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Liver Lipid Accumulation in European Bullhead (*Cottus cobio*) from a High-Mountain Lake: An Adaptive Strategy to Survive the Adverse Winter Season

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Abstract: The hypothesis that liver lipid accumulation in fish is an adaptive strategy to survive the winter in the high-altitude environment was assessed in this study. During summer 2019, specimens of *Cottus gobio* were sampled in 15 watercourses of Friuli Venezia-Giulia Region (Italy) to verify if hepatic steatosis is or not normally present in the species. To do this, hepatic vacuolization was assessed by histology using a semiquantitative score. Furthermore, *C. gobio* were also captured during the ice-free season at Dimon Lake (1872 m a.s.l.) and But Stream (520 m a.s.l.) to compare the trend in lipid accumulation: water temperature, hepatosomatic index (HSI), gonadosomatic index (GSI), Fulton's condition factor (K), and lipid area percentage (lipid %) were measured monthly. Findings revealed that liver steatosis is rather common in *C. gobio*. However, the trend in lipid accumulation of this species differed between Dimon Lake and But Stream. Based on the HSI and the GSI, the reproductive cycles differed in fish from the two environments (April–May in But Stream; May–June in Dimon Lake). While K values remained unchanged in the But Stream specimens, significant changes were recorded for Dimon specimens. The increase in lipid % from July to August in the Dimon Lake specimens coincided with greater food availability. With the rapid drop in lake water temperature in October, the lipid % decreased due to slower metabolic rate and lipid utilization from liver stores. There was a slight decrease in lipid % in the But Stream specimens, indicating that lipids were not being accumulated. Introduced years ago, the Dimon Lake bullhead population has since adapted to the winter conditions at high elevation.

Keywords: Alps; environmental adaptation; GSI; HSI; hepatic steatosis; northeast Italy; watercourses

1. Introduction

Lipids are essential nutrients for fish and provide fuel for growth [1]. Lipid metabolism in fish differs from that of other vertebrates. Fish acquire lipids from their diet and absorb them as fatty acids and triacylglycerols, which are then aggregated into chylomicron particles [2]. Lipids can also be synthesized by the liver (endogenous lipid metabolism). Fish store lipids in various depot organs, including the mesenteric membranes, the muscle, and the liver [2]. Liver lipid accumulation is induced by biochemical mechanisms: decreased hepatic lipid export, increased hepatic uptake of circulating fatty acids, decreased hepatic beta-oxidation, and increased hepatic fatty acid synthesis [2].

Lipid storage and depletion is an adaptive strategy fish employ to cope with lower metabolic rate and activity during winter [3]. Lipids are usually the first reserves to be mobilized when food resources become scarce [4]. During extended periods of starvation, these reserves are depleted; in their place, liver and muscle protein is then mobilized to fuel metabolism [5].

In winter, fish implement behavioral and physiological mechanisms or pathways to sustain activity and energy acquisition [3,4,6]. Obligate and facultative controls on energy usage during the winter season have been observed in several fish species. For example, Evans [7] reported a slower basal metabolic rate in the pumpkinseed (*Lepomis gibbosus*) during fall and winter, suggesting an adaptive metabolic strategy similar to hibernation. In addition, facultative behavior (i.e., a shift to deeper areas of the water column) has been observed for cyprinids to reduce exposure to cold temperatures [8]. During winter, the reduction in water temperature, light, and primary production limits the availability of food resources. In response, ectothermic animals allocate energy to storage before winter, especially in the form of lipids, which can then be spent to meet metabolic demands without losing structural mass [9,10]. Since fish are ectothermic organisms, metabolic pathways are influenced by water temperature: cold temperatures reduce metabolic rate, as well as the ability to forage, digest, and avoid predators.

In their preliminary study, Pastorino et al. [11] during two fish sampling campaigns (summer and autumn 2017) reported marked differences in liver alterations (hepatic steatosis) between European bullhead (*Cottus gobio*) from a high-mountain lake (Dimon Lake) and those from an Alpine stream (Degano Creek) located in Friuli Venezia-Giulia (northeast Italy). They concluded that the liver steatosis present in the specimens from a high-altitude environment might be related to adaptation to the long winter season, which may also last for several months in the Alpine environment. While steatosis can also be induced by environmental contaminants such as cadmium, this hypothesis was rejected based on published data [12]. To better understand this unusual situation, during summer 2019, we performed fish sampling campaigns in 15 watercourses of Friuli Venezia-Giulia Region (northeast Italy) to verify if hepatic steatosis is or not normally present in the European bullhead. Furthermore, fish sampling campaigns were also performed during the ice-free season at Dimon Lake (1872 m a.s.l.) and But Stream (520 m a.s.l.) (Friuli Venezia-Giulia, northeast Italy) to compare the trend in lipid accumulation in the two populations. This deepening did not include all watercourses, being the species included in Annex II of the European Directive 92/43/EEC (Habitat Directive).

