

Assessing the acclimatisation to the wild of stocked European graylings *Thymallus thymallus* by monitoring lipid dynamics and food consumption

Mladen Avramović^{1,*} , Jan Turek¹, Aleš Tomčala¹, Jan Mráz¹, Martin Bláha¹, Marek Let¹, Mariusz Szmyt² and Tomáš Randák¹

¹ University of South Bohemia in České Budějovice, Faculty of Fisheries and Protection of Waters, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Zátisí 728/II, 389 25 Vodňany, Czech Republic

² Department of Ichthyology and Aquaculture, Faculty of Animal Bioengineering, University of Warmia and Mazury in Olsztyn, Poland

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Abstract – This study investigated how 2+-year-old stocked pond-reared European graylings (*Thymallus thymallus*) acclimatised to a wild environment during six months (May–October 2019) after released. We examined the quantity and composition of lipids in the liver, muscles and visceral adipose tissues (VAT), as well as size parameters, condition factors and stomach contents. Our results showed a low post-stocking recapture rate (5.17%) of stocked fish after 6 months suggesting a poor acclimation to the wild environment. During the six months of monitoring, stocked fishes exhibited a sharp decrease in lipid content in all examined tissues, and, in the final month, lipid content was well below those of wild conspecifics. Stocked graylings preferred risky foraging behaviour and consumed numerous small drift preys with occasional hyperphagic events, thus experiencing strong bioenergetic challenges. Our study reveals that fish cultivated for extended periods struggle to acclimatise to the wild environment and that this commonly used stocking practice seems unsuccessful when aimed at strengthening wild grayling populations.

Keywords: post-stocking acclimatisation / fatty acids / restocking salmonids / foraging behaviour

1 Introduction

Over the past three decades, the European grayling (EG) (*Thymallus thymallus*) has been the focus of various stocking programmes, all of which have been largely unsuccessful (Thorfve, 2002; Turek *et al.*, 2010, 2012, 2018). The further decline in EG populations and loss of natural habitat are predicted to persist (Basen *et al.*, 2022), giving the alarm signal to elucidate the major reasons for stocking failures. In general, the lack of stocking success with reared salmonids is attributed to their dramatic reduction of fitness in captivity (Fraser *et al.*, 2011), manifested in altered feeding behaviour, responses to predators and habitat use (Einum and Fleming, 2001). Specifically, one of the most important behavioural characteristic of stocked EG is their pronounced post-stocking migration (Carlstein and Eriksson, 1996; Thorfve and Carlstein, 1998; Thorfve, 2002; Horká *et al.*, 2015). In terms of feeding behaviour, reared graylings stocked in rivers showed the ability to efficiently consume natural preys on the first day after release (Thorfve and Carlstein, 1998). However, a further study has

reported that terrestrial invertebrates represent the predominant diet of stocked grayling one week after stocking (Thorfve, 2002), which contrast with the diet composition of wild conspecifics that rely weakly on terrestrial preys (Radforth, 1940; Hellawell, 1971; Kruzhylina and Didenko, 2011). Nevertheless, information on the longer-term feeding dynamics and diet composition of post-stocking grayling is still lacking.

In the natural environment, stocked fish can experience high energy demands due to intensive post-stocking migrations, low food intake or costly feeding, which can lead to energy deficiency in stocked fish and subsequent mortality (Ersbak and Haase, 1983; Bachman, 1984; Umino *et al.*, 1991). Accordingly, better pre-stocking biometric parameters of EG can enhance the post-stocking recapture rates (Carlstein, 1997; Turek *et al.*, 2012). Lipids, the primary source of energy in fish (Cho and Kaushik, 1990), have adaptable purposes reflected in supporting energy-demanding processes (Sheridan, 1994), such as physical activity in general (Cho and Kaushik, 1990), especially migration for salmonids (Jobling *et al.*, 1998; Olsen, 1999), and responses to environmental stressors (Adams *et al.*, 1999). As the amount of lipid reserves at any given time is determined by the ratio of their utilisation and deposition (Meier and Burns, 1976), lipids

*Corresponding author: mavramovic@frov.jcu.cz

can serve as a relevant indicator that clarifies the condition and general health of fish (Weber *et al.*, 2003; Arts and Kohler, 2009).

Data on the lipid content of EG are currently limited to muscle fatty acids (FA) spectrum studies (Ahlgren *et al.*, 1994, 1999) and no other lipid depots including in the liver and visceral adipose tissues, have been studied. The FA composition of muscle in EG is mainly influenced by the quality of dietary fat, thus resulting in the divergence of reared specimens fed on commercial pellets from wild conspecifics (Ahlgren *et al.*, 1994), for example, disrupted n-3/n-6 ratio (Ahlgren *et al.*, 1999). A proper balance of this ratio in salmonids is crucial for fish health (Olsen, 1999; Glyzina *et al.*, 2009). Typically, natural EG preys are relatively rich in eicosapentaenoic acid (EPA) (20:5n-3), which is a precursor of docosahexaenoic acid (DHA) (22:6n-3) (Kaur *et al.*, 2011). It is worth noting that aquatic invertebrates are richer in n-3 highly unsaturated fatty acids (HUFAs) than terrestrial invertebrates (Hixson *et al.*, 2015; Twining *et al.*, 2019). These n-3 HUFAs – particularly DHA – play a crucial role in certain critical physiological processes in fish (Tocher, 2003) and are especially important in wild salmonids (Olsen, 1999; Gladyshev *et al.*, 2018; Makhutova and Stoyanov, 2021). Accordingly, assessing the FA composition of fish is a valuable way for exploring their acclimatisation to environmental conditions (Glyzina *et al.*, 2009), and in particular, the transitioning effects of a switch in diet caused by stocking in a natural environment.

Our study aimed to acquire insights into how post-stocking conditions influence monitored parameters such as biometrics, lipid content dynamics and FA composition. Long-term monitoring of the food composition intake by stocked graylings will provide supportive data on the quantity and quality of lipids, thereby contributing to the clarification of their eco-physiological backgrounds during acclimatisation.

