PAPER

FATTY ACIDS PROFILE IN *CARASSIUS SPP.* FROM LAKE GOPŁO, POLAND

M. STANEK*, J. DABROWSKI², G. MAIORANO³, S. TAVANIELLO³ and F. VIZZARRI³

¹Department of Animal Biochemistry and Biotechnology, Faculty of Animal Breeding and Biology, UTP University of Science and Technology, Bydgoszcz, Poland ²Chair of Ecology, Faculty of Animal Breeding and Biology, UTP University of Science and Technology, Bydgoszcz, Poland ³Department of Agricultural, Environmental and Food Sciences, University of Molise, Campobasso, Italy *Corresponding author: Tel.: +48 523749739, Fax. +48 523228158 E-mail address: winiarska@utp.edu.pl

ABSTRACT

Effects of sex and age on fatty acids profile in the meat of *Carassius* spp. were evaluated. *Carassius auratus* fillets displayed a higher content of SFA and MUFA than *Carassius carassius*. In contrast, *Carassius carassius* had a higher content of PUFA (higher proportion of linoleic, arachidonic, eicosapentaenoic, docosatetraenoic and docosapentaenoic acids) and total n-3 and n-6 PUFA, compared with *Carassius auratus*. Moreover, γ -linolenic acid was higher in fillets from *Carassius auratus*. What is more, fillets from females had a greater content of eicosapentaenoic acid than males. Finally, 4-year-old fish had a lower content of ALA, higher MUFA content and better nutritional indexes (n-3/n-6, AI) than 3-year-old fish.

Keywords: age, Carassius, fatty acids, sex

1. INTRODUCTION

Fish meat is considered a high quality-food for human consumption. It is also considered as a functional food that can promote superior health (HOSOMI *et al.*, 2012). Fish can be a great source of high amounts of protein, vitamins and minerals and most of all polyunsaturated fatty acids (PUFA) such as omega-3 (n-3) long-chain PUFA, including eicosapentaenoic acid (C20:5 n-3, EPA) or docosahexaenoic acid (C22:6 n-3, DHA), considered as 'essentials' (HUYNH and KITTS, 2009). In fact, these long-chain PUFA can be synthesized by humans from α -linolenic acid (C18:3 n-3, ALA), but due to low conversion efficiency, it is recommended to obtain EPA and DHA from additional sources, such as seafood. EPA and DHA are dietary fats with an array of health benefits. Indeed, a daily intake of 250–500 mg of EPA+DHA decreases the risk of mortality from coronary heart disease and sudden cardiac death (EFSA 2010). Moreover, EPA and DHA are also the precursors of several metabolites that are potent lipid mediators, considered by many investigators to be beneficial in the prevention or treatment of several diseases (SERHAN *et al.*, 2008). In contrast, EPA in blood is an extremely potent antithrombotic factor.

There are various studies, which have examined the effects of exogenous (SHIRAI *et al.*, 2002; LUZIA *et al.*, 2003; GULER *et al.*, 2008; KALYONCU *et al.*, 2009; JABEEN and CHAUDHRY, 2011) and endogenous (AKPINAR *et al.*, 2009; JABEEN and CHAUDHRY, 2011) factors on the fatty acids composition of fish meat. It should be noted that fish body composition is determined by different factors, such as sex (AKPINAR *et al.*, 2009), age (ALEMU *et al.*, 2013), feed availability, fishing season, location of reservoir, type of tissue and species (KALYONCU *et al.*, 2009, 2010; JABEEN and CHAUDHRY, 2011).

To our best knowledge, no research has been found on the effects of age and sex on muscle fatty acid composition in two different species of *Carassius*. Thus, the aim of this study was to analyze the impact of sex and age on the fatty acids profile in the meat of *Carassius carassius* L. and *Carsassius auratus gibelio* Bloch collected in the same exogenous conditions, in the autumn, from the Lake Gopło.

