
394 contain feed prices and increase its sustainability. Most fish are not able to synthesize
395 polyunsaturated fatty acids (PUFA) de novo, so they must introduce them through feed; for these
396 reasons, fatty acids can also be considered "biomarkers" of feeding behaviour, being generally
397 transferred in a conservative way throughout the trophic web (Prigee et al., 2012; Vasconi et al,
398 2015). The results of fatty acid analysis presented in our work show that the Dutch and Danish eels
399 within the same category did not show any statistically significative difference. Moreover, within
400 the same farm, buratelli and capitoni presented a statistically different ($p<0.05$) amount of some
401 fatty acids, in particular OA, LA, ALA, GA and CA. These results suggest that the feed used for the
402 two commercial sizes is characterized by a different fatty acids composition; particularly, the feed
403 used with RAS buratelli presented a higher amount of GA and CA. The principal source of GA and
404 CA is fish oil coming from herrings, sprats and capelins caught from North Atlantic Sea, where they
405 feed on copepods rich in these fatty acids (Pascal and Ackman, 1976). Capitoni farmed in RAS and
406 generally eels coming from Italian pond systems, both capitoni and buratelli, presented a
407 statistically significant ($p<0.05$) higher amount of OA and LA, that are fatty acids markers of
408 muscle of fish fed with a plant derivate oils based, like soybean, rapeseed or sunflower.
409 Focussing on differences between capitoni and buratelli, regardless of the farm type, we obtained a
410 satisfactory classification in the discriminant model. Fatty acids were highlighted as the main
411 markers for discriminate between capitoni and buratelli.
412 In addition, differences in the fatty acid profile allowed to clearly differentiate wild from farmed
413 eels. However, if we compare wild and farmed eels there is not an extreme difference regarding the

414 fatty acids profile, as is obtained when comparing an intensive farmed fish with its wild counterpart.
415 This aspect is primarily due to the scarce presence of n-3 PUFA in muscle of wild eels. In a study in
416 which the fatty acid composition of wild and farmed eel muscle was compared (Abrami et al.,
417 1992), farmed eels, especially those fed with an addition of cod liver oil, resulted richer of DHA
418 than wild eels. Similar results were achieved measuring the fatty acid content of wild and farmed
419 Japanese eels. Japanese eels are characterized by the presence of MUFA as main fatty acid category
420 and farmed eels were found to be richer of n-3 PUFA (Oku et al., 2009). These two studies, carried
421 out in a period in which fish feed where richer of fish oil (if compared with the inclusion levels of
422 today), prove the efficiency of eel metabolic paths in the accumulation of high valuable fatty acid
423 when they are included in their diet.
424 Lagoon eels and sea eels present a different fatty acid profile and both of them differ from fed eels.
425 Several of them were found to be potential marker for differentiate both group of samples, as stated
426 before. Lagoon eels have n-6 PUFA amount statistically comparable with fed eels, but they present
427 the highest ARA content ($p<0.05$). Conversely, the lower n-6 PUFA content is recorded in sea eels.
428 Differences are notable also in n-3 PUFA. Sea eels present a statistically higher amount of EPA
429 respect to DHA ($p<0.05$), in opposite to lagoon eels. The differences in samples collected from two
430 very close areas are probably related to the variation of the two environmental condition. The lipids
431 coming from the food web of a Mediterranean coastal lagoon appear to be richer in SFA and n-6
432 PUFA if compared to open sea food web and fish that live in lagoons are richer in ARA, with
433 $p<0.05$. Consequently, they present some differences in their fatty acid profile if compared to the
434 one observed in marine species, caught in open waters (Koussoroplis et al., 2011). The main
435 difference existing from lagoon and open sea consists in the salinity of water. The salinity affects
436 the EPA/DHA ratio of fish tissue. Haliloğlu et al. (2004) have fed two groups of rainbow trout
437 reared at different salinity, freshwater and marine water, with the same feed. They found
438 insignificant effects of salinity in total SFA, MUFA and PUFA composition, but they evidenced

some differences in EPA/DHA ratio. Freshwater trout presented the lowest ratio and seawater trout the highest; a similar trend is present in eels examined in the current work, where EPA is dominant in sea eels.

