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Effects of salinity on lipids reserves, survival and growth of flathead grey mullet *Mugil cephalus* (Linnaeus, 1758)

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Mullet juvenile response to low salinity

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Abstract

Salinity is one of the main factors affecting teleost fish physiological performances. Its influence on survival and growth rate of juvenile (five- month- old) *Mugil cephalus* (25.33 ± 1.01 mm length) was tested under hatchery conditions. After acclimation, fish were reared for three months at two salinities: freshwater (FW, 0 ppt) and saltwater (SW, 36 ppt). At the end of the experiment, growth, survival, and lipids deposition in the liver and carcasses were evaluated by histological and biochemical methods.

Survival was high and similar between treatments. Fish reared in saltwater showed a significantly higher specific growth rate for body weight while Condition Index was similar. Lipid accumulation in the liver and total lipids and fatty acid profiles in the whole body were also similar among salinity treatments, while the levels of bioactive metabolite N-acylethanolamines were significantly higher in fish reared in FW.

The high survival rate, a similar Condition Index and lipid deposition in both treatments strongly suggest that the transfer of mullet juveniles from saltwater hatchery conditions to freshwater is a viable commercial scale production strategy. These results contribute to our understanding of mullet juveniles' performances under different salinities and indicate that FW conditions provide a suitable environment for mullet juveniles.

Keywords

Mugil cephalus; salinity; growth; survival; lipids.

1. Introduction

One of the main purposes of aquaculture is to sustainably maximize fish production outputs minimizing resources inputs (Lisboa, Barcarolli, Sampaio, & Bianchini, 2015). Salinity is a crucial abiotic factor with the potential to significantly influence fish growth (Cardona, 2000; Boeuf & Payan, 2001; Ruiz-Jarabo et al., 2019), by affecting feed consumption rates, digestibility and Feed

Conversion Ratios (FCR) (Resley, Webb, & Holt, 2006). In addition, the optimal salinity for growth and metabolic rates depends on the species life history strategies and developmental stages (Morgan & Iwama, 1991).

Euryhaline teleosts are able to synthesize new salt-transporting proteins during the passage from salt to fresh water and vice versa (Kidder III, Petersen, & Preston, 2006). Consequently, they can live in environments with a wide range of salinities by using energy demanding osmoregulatory mechanisms (Sampaio & Bianchini, 2002). Therefore, the growth of euryhaline species is clearly affected by salinity since the energy used for regulating homeostasis is not available for growth (Cardona, 2000). At their optimum salinity, euryhaline fish reach the highest growth rate at the lowest osmoregulation cost. Exposure to less-than-optimal salinity, and the associated metabolic costs of osmoregulation, may influence the fish energy status and the lipids dynamics between the liver, the muscular mass and body fats reserves (Rabeh, Telahigue, Boussoufa, Besbes, & El Cafsi, 2015), which in turn may compromise growth performances. Indeed, liver lipids deposition is recognised as a good indicator for the nutritional and physiological status of the fish (Caballero, López-Calero, Socorro, Roo, Izquierdo, 1999; Loi, Papadakis, Leggieri, Giménez Papiol, & Vallainc, 2020), as it can highlight the effects of malnutrition during the rearing process (Papadakis et al., 2009).

~~Thanks~~ Due to their euryhalinity and their low trophic status, mullets have recently become a species of interest for aquaculture production. The flathead grey mullet *Mugil cephalus* is a coastal species with a worldwide distribution (Crosetti & Blaber, 2016) and tolerates a wide range of salinities (Whitfield, Panfili, & Durand, 2012). Among mullets, the flathead grey mullet is appreciated especially for its salted and dried egg roe, a traditional product highly valued as a luxury food in many regions of the world (Crosetti & Blaber, 2016). Due to its omnivorous feeding habits, *M. cephalus* plays an ecologically important role in tropical and temperate estuaries and is suitable for both mono and polyculture practices (Biswas et al., 2012).

Currently, aquaculture of *M. cephalus* is at its dawn and is based on the collection of wild fry and their subsequent rearing up to marketable size (Biswas et al., 2012; Whitfield et al., 2012; El-Dahhar,

Salama Moustafa, & Elmorshedy, 2014), equal to 0.5 – 1 kg in flathead grey mullets cultured in Italian *valli* (Cataudella & Monaco, 1983). Juveniles are commonly cultured in extensive systems, such as coastal lagoons in the Mediterranean region, as well as in semi-intensive and intensive systems (Crosetti & Blaber, 2016).

Despite the ecological and economical importance of flathead grey mullet, little is currently known about their physiological performances under culture conditions. In particular, reports on the effects of salinity on this species growth are often contradictory (De Silva & Perera, 1976; Rodriguez, Flores, & Martinez, 1993; Cardona, 2000).

The impact of salinity on physiological performances and growth rates, therefore, remains of significant interest, also due to the business development opportunities that could arise from evidence of similar growth and survival rates between fresh and saltwater and the subsequent potential use of freshwater reservoirs for *M. cephalus* production. Accordingly, the aim of this study was to test the effects of two salinities on the growth performances and nutritional status of juvenile *M. cephalus* born and raised under laboratory conditions.

2. Materials and methods

2.1 Ethics statement

The present paper reports results originated from established protocols for the commercial rearing of grey mullet in captivity and all tested environmental conditions fall within the ecological range of the species. Hence, this trial does not fall into the regulation for the use of animals for experimental procedures (Directive 2010/63/UE art. 1 comma 5 letter e; Decree Law 26/2014 art. 2, comma 1 letter f). Nonetheless, all procedures during this trial were carried out in accordance with the Directive 2010/63/EU and the experimental design followed the 3Rs principles. It was conducted in the farming establishments of the IMC, authorized by the Sardinia Region with authorization number IT038OR501 and under veterinary supervision.

105 **2.2 Experimental set-up and juvenile rearing**

106 A total of 380 five- month- old juveniles (25.33 ± 1.01 mm length and 0.18 ± 0.03 g weight, mean ±
107 standard error), from the same egg batch and reared in intensive conditions according to Vallainc et
108 al. (2021) ~~took part~~were included in the experiment. Two groups of 190 individuals were removed
109 from the hatchery population and subjected to a 30 days acclimation period into two 1 m^3 tanks
110 connected to two distinct recirculating aquaculture systems (RAS).
111 During the acclimation period salinity was gradually reduced (1.2 ppt day^{-1}) in one of the acclimation
112 RAS from 36 ppt to 0 ppt. At the end of the acclimation period, the two populations were moved from
113 the acclimation tanks and distributed into two experimental RAS each consisting of three circular,
114 truncated, cone-shaped tanks of 300 L volume and equipped with a 500 µm net-mesh banjo filter,
115 supplied with biological and mechanical filtration (5 µm), a UV lamp (80 W), a protein skimmer
116 (Deltec, 12 W), water chiller (Teko, TK 1000) and aeration. Water recirculation flow in the tanks was
117 set to produce a stepwise increase during the experiment, 3 L per minute were ~~furnished~~ supplied
118 during the first month, 6 L per minute during the second and 9 L per minute for the last month of the
119 experiment. During the transfer from the acclimation system to the experimental tanks, a sample of
120 10 individuals from each population was collected to establish initial total body length and body
121 weight. Each experimental tank was then stocked with a density of 0.2 individuals per litre (60
122 individuals). Fish were fed *ad libitum* with artificial dry feed (Classic C-22, Skretting, 28% protein,
123 7% lipid, 6.5% ash, 4% fibre) throughout the acclimation period and the trial. The experiment was
124 conducted for three months.
125 Water parameters (Salinity, Temperature and Dissolved oxygen) were monitored daily with a digital
126 probe (Hach Lange HQ 40 d). The salinity was maintained at 0.2 ± 0.02 and 36.2 ± 0.09 ppt in FW
127 and SW respectively, the temperature was set at 22.4 ± 0.03 °C, and dissolved oxygen was 96.4 ±
128 0.13%. Ammonia and nitrite were monitored every 10 days and were kept below 0.25 mg l^{-1},
129 performing a 30% water exchange when necessary.
130 Faeces and uneaten feed were siphoned out of the tanks daily and discarded. Dead individuals were

also removed during siphoning operations and mortality in each tank was recorded.

2.3 Survival and Growth analysis

At the beginning of the trial ("time: zero", t0), a total of 10 juveniles per treatment were randomly collected and lethally anesthetised with clove oil (Erboristeria Magentina®, 0.1%). The samples were quickly transferred on a scale (Kern PLJ 2100-2M) to record their wet body ~~wet~~ weight (BW, g) and then photographed with a Canon G15 camera. Subsequently, the total length (TL, mm) of each fish was measured through image analysis software (ImageJ NIH, USA). At the end of the experiment ("time: final", tf), all individuals were counted to calculate final survival in each tank according to the formula [(number of survivors / initial number of fish in the tank) x 100], and then the mean value was calculated for each treatment. Additionally, total length and weight were recorded as described before. Growth was assessed based on mean TL and BW. These measurements were used to calculate the mean Specific Growth Rate (SGR, % day^{-1}) for total length and weight according to the formula [(ln final TL or BW – ln initial TL or BW) / days of experimental period x 100], for each replicate of each treatment. To establish if fish growth was isometric, the length-weight relationship or Condition Index (CI) was also calculated for each replicate at the end of the experiment, as the coefficient b (slope) of the regression line between the logarithmically transformed length and weight data according to Putra, Restu, & Kartika (2021).

2.4 Liver histology

~~Half of the individuals sampled for morphometry at the end of the acclimation period (N=5 for each acclimation tank)~~ A number of 5 fish for each acclimation tank at the end of the acclimation period and 5 fishes per replicate in each treatment (15 individuals per treatment) at the end of the trial, were preserved for histology in 10% buffered formalin. Livers were carefully extracted from the body, dehydrated in increasing concentrations of ethanol solutions (70–96%), and then embedded in methacrylate resin (Technovit 7100®, Kulzer, Germany). Sections of 4 µm were cut with a manual

rotary microtome (Leica RM2125, Germany), and then stained with Methylene Blue (Alfa Aesar, Thermo Fisher GmbH, Germany)/Azure II (Sigma-Aldrich, Germany)/Basic Fuchsin (Sigma-Aldrich, Germany) (Bennett, Wyrick, Lee, & McNeil, 1976). All the sections were examined under a microscope (Leica DM2000, Germany) connected to a digital camera (Leica DMC2900, Germany). Six microphotographs of histological sections of liver were taken at 100× magnification (Fig. 1A) for the estimation of the area covered with lipid vacuoles in the liver (ACLV%), according to Papadakis et al. (2009). The image analysis was performed using ImageJ. Photographs were firstly converted to grey scale to highlight the contrast between lipid vacuoles (in white) and the rest of the hepatic tissue (in dark grey). Tissue structures other than lipid vacuoles, such as blood vessels, were manually removed from the analysis (Fig. 1B). Thereafter, the boundaries of all lipid vacuoles were marked manually (Fig. 1C) and their total area was automatically calculated by the image analysis software (Fig. 1D).

2.5 Lipid analyses

The remaining half of the individuals used for initial biometrical measurements (N=5 for each acclimation tank), and 5 fishes per replicate in each treatment (15 individuals per treatment) at the end of the experiment, were frozen at -80°C for further analysis.