2 Material and methods

2.1 Study location

The experiment took place on the river Blanice in South Bohemia (Czech Republic), a 93 long watercourse with a catchment area of 860 km². We used six experimental stretches of river (150–190 m) over 6 km of the river's course (river km 46.5–52.6) downstream from the Husinec Dam (37 ha; 2.5×10^6 m³; 49° 02' 11.1" N 13° 59' 02.8" E). All chosen stretches had widths of 7–11 m and depths of 10–80 cm. In this tailwater area, fishing for grayling is prohibited and no effects of hydropower plant hydropeaking exist. The observed ichthyofauna is typical of salmonid waters with a clear dominance of brown trout *Salmo trutta*. The river has abundant rapids that are characterised by large rocks and pebbles but there are also deeper whirlpools with slower flows and gravelly bottoms. The banks are composed of natural gravel with well-preserved and developed riparian vegetation that is continued by continuous meadows ~500 m.

2.2 Stocking procedure

We used a total of 1,200 randomly selected pond-reared 2+ years old graylings taken from a small rearing pond located on a small stream (a tributary of the river Blanice) in the town of

Prachatice (southern Czech Republic; 49° 00' 29.9" N 13° 59' 58.8" E). These fishes (the progeny of wild broodstock originating from the river Blanice) were first fed (fingerling stage) using conventional dry-food floating pellets designed for rainbow trout. Afterwards, food was transitioned to a reduced-fat diet (American catfish ALLER BONA FLOAT), also in the form of dry floating pellets. We divided the 1,200 2+ years old graylings into six equal groups. Fishes were then anaesthetised with 2-phenoxy-ethanol (2-PHE; 0.2 mL · L⁻¹), measured (standard length SL, cm) and weighed (W, g) using a Kern balance (model EMB 1200-1; max. 1200 g, d = 0.1 g). We then tagged all fishes with visible implant elastomer (VIE) tags (Northwest Marine Technology, Ltd., USA), each group having a different colour of VIE tag. The six different colours of the tags corresponded to the six river stretches where fishes were released in April 2019.

2.3 Sampling

We conducted monthly recapture of 8 tagged stocked fish, six times in total (May–October 2019), by using two backpack pulsed-DC electrofishing units (FEG 1500, EFKO-Germany). Simultaneously, we sampled eight grayling per month from the rearing pond. During the final sampling event (October 2019), we electro-fished a 6-km-portion of the river Blanice, including all six experimental (stocked) stretches, and recorded the total number of recaptured stocked graylings. Additionally, in October 2019, we captured and analysed six wild graylings from the river Blanice. The wild fishes could not be sampled and analysed during all sampling periods due to their very low numbers in the river. The biometric parameters of every sampled fish were measured, including standard length and body weight, and the condition factor $K = 100 \times (W/L^3)$ was calculated to estimate the initial and post-stocking performances. The monitoring in October of the whole experimental area provided data on “stretch fidelity”, a term used to describe the recapture of fishes at stretches where they have been initially released. We also observed the direction of the post-stocking dispersal (upstream or downstream).

2.4 Ethical statement

This study was conducted in accordance with the ethical guidelines of the Czech Republic and received approval from the relevant ethics committee. The treatment and welfare of fish fully adhered to the legal requirements in the Czech Republic (§ 7 Law No. 114/1992 on The Protection of Nature and Landscape and § 6, 7, 9, and 10 Regulation No. 419/2012 on the Care, Breeding, and Use of Experimental Animals).

2.5 Tissues targeted for analysis

For the analysis of lipids, we sampled dorsal muscle in the area between the head and dorsal fin. Then, the fish was abdominally opened to sample the liver and visceral fat. VAT samples was weighed. Tissue samples were stored at –80 °C until processing in the laboratory. The stomach content was sampled by cutting the stomach longitudinally and flushing its content with 96% ethanol into 25 ml vials for subsequent examination.

2.6 Lipid analysis

Lipid extraction was performed according to the method of Hara and Radin (Hara, 1978), with slight modifications by Mráz and Pickova (Mráz and Pickova, 2009). In brief, an Ultra Turrax homogeniser (T25, Janke and Kunkel, IKA Werke, Germany) was used to homogenise an approximately 1 g sample in 10 ml of hexane-isopropanol (3:2); 6 ml of Na₂SO₄ (6.67%) was added to the obtained homogenates and mixed. After centrifugation, the upper lipid phase was transferred into pre-weighed tubes and subsequently evaporated under nitrogen. The lipid content was quantified gravimetrically (Mettler Toledo XP2U Excellence Plus XP Ultra Micro Balances 2 g × 0.1 µg, Germany). For FA analyses, the methylation of total lipids was performed according to the methods of Appelqvist *et al.* (Appelqvist, 1968). The fatty acid methyl ester (FAME) C 23:0 was used as an internal standard. FA composition was analysed using gas chromatography (GC) (Trace Ultra FID; Thermo Scientific, Milan, Italy) with a BPX-70 50 m fused silica capillary column (id. 0.22 mm, 0.25 µm film thickness, SGE, USA). The temperature gradient started at 70 °C and was held for 0.5 min. Then, the temperature was increased by 30 °C per minute until it reached 150 °C. Next, the temperature was increased to 220 °C at a rate of 1.5 °C per minute and held there for 11 min. The whole analysis took 60 min. The temperature of the PVT injector was 170 °C and that of the detector 260 °C. The peaks were identified and quantification was achieved with Thermo Xcalibur 3.0.63 (Thermo Fisher Scientific Inc.) by comparing sample retention times and peak areas to retention times and peak area at seven levels (1000 µg/ml–15 µg/ml) of the standard mixture Supelco 37 Component FAME mix (Sigma-Aldrich).

2.7 Food contents analysis

The food analysis was performed using a stereoscopic light microscope (Olympus SZX16 with Olympus DP74 camera) to determine the abundance, size category and taxonomy of prey items. A simple numerical method (Hyslop, 1980) was employed to record the total number of digestible food items in the stomachs. The problem of determining partially digested soft-bodied prey was overcome by counting their non-digested chitinised parts (Blake, 1977). Taxa were identified to order and family levels. The scale for the size of the prey body length was as follows: small, up to 8 mm; medium, 8.1–15 mm; and large, over 15 mm. Given that the size of some prey items depended on their current developmental stage, we also separated the same taxa into different size groups. The obtained data aimed to clarify a picture of stocked grayling foraging strategies and their prey preferences.