The stable isotope analysis confirms partially what has been demonstrated using the fatty acid composition. Considering that the carbon and nitrogen ratio have been calculated on the defatted eels muscle, the results of this analysis could provide interesting information about the protein composition of diet, while fatty acid analysis provided information about lipid composition. On the basis of our results, it can be argued that feed that was administered to Danish and Dutch buratelli had a different protein composition respect to the one used to feed capitoni in the same farms. In the other hand, the diet fed in the pond farming system seems to have a similar isotopic composition for both the two commercial categories (buratelli/capitoni) and it was quite similar to the one used in capitoni farmed in RAS. The comparison between the stable isotope composition of sea and lagoon eels shows large differences, even greater than the ones highlighted in the lipid composition. In point of fact, $\delta\ ^{13}C$ and $\delta\ ^{15}N$ were selected as the most predictive variables for differentiate between lagoon and sea eel samples (Figure 2). The enrichment of $\delta^{15}N$ in sea eels, comparing to $\delta^{15}N$ values in eels coming from the lagoon, suggests an higher position in the trophic structure, as experimented by Perkins et al (2014) who measured $\delta\ ^{13}C$ and $\delta\ ^{15}N$ at four different trophic level in and experimental aquatic environment. Lagoon eels could have suffered by the reduction of their potentially preys during the sampling season. Eel is an opportunistic predator, with a high ability to change its diet according to environmental availability. Bouchereau et al. (2009) studied the diet of eels caught in the Mauguio lagoon (Mediterranean sea, France) and discovered a switch in eels' diet. During the winter, preys found in higher occurrence in eels' gut were polychaeta, while during spring and autumn fish and *gammarus* were the dominant preys. We can speculate that the statistically lower $\delta^{15}N$ value of lagoon eel ($p<0.05$), compared with sea eels, could be attributed to

463 a switch in their diet, which in the closed and limited environment of the lagoon had been based on
464 preys with a lower trophic position compared with sea eels.
465 Moreover, carbon isotopes values in fish muscle reflect relevant differences if we consider the
466 presence of vegetal or animal raw materials in commercial diets. Fish with a diet based on vegetal
467 origin meals and oils showed lower isotopic ratio values (Moreno-Rojas et al., 2008). According to
468 Bell et al. (2007) and Dempson and Power (2004), in our study we have observed that, generally,
469 wild eels had a lighter carbon composition (lower ^{13}C content and more negative values of $\delta^{13}C$) in
470 contrast to farmed eels, which present higher (less negative) values for $\delta^{13}C$ ($p<0.05$).
471

472 **5. Conclusions**

473 The present study contributed to the characterization of muscle tissue from eels coming from
474 different environment and different farming techniques. To the authors' knowledge, it is the first
475 time that a comparison of different origin of eels and production method was performed using the
476 combination of fatty acid and stable isotope analysis. The results have mainly demonstrated that the
477 fatty acid profile and stable isotopic composition were mainly influenced by diet provided to fish
478 and it was not possible to clearly discriminate eels coming from different farming system, that were
479 probably fed with feed based on the same ingredients. In contrast, eels that did not receive any
480 commercial feed, even if they were sampled in very close sites, could be distinct according to their
481 chemical characteristics.
482 This work has developed new instrumental biomarkers for assessing the authenticity of European
483 farmed and wild eel. Using a multidisciplinary approach, this study expanded our fundamental
484 knowledge of eel authenticity, whilst providing significant practical benefits with respect to
485 producers and final consumers, facilitating an economically sustainable growth of this important
486 regional industry.

At the consumer level, the described biomarkers could be a useful tool for developing a labeling protocol of marketed eels that is essential to inform consumers on what kind of fish they are buying. At farmers' level, they can use these tools to promote their products. As an example, the farmers who operate in a lagoon system, a valuable traditional and environmentally friendly farming technique, can take advantage of a tool that demonstrate the origin of their eels.

As a third aspect we have to take into account that the chemical characterization of eels is an important factor in order to distinguish farmed and wild samples, considering eels trade limitation linked to its ranking in CITES appendix II.

Our results could enable farmers to achieve higher product quality benchmarks, towards the ultimate goal of improving the marketability of eels both locally and in the European market.

Competing interests

Declarations of interest: none.

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