2.5.1 Total lipids and fatty acid (FA) analysis

Total lipids were extracted from homogenized whole body according to the method of Folch, Lees, & Sloane Stanley (1957) by a chloroform/methanol 2:1 (v/v) solution. Total lipid quantification was performed by the method of Chiang, Gessert, & Lowry (1957). Aliquots of the lipid fraction were mildly saponified in order to obtain free FA for High Performance Liquid Chromatograph (HPLC) and Gas Chromatography (GC) analysis. The separation and identification of unsaturated FA (UFA) was carried out using an Agilent 1100 HPLC System (Agilent, Palo Alto, CA, USA) equipped with a diode array detector (DAD) as previously reported (Banni et al., 1996). Saturated FA (SFA) were

183 measured, after methylation, as fatty acid methyl esters (FAME) by a GC (Agilent, Model 6890, Palo
184 Alto) equipped with a flame ionization detector (FID) (Batetta et al., 2009).
185
186 *2.5.2 N-acylethanolamines (NAE)*
187 Deuterated NAE were added as internal standards for their quantification to the samples before tissue
188 total lipids extraction. Analyses of NAE from chloroform were carried out by an Agilent UPLC
189 system (Agilent, Palo Alto, CA, USA) equipped with a mass spectrometry Agilent Technologies
190 QQQ triple quadrupole 6420 (LC-MS/MS) with an ESI source, using positive mode (ESI+) for the
191 compounds and their deuterated homologs quantification as described in Manca et al. (2021).
192
193 **2.6 Data analysis**
194 Data of survival, total lengths and body weights, SGR, Condition Index and lipids (liver coverage,
195 total lipids and fatty acids) were checked for the normality of distribution and the homoscedasticity
196 of variances.
197 When normality and homoscedasticity of variance were not satisfied, a Kruskal-Wallis one-way
198 analysis of variance on ranks or a General Linear Model (GLM) were preferred to a one-way or a
199 two-way ANOVA, respectively. For total lipids, FA and NAE analyses the statistical significance
200 among groups was assessed using the nonparametric Mann-Whitney test, two tail. Comparisons were
201 considered statistically significant when $P < 0.05$. All data expressed in percentage were transformed
202 prior to analysis using the arcsine of the square root transformation.
203 The analyses were performed using SigmaPlot 11, Statistica 6.1 StatSoft, Inc. (2004) and GraphPad
204 Prism 8 (La Jolla, CA, USA) statistical packages. Data in this paper are presented as mean ± standard
205 error (SE).
206
207 **3. Results**
208 **3.1 Survival and growth**

Survival at the end of experiment was 70.56 ± 11.95% in FW and 68.89 ± 5.88% in SW, resulting similar ($P > 0.05$) between the two treatments (Fig. 2).

During the three-month experiment, *M. cephalus* juveniles significantly grew both in length (Fig. 3A) and body weight (Fig. 3B) ($P < 0.05$), reaching 47.32 ± 1.08 mm and 1.21 ± 0.08 g in FW and 48.33 ± 0.83 mm and 1.29 ± 0.07 g in SW, respectively, with no statistical differences between the two treatments (Fig. 3).

SGR% in FW treatment was 0.66 ± 0.03 for TL (Fig. 4A) and 1.86 ± 0.08 for BW (Fig. 4B), while in SW reached higher values for both TL and BW (0.74 ± 0.02, Fig. 4A, and 2.28 ± 0.06, Fig. 4B, respectively). However, statistical analysis showed a significant difference only for SGR% for BW (Fig. 4B).

The Condition Index was slightly higher in FW (2.95 ± 0.07 versus 2.55 ± 0.13 in SW), although no statistical difference was highlighted by the analysis (Fig. 5). Growth was more isometric in FW, while juveniles were slightly longer than robust in SW.

3.2 Lipid analysis

3.2.1 Liver lipid analysis

ACLV% grew along the experiment and changed from 31.70 ± 5.20% to 32.80 ± 1.51% in FW, and from 29.90 ± 2.71% to 38.19 ± 0.65% in SW. Although the trend seemed increasing throughout time especially for SW, a two-way ANOVA did not detect differences between the initial and final values nor between treatments (Fig. 6).

3.2.2 Whole body lipid analysis

Total lipid content increased with growth in *M. cephalus* juveniles in SW (40.34 ± 10.31 ~~and~~ to 81.48 ± 8.86 mg/g tissue), however no changes were detected between SW and FW at the end of three-months (Tab. 1). Accordingly, total FA content in FW or SW showed no differences at the end of the study (Tab. 2). This is confirmed also for total UFA, while SFA were rather stable. n3 and n6 PUFA

families showed the same pattern of UFA and in particular 20:5n3 and 22:6n3. At tf, only 18:3n3 levels were higher in SW compared to FW, while 20:4n6, 22:5n6 and hydroperoxides (HP) were lower (Tab. 2).

Fish in SW had lower levels of anandamide (AEA, Fig. 7A), oleoylethanolamide (OEA, Fig. 7B) and docosahexaenoyl ethanolamide (DHEA, Fig. 7C) respect to FW at the end of the trial.

4. Discussion

Either fresh- and saltwater resulted suitable for *M. cephalus* juvenile survival. This result is supported by previous observations pointing toward the ability of mullet species to cope well with salinity fluctuations soon after hatching or even during the early embryos development stages (Sylvester, Nash, & Emberson, 1975; Lee & Menu, 1981; Walsh, Swanson, Lee, Banno, & Eda, 1989). Other investigators have suggested that newly-hatched *M. cephalus* larvae can survive in brackish water (17 to 28 ppt), for several days (Murashige et al., 1991); however, the ability to tolerate freshwater is directly proportional to their size (Rodriguez et al., 1993) as shown by Nordlie, Szelistowski, & Nordlie (1982) and Liao (1981), who compared the survival in freshwater of *M. cephalus* juveniles, recording lower survival rates for the smaller cohorts. Our results are however in contrast with those reported by Rodriguez and colleagues (1993) who exposed *M. cephalus* juveniles, to 0, 8, and 35 ppt ~~water~~ salinity level for three months recording very low survival in freshwater (less than 20%). Interestingly, this experiment was conducted with larger (ranging from 75 to 85 mm length) and likely older individuals compared to those used in the present study, which should have therefore better coped with low salinities. Conversely, the survival recorded in the present study is closer to that reported for juvenile *Mugil liza* (32.7 ± 0.10 mm length, 0.48 ± 0.01 g) reared in waters ranging from 0 to 24 ppt salinity level, which, being close to 100% in all treatments, does not appear to negatively impact survival in this species (Lisboa et al., 2015).

Available literature on the effect of salinity on mullet growth is often contradictory. Growth resulted higher in juvenile (Rodriguez et al., 1993) and adult (Liao, 1981) flathead grey mullet reared in marine

and brackish waters than in freshwater. In juvenile *M. liza*, final body weight and SGR were influenced by water salinity, being significantly higher in saltwater (24 ppt) than in freshwater (Lisboa et al., 2015). Conversely, *M. cephalus* juveniles (0.1-3 g BW) showed a more efficient growth in terms of BW and SGR in waters at intermediate salinities (10 and 20 ppt) rather than in fresh- or saltwater (De Silva & Perera, 1976). Finally, growth performance of *M. cephalus* juveniles (50 mm mean initial TL) was negatively affected by salinities above 5 ppt (Cardona, 2000).

This variability in reports is possibly the result of the different developmental stages of the fish being used in the different experiments, which can modify the energy cost associated with the maintenance of homeostasis (Lisboa et al., 2015).

In our experiment, salinity did not affect growth, in terms of total length and body weight. No statistical differences were observed between treatments, and at the end of the trial fish from both salinities doubled their TL, while their BW increased by almost six folds. However, the two treatments differed for SGR for BW, which was higher ($P < 0.05$) in saltwater fish. The lower growth observed in juveniles reared in freshwater may be related to the higher cost of the osmoregulatory mechanisms under this environmental condition (Sampaio & Bianchini, 2002), which diverts energy deriving from the feed from growth to osmoregulation (Cardona, 2000).

However, Condition Index did not differ between treatments. Generally, a Condition Index below 3 indicates that individuals are slim and have grown more in length than in weight, while when CI is above 3, individuals are considered to be rounder than longer; finally, a CI of 3 indicates isometric growth (Putra et al., 2021). According to Crosetti & Blaber (2016), in *M. cephalus* individuals the value of the slope is equal to 3 when fish present the optimal proportion between TL and BW. The Condition Index recorded in the present study (2.95 in FW and 2.55 in SW) indicates that mullet juveniles grew almost isometrically under the tested rearing conditions. In addition to being influenced by the environmental factors, the growth pattern of teleosts is affected by swimming ~~behaviour~~ patterns (Muchlisin, Musman, & Siti-Azizah, 2010). Therefore, the similar, isometric

growth observed in fish from both treatments suggests that the ~~behaviour~~ swimming patterns of fish was not affected by the different salinity.

The area covered by lipid vacuoles (ACLV%) in liver from FW was similar to the area occupied by lipids in SW, even if values were slightly higher (not significant) in the latter treatment. This indicates that fish from both treatments had accumulated a similar content of reserves in this tissue and that salinity may not have affected the lipid absorption and storage in hepatic tissue. Liver is the main metabolic organ and fulfils important functions in detoxification, digestive processes and lipid storage (Zambonino-Infante et al., 2008). No previous record is available on the comparison of liver area covered by lipids in different experimental salinities in the mugilidae family; however, the proximate composition of liver of *Chelon labrosus* (30-40 g), reared for 30 days in SW and FW, showed that total lipids were statistically higher in the latter treatment (Rabeh et al., 2015). Conversely, Rodriguez et al. (1993) observed a 30% decrease in liver total lipids of *M. cephalus* juveniles reared in freshwater, compared to fish maintained in waters with intermediate and marine salinities. Our lipid and fatty acid analysis, revealed some differences between FW and SW at tf, with higher levels of 18:3n3, and lower levels of 20:4n6 and 22:5n6 in SW compared to FW. Furthermore, the fish reared in saltwater had lower percentage of lipid HP, a marker of oxidative stress (Banni et al., 1996). However, these PUFA represent a minor lipid component thereby these changes did not modify substantially the fatty acid profile. On the other hand, the bioactive fatty acid derivatives N-acylethanolamines (NAEs) were found significantly increased in fish reared in FW with respect to those in SW.

Changes in NAEs levels suggest a different activity of the fatty-acid amide hydrolase 2 (FAAH2) and their relative degrading enzyme, which has been shown to mediate the hyperosmotic stress responses in non-rodent vertebrate (Krug et al., 2018). Future studies will aim at evaluating whether these lipid bioactive compounds contribute to better cope with different salinity environment by optimising the energy expenditure used for the osmoregulatory mechanisms without affecting the physiological growth.

In conclusion, survival, growth rate and Condition Index, as well as lipid deposition described in fish reared at the salinity of 0 ppt confirm that their performances are similar to those reared under marine conditions. Therefore, the transfer from saltwater hatchery conditions to freshwater would seem a viable commercial scale production strategy. The rearing in freshwater could increase the number of suitable spaces available for *M. cephalus* aquaculture, and could give a substantial boost to the development of a mullet aquaculture industry.

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Data availability statements

Raw data underpinning the analyses will be provided by the corresponding author upon reasonable request.

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444 B.G. Kapoor (Eds.), Feeding and Digestive Functions of Fishes (pp. 281–348). Enfield (NH), Science
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446

447 Table 1. Total lipids (mg/g tissue), total fatty acids (µg/mg lipids), total saturated fatty acids (µg/mg lipids),
448 and total unsaturated fatty acids (µg/mg lipids) measured in the whole body of *M. cephalus* juveniles, in the
449 two treatments (Mann-Whitney test, data expressed as mean ± SE). t0: start of the experiment; tf: end of the
450 experiment.