2.8 Statistical analyses

GraphPad Prism (version 9.1.0) software was employed to conduct a range of analyses of the numerical abundance of prey in the stomach contents of the stocked fish. Subsequently, we applied Student's *t*-test to compare abundance and size of food items and to assess differences in individual FA contents between stocked and wild fish. The χ^2 test was performed to test for differences in the ratio of aquatic to terrestrial prey

between in the stocked and wild groups in October and to assess the movement of tagged stocked fish. Prior to analysis, the pooled data of each fatty acid underwent a Shapiro-Wilk normality test. Based on the normality assessment results, either a parametric test (ANOVA with post hoc Tukey HSD) or a non-parametric test (Kruskal-Wallis rank sum test followed by post hoc Dunn's test with Bonferroni correction) was selected for each fatty acid. Significant differences were determined at $\alpha=0.05$. RStudio (version 1.3.1093) using R (version 4.1.1) software was employed to model temporal dynamics in fish biometrics (weights, lengths and condition factors) and contents of four FA groups (SFA, MUFA, PUFA, and n-3 HUFA) distributed within each sample (different tissues and fish origin used as factors with fixed effects). Generalised linear models (GLMs) with a Gamma distribution with a log link function and linear mixed-effect models (LMEs) with the identities of individuals as factors and a random effect on the intercept, were run for these purposes, respectively. The assumptions of homoscedasticity and the normality of the residuals were checked visually and considered sufficient after a log-transformation of the response variables. Non-linear patterns in FA dynamics were modelled using polynomial regressions while the significance of improvements was tested with likelihood ratio tests. A similar approach was used to compare all experimental groups at the final sampling time when the wild fishes were also sampled.

3 Results

Six months after stocking with 1,200 individuals, the total recapture rate was 62 grayling (5.17%). Post-stocking dispersals were significant ($\chi^2=17.84$, $df=2$, $p < 0.001$), with average downstream and upstream migrations of 59.2% and 8.6%, respectively. The "stretch fidelity" of recaptured fish along the six stretches ranged from 0–100% (mean 32.1%). Both stocked and pond groups increased significantly in standard length during April–October; the differences between the two groups were non-significant. Pond fishes were significantly heavier than stocked fishes ($p < 0.01$), although both fish groups exhibited the same temporal weight dynamic ($p=0.32$), with a peak of maximum weights between July and August. However, the dynamics of Fulton's condition factor (*K*) differed between groups ($F_{6,98}=3.78$, $p=0.002$) being significantly higher for pond than for stocked fishes in May and October (Supplementary file, Fig. S1). The *K* comparisons for October (Tab. 1) showed that wild fishes had significantly lower *K* than pond fishes but higher *K* than the stocked fishes (Tab. 1).

3.1 Stomach contents analysis

During the first month of sampling the stomach content of stocked graylings revealed the appearance of many artificial objects such as macroplastics and pellet-like non-food items including pieces of wood or gravel. The numerical abundance of prey items in sampled stocked fish varied significantly over the six months ($p < 0.001$).

The monthly food composition of stocked fishes (May–October) and one group of wild fishes (Oct*) is shown in Figure 1. The percentage of terrestrial preys ingested by

Table 1. Biometric data of for three grayling groups obtained during the final sampling conducted in October. The table includes the mean values ($\pm$ s.e.m) for standard length (SL), weight (W), and Fulton's condition factor (K), and were analysed using a GLM with a Gamma distribution and logarithmic link for SL, W and K and tested with a partial F-test. Significant differences between groups were detected for all biometrics. Tukey's method was employed for post-hoc comparisons. Superscripts 'a', 'b', and 'c' denote significant differences ($p < 0.05$).

Group	n	SL (cm)	W (g)	K
Stocked	8	21.6 $\pm$ 0.6 ^a	103.4 $\pm$ 5.9 ^a	0.65 $\pm$ 0.02 ^a
Pond	8	19.0 $\pm$ 1.1 ^b	100.1 $\pm$ 18.2 ^b	0.89 $\pm$ 0.03 ^b
Wild	6	23.3 $\pm$ 0.4 ^a	129.8 $\pm$ 9.7 ^a	0.75 $\pm$ 0.03 ^c

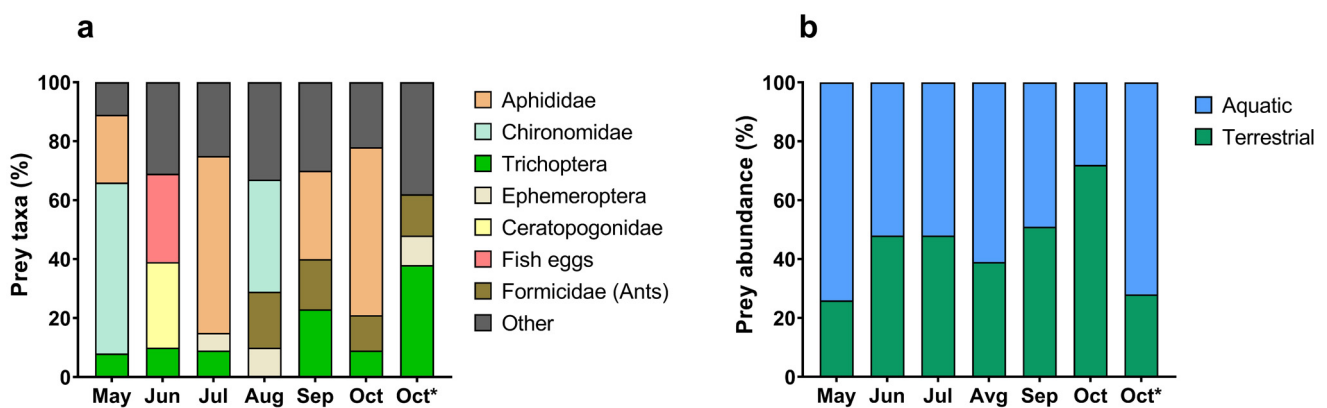


Fig. 1. Representation of the monthly food content in the stomachs of stocked (May–Oct) and wild (Oct*) European grayling (*Thymallus thymallus*). The plots depict ratios of ingested prey taxa (a) and ratios of aquatic and terrestrial prey abundance (b).

stocked fishes ranged from 26% in May to 72% in October (Fig. 1b). Calculations of the monthly proportions of prey taxa (%) in the stomachs of stocked grayling (Fig. 1a) revealed that Aphididae were the dominant prey items in July, September and October; larvae and pupae of Chironomidae were dominant in May and August; and fish eggs were the predominant food type in June. A comparison of stocked and wild fishes in October showed a higher ratio of terrestrial invertebrates in the stomachs of stocked fishes ($\chi^2 = 38.72$, $df = 1$, $p < 0.001$) (Fig. 1b). The numerical abundance of monthly ingested preys (Tab. S9 in the Supplementary file) indicates that wild fishes ingested significantly more preys than stocked fishes ($p < 0.001$).