2. Materials and Methods

2.1. Study Area

The presence of hepatic steatosis in *C. gobio* was assessed in 15 watercourses of Friuli Venezia-Giulia Region (northeast Italy) located at different elevations (Figure 1a, Table 1).

In the second part of this work, the main study site was Dimon Lake (Figure 1a,b), a high-mountain lake located in the Carnic Alps (municipality of Ligosullo, Friuli Venezia-Giulia, northeast Italy) at 1857 m a.s.l. (46°34′05.4″ N; 13°03′45.8″ E). Dimon Lake is a typical glacial-origin lake included within a site of community importance and special area of conservation (sCI/SAC-IT3320002 "Monti Dimon e Paularo"). The lake lies on sandstone and volcanic rock [13–15]; it measures 376 m in perimeter, 0.6 ha in surface area, and has a maximum depth of 4.27 m. The Carnic Alps are among the most remote areas in Italy, and anthropic impact is extremely limited, except for pasturing activity. Originally a fishless lake, fish were introduced for recreational fishing in the past [16]. For the purposes of comparison, *C. gobio* individuals were captured from But Stream (length, 33 km; basin catchment area, 330 km^2), a tributary of the Tagliamento River, the main watercourse of the region [17]. The sampling site at But Stream was located in Sutrio Municipality (520 m a.s.l.), near Noiaris, downstream of Sutrio (Figure 1a,c) (46°29′55.08″ N; 13° 0′3.59″ E).
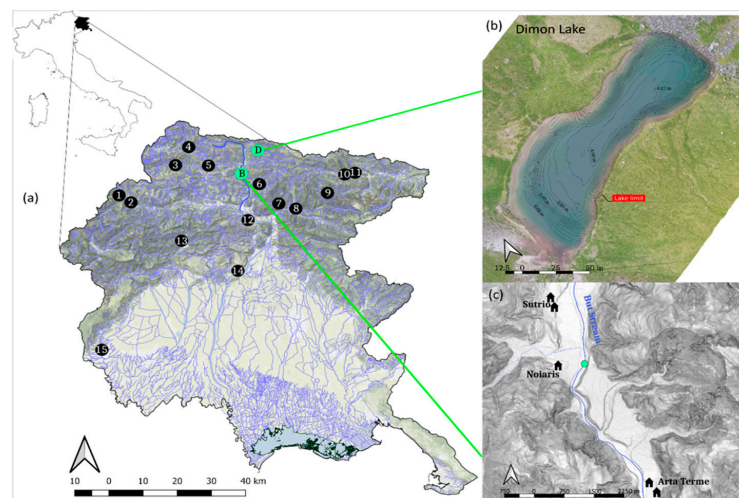
Figure 1. Location of the analyzed watercourses in Friuli Venezia-Giulia Region to verify if hepatic steatosis is or not normally present in the European bullhead (**a**); location of the Dimon Lake (46°34′05.4″ N; 13°03′45.8″ E) and But Stream (46°29′55.08″ N; 13° 0′3.59″ E) in Friuli Venezia-Giulia, northeast Italy (**a**); bathymetric profile of Dimon Lake (**b**); detailed location of the But Stream site (**c**). Figure (**b**) is kindly provided by GeoStudio RC (Giaveno, Italy).

Table 1. Map code (Figure 1a), name, latitude, longitude and elevation of selected watercourses in Friuli Venezia-Giulia Region (northeast Italy). The liver alteration score (range 0–3) observed in *Cottus gobio* sampled during summer 2019 is also reported. Liver alteration: 0—not observed; 1—mild; 2—moderate; 3—severe.