2. MATERIALS AND METHODS

2.1. Study area

Lake Gopło is located in the southern part of Kuyavian-Pomeranian province. The western part of this lake is a strict nature reservoir. The main morphometric indicators of Lake Gopło are as follows; surface area: 22 km², maximum depth: 16 m, average depth ca 4.7 m and length of shoreline: 90 km (ŁUCZYŃSKA *et al.*, 2008). Based on the limnological classification, it is an eutrophic reservoir, and, based on fishing classification, it is a zander type of the lake.

2.2. Biological material collecting

The study involved 56 individuals of *Carassius* (28 individuals of *Carassius carassius* L. and 28 *Carassius auratus gibelio* Bloch), selected according to the age (n=28 for fishes > 3-year-old and n=28 for fishes > 4-year-old) and sex (females: n=28; males: n=28). Fish were caught in natural conditions (Lake Gopło), in autumn (November 2015) and body weight and length were determined for each fish (Table 1).

	Carassius ca	arassius L.	Carassius auratus gibelio B.				
	Body weight (g)	Length (cm)	Body weight (g)	Length (cm)			
Age							
>3 years	176.9-206.4	16.3-17.4	176.5-209.0	16.0-17.5			
>4 years	267.1-297.5	18.0-19.6	255.3-317.0	19.5-20.8			
Sex							
Male	176.9-287.8	16.3-19.1	176.5-288.6	16.0-19.8			
Female	178.6-297.5	16.6-19.6	175.1-317.0	16.4-20.8			

Table 1. Body weight and length of *Carassius carassius* L. and *Carassius auratus gibelio* B. according to the age and sex.

The fillet samples for analyses were taken from the large side muscle of the fish body above the lateral line. The samples were vacuum packaged and stored frozen (-20°C) until analyzed for fatty acid composition.

2.3. Fatty acids analyses

Lipid extraction from muscle samples was performed by modification of the BLIGH and DYER (1959) and FOLCH et al. (1957) methods. Briefly, 100 mg of freeze-dried muscle was treated with chloroform/methanol/water (1:2:0.8) and stirred for 4 hours. Then, to obtain a better separation between the two phases, chloroform, 2N KCl/0.3 N HCl, and H₂O were added consecutively, and the samples were centrifuged for 5 min at 3000 g. The lower lipid-containing phase was separated from the upper phase, and retained for use. Subsequently, another extraction with chloroform was repeated. The extracted lipids were esterified and then analyzed by gas chromatography (GC Trace 2000 ThermoQuest EC Instruments) equipped with a flame ionization detector (260°C) and a fused silica capillary Column (Omegawax 320, Phenomenex, Torrance, CA, USA) 30 m x 0.32 mm x 0.25 µm film thickness. Helium was used as the carrier gas at a flow rate of 1.0 mL/min with constant flow compensation. GC inlets were held at a temperature of 240°C, and the detector was maintained at a temperature of 250°C. The oven temperature was programmed from 150°C, and was followed by a ramp-up at a rate of 5°C/min till 240°C with a final hold of 15 min (the total analysis time was 33.00 min). The individual fatty acids peaks were identified by comparison of retention times with those of known mixtures of standard fatty acids (PUFA-2, Supelco, Bellofonte, PA, USA) run under the same operating conditions. Results were expressed as percentage of the total fatty acids identified. An example of gas chromatography analysis (GC profile) is presented in Fig. 1. To assess the nutritional implications, the n-6 PUFA/n-3 PUFA and the PUFA/SFA ratios were calculated. The group of the fatty acids analyzed included saturated fatty acids (SFA) (C14:0, C16:0, C18:0), monounsaturated fatty acids (MUFA) (C16:1 n-7, C18:1 n-9, C18:1 n-7, C20:1 n-9,) and polyunsaturated fatty acids (PUFA) (C18:2 n-6, C18:3 n-6, C18:3 n-3, C20:3 n-3, C20:4 n-6, C20:5 n-3, C22:4 n-6, C22:5 n-3, C22:6 n-3). In order to evaluate the risk of atherosclerosis and the potential aggregation of blood platelets, respectively, the atherogenic index (AI) and the thrombogenic index (TI) were calculated, according to the formulas suggested by ULBRICHT and SOUTHGATE (1991). AI=[12:0+(4×14:0)+16:0]/[n-6 PUFA+n-3PUFA+MUFA]; TI=[14:0+16:0+18:0]/[(0.5×MUFA)+(0.5×n-6 PUFA)+(3×n-3PUFA)+(n-3 PUFA/n-6 PUFA)].