451

	t0		tf	
	FW	SW	FW	SW
Total lipids (mg/g tissue)	34.26 ± 3.30	40.34 ± 10.31	55.79 ± 8.38	81.48 ± 8.86
Total FA (µg/mg lipids)	605.78 ± 133.26	436.48 ± 147.03	901.19 ± 36.28	911.90 ± 50.60
Total SFA (µg/mg lipids)	252.30 ± 27.02	217.83 ± 40.12	286.20 ± 11.78	288.74 ± 16.54
Total UFA (µg/mg lipids)	353.50 ± 110.00	218.66 ± 115.12	614.97 ± 24.77	623.17 ± 36.80

452 † FA: fatty acids; SFA: saturated fatty acids; UFA: unsaturated fatty acids
453 ‡ * $p<0.05$; ** $p<0.005$; *** $p<0.001$

454

455 Table 2. Fatty acids composition, expressed in mol%/total fatty acids, measured in the whole body of *M.*
456 *cephalus* juveniles, in the two treatments (Mann-Whitney test, data expressed as mean ± SE). t0: start of the
457 experiment; tf: end of the experiment.

	t0		tf	
	FW	SW	FW	SW
10:0	0.09 ± 0.05	0.16 ± 0.08	0.04 ± 0.02	0.09 ± 0.04
12:0	0.14 ± 0.05	0.18 ± 0.05	0.06 ± 0.00	0.05 ± 0.00

14:0	4.39 ± 0.37	5.40 ± 0.48	3.67 ± 0.31	4.53 ± 0.29
15:0	0.73 ± 0.12	0.90 ± 0.12	0.48 ± 0.01	0.46 ± 0.04
16:0	30.29 ± 3.48	38.04 ± 3.59	23.09 ± 0.47	23.76 ± 0.89
17:0	0.58 ± 0.14	0.98 ± 0.27	0.43 ± 0.04	0.39 ± 0.02
18:0	10.63 ± 2.31	13.40 ± 2.74	5.48 ± 0.69	3.95 ± 0.46
20:0	2.31 ± 1.42	0.29 ± 0.05	0.21 ± 0.01	0.19 ± 0.01
22:0	0.14 ± 0.03	0.12 ± 0.01	0.16 ± 0.01	0.14 ± 0.01
16:1	5.18 ± 0.35	6.35 ± 1.02	6.88 ± 0.66	8.07 ± 0.64
18:1	15.72 ± 1.79	18.98 ± 0.82	14.26 ± 0.39	14.74 ± 0.67
20:1	1.2 ± 0.11	1.16 ± 0.11	1.18 ± 0.05	1.12 ± 0.06
18:3n3	1.48 ± 0.49	0.63 ± 0.42	1.74 ± 0.17	2.13 ± 0.15*
18:4n3	0.74 ± 0.30	0.42 ± 0.25	1.13 ± 0.14	1.38 ± 0.11
20:5n3	3.17 ± 1.23	1.11 ± 0.84	5.17 ± 0.22	4.69 ± 0.28
22:5n3	1.80 ± 0.53	1.50 ± 0.73	3.30 ± 0.14	3.30 ± 0.18
22:6n3	5.95 ± 2.36	1.81 ± 1.23	10.60 ± 1.33	8.10 ± 1.25
18:2n6	13.26 ± 3.47	7.68 ± 2.78	17.65 ± 0.81	19.48 ± 0.96
18:3n6	0.18 ± 0.04	0.11 ± 0.05	0.21 ± 0.02	0.26 ± 0.02
20:2n6	0.90 ± 0.14	0.78 ± 0.29	1.21 ± 0.11	1.02 ± 0.11
20:3n6	0.80 ± 0.16	0.50 ± 0.15	0.82 ± 0.03	0.85 ± 0.07
20:4n6	0.74 ± 0.24	0.29 ± 0.11	1.51 ± 0.30	0.81 ± 0.19**
22:5n6	0.26 ± 0.07	0.11 ± 0.07	0.57 ± 0.09	0.29 ± 0.03***
22:4n6	0.17 ± 0.01	0.17 ± 0.00	0.28 ± 0.03	0.22 ± 0.02
20:3n9	0.02 ± 0.01	0.03 ± 0.00	0.04 ± 0.01	0.03 ± 0.01
Total HP	2.14 ± 0.75	2.91 ± 0.74	0.30 ± 0.03	0.18 ± 0.01***
Total MUFA	21.92 ± 1.49	26.49 ± 0.79	22.32 ± 1.05	23.92 ± 1.29
Total UFA	50.70 ± 7.45	40.52 ± 6.63	66.39 ± 0.21	66.45 ± 1.06
Total SFA	49.30 ± 7.46	59.48 ± 6.63	33.61 ± 0.21	33.55 ± 1.06
PUFAn3	13.51 ± 4.62	4.45 ± 3.16	21.41 ± 1.27	19.09 ± 1.45
PUFAn6	16.24 ± 4.08	9.38 ± 3.05	22.25 ± 0.41	22.93 ± 0.71
PUFAn9	15.73 ± 1.79	18.99 ± 0.82	14.30 ± 0.39	14.76 ± 0.67

458 † HP: hydroperoxides; SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; UFA:
459 unsaturated fatty acids; PUFA: polyunsaturated fatty acids
460 ‡ * $p<0.05$; ** $p<0.005$; *** $p<0.001$
461

462 Figure 1. Image analysis of liver microphotographs (100×). A) Rough photo of liver tissue stained
463 with Methylene Blue/Azure II/Basic Fuchsin. B) After the conversion to grey scale, blood vessels
464 (black spot) are manually removed. C) The boundaries of lipid vacuoles are marked manually (in
465 red). D) Total area of lipid vacuoles (in black) is automatically calculated by the software.
466
467 Figure 2. Survival (%) of *M. cephalus* juveniles in the two treatments (one-way ANOVA, data
468 expressed as mean ± SE, N=3).
469
470 Figure 3. Growth of *M. cephalus* juveniles in terms of A) total length (TL) and B) body weight (BW)
471 in the two treatments. Significant differences between means are indicated with different letters
472 (General Linear Model, data expressed as mean ± SE, N=3). t0: start of the experiment; tf: end of the
473 experiment.
474
475 Figure 4. Specific Growth Rate (SGR%) of A) total length (TL) and B) body weight (BW) of *M.*
476 *cephalus* juveniles in the two treatments. Significant differences between means are indicated with
477 different letters (Kruskal-Wallis one-way analysis of variance on ranks, data expressed as mean ± SE,
478 N=3).
479
480 Figure 5. Condition Index of *M. cephalus* juveniles in the two treatments (one-way ANOVA, data
481 expressed as mean ± SE, N=3).
482
483 Figure 6. Area covered by lipid vacuoles (ACLV%) of *M. cephalus* juveniles in the two treatments
484 (two-way ANOVA, data expressed as mean ± SE, N=3). t0: start of the experiment; tf: end of the
485 experiment.
486

Figure 7. N-acylethanolamines (NAE), expressed as mol% of total FA, measured in the whole body of *M. cephalus* juveniles, in the two treatments. A) arachidonoylethanolamide (AEA), B) oleoylethanolamide (OEA), and C) docosahexaenoylethanolamide (DHEA) Values are presented as boxes (mean value) and whiskers (higher and lower values) (Mann-Whitney test). t0: start of the experiment; tf: end of the experiment.

Effects of salinity on lipids reserves, survival and growth of flathead grey mullet *Mugil cephalus* (Linnaeus, 1758)

Mullet juvenile response to low salinity

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Abstract

Salinity is one of the main factors affecting teleost fish physiological performances. Its influence on survival and growth rate of juvenile (five month old) *Mugil cephalus* (25.33 ± 1.01 mm length) was tested under hatchery conditions. After acclimation, fish were reared for three months at two salinities: freshwater (FW, 0 ppt) and saltwater (SW, 36 ppt). At the end of the experiment, growth, survival, and lipids deposition in the liver and carcasses were evaluated by histological and biochemical methods.

Survival was high and similar between treatments. Fish reared in saltwater showed a significantly higher specific growth rate for body weight while Condition Index was similar. Lipid accumulation in the liver and total lipids and fatty acid profiles in the whole body were also similar among salinity treatments, while the levels of bioactive metabolite N-acylethanolamines were significantly higher in fish reared in FW.

The high survival rate, a similar Condition Index and lipid deposition in both treatments strongly suggest that the transfer of mullet juveniles from saltwater hatchery conditions to freshwater is a viable commercial scale production strategy. These results contribute to our understanding of mullet juveniles' performances under different salinities and indicate that FW conditions provide a suitable environment for mullet juveniles.

Keywords

Mugil cephalus; salinity; growth; survival; lipids.

1. Introduction

One of the main purposes of aquaculture is to sustainably maximize fish production outputs minimizing resources inputs (Lisboa, Barcarolli, Sampaio, & Bianchini, 2015). Salinity is a crucial abiotic factor with the potential to significantly influence fish growth (Cardona, 2000; Boeuf & Payan, 2001; Ruiz-Jarabo et al., 2019), by affecting feed consumption rates, digestibility and Feed

Conversion Ratios (FCR) (Resley, Webb, & Holt, 2006). In addition, the optimal salinity for growth and metabolic rates depends on the species life history strategies and developmental stages (Morgan & Iwama, 1991).

Euryhaline teleosts are able to synthesize new salt-transporting proteins during the passage from salt to fresh water and vice versa (Kidder III, Petersen, & Preston, 2006). Consequently, they can live in environments with a wide range of salinities by using energy demanding osmoregulatory mechanisms (Sampaio & Bianchini, 2002). Therefore, the growth of euryhaline species is clearly affected by salinity since the energy used for regulating homeostasis is not available for growth (Cardona, 2000). At their optimum salinity, euryhaline fish reach the highest growth rate at the lowest osmoregulation cost. Exposure to less-than-optimal salinity, and the associated metabolic costs of osmoregulation, may influence the fish energy status and the lipids dynamics between the liver, the muscular mass and body fats reserves (Rabeh, Telahigue, Boussoufa, Besbes, & El Cafsi, 2015), which in turn may compromise growth performances. Indeed, liver lipids deposition is recognised as a good indicator for the nutritional and physiological status of the fish (Caballero, López-Calero, Socorro, Roo, Izquierdo, 1999; Loi, Papadakis, Leggieri, Giménez Papiol, & Vallainc, 2020), as it can highlight the effects of malnutrition during the rearing process (Papadakis et al., 2009).

Due to their euryhalinity and their low trophic status, mullets have recently become a species of interest for aquaculture production. The flathead grey mullet *Mugil cephalus* is a coastal species with a worldwide distribution (Crosetti & Blaber, 2016) and tolerates a wide range of salinities (Whitfield, Panfili, & Durand, 2012). Among mullets, the flathead grey mullet is appreciated especially for its salted and dried egg roe, a traditional product highly valued as a luxury food in many regions of the world (Crosetti & Blaber, 2016). Due to its omnivorous feeding habits, *M. cephalus* plays an ecologically important role in tropical and temperate estuaries and is suitable for both mono and polyculture practices (Biswas et al., 2012).

Currently, aquaculture of *M. cephalus* is at its dawn and is based on the collection of wild fry and their subsequent rearing up to marketable size (Biswas et al., 2012; Whitfield et al., 2012; El-Dahhar,

Salama Moustafa, & Elmorshedy, 2014), equal to 0.5 – 1 kg in flathead grey mullets cultured in Italian *valli* (Cataudella & Monaco, 1983). Juveniles are commonly cultured in extensive systems, such as coastal lagoons in the Mediterranean region, as well as in semi-intensive and intensive systems (Crosetti & Blaber, 2016).

Despite the ecological and economical importance of flathead grey mullet, little is currently known about their physiological performances under culture conditions. In particular, reports on the effects of salinity on this species growth are often contradictory (De Silva & Perera, 1976; Rodriguez, Flores, & Martinez, 1993; Cardona, 2000).

The impact of salinity on physiological performances and growth rates, therefore, remains of significant interest, also due to the business development opportunities that could arise from evidence of similar growth and survival rates between fresh and saltwater and the subsequent potential use of freshwater reservoirs for *M. cephalus* production. Accordingly, the aim of this study was to test the effects of two salinities on the growth performances and nutritional status of juvenile *M. cephalus* born and raised under laboratory conditions.

2. Materials and methods

2.1 Ethics statement

The present paper reports results originated from established protocols for the commercial rearing of grey mullet in captivity and all tested environmental conditions fall within the ecological range of the species. Hence, this trial does not fall into the regulation for the use of animals for experimental procedures (Directive 2010/63/UE art. 1 comma 5 letter e; Decree Law 26/2014 art. 2, comma 1 letter f). Nonetheless, all procedures during this trial were carried out in accordance with the Directive 2010/63/EU and the experimental design followed the 3Rs principles. It was conducted in the farming establishments of the IMC, authorized by the Sardinia Region with authorization number IT038OR501 and under veterinary supervision.