We also found differences in prey size preferences between the same groups: stocked fishes fed more on small preys ($p < 0.005$), while wild fishes preferred medium-sized prey ($p < 0.003$); there were no significant preferences for large preys (stocked: 27 ± 14 , wild: 31 ± 19).

3.2 Lipid content

The lipid content of muscle, liver and VAT is shown in Figure 2. Throughout the monitoring period, pond fishes consistently maintained higher amounts of lipid in all three tissues with significant fluctuations in muscle ($p < 0.006$), liver ($p < 0.032$) and VAT ($p < 0.001$). Stocked fishes, on the other hand, exhibited a significant depletion in lipid contents across all analysed tissues. The first significant drop in lipid content in the muscle of stocked fish compared to the initial

state occurred in August ($p < 0.027$). This depletion trend continued until the end of the experiment, where fishes had a 4.4-fold lower lipid content in muscle than prior to stocking ($p < 0.005$). Lipid content in the liver decreased significantly from July onwards ($p < 0.022$), an approximately three-fold depletion compared to April. Finally, the lipid content in VAT began to significantly decrease from its initially state in July ($p < 0.004$), a decline that persisted until October ($p < 0.023$), resulting in a total decrease of 2.9-fold relative to the initial state.

3.3 FA composition

3.3.1 Muscle

Comparisons of dynamics in pond and stocked fishes are given in Figures 3b and 3d. In stocked fishes, levels of monounsaturated fatty acids (MUFAs) and saturated fatty acids (SFAs) dropped significantly in July, and by October had fallen 9.6- and 3.9-fold compared to the initial sampling, respectively. By contrast, the depletion of polyunsaturated fatty acids (PUFAs) and n-3 HUFAs was more conservative and declined more slowly in content. PUFA showed a significant decrease in October and end 2.3-times lower in October than in the initial sampling; while n-3 HUFAs did not decrease during the monitoring period. The quantitatively dominant FA was oleic acid (18:1n-9) in April–September (16.05–2.84 mg/g w. w.), whereas in October DHA had the highest values (2.91 mg/g w. w.) in muscle lipid content. Analysing DHA separately, it significantly decreased in July

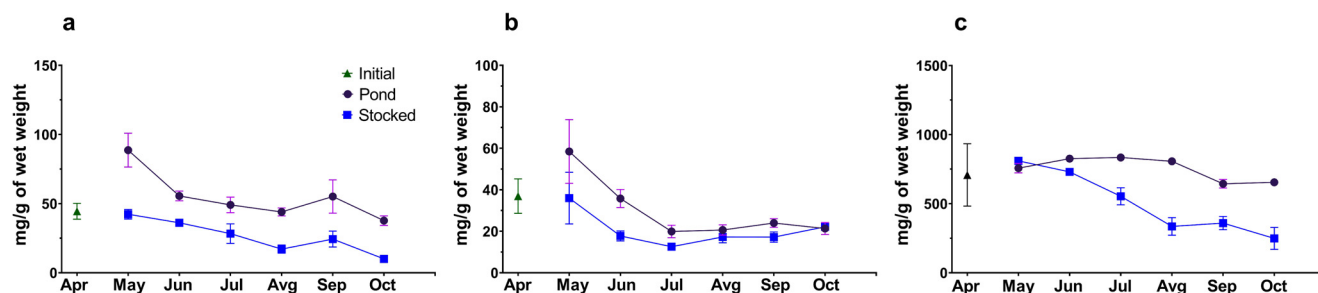


Fig. 2. The total lipid content dynamics (Apr-Oct) in muscle (a), liver (b) and visceral adipose tissue (c) in pond-reared and stocked European grayling. The initial grayling group from April was sampled before stocking. Values are expressed as mg/g of wet weight; standard deviation bars are shown (Top of Form).