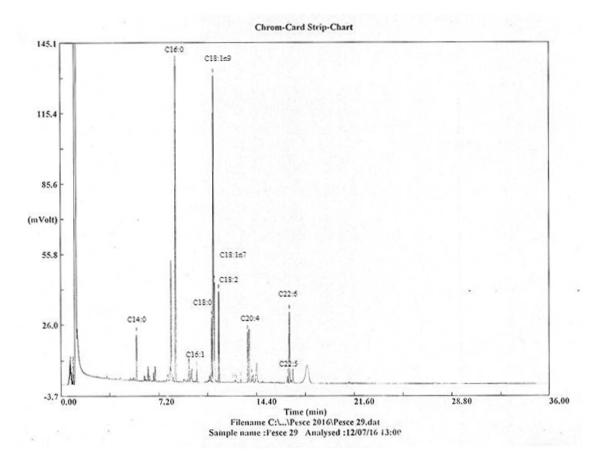


Figure 1. An example of gas chromatography profile.

2.4. Statistical analyses

Fatty acid composition and nutritional ratio data were analysed by $2\times2\times2$ factorial ANOVA with interactions, where species, sex and age were the main factors. Each individual fish was considered as the experimental unit. Data analysis was performed using the SPSS package (SPSS, 2010). Data are presented as means±SEM, and a value of p<0.05 was used to indicate statistical significance.

3. RESULTS

The fillet fatty acids composition and nutritional ratios are presented in Table 2. Overall, the MUFA were the most abundant acids ($39.86\pm0.42\%$), followed by SFA ($31.79\pm0.39\%$) and PUFA ($27.22\pm0.38\%$). The factor of greatest relevance affecting SFA content was fish species. *C. auratus* recorded significantly higher (+3%) SFA concentration compared to *C. carassius* (p<0.001), and particularly, higher proportions (p<0.001) of myristic (C14:0) and stearic (C18:0) acids, as well as palmitic acid (C16:0; p>0.05). As analyses indicated, sex did not significantly affect the total content of SFA or the content of the single SFA. The age factor affected only the palmitic acid content, which was higher (p<0.05) in fillets from 3-year-old fish compared to 4-year-old ones. Age did not influence the total content of SFA. In general, the palmitic acid was the most abundant ($25.7\pm0.37\%$) among SFA, followed by the stearic ($4.0\pm0.10\%$) and myristic acids ($2.1\pm0.09\%$).