105 **2.2 Experimental set-up and juvenile rearing**

106 A total of 380 five month old juveniles (25.33 ± 1.01 mm length and 0.18 ± 0.03 g weight, mean ±
107 standard error), from the same egg batch and reared in intensive conditions according to Vallainc et
108 al. (2021) were included in the experiment. Two groups of 190 individuals were removed from the
109 hatchery population and subjected to a 30 days acclimation period into two 1 m^3 tanks connected to
110 two distinct recirculating aquaculture systems (RAS).

111 During the acclimation period salinity was gradually reduced (1.2 ppt day^{-1}) in one of the acclimation
112 RAS from 36 ppt to 0 ppt. At the end of the acclimation period, the two populations were moved from
113 the acclimation tanks and distributed into two experimental RAS each consisting of three circular,
114 truncated, cone-shaped tanks of 300 L volume and equipped with a 500 µm net-mesh banjo filter,
115 supplied with biological and mechanical filtration (5 µm), a UV lamp (80 W), a protein skimmer
116 (Deltec, 12 W), water chiller (Teko, TK 1000) and aeration. Water recirculation flow in the tanks was
117 set to produce a stepwise increase during the experiment, 3 L per minute were supplied during the
118 first month, 6 L per minute during the second and 9 L per minute for the last month of the experiment.
119 During the transfer from the acclimation system to the experimental tanks, a sample of 10 individuals
120 from each population was collected to establish initial total body length and body weight. Each
121 experimental tank was then stocked with a density of 0.2 individuals per litre (60 individuals). Fish
122 were fed *ad libitum* with artificial dry feed (Classic C-22, Skretting, 28% protein, 7% lipid, 6.5% ash,
123 4% fibre) throughout the acclimation period and the trial. The experiment was conducted for three
124 months.

125 Water parameters (Salinity, Temperature and Dissolved oxygen) were monitored daily with a digital
126 probe (Hach Lange HQ 40 d). The salinity was maintained at 0.2 ± 0.02 and 36.2 ± 0.09 ppt in FW
127 and SW respectively, the temperature was set at 22.4 ± 0.03 °C, and dissolved oxygen was 96.4 ±
128 0.13%. Ammonia and nitrite were monitored every 10 days and were kept below 0.25 mg l^{-1},
129 performing a 30% water exchange when necessary.

130 Faeces and uneaten feed were siphoned out of the tanks daily and discarded. Dead individuals were

also removed during siphoning operations and mortality in each tank was recorded.

2.3 Survival and Growth analysis

At the beginning of the trial ("time: zero", t0), a total of 10 juveniles per treatment were randomly collected and lethally anesthetised with clove oil (Erboristeria Magentina®, 0.1%). The samples were quickly transferred on a scale (Kern PLJ 2100-2M) to record their wet body weight (BW, g) and then photographed with a Canon G15 camera. Subsequently, the total length (TL, mm) of each fish was measured through image analysis software (ImageJ NIH, USA). At the end of the experiment ("time: final", tf), all individuals were counted to calculate final survival in each tank according to the formula [(number of survivors / initial number of fish in the tank) x 100], and then the mean value was calculated for each treatment. Additionally, total length and weight were recorded as described before. Growth was assessed based on mean TL and BW. These measurements were used to calculate the mean Specific Growth Rate (SGR, % day^{-1}) for total length and weight according to the formula [(ln final TL or BW – ln initial TL or BW) / days of experimental period x 100], for each replicate of each treatment. To establish if fish growth was isometric, the length-weight relationship or Condition Index (CI) was also calculated for each replicate at the end of the experiment, as the coefficient b (slope) of the regression line between the logarithmically transformed length and weight data according to Putra, Restu, & Kartika (2021).

2.4 Liver histology

A number of 5 fish for each acclimation tank at the end of the acclimation period and 5 fishes per replicate in each treatment (15 individuals per treatment) at the end of the trial, were preserved for histology in 10% buffered formalin. Livers were carefully extracted from the body, dehydrated in increasing concentrations of ethanol solutions (70–96%), and then embedded in methacrylate resin (Technovit 7100®, Kulzer, Germany). Sections of 4 µm were cut with a manual rotary microtome (Leica RM2125, Germany), and then stained with Methylene Blue (Alfa Aesar, Thermo Fisher

GmbH, Germany)/Azure II (Sigma-Aldrich, Germany)/Basic Fuchsin (Sigma-Aldrich, Germany) (Bennett, Wyrick, Lee, & McNeil, 1976). All the sections were examined under a microscope (Leica DM2000, Germany) connected to a digital camera (Leica DMC2900, Germany). Six microphotographs of histological sections of liver were taken at 100× magnification (Fig. 1A) for the estimation of the area covered with lipid vacuoles in the liver (ACLV%), according to Papadakis et al. (2009). The image analysis was performed using ImageJ. Photographs were firstly converted to grey scale to highlight the contrast between lipid vacuoles (in white) and the rest of the hepatic tissue (in dark grey). Tissue structures other than lipid vacuoles, such as blood vessels, were manually removed from the analysis (Fig. 1B). Thereafter, the boundaries of all lipid vacuoles were marked manually (Fig. 1C) and their total area was automatically calculated by the image analysis software (Fig. 1D).

2.5 Lipid analyses

The remaining half of the individuals used for initial biometrical measurements (N=5 for each acclimation tank), and 5 fishes per replicate in each treatment (15 individuals per treatment) at the end of the experiment, were frozen at -80°C for further analysis.

2.5.1 Total lipids and fatty acid (FA) analysis

Total lipids were extracted from homogenized whole body according to the method of Folch, Lees, & Sloane Stanley (1957) by a chloroform/methanol 2:1 (v/v) solution. Total lipid quantification was performed by the method of Chiang, Gessert, & Lowry (1957). Aliquots of the lipid fraction were mildly saponified in order to obtain free FA for High Performance Liquid Chromatograph (HPLC) and Gas Chromatography (GC) analysis. The separation and identification of unsaturated FA (UFA) was carried out using an Agilent 1100 HPLC System (Agilent, Palo Alto, CA, USA) equipped with a diode array detector (DAD) as previously reported (Banni et al., 1996). Saturated FA (SFA) were measured, after methylation, as fatty acid methyl esters (FAME) by a GC (Agilent, Model 6890, Palo

183 Alto) equipped with a flame ionization detector (FID) (Batetta et al., 2009).

184

185 *2.5.2 N-acylethanolamines (NAE)*

186 Deuterated NAE were added as internal standards for their quantification to the samples before tissue
187 total lipids extraction. Analyses of NAE from chloroform were carried out by an Agilent UPLC
188 system (Agilent, Palo Alto, CA, USA) equipped with a mass spectrometry Agilent Technologies
189 QQQ triple quadrupole 6420 (LC-MS/MS) with an ESI source, using positive mode (ESI+) for the
190 compounds and their deuterated homologs quantification as described in Manca et al. (2021).

191

192 **2.6 Data analysis**

193 Data of survival, total lengths and body weights, SGR, Condition Index and lipids (liver coverage,
194 total lipids and fatty acids) were checked for the normality of distribution and the homoscedasticity
195 of variances.

196 When normality and homoscedasticity of variance were not satisfied, a Kruskal-Wallis one-way
197 analysis of variance on ranks or a General Linear Model (GLM) were preferred to a one-way or a
198 two-way ANOVA, respectively. For total lipids, FA and NAE analyses the statistical significance
199 among groups was assessed using the nonparametric Mann-Whitney test, two tail. Comparisons were
200 considered statistically significant when $P < 0.05$. All data expressed in percentage were transformed
201 prior to analysis using the arcsine of the square root transformation.

202 The analyses were performed using SigmaPlot 11, Statistica 6.1 StatSoft, Inc. (2004) and GraphPad
203 Prism 8 (La Jolla, CA, USA) statistical packages. Data in this paper are presented as mean ± standard
204 error (SE).

205

206 **3. Results**

207 **3.1 Survival and growth**

Survival at the end of experiment was 70.56 ± 11.95% in FW and 68.89 ± 5.88% in SW, resulting similar ($P > 0.05$) between the two treatments (Fig. 2).

During the three-month experiment, *M. cephalus* juveniles significantly grew both in length (Fig. 3A) and body weight (Fig. 3B) ($P < 0.05$), reaching 47.32 ± 1.08 mm and 1.21 ± 0.08 g in FW and 48.33 ± 0.83 mm and 1.29 ± 0.07 g in SW, respectively, with no statistical differences between the two treatments (Fig. 3).

SGR% in FW treatment was 0.66 ± 0.03 for TL (Fig. 4A) and 1.86 ± 0.08 for BW (Fig. 4B), while in SW reached higher values for both TL and BW (0.74 ± 0.02, Fig. 4A, and 2.28 ± 0.06, Fig. 4B, respectively). However, statistical analysis showed a significant difference only for SGR% for BW (Fig. 4B).

The Condition Index was slightly higher in FW (2.95 ± 0.07 versus 2.55 ± 0.13 in SW), although no statistical difference was highlighted by the analysis (Fig. 5). Growth was more isometric in FW, while juveniles were slightly longer than robust in SW.

3.2 Lipid analysis

3.2.1 Liver lipid analysis

ACLV% grew along the experiment and changed from 31.70 ± 5.20% to 32.80 ± 1.51% in FW, and from 29.90 ± 2.71% to 38.19 ± 0.65% in SW. Although the trend seemed increasing throughout time especially for SW, a two-way ANOVA did not detect differences between the initial and final values nor between treatments (Fig. 6).

3.2.2 Whole body lipid analysis

Total lipid content increased with growth in *M. cephalus* juveniles in SW (40.34 ± 10.31 to 81.48 ± 8.86 mg/g tissue), however no changes were detected between SW and FW at the end of three-months (Tab. 1). Accordingly, total FA content in FW or SW showed no differences at the end of the study (Tab. 2). This is confirmed also for total UFA, while SFA were rather stable. n3 and n6 PUFA families

showed the same pattern of UFA and in particular 20:5n3 and 22:6n3. At tf, only 18:3n3 levels were higher in SW compared to FW, while 20:4n6, 22:5n6 and hydroperoxides (HP) were lower (Tab. 2). Fish in SW had lower levels of anandamide (AEA, Fig. 7A), oleoylethanolamide (OEA, Fig. 7B) and docosahexaenoyl ethanolamide (DHEA, Fig. 7C) respect to FW at the end of the trial.

4. Discussion

Either fresh- and saltwater resulted suitable for *M. cephalus* juvenile survival. This result is supported by previous observations pointing toward the ability of mullet species to cope well with salinity fluctuations soon after hatching or even during the early embryos development stages (Sylvester, Nash, & Emberson, 1975; Lee & Menu, 1981; Walsh, Swanson, Lee, Banno, & Eda, 1989). Other investigators have suggested that newly-hatched *M. cephalus* larvae can survive in brackish water (17 to 28 ppt), for several days (Murashige et al., 1991); however, the ability to tolerate freshwater is directly proportional to their size (Rodriguez et al., 1993) as shown by Nordlie, Szelistowski, & Nordlie (1982) and Liao (1981), who compared the survival in freshwater of *M. cephalus* juveniles, recording lower survival rates for the smaller cohorts. Our results are however in contrast with those reported by Rodriguez and colleagues (1993) who exposed *M. cephalus* juveniles, to 0, 8, and 35 ppt salinity level for three months recording very low survival in freshwater (less than 20%). Interestingly, this experiment was conducted with larger (ranging from 75 to 85 mm length) and likely older individuals compared to those used in the present study, which should have therefore better coped with low salinities. Conversely, the survival recorded in the present study is closer to that reported for juvenile *Mugil liza* (32.7 ± 0.10 mm length, 0.48 ± 0.01 g) reared in waters ranging from 0 to 24 ppt salinity level, which, being close to 100% in all treatments, does not appear to negatively impact survival in this species (Lisboa et al., 2015).