Map Code	Watercourse	Latitude	Longitude	Elevation (m a.s.l.)	Liver Alteration Score	
					Min	Max
1	Giaf Creek	46°25′38″ N	12°32′19″ E	1050	2	2
2	Tagliamento River	46°24′29″ N	12°35′07″ E	870	0	1
3	Pesarina Stream	46°31′09″ N	12°44′59″ E	820	0	1
4	Alpo Creek	46°34′20″ N	12°47′53″ E	815	0	1
5	Margò Stream	46°31′12″ N	12°52′32″ E	555	0	0
6	Ambruseit Creek	46°28′12″ N	13°04′17″ E	458	2	3
7	Glagnò Stream	46°24′54″ N	13°08′50″ E	334	0	1
8	Alba Creek	46°24′05″ N	13°12′46″ E	316	0	1
9	Dogna Stream	46°26′55″ N	13°19′54″ E	453	1	3
10	Bianco Creek	46°30′11″ N	13°23′45″ E	767	0	3
11	Malborghetto Creek	46°30′31″ N	13°26′06″ E	713	0	2
12	Faeit Stream	46°21′55″ N	13°01′56″ E	290	0	1
13	Meduna Stream	46°18′03″ N	12°46′54″ E	365	1	3
14	Molino Millrace	46°13′04″ N	12°59′56″ E	170	1	3
15	Bodegan Creek	45°58′45″ N	12°39′37″ E	20	0	2

2.2. Sampling Design

During the summer (June–August) 2019, five fish for each of the 15 watercourses were captured using a backpack electrofisher (Model IG200–2; Hans–Grassl GmbH) to assess the presence of hepatic steatosis. Fish were suppressed with an anesthetic overdose of tricaine methane-sulfonate MS-222 (50 mg kg^{-1}) [18] and transported to the laboratory. Livers were then dissected, partly fixed in 10% neutral-buffered formalin and partly frozen [11].

Fish sampling at Dimon Lake was performed monthly from June to October 2019, during the ice-free period when access to the area is possible. At each sampling event, 20 bullhead specimens were captured by electrofishing (backpack electrofisher, model ELT62 II 135 GI, Hans–Grassl GmbH, Schönau am Königssee, Germany), acting from the lakeshore. Fish sampling at But Stream was performed monthly from March to October 2019 in order to cover the ice-free period at the Dimon Lake and to include the reproduction period, generally between February and May/June [19,20]. At each event, 20 specimens were caught using a battery-powered backpack electrofisher (Model IG200–2; Hans–Grassl GmbH). All specimens were placed in a tank and anesthetized with MS-222 [18] to minimize damage during manipulation. Total length (L_T; $x \pm 0.1$ cm) and weight (W; $x \pm 0.1$ g) were measured. The fish were suppressed with an anesthetic overdose (50 mg kg^{-1}) and placed in frozen bags for transport to the laboratory. Ovaries and livers were dissected. Fresh gonad weight and fresh liver weight were measured immediately after dissection ($x \pm 0.0001$ g) with an analytical balance. Sex was determined by gonadal inspection after dissection. The gonadosomatic index (GSI%), the hepatosomatic index (HSI%), and Fulton's condition factor (K) were calculated as previously reported [11,21].

2.3. Histological, Cytological Analysis and Lipid Content

The fixed livers of fish captured in the 15 watercourses were embedded in paraffin, cut into sections (4 ± 2 μm) and stained with hematoxylin and eosin (HE) for microscopic evaluation, following the methods previously reported [11]. Histological changes (nuclear displacement and cytoplasm vacuolization) to define liver steatosis were evaluated using a semiquantitative severity score (0—not observed; 1—mild; 2—moderate; 3—severe [11,22]. Sudan III stain to confirm lipids (presence of orange lipidic deposits in hepatocyte cytoplasm) was also performed on frozen samples [23]. Slides were evaluated microscopically at increasing magnification on a Zeiss Axio Scope.A1 microscope.

Liver samples from Dimon Lake and But Stream were fixed in SPAFG fixative (2.5% glutaraldehyde, 0.8% paraformaldehyde, 7.5% saturated aqueous solution of picric acid in 0.15 M PBS, pH 7.4, with 1.5% sucrose) [24] and stored at 4 °C until resin inclusion. The samples were then serially washed in 0.15 M PBS, pH 7.4, and postfixed in 1% osmium tetroxide in the same buffer, serially dehydrated in ethanol and embedded, via propylene oxide, in Embed812/Araldite (Electron Microscopy Sciences, Fort Washington, PA, USA). Sections (1 μm) were stained with toluidine blue and examined with an Olympus BX50 microscope; images were acquired with a digital Olympus PEN E-P1 camera (100×). Six specimens (3 males and 3 females) were chosen after HSI distribution analysis. Lipid droplets in liver tissues were identified, and their surface was measured. The sum of lipid droplet areas was calculated, and steatosis (lipid %) was evaluated according to the formula:

$$\text{Lipid \%} = \frac{\text{Lipid droplet area}}{\text{Sample area}} \times 100 \qquad (1)$$

For transmission electron microscopy, ultrathin sections (120 nm) were cut with a Pabisch TOP Ultra 150, stained with uranyl acetate and lead citrate, and examined with a Philips EM 208 electron microscope at 100 kV; images were acquired with a Quemesa bottom-mounted TEM CCD camera (Olympus, Germany) provided with an iTEM imaging platform and saved in TIF format.