Fatty acids‡	Species (SP)†		Sex (S)		Age (A)		SEM	Significance						
	СС	CA	Male	Female	>3 years	>4 years	SEM	SP	S	Α	SPxS	SPxA	SxA	SPxSxA
C14:0	1.65	2.56	1.99	2.22	2.14	2.07	0.09	***	ns	ns	ns	*	ns	ns
C16:0	25.14	26.23	25.48	25.90	26.46	24.92	0.37	ns	ns	*	ns	ns	ns	ns
C18:0	3.50	4.50	4.13	3.87	3.91	4.08	0.10	***	ns	ns	ns	**	ns	ns
C16:1	3.66	3.02	3.41	3.27	3.49	3.19	0.05	***	ns	**	ns	ns	ns	ns
C18:1n-9	28.06	29.49	28.75	28.80	27.22	30.33	0.41	ns	ns	***	ns	ns	ns	ns
C18:1n-7	5.72	7.11	6.36	6.48	6.52	6.32	0.11	***	ns	ns	ns	ns	ns	ns
C20:1n-9	1.31	1.35	1.24	1.41	1.32	1.33	0.05	ns	ns	ns	ns	ns	ns	ns
C18:2n-6	10.72	8.23	9.57	9.38	9.84	9.11	0.19	***	ns	ns	*	ns	ns	ns
C18:3n-6	0.26	0.36	0.32	0.30	0.32	0.30	0.02	*	ns	ns	ns	ns	*	ns
C18:3n-3	0.18	0.24	0.23	0.19	0.26	0.16	0.02	ns	ns	*	ns	ns	**	ns
C20:3n-3	0.43	0.37	0.43	0.37	0.43	0.37	0.03	ns	ns	ns	ns	ns	ns	ns
C20:4n-6	4.95	4.41	4.60	4.76	4.85	4.51	0.10	*	ns	ns	ns	ns	ns	ns
C20:5n-3	1.67	0.57	0.95	1.29	1.15	1.09	0.06	***	**	ns	**	ns	ns	**
C22:4n-6	0.32	0.24	0.29	0.28	0.30	0.26	0.01	*	ns	ns	**	ns	ns	ns
C22:5n-3	1.12	0.72	0.97	0.86	0.90	0.94	0.05	***	ns	ns	*	ns	**	ns
C22:6n-3	10.13	9.52	9.80	9.85	9.62	10.02	0.17	ns	ns	ns	ns	ns	ns	ns
Partial sums														
SFA	30.28	33.29	31.59	31.99	32.51	31.07	0.39	***	ns	ns	ns	ns	ns	ns
MUFA	38.76	40.97	39.76	39.96	38.56	41.17	0.42	*	ns	**	ns	ns	ns	ns
PUFA	29.79	24.66	27.16	27.29	27.68	26.77	0.38	***	ns	ns	**	ns	ns	ns
Total n-6	16.25	13.25	14.78	14.72	15.31	14.19	0.25	***	ns	*	*	ns	ns	ns
Total n-3	13.53	11.41	12.38	12.57	12.36	12.58	0.22	***	ns	ns	**	ns	ns	ns
Nutritional ratios														
n-3/n-6	0.84	0.87	0.85	0.87	0.82	0.90	0.02	ns	ns	*	ns	ns	ns	**
P/S	1.00	0.75	0.88	0.87	0.87	0.88	0.02	***	ns	ns	**	ns	ns	ns
AI	0.46	0.56	0.50	0.52	0.53	0.49	0.01	***	ns	*	ns	ns	ns	ns
TI	0.44	0.54	0.49	0.50	0.51	0.47	0.01	***	ns	ns	*	ns	ns	ns

Table 2. Effects of species, sex and age on fatty acids composition (% of total fatty acids) and nutritional ratios of fish fillets.

+CC = Carassius carassius L; CA = Carassius auratus gibelio Bloch; +SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids; P/S = PUFA/SFA ratio; AI = Atherogenic index; TI = Thrombogenic index. Significance: *** p<0.001; ** p<0.01; ** p<0.05; ns: not significant.

Age effect was evident in the oldest *C. auratus* fish for both myristic and stearic acid contents, which were in lower (p<0.05 and p<0.01) amounts in 4-year-old fish.

The total MUFA content was higher (p<0.05) in *C. auratus* fillets compared to those of *C. carassius* (+2.21%), with higher (p<0.001) content of vaccenic acid (C18:1n-7) and slightly higher (p>0.05) oleic acid (C18:1n-9). In contrast, percentage content of palmitoleic acid (C16:1) was lower in *C. auratus* fillets compared to those of *C. carassius* (p<0.001). Sex did not significantly affect total MUFA and the proportions of the single MUFA. As opposed to *C. carassius*, age has been a relevant factor for the total MUFA content. In fact, with the increase of the age of the fish, total MUFA content increased (<0.01), as did oleic acid (p<0.001), whereas palmitoleic acid decreased (p<0.01).