Available literature on the effect of salinity on mullet growth is often contradictory. Growth resulted higher in juvenile (Rodriguez et al., 1993) and adult (Liao, 1981) flathead grey mullet reared in marine and brackish waters than in freshwater. In juvenile *M. liza*, final body weight and SGR were

influenced by water salinity, being significantly higher in saltwater (24 ppt) than in freshwater (Lisboa et al., 2015). Conversely, *M. cephalus* juveniles (0.1-3 g BW) showed a more efficient growth in terms of BW and SGR in waters at intermediate salinities (10 and 20 ppt) rather than in fresh- or saltwater (De Silva & Perera, 1976). Finally, growth performance of *M. cephalus* juveniles (50 mm mean initial TL) was negatively affected by salinities above 5 ppt (Cardona, 2000).

This variability in reports is possibly the result of the different developmental stages of the fish being used in the different experiments, which can modify the energy cost associated with the maintenance of homeostasis (Lisboa et al., 2015).

In our experiment, salinity did not affect growth, in terms of total length and body weight. No statistical differences were observed between treatments, and at the end of the trial fish from both salinities doubled their TL, while their BW increased by almost six folds. However, the two treatments differed for SGR for BW, which was higher ($P < 0.05$) in saltwater fish. The lower growth observed in juveniles reared in freshwater may be related to the higher cost of the osmoregulatory mechanisms under this environmental condition (Sampaio & Bianchini, 2002), which diverts energy deriving from the feed from growth to osmoregulation (Cardona, 2000).

However, Condition Index did not differ between treatments. Generally, a Condition Index below 3 indicates that individuals are slim and have grown more in length than in weight, while when CI is above 3, individuals are considered to be rounder than longer; finally, a CI of 3 indicates isometric growth (Putra et al., 2021). According to Crosetti & Blaber (2016), in *M. cephalus* individuals the value of the slope is equal to 3 when fish present the optimal proportion between TL and BW. The Condition Index recorded in the present study (2.95 in FW and 2.55 in SW) indicates that mullet juveniles grew almost isometrically under the tested rearing conditions. In addition to being influenced by the environmental factors, the growth pattern of teleosts is affected by swimming patterns (Muchlisin, Musman, & Siti-Azizah, 2010). Therefore, the similar, isometric growth observed in fish from both treatments suggests that the swimming patterns of fish was not affected by the different salinity.

The area covered by lipid vacuoles (ACLV%) in liver from FW was similar to the area occupied by lipids in SW, even if values were slightly higher (not significant) in the latter treatment. This indicates that fish from both treatments had accumulated a similar content of reserves in this tissue and that salinity may not have affected the lipid absorption and storage in hepatic tissue. Liver is the main metabolic organ and fulfils important functions in detoxification, digestive processes and lipid storage (Zambonino-Infante et al., 2008). No previous record is available on the comparison of liver area covered by lipids in different experimental salinities in the mugilidae family; however, the proximate composition of liver of *Chelon labrosus* (30-40 g), reared for 30 days in SW and FW, showed that total lipids were statistically higher in the latter treatment (Rabeh et al., 2015). Conversely, Rodriguez et al. (1993) observed a 30% decrease in liver total lipids of *M. cephalus* juveniles reared in freshwater, compared to fish maintained in waters with intermediate and marine salinities. Our lipid and fatty acid analysis, revealed some differences between FW and SW at tf, with higher levels of 18:3n3, and lower levels of 20:4n6 and 22:5n6 in SW compared to FW. Furthermore, the fish reared in saltwater had lower percentage of lipid HP, a marker of oxidative stress (Banni et al., 1996). However, these PUFA represent a minor lipid component thereby these changes did not modify substantially the fatty acid profile. On the other hand, the bioactive fatty acid derivatives N-acylethanolamines (NAEs) were found significantly increased in fish reared in FW with respect to those in SW.

Changes in NAEs levels suggest a different activity of the fatty-acid amide hydrolase 2 (FAAH2) and their relative degrading enzyme, which has been shown to mediate the hyperosmotic stress responses in non-rodent vertebrate (Krug et al., 2018). Future studies will aim at evaluating whether these lipid bioactive compounds contribute to better cope with different salinity environment by optimising the energy expenditure used for the osmoregulatory mechanisms without affecting the physiological growth.

In conclusion, survival, growth rate and Condition Index, as well as lipid deposition described in fish reared at the salinity of 0 ppt confirm that their performances are similar to those reared under marine

conditions. Therefore, the transfer from saltwater hatchery conditions to freshwater would seem a viable commercial scale production strategy. The rearing in freshwater could increase the number of suitable spaces available for *M. cephalus* aquaculture, and could give a substantial boost to the development of a mullet aquaculture industry.

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Data availability statements

Raw data underpinning the analyses will be provided by the corresponding author upon reasonable request.

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Table 1. Total lipids (mg/g tissue), total fatty acids (µg/mg lipids), total saturated fatty acids (µg/mg lipids), and total unsaturated fatty acids (µg/mg lipids) measured in the whole body of *M. cephalus* juveniles, in the two treatments (Mann-Whitney test, data expressed as mean ± SE). t0: start of the experiment; tf: end of the experiment.

	t0		tf	
	FW	SW	FW	SW
Total lipids (mg/g tissue)	34.26 ± 3.30	40.34 ± 10.31	55.79 ± 8.38	81.48 ± 8.86
Total FA (µg/mg lipids)	605.78 ± 133.26	436.48 ± 147.03	901.19 ± 36.28	911.90 ± 50.60
Total SFA (µg/mg lipids)	252.30 ± 27.02	217.83 ± 40.12	286.20 ± 11.78	288.74 ± 16.54
Total UFA (µg/mg lipids)	353.50 ± 110.00	218.66 ± 115.12	614.97 ± 24.77	623.17 ± 36.80

† FA: fatty acids; SFA: saturated fatty acids; UFA: unsaturated fatty acids
‡ * p<0.05; ** p<0.005; *** p<0.001

Table 2. Fatty acids composition, expressed in mol%/total fatty acids, measured in the whole body of *M. cephalus* juveniles, in the two treatments (Mann-Whitney test, data expressed as mean ± SE). t0: start of the experiment; tf: end of the experiment.

	t0		tf	
	FW	SW	FW	SW
10:0	0.09 ± 0.05	0.16 ± 0.08	0.04 ± 0.02	0.09 ± 0.04
12:0	0.14 ± 0.05	0.18 ± 0.05	0.06 ± 0.00	0.05 ± 0.00
14:0	4.39 ± 0.37	5.40 ± 0.48	3.67 ± 0.31	4.53 ± 0.29
15:0	0.73 ± 0.12	0.90 ± 0.12	0.48 ± 0.01	0.46 ± 0.04
16:0	30.29 ± 3.48	38.04 ± 3.59	23.09 ± 0.47	23.76 ± 0.89

17:0	0.58 ± 0.14	0.98 ± 0.27	0.43 ± 0.04	0.39 ± 0.02
18:0	10.63 ± 2.31	13.40 ± 2.74	5.48 ± 0.69	3.95 ± 0.46
20:0	2.31 ± 1.42	0.29 ± 0.05	0.21 ± 0.01	0.19 ± 0.01
22:0	0.14 ± 0.03	0.12 ± 0.01	0.16 ± 0.01	0.14 ± 0.01
16:1	5.18 ± 0.35	6.35 ± 1.02	6.88 ± 0.66	8.07 ± 0.64
18:1	15.72 ± 1.79	18.98 ± 0.82	14.26 ± 0.39	14.74 ± 0.67
20:1	1.2 ± 0.11	1.16 ± 0.11	1.18 ± 0.05	1.12 ± 0.06
18:3n3	1.48 ± 0.49	0.63 ± 0.42	1.74 ± 0.17	2.13 ± 0.15*
18:4n3	0.74 ± 0.30	0.42 ± 0.25	1.13 ± 0.14	1.38 ± 0.11
20:5n3	3.17 ± 1.23	1.11 ± 0.84	5.17 ± 0.22	4.69 ± 0.28
22:5n3	1.80 ± 0.53	1.50 ± 0.73	3.30 ± 0.14	3.30 ± 0.18
22:6n3	5.95 ± 2.36	1.81 ± 1.23	10.60 ± 1.33	8.10 ± 1.25
18:2n6	13.26 ± 3.47	7.68 ± 2.78	17.65 ± 0.81	19.48 ± 0.96
18:3n6	0.18 ± 0.04	0.11 ± 0.05	0.21 ± 0.02	0.26 ± 0.02
20:2n6	0.90 ± 0.14	0.78 ± 0.29	1.21 ± 0.11	1.02 ± 0.11
20:3n6	0.80 ± 0.16	0.50 ± 0.15	0.82 ± 0.03	0.85 ± 0.07
20:4n6	0.74 ± 0.24	0.29 ± 0.11	1.51 ± 0.30	0.81 ± 0.19**
22:5n6	0.26 ± 0.07	0.11 ± 0.07	0.57 ± 0.09	0.29 ± 0.03***
22:4n6	0.17 ± 0.01	0.17 ± 0.00	0.28 ± 0.03	0.22 ± 0.02
20:3n9	0.02 ± 0.01	0.03 ± 0.00	0.04 ± 0.01	0.03 ± 0.01
Total HP	2.14 ± 0.75	2.91 ± 0.74	0.30 ± 0.03	0.18 ± 0.01***
Total MUFA	21.92 ± 1.49	26.49 ± 0.79	22.32 ± 1.05	23.92 ± 1.29
Total UFA	50.70 ± 7.45	40.52 ± 6.63	66.39 ± 0.21	66.45 ± 1.06
Total SFA	49.30 ± 7.46	59.48 ± 6.63	33.61 ± 0.21	33.55 ± 1.06
PUFAn3	13.51 ± 4.62	4.45 ± 3.16	21.41 ± 1.27	19.09 ± 1.45
PUFAn6	16.24 ± 4.08	9.38 ± 3.05	22.25 ± 0.41	22.93 ± 0.71
PUFAn9	15.73 ± 1.79	18.99 ± 0.82	14.30 ± 0.39	14.76 ± 0.67

† HP: hydroperoxides; SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; UFA: unsaturated fatty acids; PUFA: polyunsaturated fatty acids

‡ * $p<0.05$; ** $p<0.005$; *** $p<0.001$

Figure 1. Image analysis of liver microphotographs (100×). A) Rough photo of liver tissue stained with Methylene Blue/Azure II/Basic Fuchsin. B) After the conversion to grey scale, blood vessels (black spot) are manually removed. C) The boundaries of lipid vacuoles are marked manually (in red). D) Total area of lipid vacuoles (in black) is automatically calculated by the software.

464

465 Figure 2. Survival (%) of *M. cephalus* juveniles in the two treatments (one-way ANOVA, data
466 expressed as mean ± SE, N=3).

467

468 Figure 3. Growth of *M. cephalus* juveniles in terms of A) total length (TL) and B) body weight (BW)
469 in the two treatments. Significant differences between means are indicated with different letters
470 (General Linear Model, data expressed as mean ± SE, N=3). t0: start of the experiment; tf: end of the
471 experiment.

472

473 Figure 4. Specific Growth Rate (SGR%) of A) total length (TL) and B) body weight (BW) of *M.*
474 *cephalus* juveniles in the two treatments. Significant differences between means are indicated with
475 different letters (Kruskal-Wallis one-way analysis of variance on ranks, data expressed as mean ± SE,
476 N=3).

477

478 Figure 5. Condition Index of *M. cephalus* juveniles in the two treatments (one-way ANOVA, data
479 expressed as mean ± SE, N=3).