2.4. Determination of Water Temperature in Dimon Lake and but Stream

Water temperature was recorded at each site during each sampling campaign with field meters (HI 9125 pH/ORP meter, manufactured by Hanna Instruments Inc., Woonsocket, RI, USA) and related to the bullhead life cycle. Three replicates were obtained at each sampling event.

2.5. Statistical Analysis

Differences in GSI, HSI, and K indexes were analyzed using the nonparametric Kruskal–Wallis test since the null hypothesis for the homogeneity of variance and/or for normal distribution could not be rejected. Significant differences in lipid area percentage were tested using the Kruskal–Wallis test Post hoc tests were the Dunn test or the Conover test (when *n* < 20) [25,26]. Differences in lipid area percentages between the two sites were analyzed using the Wilcoxon nonparametric test. All analyses were performed using RStudio version 3.4.3. Figures were produced with RStudio and processed with Inkscape software version 0.92; maps were created using QGis version 3.2.2–Bonn; histological images were analyzed using ImageJ 1.50i [27]. Statistical significance was set at *p* < 0.05.

2.6. Ethical Statement

All activities described in the present study were performed according to European (Directive 2010/63/EU), National (D. Lgs. 26/14), and regional laws (Ente Tutela Patrimonio Ittico del Friuli- Venezia Giulia) dealing with procedures for the protection of animals used for scientific purposes. Permission for fish sampling and fish dissection was obtained from the Ente Tutela Patrimonio Ittico del Friuli Venezia-Giulia (authorization No. 258-12/04/2019).

3. Results

3.1. Hepatic Steatosis in Specimens of Bullhead from Watercourses

On gross inspection, the liver often appeared pale to yellowish in color and friable in consistency in the specimens from all sites, except those captured in Margò Stream that showed a reddish-brown color. Table 1 reports the liver alteration score observed in *C. gobio*. Histology of the samples showed a range of cytoplasm vacuolization of the hepatocytes from score 0 (not observed) to score 3 (severe). Sudan III stain was positive in all the samples that showed vacuolization of hepatocytes (score from 1 to 3), confirming the presence of lipids. Generally, at least one liver sample for each site showed hepatic vacuolization, except samples from Margò Stream, which did not show any alterations.

3.2. Water Temperature in Dimon Lake and But Stream

Figure 2 presents the trends for water temperature at the sampling sites. A minimum mean water temperature of 8.3 °C was recorded in June at Dimon Lake; snow and ice still covered the area and partially limited access; the mean temperature for July was 16.1 °C. A slight decrease was noted for August and September, followed by a drop to 9.7 °C in October. The optimum water temperature for bullhead spawning was recorded at the first sampling event in June. A minimum mean water temperature of 6.6 °C was recorded in March at But Stream. The water temperature increased until August, before decreasing to 11.8 °C recorded in October. The optimum water temperature for bullhead spawning was observed between April and May (8.8–9.7 °C).

3.3. Biometric Features, Gonadosomatic Index (GSI), Hepatosomatic Index (HSI) and K Index of Bullhead from Dimon Lake and But Stream

On gross inspection, the liver often appeared pale to yellowish in color and friable in consistency in the specimens from both sites throughout the investigated period. Table 2 presents the biometric features (total length and total weight); Figure 3a,b presents the GSI; the maximum for the female specimens from Dimon Lake was recorded in June (range, 17.2–24.5%), which then dramatically decreased in July and continued to decrease between July and August before reaching a plateau in September; a slight albeit significant increase was observed in October (Kruskal–Wallis test, *H* = 29.82, df = 4, *p* < 0.001; Conover test, *p* < 0.05 for significant comparisons). The maximum GSI for the male specimens was recorded in June (0.9–1.7%), which was decreased in July (Kruskal–Wallis test, *H* = 21.94, df = 4, *p* < 0.001; Dunn test, *p* < 0.001). No significant differences were recorded for the following months (Dunn test, *p* > 0.07 for all comparisons). In the But Stream specimens, there was a significant