C. carassius also had higher content of total PUFA (+5.13%), in comparison with fillets of *C*. *auratus*, and this difference was statistically significant at p<0.001. A higher percentage (p<0.05 and p<0.001) content of linoleic (LA, C18:2n-6), arachidonic (AA, C20:4n-6), (EPA, C20:5n-3), docosatetraenoic (DTA, C22:4n-6) eicosapentaenoic and docosapentaenoic (DPA, C22:5n-3) acids, and content of total n-3 and n-6 fatty acids was found in the fillets of C. carassius, while γ -linolenic acid (GLA, C18:3 n-6) was higher (p < 0.05) in fillets from *C. auratus*. A high content of docosahexaenoic acid (DHA, C22:6 n-3) was also detected in both species, but no significant differences were found. Sex and age had a minimal influence on PUFA composition. Fillets from females had a higher (p < 0.01) content of EPA than males. Comparison among ages showed that fillets of 3-year-old fish had a higher (p < 0.05) content of α -linolenic (ALA, C18:3 n-3) and total n-6 fatty acids, compared to those of 4-year-old fish. Significant interactions were found between species and sex for total PUFA (p < 0.01), LA (p < 0.05), EPA (p < 0.01), DTA (p < 0.01), DPA (p < 0.05), total n-6 (p<0.05) and n-3 (p<0.01), between sex and age for GLA (p<0.05), ALA (p<0.01) and DPA (p < 0.01), as well as among species, sex and age for EPA (p < 0.01). Analyses indicated that the n-3/n-6 ratio (ranging from 0.82 to 0.90) was affected only by the age, being higher (p<0.01) in 4-year-old fish than in 3-year-old. In addition, significant interaction was found among species, sex and age in n-3/n-6 ratio (p<0.01). The PUFA/SFA ratio was affected only by species, being higher (*p*<0.001) in *C. carassius* than in *C. auratus* fish. However, a significant interaction (*p*<0.01) was found among species and sex for PUFA/SFA ratio. In the present study, AI and TI were lower (p < 0.001) in C. carassius than in C. auratus fish, and AI was lower (p < 0.05) in 4-year-old fish than in younger individuals. In addition, a significant (p < 0.05) interaction was found among species and sex for TI.

4. DISCUSSION

The results on the fillet fatty acids composition (31.79% of SFA, 39.86% of MUFA and 27.22% of PUFA) are in line with the values obtained by KOŁAKOWSKA *et al.* (2000) in roach (*Rutilus rutilus* L.), caught in the Odra and Regalica River (Poland), with higher amounts of total SFA (34.98%) and MUFA (46.83%) than total PUFA (18.19%). Different trends were reported by POLAK-JUSZCZAK and KOMAR-SZYMCZAK (2009), on roach caught from the Vistula Lagoon (25.10% of SFA, 32.49% of MUFA and 42.41% of PUFA), and by STANEK *et al.* (2008) on perch (*Perca fluviatilis* L.) from Włocławski Reservoir (46.02-47.53% of SFA, 23.83-32.24% of MUFA and 21.73-28.56% of PUFA).

Regarding the proportion of single SFA, palmitic acid was the most abundant, followed by stearic and myristic acids, respectively. The highest presence of palmitic acid (14.6-16.6%) was found in the meat of carp (*Cyprinus carpio* L.), caught in all four seasons (GULER *et al.*, 2008). In the meat of roach originating from the Mazurian Great Lakes region (Poland), palmitic acid content was the dominant SFA (25.38%) and the total amount of SFA for this

species accounting for 35.38% (ŁUCZYŃSKA *et al.* 2008). The same results were confirmed by ÖZOGUL *et al.* (2007) for the fish called kutum (*Rutilus frisii*) caught in the Seyhan Dam Lake in Adana (Turkey).