480

481 Figure 6. Area covered by lipid vacuoles (ACLV%) of *M. cephalus* juveniles in the two treatments
482 (two-way ANOVA, data expressed as mean ± SE, N=3). t0: start of the experiment; tf: end of the
483 experiment.

484

485 Figure 7. N-acylethanolamines (NAE), expressed as mol% of total FA, measured in the whole body
486 of *M. cephalus* juveniles, in the two treatments. A) arachidonoylethanolamide (AEA), B)
487 oleoylethanolamide (OEA), and C) docosahexaenoylethanolamide (DHEA) Values are presented as
488 boxes (mean value) and whiskers (higher and lower values) (Mann-Whitney test). t0: start of the
489 experiment; tf: end of the experiment.

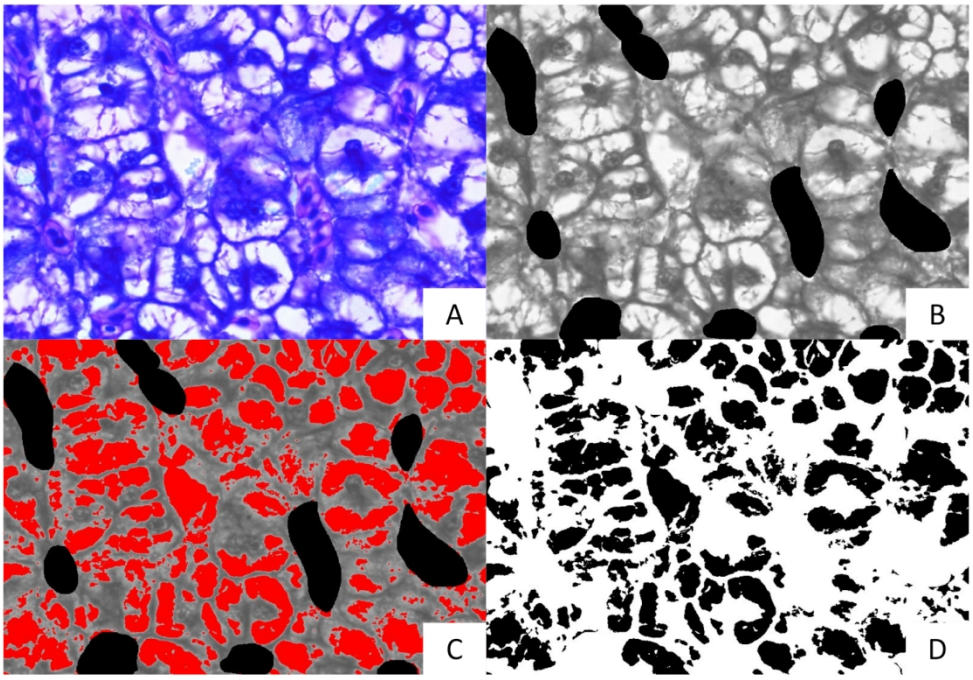
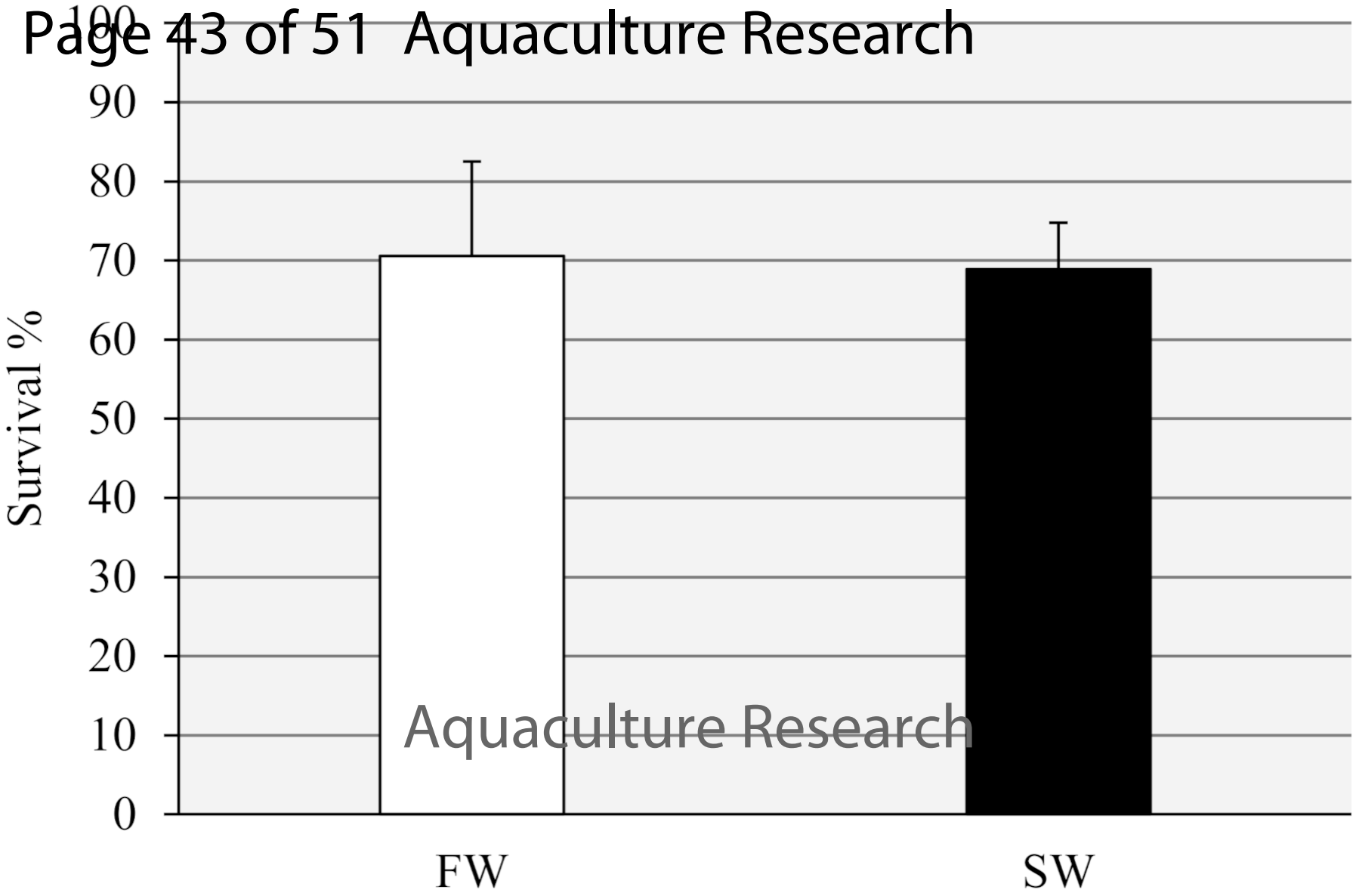


Image analysis of liver microphotographs (100×). A) Rough photo of liver tissue stained with Methylene Blue/Azure II/Basic Fuchsin. B) After the conversion to grey scale, blood vessels (black spot) are manually removed. C) The boundaries of lipid vacuoles are marked manually (in red). D) Total area of lipid vacuoles (in black) is automatically calculated by the software.

160x111mm (300 x 300 DPI)