increase in the GSI for the female specimens between March and April, with a peak of 21.0–24.5%, before decreasing from May to July. A significant increase was recorded in August, after which the levels remained constant (Kruskal–Wallis chi-squared $H = 27.85$, df = 7, $p < 0.001$; Dunn test, $p < 0.05$ for significant comparisons). The maximum for the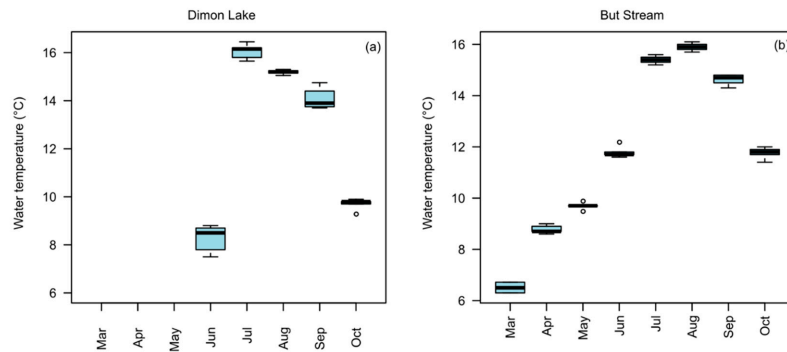 male specimens was recorded in April (1.2–3.2%), which then decreased from May to June, and remained fairly constant thereafter (Kruskal–Wallis chi-squared $H = 33.04$, df = 7, $p < 0.001$; Dunn test, $p < 0.05$ for significant comparisons).

Figure 2. Boxplots of water temperature (°C) monitored at Dimon Lake (**a**) and But Stream (**b**) during the study period.
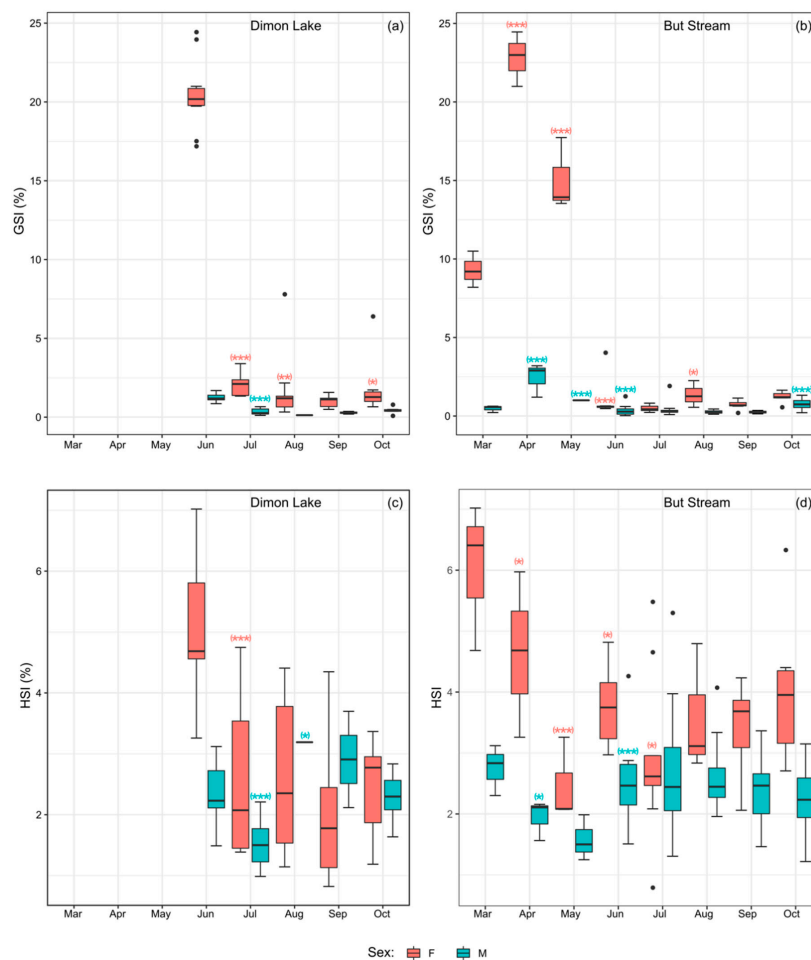

Figure 3. Trends in the gonadosomatic index of specimens from Dimon Lake (**a**) and But Stream (**b**) and trends in the hepatosomatic index in specimens from Dimon Lake (**c**) and But Stream (**d**) ($n = 20$ per sample). Asterisks (* = p-level < 0.05; ** = p-level < 0.02; *** = p-level < 0.001; red for female and blue for males) indicate significant differences with the previous monthly values.