It is reported that SFA with 12-16 carbon atoms increase serum concentrations of LDL cholesterol (MENSINK and KATAN, 1992). In particular, palmitic acid increases total serum cholesterol, but its effect is lower than that of the myristic acids (DALEY *et al.*, 2010), which, in the present study, is approximately 2%. Stearic acid is considered a "neutral" fatty acid since it does not affect the plasmatic level of LDL or HDL cholesterol in humans (MENSINK and KATAN, 1992; WILLIAMSON *et al.*, 2005). This effect of stearic acid has been attributed to its reduced digestibility and easy desaturation into oleic acid. From a nutritional point of view, the oleic acid, the most common MUFA present in considerable quantities in both animal and plant sources, has a relevant importance in the human diet because it acts on lipaemia, reducing both LDL cholesterol and the triglycerides (MOZAFFARIAN and CLARKE, 2009) and providing other health benefits (reviewed in SALES-CAMPOS *et al.*, 2013).

In the present study, oleic acid ranged from 27.2% to 30.3 %, with the highest values in the fillet of 4-year-old fish. Regarding the composition of the single PUFA, the high amount of DHA in comparison with the low level of α -linolenic acid observed in *Carassius* is in agreement with the results obtained by MURZINA et al. (2016). DHA cannot be synthesized *de novo* in vertebrates, it is biosynthesized from its precursor α -linolenic acid during desaturation and elongation on biochemical pathways (STEFFENS and WIRTH, 2005; TOCHER, 2015). It is well known that the freshwater food system contains higher levels of linoleic and α -linolenic acids (TOCHER, 2003, 2010) leading to evident differences between freshwater and marine fish in terms of fatty acids distribution. In fact, the marine food chain is rich in EPA and DHA. However, some fish species from freshwater can also be a valuable source of EPA and DHA (STEFFENS and WIRTH, 2005; REITER and GRIMM, 2012), which derive from freshwater plankton (BRETT et al., 2009) and by endogenous metabolism (TOCHER, 2003). The dominant PUFA found in roach from Brda River (Poland) was linoleic acid (7.41-10.11%) (STANEK et al., 2012). Other studies in the meat of roach have found, among PUFA, the highest proportion of EPA, ranging from 7 to 12% (AHLGREN et al., 1994), 10% (GRAHL-NIELSEN et al., 2011) and 9% (UYSAL et al., 2008). However, variable values (ranging from 1.54 to 20.15%) were also found in 9 freshwater fish species from the Tigris River (CENGIZ et al., 2010). A diet rich in n-3 EPA is believed to shift the physiological state to one that is less inflammatory than that of a diet containing high amounts of n-6. In addition, marine-derived omega-3 fatty acids are recommended for the treatment and prevention of many chronic diseases, including cardiovascular disease and metabolic syndrome (HARRIS et al., 2010).

As analyses indicated, sex and age of *Carassius* had a minimal influence on PUFA composition. Analyses carried out by STANEK *et al.* (2012), concerning roach caught in fall and spring, indicated that the total PUFA content in the fish meat, ranged from 19.96 to 27.42%, and was not affected by sex.