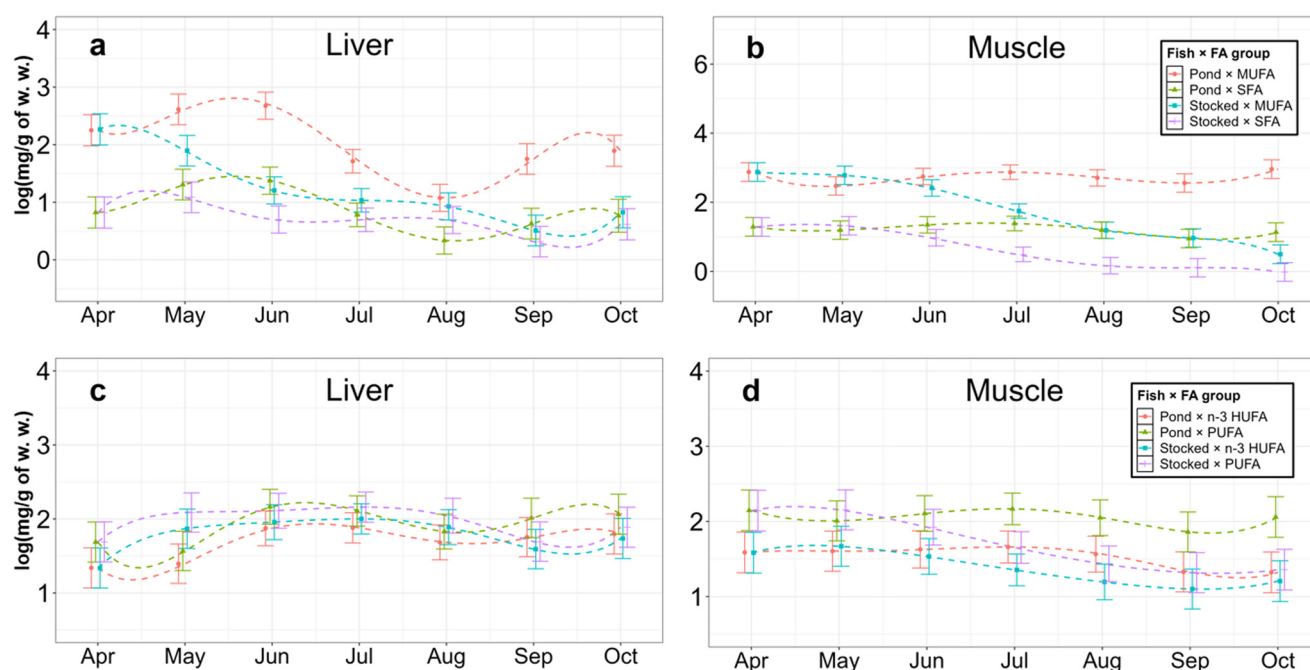


Fig. 3. Linear Mixed-Effect Model (LME) of the dynamics of fatty acid groups in the liver (a, c) and muscle (b, d) tissues of stocked and pond-reared European grayling (*Thymallus thymallus*) over a monitored period (April–October). Units are natural logarithms of values of mg/g of wet weight samples. The LME shows relationships and trends in fatty acid content over time.

and stayed at that level until October but with a significantly higher value than in wild fishes in the same month (means: 2.91–2.23 mg/m w. w., $p < 0.001$). DHA increased and dominated over EPA, with a DHA:EPA ratio of 1.5 by October. Arachidonic acids similarly tended to decline in both environments (pond and river), with a quantitative drop in September. C18 PUFA, linoleic acid (18:2n-6; LA) and alpha-linolenic acid (18:3n-3) all had similar dynamics and they decreased 8.4-times by October. Following these changes, the initial n-3/n-6 ratio significantly rose from 1.84 in April to 8.26 in October.

3.3.2 Liver

Comparisons of pond and stocked dynamics are given in Figures 3a and 3c. All the FAs in the liver of stocked fishes varied significantly. Oleic acid was quantitatively dominant from April to June but thereafter declined continually and

significantly, to reach its lowest value in September (8.5 times lower). Conversely, DHA content rose significantly from April onwards and became the quantitatively dominant FA in the liver in June–October. Moreover, the DHA content remained consistent with that of the pond fishes. EPA content showed a significant 3-fold increase one month after release in April–May of 0.42–1.28 mg/g w. w. ($p < 0.001$) but did not then significantly decrease in the following months. Consequently, this significantly increased the n-3 HUFAs content in these initial two months, from 3.93 to 7.94 mg/g w. w., which did not differ from these levels in the following months (June–October). The LA content significantly decreased from April to September and increased in October. The n-3/n-6 ratio significantly increased in both pond and stocked groups by 2.5-fold in April–May. Unlike pond fishes, over the following months the n-3/n-6 values in stocked fishes were more than twice as high as in the initial pond sampling in April.

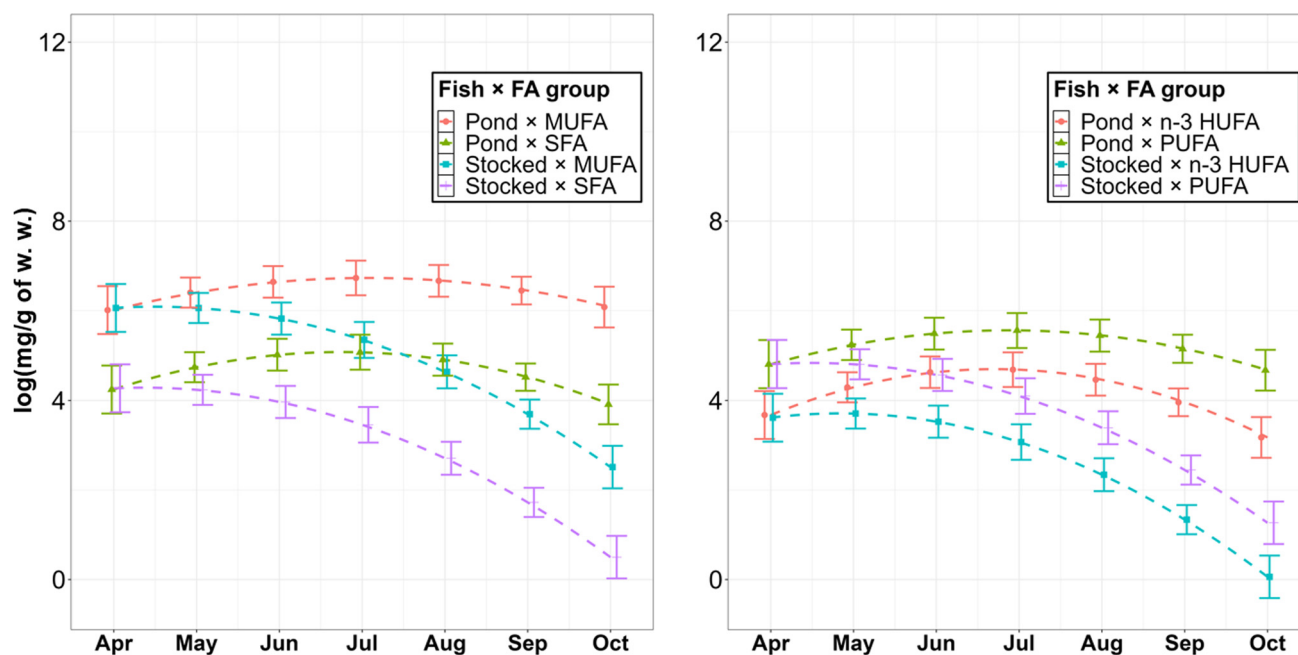


Fig. 4. Linear Mixed-Effect Model (LME) of all detected fatty acids in visceral adipose tissue of European grayling (*Thymallus thymallus*), summed in classified FA groups (SFA, MUFA, PUFA and n-3 HUFA). These groups represent the FA content in the entire VAT (FA content * VAT weight). Throughout the monitored period, stocked fish exhibited a significant decrease in every FA group, starting from June and maintaining a consistent downward trend. By contrast, pond grayling did not display any pronounced variations in any FA group over time. The error bars represent 95% confidence intervals.