100
90
80
70
60
50
40
30
20
10
0
Survival %
FW
SW
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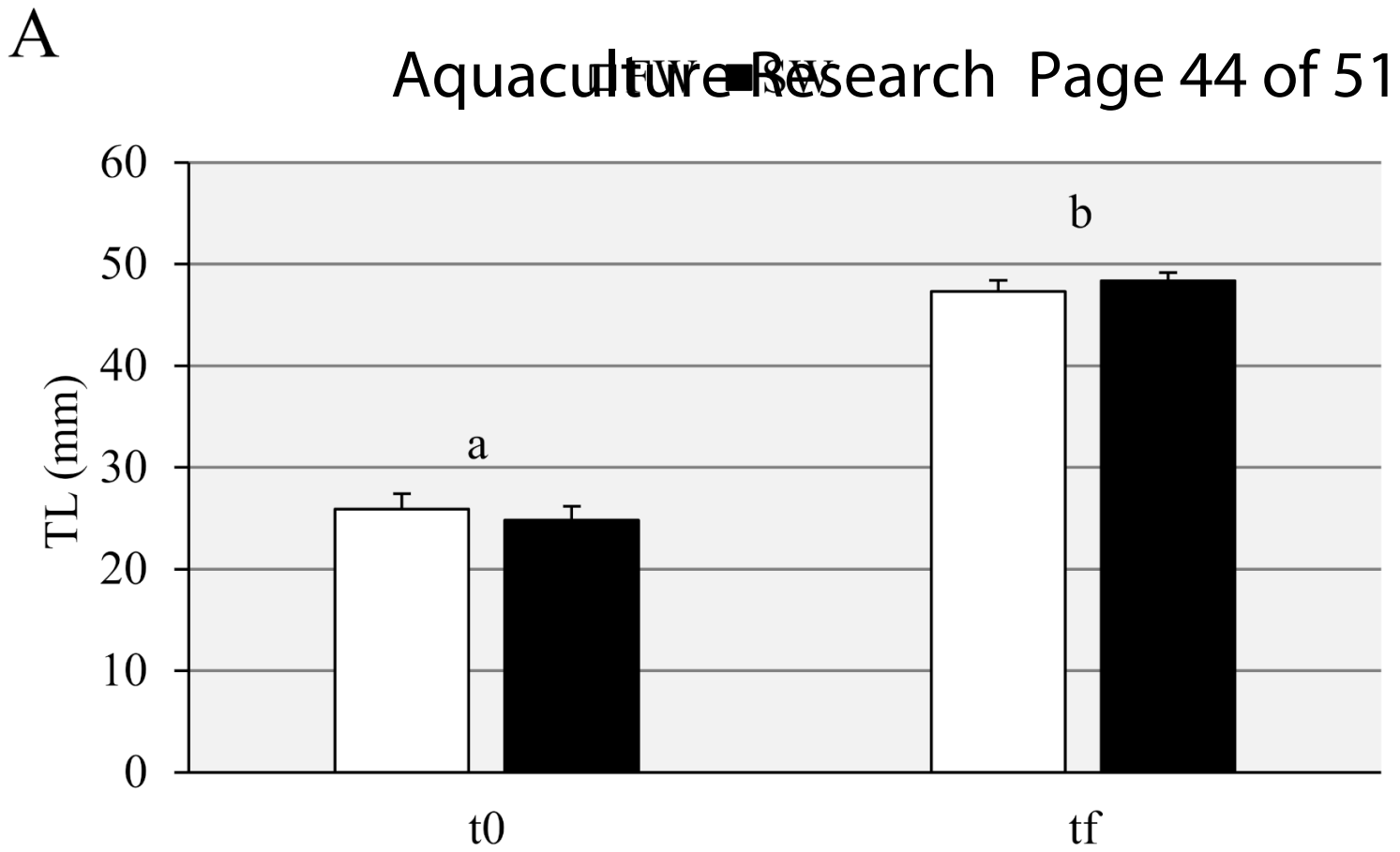
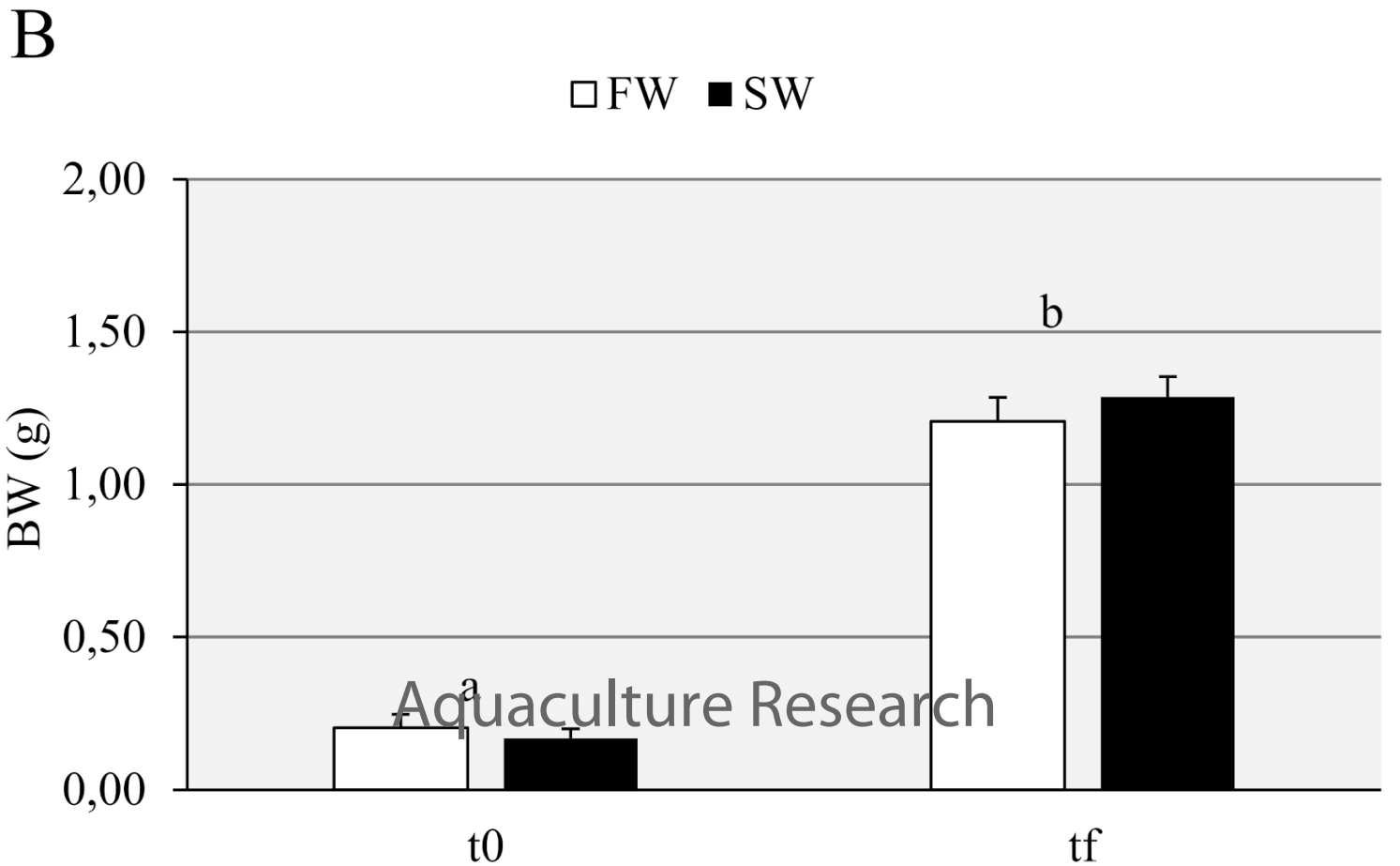
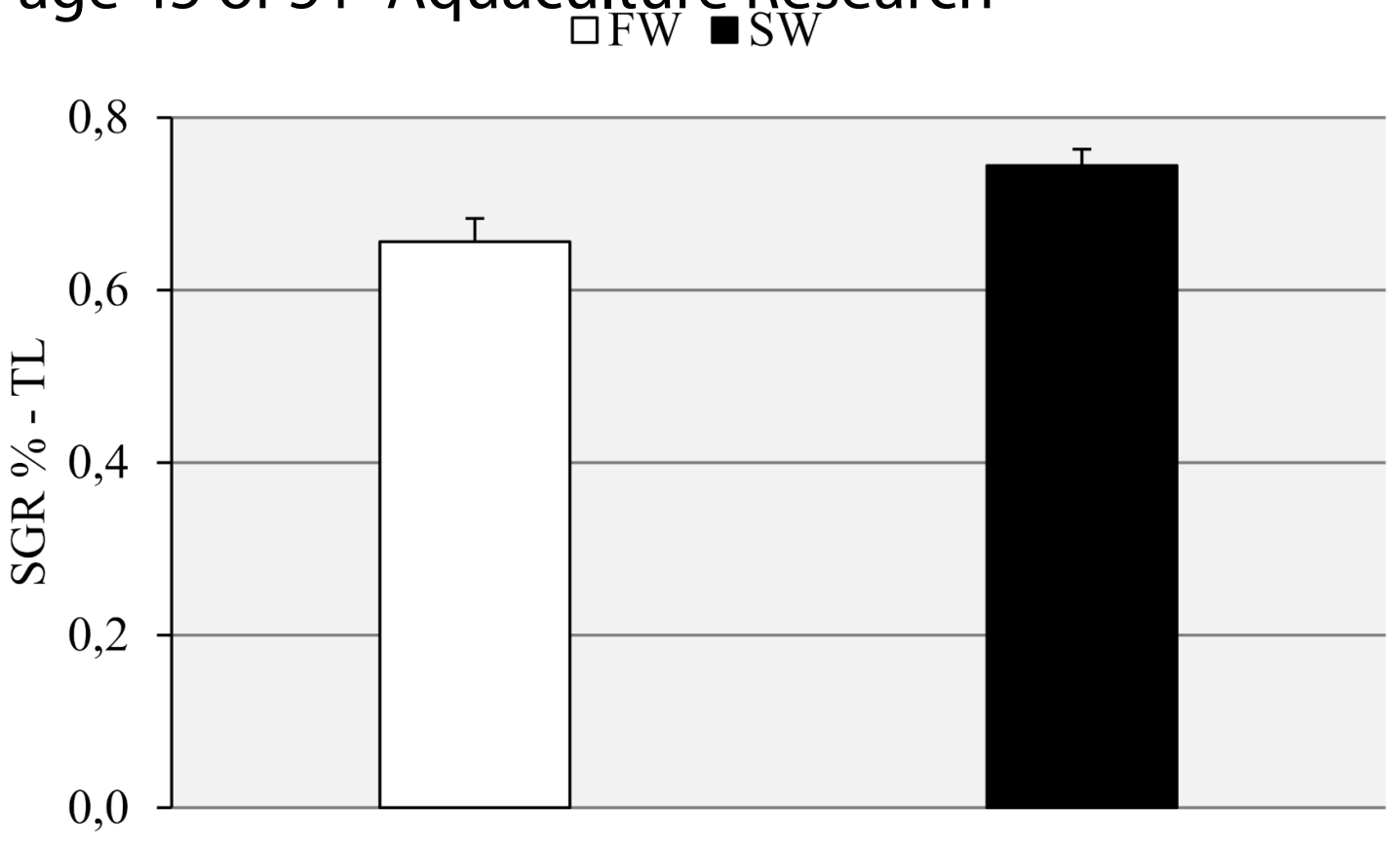
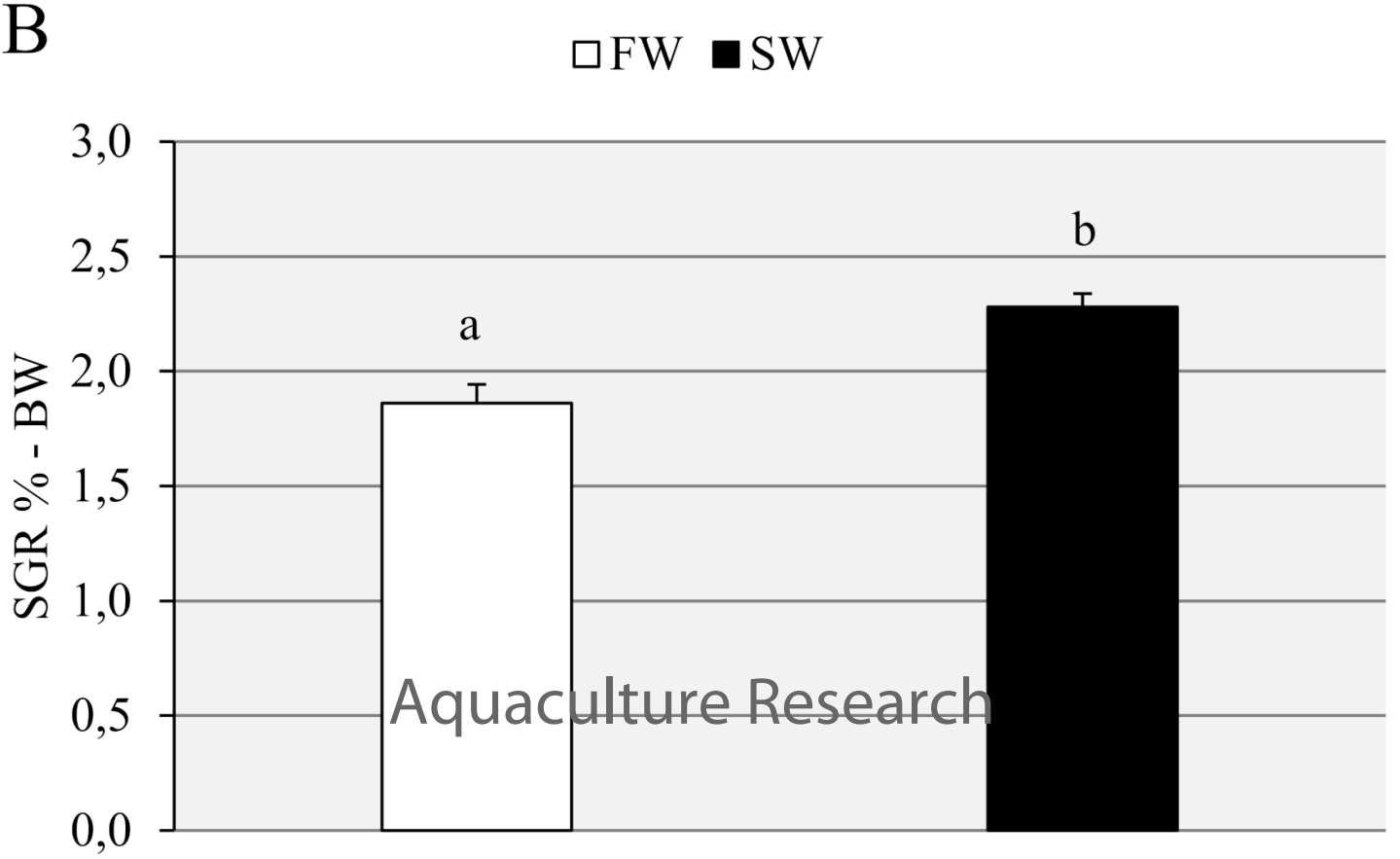