Concerning the nutritional ratios, the n-3/n-6 ratio (ranging from 0.82 to 0.90) was affected only by age, being higher in 4-year-old fish. The value of n-3/n-6 ratio was found low (0.23) in the fillet of Nile tilapia (*Oreochromis niloticus*) (HERATH *et al.*, 2016) and in common carp (0.47) reared in natural temperatures with water from Lake Trasimeno (GERI *et al.*, 1995). GULER *et al.*, (2008) found in fillets of carp caught from Lake Beysehir, values of n-3/n-6 ratio near 1 in winter, spring and summer and 0.5 in autumn. In contrast, in the meat of carp, caught in Ivriz Dam Lake, n-3/n-6 ratio were found to be 1.08, 1.43, 1.64 and 1.60 in spring, summer, autumn and winter, respectively (KALYONCU *et al.*, 2010). The n-3/n-6 ratio has been suggested to be a useful indicator for comparing relative nutritional values of fish oils (PIGOTT and TUCKER, 1990). Furthermore, an increase in the human dietary n-3/n-6 fatty acids ratio is essential to prevent cardiomyopathy (by reducing plasma lipids) and to reduce cancer risk. Until recently, the dietary balance between n-3 and n-6 PUFA has been evenly balanced with a n-3/n-6 ratio of approximately 1; the modern western diet is now dominated by a n-3/n-6 ratio of approximately 0.06 (SIMOPOULOS, 2002). The ratio of n-3/n-6 PUFA in lipids of freshwater fish varies mostly between 0.5 and 3.8, whereas it varies between 4.7 and 14.4 in marine fish (GULER *et al.*, 2008).

The PUFA/SFA is used for the assessment of lipids on the basis of the proportions of the different fatty acid groups. In our study, the PUFA/SFA ratio was affected only by species, being higher in *C. carassius* than in *C. auratus* fish. The PUFA/SFA ratios obtained in the present study are similar to those reported in fillets of Nile tilapia (0.83–1.32; HERATH *et al.*, 2016). However, the PUFA/SFA ratio value of the fillets of the present study, ranged from 0.75 to 1.00, is well above the minimum value of 0.45 recommended by the Department of Health of the UK (HMSO, 1994) and confirms that these freshwater fish species are suitable for human consumption.

The AI and TI represent the criteria for evaluating the level and interrelation through which some fatty acids may have atherogenic or thrombogenic properties, respectively. In particular, these indexes take into account the different effects that single fatty acid might have on human health, and, in particular, on the probability of increasing the incidence of pathogenic phenomena, such as atheroma and/or thrombus formation (GARAFFO *et al.*, 2011). In the present study, AI and TI were lower in *C. carassius* than in *C. auratus* fish, and AI was lower in 4-year-old fish than in younger. The AI (ranging from 0.46 to 0.56) and TI (ranging from 0.44 to 0.54) values found in the current study can be considered low according with literature reports (ULBRICHT and SOUTHGATE, 1991; JANKOWSKA *et al.*, 2010; STANEK *et al.*, 2012), and not comparable with the values reported in the meat of ruminants (AI: 1.29 ± 0.26 , TI: 1.54 ± 0.179 , D'ALESSANDRO *et al.*, 2012) but is similar to poultry meat (AI: 0.56 ± 0.13 , TI: 0.55 ± 0.14 , LAUDADIO and TUFARELLI, 2010; AI: 0.43 ± 0.02 , HE *et al.*, 2015; AI: 0.42 ± 0.01 , TAVANIELLO *et al.*, 2018).

5. CONCLUSIONS

In conclusion, the results of this study indicate that the meat from *C. auratus* and *C. carassius* is characterized by a favourable fatty acids profile from the human health point of view. Considering that fish were caught in the same season, sex had a negligible effect on fatty acids composition, while species and age had a marked effect on it. Furthermore, *C. auratus* fillets displayed significantly higher contents of SFA and MUFA, and a lower content of PUFA compared to those of *C. carassius*. Fillets of 4-year-old fish had a lower content of ALA, higher MUFA content and better nutritional indexes (n-3/n-6 and AI) compared to those of 3-year-old fish. Both species of *Carassiuss* lightly differ in their feeding trait and trophic level. This may cause differences in the fatty acid composition. In light of the obtained results, further experimental investigations are needed to deepen knowledge on these freshwater fish species, considering the different place and different season in which they were caught, in view of their potential exploitation for human diet or aquaculture.

All procedures performed in studies were in accordance with the ethical standards of the institution or practice at which the studies were conducted. The authors declare that they have no conflict of interest. The study was supported by a departmental funding alone.

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