3.3.3 Visceral adipose tissue

The dynamics of FA groups in the VAT of stocked fishes are presented in the Figure 4. All FA groups in VAT showed similar dynamics, which from June onwards differed significantly from their conspecifics in the pond (April) and were characterised by the simultaneous depletion of major FA groups throughout the whole experimental period. Among SFAs, the C16:0 content was depleted the most, approximately four-fold from its initial state. The pronounced drop in MUFAs as a dominant FA group occurred in June–July, with the lowest level found in October, matching the oleic acid dynamics. Similarly, linoleic acid was the second-most quantitatively important FA, with a substantial decrease in July and August. The levels of n-3 HUFAs were highest in June and decreased significantly until October. The n-3/n-6 ratio during the experiment did not vary, with an April–October mean of 0.95. The differences between pond, stocked and wild grayling groups were clearest at the end of the experiment and are shown in Figure 5.

In both stocked and pond graylings, we detected changes in FA composition based on a significant variation in individual FA values between the initial and final states. The quantified absolute values (mg/g of wet weight sample) of individual FAs are shown in Supplementary Tables S2–S7.

4 Discussion

This study represents the first attempt to characterise transitional changes in pond-reared 2+ European grayling after translocation to a natural environment. Compared to

similar previous studies (Carlstein and Eriksson, 1996; Thorfve, 2002; Turek *et al.*, 2010, 2012, 2018), our results provide novel explanations of stocked graylings acclimatisation processes via insights into lipid content dynamics, changes in FA composition and diet of stocked fishes.

Our stocking attempt resulted in a low post-stocking recapture rate in the river compared to previous attempts in the same locality but using younger age graylings (Turek *et al.*, 2010; 2012), which implies that grayling that has been reared for a longer time are less prone to acclimation. The observed spatial post-stocking distribution also indicates that intensive migration took place over the six months, confirming the migratory nature of reared EG (Thorfve, 2002; Turek *et al.*, 2010, 2018), which are further more prone to disperse downstream than upstream compared to their wild relatives (Carlstein and Eriksson, 1996; Thorfve and Carlstein, 1998; Horká *et al.*, 2015).

4.1 Bioenergetic challenges during acclimatisation

The acclimatisation process showed that lipid reserves in stocked fishes underwent an intensive reduction. Muscle lipids were the supportive energy source for the stocked fish and were intensively catabolised, similar to what expected in starvation conditions (Einen *et al.*, 1998). This confirms that muscle lipids can be easily mobilised for energy-demanding processes (Jezierska *et al.*, 1982; Jobling *et al.*, 1998). By contrast, the grayling in the rearing pond (under a stable feeding regime with high-energy dry pellets) developed lipid-rich visceral adipose tissue (VAT), typical of salmonids

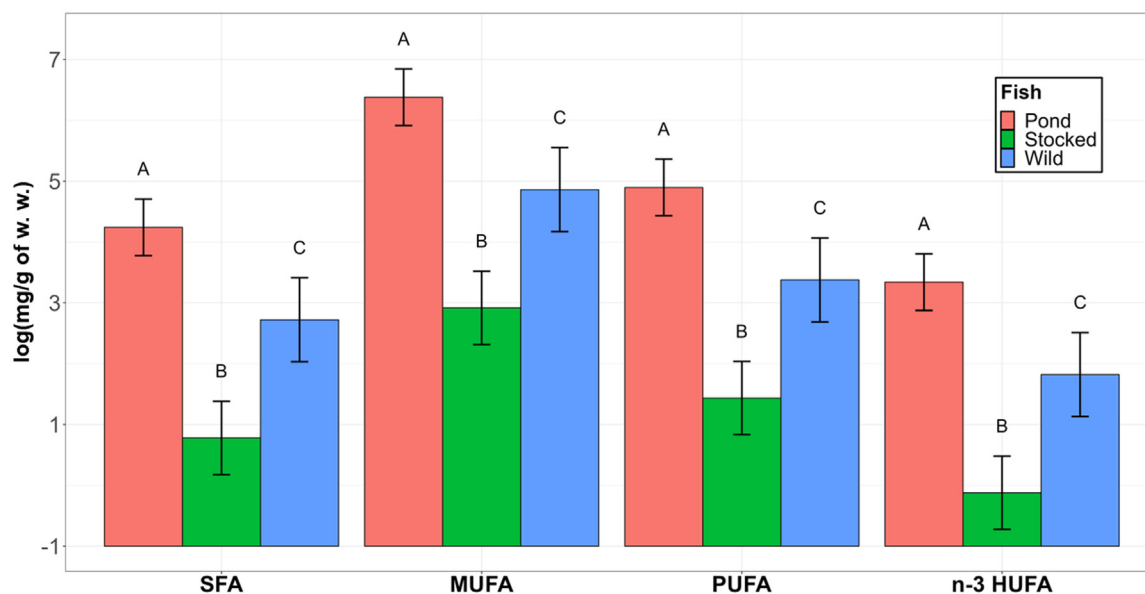


Fig. 5. Linear mixed-effects model (LME) comparing four FA groups in three tissues (SFA, MUFA, PUFA and n-3 HUFA) in three groups of European grayling captured in October (Pond, Stocked and Wild). Values expressed in log(mg/g of w. w.) (fitted means with 95% confidence intervals as error bars) and represent the FA content in the entire VAT (FA content * VAT weight). The plot highlights significant differences with different letters (A, B, C) denoting the variations between the pond, stocked and wild fish groups.

(Henderson and Tocher, 1987). As expected, the largest decline in lipid content during the monitoring period was in VAT, consistent with findings for starved rainbow trouts (Jezińska *et al.*, 1982). After three months in the river (July), bioenergetic challenges had led to a significant drop in lipid content in muscle and VAT compared to their initial levels in April. Specifically, VAT exhibited a nearly complete depletion of lipid reserves in stocked fishes by October compared to the wild graylings. This drop in lipid reserves was preceded by a 50% reduction in ingested food items in June–July, possibly due to a shift in preferred food items from fish eggs to aphids (Aphididae). This dietary change coincided with low values for the condition factor (K) in August which, nonetheless, remained at similar levels to those found in wild conspecifics in October. Based on our results, the lipid content in liver appeared to respond to feeding intensity and reaches its lowest point during the warmest months. This underlines its less significant role in long-term energy-demanding processes (Jezińska *et al.*, 1982; Henderson and Tocher, 1987; Einen *et al.*, 1998).