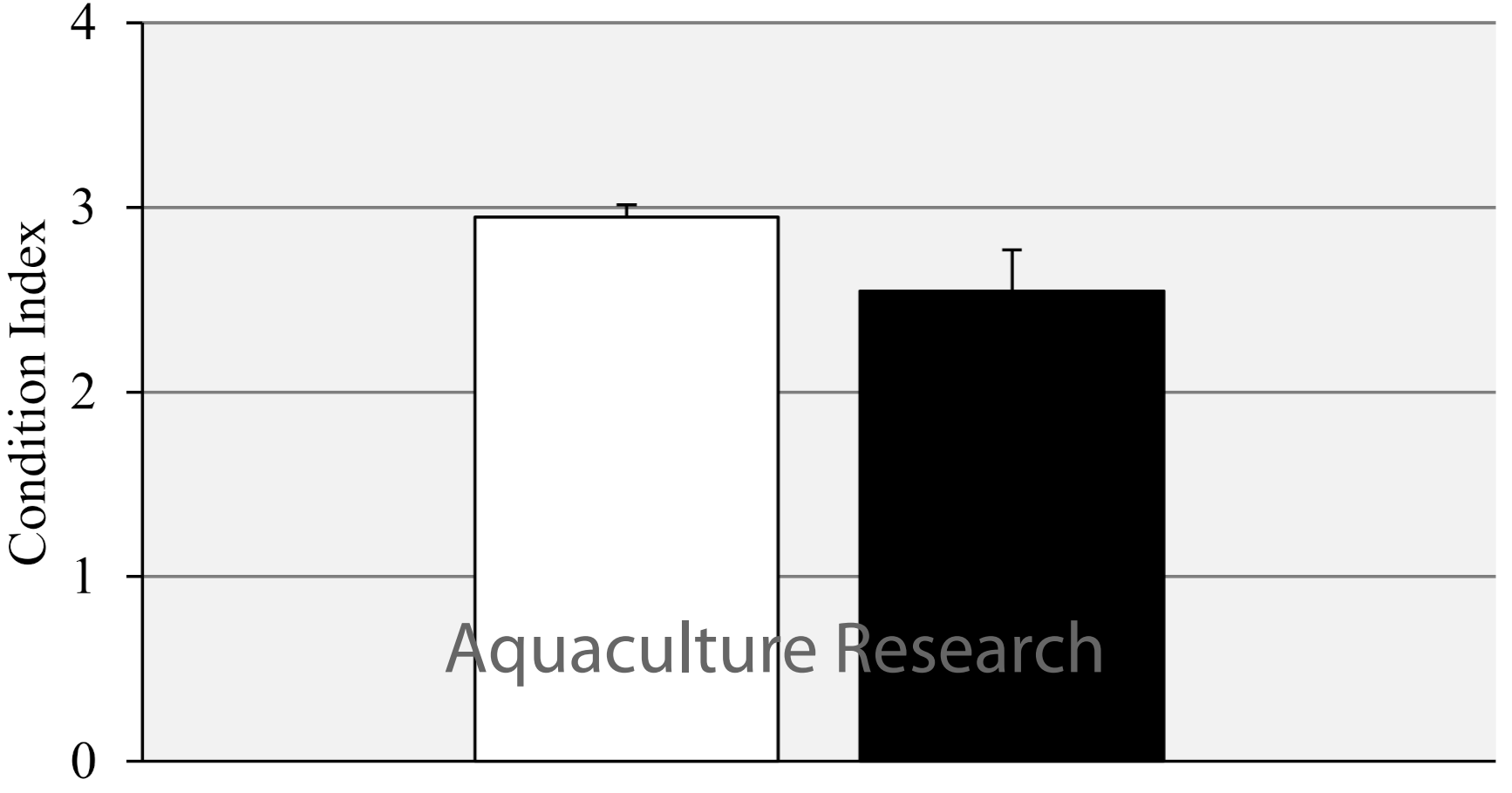


A



B




4
3
2
1
0
Condition Index
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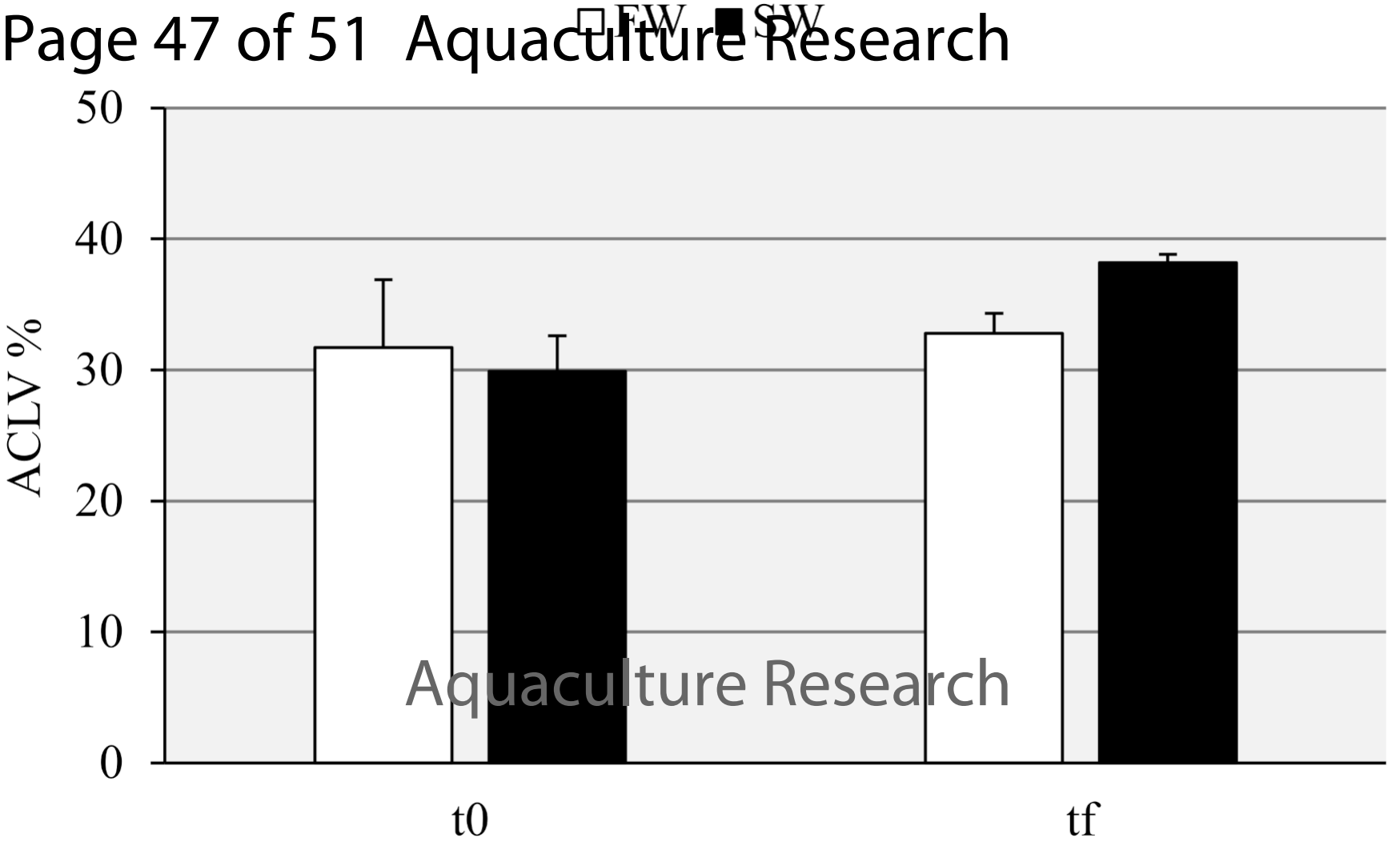
FW
SW
50
40
30
20
10
0
ACLV %
t0
tf
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Table 1. Total lipids (mg/g tissue), total fatty acids (µg/mg lipids), total saturated fatty acids (µg/mg lipids), and total unsaturated fatty acids (µg/mg lipids) measured in the whole body of *M. cephalus* juveniles, in the two treatments (Mann-Whitney test, data expressed as mean ± SE). t0: start of the experiment; tf: end of the experiment.

	t0		tf	
	FW	SW	FW	SW
Total lipids (mg/g tissue)	34.26 ± 3.30	40.34 ± 10.31	55.79 ± 8.38	81.48 ± 8.86
Total FA (µg/mg lipids)	605.78 ± 133.26	436.48 ± 147.03	901.19 ± 36.28	911.90 ± 50.60
Total SFA (µg/mg lipids)	252.30 ± 27.02	217.83 ± 40.12	286.20 ± 11.78	288.74 ± 16.54
Total UFA (µg/mg lipids)	353.50 ± 110.00	218.66 ± 115.12	614.97 ± 24.77	623.17 ± 36.80

† FA: fatty acids; SFA: saturated fatty acids; UFA: unsaturated fatty acids
‡ * $p<0.05$; ** $p<0.005$; *** $p<0.001$

Table 2. Fatty acids composition, expressed in mol%/total fatty acids, measured in the whole body of *M. cephalus* juveniles, in the two treatments (Mann-Whitney test, data expressed as mean ± SE). t0: start of the experiment; tf: end of the experiment.

	t0		tf	
	FW	SW	FW	SW
10:0	0.09 ± 0.05	0.16 ± 0.08	0.04 ± 0.02	0.09 ± 0.04
12:0	0.14 ± 0.05	0.18 ± 0.05	0.06 ± 0.00	0.05 ± 0.00
14:0	4.39 ± 0.37	5.40 ± 0.48	3.67 ± 0.31	4.53 ± 0.29
15:0	0.73 ± 0.12	0.90 ± 0.12	0.48 ± 0.01	0.46 ± 0.04
16:0	30.29 ± 3.48	38.04 ± 3.59	23.09 ± 0.47	23.76 ± 0.89
17:0	0.58 ± 0.14	0.98 ± 0.27	0.43 ± 0.04	0.39 ± 0.02
18:0	10.63 ± 2.31	13.40 ± 2.74	5.48 ± 0.69	3.95 ± 0.46
20:0	2.31 ± 1.42	0.29 ± 0.05	0.21 ± 0.01	0.19 ± 0.01
22:0	0.14 ± 0.03	0.12 ± 0.01	0.16 ± 0.01	0.14 ± 0.01
16:1	5.18 ± 0.35	6.35 ± 1.02	6.88 ± 0.66	8.07 ± 0.64
18:1	15.72 ± 1.79	18.98 ± 0.82	14.26 ± 0.39	14.74 ± 0.67
20:1	1.2 ± 0.11	1.16 ± 0.11	1.18 ± 0.05	1.12 ± 0.06
18:3n3	1.48 ± 0.49	0.63 ± 0.42	1.74 ± 0.17	2.13 ± 0.15*
18:4n3	0.74 ± 0.30	0.42 ± 0.25	1.13 ± 0.14	1.38 ± 0.11
20:5n3	3.17 ± 1.23	1.11 ± 0.84	5.17 ± 0.22	4.69 ± 0.28
22:5n3	1.80 ± 0.53	1.50 ± 0.73	3.30 ± 0.14	3.30 ± 0.18
22:6n3	5.95 ± 2.36	1.81 ± 1.23	10.60 ± 1.33	8.10 ± 1.25
18:2n6	13.26 ± 3.47	7.68 ± 2.78	17.65 ± 0.81	19.48 ± 0.96
18:3n6	0.18 ± 0.04	0.11 ± 0.05	0.21 ± 0.02	0.26 ± 0.02
20:2n6	0.90 ± 0.14	0.78 ± 0.29	1.21 ± 0.11	1.02 ± 0.11
20:3n6	0.80 ± 0.16	0.50 ± 0.15	0.82 ± 0.03	0.85 ± 0.07
20:4n6	0.74 ± 0.24	0.29 ± 0.11	1.51 ± 0.30	0.81 ± 0.19**
22:5n6	0.26 ± 0.07	0.11 ± 0.07	0.57 ± 0.09	0.29 ± 0.03***
22:4n6	0.17 ± 0.01	0.17 ± 0.00	0.28 ± 0.03	0.22 ± 0.02
20:3n9	0.02 ± 0.01	0.03 ± 0.00	0.04 ± 0.01	0.03 ± 0.01
Total HP	2.14 ± 0.75	2.91 ± 0.74	0.30 ± 0.03	0.18 ± 0.01***
Total MUFA	21.92 ± 1.49	26.49 ± 0.79	22.32 ± 1.05	23.92 ± 1.29
Total UFA	50.70 ± 7.45	40.52 ± 6.63	66.39 ± 0.21	66.45 ± 1.06
Total SFA	49.30 ± 7.46	59.48 ± 6.63	33.61 ± 0.21	33.55 ± 1.06

PUFAn3	13.51 ± 4.62	4.45 ± 3.16	21.41 ± 1.27	19.09 ± 1.45
PUFAn6	16.24 ± 4.08	9.38 ± 3.05	22.25 ± 0.41	22.93 ± 0.71
PUFAn9	15.73 ± 1.79	18.99 ± 0.82	14.30 ± 0.39	14.76 ± 0.67

† HP: hydroperoxides; SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; UFA: unsaturated fatty acids; PUFA: polyunsaturated fatty acids

‡ * $p<0.05$; ** $p<0.005$; *** $p<0.001$