4.2 FA composition

The initial pond sampling revealed the unnatural impact of rearing conditions on muscle FA composition when compared to wild conspecifics (Ahlgren *et al.*, 1994, 1999) and other wild *Thymallus* spp. (Sushchik *et al.*, 2006, Sushchik 2007; Glyzina *et al.*, 2009; Gladyshev *et al.*, 2012; Makhutova and Stoyanov, 2021). The dietary conditions that the stocked grayling faced in the river clearly changed the FA composition of the analysed tissues. The general pattern of observed changes in the FA composition mirrors processes similar to those occurring during starvation (Jezińska *et al.*, 1982) or stressful and fasting periods (Bermejo-Poza *et al.*, 2020). Evidently, the

elevated MUFAs in all tissues were primarily utilised in the wild environment and, in particular, oleic acid (18:1n-9) was used in the greatest quantities, which is typical of conditions such as starvation (Tocher, 2003). The depletion of SFAs and PUFAs followed this.

4.3 Muscle FA composition

In muscle DHA depletion rate was slower than in the majority of the other FAs, thereby suggesting the existence of a conservative metabolism that protects expensive long-chain PUFAs from oxidation (Bermejo-Poza *et al.*, 2020; Jezińska *et al.*, 1982). Accordingly, in October DHA emerged as the quantitatively primary muscle fatty acid, aligning with FA dynamics in *T. arcticus* (Sushchik *et al.*, 2007) and indicates the successful acclimatisation of FA metabolism in stocked fishes. Contrary to a previous study (Ahlgren *et al.*, 1999), we found adequate levels of arachidonic acid (AA) in the muscle of pond-reared grayling that closely match levels in wild *T. arcticus* (Sushchik *et al.*, 2006, 2007). Like the DHA dynamics, AA seems to be well preserved from depletion in the stocked graylings. The level of AA in stocked graylings was not far from pond grayling levels and did not differ from wild fishes in October, which reflects the strong metabolic conservation of AA that secures optimal levels of this critical PUFA for fish health (Le *et al.*, 2009; Hixson *et al.*, 2015). The consistently high LA content in pond fishes muscle could be attributed to commercial food pellets (American catfish ALLER BONA FLOAT), which contain a considerable amount of vegetable oil rich in LA content. Consequently, elevation in this n-6 FA contributed to the unusually low n-3/n-6 ratio in pond graylings muscle that was under the levels of wild *T. thymallus* or *T. arcticus* (Ahlgren *et al.*, 1994; Ahlgren 1999;

Sushchik *et al.*, 2006) and considerably lower than the values reported for *T. baicalensis* (Makhutova and Stoyanov, 2021). On the other hand, stocked fishes in the wild typically cannot find LA-rich food comparable to feeding pellets (Ahlgren *et al.*, 1994), while demands for this acid increases during energy-demanding periods and for AA synthesis (Jezińska *et al.*, 1982; Bermejo-Poza *et al.*, 2020;). Therefore, LA content of stocked graylings underwent significant depletion compared to the initial state. This LA content depleted more than the quantitatively important n-3 FAs, which provoked a constant increase in the n-3/n-6 ratio that peaked in October and aligned with wild fishes.

4.4 Liver FA composition

The FA composition of liver and VAT has never been described before for EG. The FA composition in liver underwent significant changes earlier than in muscle, possibly because dietary FAs are transported to the liver where they affect FA composition (Olsen *et al.*, 1991) aided by a pronounced *de novo* synthesis of lipids (Lin *et al.*, 1977; Sheridan, 1994). Conversely, and supported by our results, alterations of FA levels in muscles may be delayed due to their poor assimilation rates of certain FAs, especially eicosapentaenoic acid (EPA) (Gladyshev *et al.*, 2012).

The remarkable changes in liver FA composition are characterised by an increase in PUFAs and n-3 HUFAs and a decrease in MUFAs even after just one month. Apparently, dietary FAs obtained from natural food provide sufficient precursors for n-3 HUFAs biosynthesis and help maintain a high n-3/n-6 ratio. Predominant prey taxa during this period included Chironomidae, rich in EPA (Sushchik *et al.*, 2006), and fish eggs, rich in both EPA and DHA (Kaitaranta and Linko, 1984). In general, grayling prey items are relatively rich in EPA (Ahlgren *et al.*, 1999; Sushchik *et al.*, 2006), which is further involved in DHA synthesis (Kaur *et al.*, 2011; Makhutova and Stoyanov, 2021; Pilecky *et al.*, 2022). Furthermore, we observed that DHA dynamics did not precisely follow the dynamics of EPA in the liver. An increase in DHA was preceded by an increase in EPA, supporting the above mentioned DHA biosynthetic pathway scenario. The general pattern of reduction in EPA and increase in DHA in the liver might occur under starvation conditions as previously reported for salmon (Einen *et al.*, 1998). Nevertheless, this reduction also highlights the importance of higher DHA levels in salmonids in wild environments (Olsen, 1999), particularly for wild grayling (Makhutova and Stoyanov, 2021). The evidenced simultaneous processes of increased biosynthesis of n-3 HUFAs and LA depletion in the river environment approximated the n-3/n-6 six-month mean (May–October) ratio from the initial state to the mean values reported for the wild *T. baicalensis* (Glyzina *et al.*, 2021).

4.5 VAT FA composition

The dynamics of the FA composition of VAT were more uniform. Unlike the muscle and, especially, the liver, the FAs in VAT revealed that no individual FA was protected from utilisation during the acclimatisation process (Fig. 4). We also found that energy-demanding periods in the river tended to

mobilise SFA from VAT to a greater extent than from the other FA groups, thereby confirming the preferred use of C16:0 acid (Jezińska *et al.*, 1982). However, this reduction in C16:0 persisted consistently over the six months of the experiment, possibly due to a limited assimilation from natural food and inadequate storage in VAT (Gladyshev *et al.*, 2012). At the same time, under rearing conditions, C16:0 is consumed in high quantities by salmonids and intensively catabolised for energy (Tocher, 2003), and was maintained at a high level in our pond fishes. In addition to the significant presence of oleic acid in VAT, which explains preponderance in storage as in *T. arcticus* (Gladyshev *et al.*, 2012), we also observed considerable LA accumulation in EG. The importance of VAT lipids in the energy support process is reflected in the fact that neither PUFAs nor n-3 HUFAs were spared from depletion over six months in the river unlike in muscle tissue. In general, there are interspecies differences within salmonids in the extent of reliance on VAT lipids under energy-demanding conditions (Einen *et al.*, 1998). However, our study reveals the near exhaustion of TL in VAT in stocked fishes, thereby suggesting the importance of having a high content of VAT lipids prior to stocking. Finally, we agree that in post-stocking acclimation, higher lipid levels play a critical role (Umino *et al.*, 1991; Czesny *et al.*, 2003).

4.6 Feeding strategies of stocked EG

The contribution of terrestrial invertebrate input seems to be important for stocked graylings during the spring, summer and early autumn seasons. In general, terrestrial invertebrates constitute a large part of the diet of salmonids (Allan *et al.*, 2003). Indeed, they can contribute over 50% of the diet of drift-feeding salmonids during the summer (Baxter *et al.*, 2005), a percentage that is close to the six-month mean (46%) of terrestrial items observed in the diet of our stocked graylings. This also concurs with previous records for stocked EG diets (Thorfve, 2002). However, these general findings contrast with data on wild EG diets, which contain a low proportion of terrestrial species (Radforth, 1940; Hellawell, 1971; Kruzhylna and Didenko, 2011). We detected 28% of terrestrial invertebrates in the wild graylings diet in October, which is when the input of terrestrials typically peaks (Baxter *et al.*, 2005). This percentage closely coincides with the diet of wild EG in spring and summer (26%) (Frossard *et al.*, 2021). Our observations indicate a divergence between stocked and wild grayling diet preferences in October, with the former preferring smaller preys and having a higher dependence on terrestrial invertebrates and a lower preference for typical benthic organisms. As well, feeding is more focused on drifting preys which usually occur in the upper layer of the water column, and may be attributed to stocked graylings prior experiences in captivity (Brown and Day, 2002). The preference for adult aphids seems to be due to their long drift distances based on their small, winged bodies with no ability to drag (Rader, 1997) and high abundance, which saturates drifts and might trigger the initiation of feeding by stocked fishes as they are easily visible prey. Accordingly, a similar finding has been reported in reared sea trout (Krepeski and Czerniawski, 2019), in which the group of trout fed with sinking pellets had a post-stocking diet composed of more

benthic preys than the group fed on floating pellets. However, the frequent consumptions of small preys by stocked grayling could be a growth-limiting factor in the subsequent period. A bioenergetic model predicts that as trout size increases, feeding to satiation with small drift preys requires more foraging movements, so negatively influencing growth (Hayes *et al.*, 2000). Additionally, the drift of preys in the upper water column at higher velocities may reduce the total energy obtained from food (Brown and Day, 2002), while the high feeding frequency of stocked fish might also make them more susceptible to aerial predators.

4.7 Concerns and conclusions for future management attention

The observed high post-stocking feeding frequency of stocked graylings with the drifting preys, raises concern about their vulnerability to predators in the natural environment. To help mitigate these rearing-related traits, conventional rearing methods (floating pellets) should be modified to decrease these movements, for example, switching to sinking pellets to strengthen bottom-feeding skills. In addition, intensive migrations of stocked graylings after stocking have distanced them from the behaviour of wild graylings and led to their unfavourable bioenergetic status. This is reflected in the exhaustion of VAT lipid reserves and implies their choice for inappropriate trade-offs between foraging efforts and energy gain, as well as high swimming costs. Therefore, we recommend culturing under a stable feeding regime to secure the fish with higher lipid levels before stocking, which will support their acclimatisation in the wild. Also, periodic exposure to experimental stream flow during the cultivation could simulate the natural conditions and seem valuable in preventing energy-costly post-stocking migration. Lastly, streams stocked with EG should be carefully monitored by fishery managers to prevent any destruction of the riparian vegetation and to ensure the natural level of input of terrestrial insects that are so valuable for graylings.

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Additional information competing interests

The authors declare no competing interests.

Author contributions

Conceptualisation, M.A., and T.R.; Formal Analysis, M.A., J.T., A.T., M. B. and M.L.; Investigation, M.A., J.T., T.R., J.M., A.T., M.B., M.L., M.S.; Resources, T.R.; Writing – Original Draft, M.A.; Writing – Review and Editing, M.A., J.M., T.R., J.T., M.B., A.T., M.L.; Visualization, M.A., and M. L.; Project Administration, M.A. and T.R.; Funding Acquisition, T.R.

Supplementary materials

Figure S1. Generalised linear model (GLM) of Fulton's condition factor (K) with two European grayling (*Thymallus thymallus*) groups during the monitored period (April–October).

Table S1. Lipid content in European grayling of three groups, pond-reared, stocked and wild (Oct*) during the monitored period. Values represent mean $\pm$ s.e.m. of lipid content (mg/g of wet weight).

Table S2. Values of individual fatty acids (mg/g of w. w. $\pm$ s.e.m.) and FA groups (SFA, MUFA, PUFA, n-3/n-6, n-3 HUFA) in the muscle tissue of pond-reared European grayling.

Table S3. Values of individual fatty acids (mg/g of w. w. $\pm$ s.e.m.) and FA groups (SFA, MUFA, PUFA, n-3/n-6, n-3 HUFA) in the muscle tissue of stocked and wild European grayling.

Table S4. Values of individual fatty acids (mg/g of w. w. $\pm$ s.e.m.) and FA groups (SFA, MUFA, PUFA, n-3/n-6, n-3 HUFA) in the liver tissue of pond-reared European grayling.

Table S5. Values of individual fatty acids (mg/g of w. w. $\pm$ s.e.m.) and FA groups (SFA, MUFA, PUFA, n-3/n-6, n-3 HUFA) in the liver tissue of stocked and wild European grayling.

Table S6. Values of FAs and FA groups (SFA, MUFA, PUFA, n-3/n-6, n-3 HUFA) in the visceral adipose tissue of pond-reared European grayling, expressed in mg/g of w. w. $\pm$ s.e.m.

Table S7. Values of FAs and FA groups (SFA, MUFA, PUFA, n-3/n-6, n-3 HUFA) in the in the visceral adipose tissue of stocked (Apr*–Oct) and wild (Oct*) European grayling, expressed in mg/g of w. w. $\pm$ s.e.m.

Table S8. Numerical prey abundance (mean $\pm$ s.e.m.) in the stomach content of stocked (May–Oct) and wild European grayling (Oct*).

The Supplementary Material is available at <https://www.kmae-journal.org/10.1051/kmae/2024008/olm>.

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