Table 2. Total length (cm), total weight (g) and Fulton's condition factor (K) in male (M) and female (F) of *Cottus gobio* captured in But Stream and Dimon Lake. Lowercase letters denote differences in K index revealed by Conover-Iman post hoc test. Plus-minus values are the mean ± standard deviation.

	But Stream											
	Female						Male					
	Total Length (cm)		Total Weight (g)		K Value		Total Length (cm)		Total Weight (g)		K Value	
March	11.57	±2.24	18.14	±8.75	1.19 [a]	±0.18	13.83	±0.71	31.42	±4.48	1.18 [a]	±0.02
April	10.87	±2.53	19.12	±4.48	1.39 [a]	±0.13	11.30	±1.65	22.07	±4.69	1.44 [a]	±0.08
May	12.40	±1.49	25.98	±9.70	1.31 [a]	±0.08	10.97	±0.99	18.15	±4.70	1.36 [a]	±0.02
June	8.03	±1.14	6.34	±0.97	1.23 [a]	±0.17	9.82	±1.14	12.40	±3.22	1.31 [a]	±0.20
July	8.87	±3.62	9.66	±3.62	1.34 [a]	±0.10	9.24	±1.27	11.69	±4.33	1.42 [a]	±0.11
August	11.40	±2.91	17.91	±9.95	1.15 [a]	±0.19	16.00	±1.20	16.00	±4.54	1.30 [a]	±0.16
September	9.23	±2.89	9.93	±2.89	1.22 [a]	±0.06	11.15	±0.98	19.04	±4.60	1.35 [a]	±0.09
October	10.05	±2.03	13.60	±6.86	1.22 [a]	±0.06	11.35	±2.03	20.77	±8.32	1.31 [a]	±0.11
	Dimon Lake											
	Female						Male					
	Total Length (cm)		Total Weight (g)		K Value		Total Length (cm)		Total Weight (g)		K Value	
June	11.61	±2.14	20.68	±9.91	1.23 [a]	±0.14	12.36	±1.47	26.44	±8.07	1.32 [a]	±0.12
July	11.08	±1.66	14.69	±6.31	1.07 [ab]	±0.09	12.37	±1.65	21.05	±7.74	1.08 [ab]	±0.11
August	10.05	±1.41	13.55	±10.41	1.04 [b]	±0.16	7.25	±0.35	3.91	±1.05	1.01 [b]	±0.13
September	10.75	±2.59	15.43	±9.50	1.06 [ab]	±0.18	9.20	±3.25	8.47	±7.71	0.99 [b]	±0.05
October	9.82	±2.12	10.68	±6.39	0.98 [b]	±0.11	9.30	±0.91	7.99	±2.00	0.97 [b]	±0.03

Figure 3c,d presents the HSI. In the Dimon Lake female specimens, the HSI decreased significantly from June to July (Kruskal–Wallis chi-squared $H = 23.83$, df = 4, $p < 0.001$; Dunn test, $p < 0.01$ for significant comparisons). The maximum levels were recorded for June (3.6–7.0%). No major changes in the HSI were noted for the other months for female specimens. Regarding males specimens in the Dimon Lake, a significant decrease from June to July and an increase in August (Kruskal-Wallis chi-squared $H = 17.61$, df = 4, $p < 0.001$; Dunn test, $p < 0.02$ for significant comparisons) were observed. In the But Stream specimens, the HSI for the females decreased significantly from March to May, followed by fluctuations between May and July; the Dunn test showed no major changes from July afterward (Kruskal–Wallis chi-squared $H = 22.20$, df = 7, $p < 0.001$; Dunn test, $p < 0.05$ for significant comparisons). A similar trend in the HSI was noted for the males, with a significant decrease from March to May, followed by a significant increase from May to June (Kruskal–Wallis chi-squared $H = 25.02$, df = 7, $p < 0.001$; Dunn test, $p < 0.05$ for significant comparisons), after which the HSI remained fairly constant.

Table 2 presents the K index for the specimens from both sites. The But Stream specimens showed some albeit non-significant fluctuations (Kruskal–Wallis $p > 0.05$), whereas significant changes in the K index were recorded for the male (Kruskal–Wallis chi-squared $H = 19.70$, df = 5, $p < 0.001$; Conover test, $p < 0.05$ between June and August, June and September, and June and October) and the female specimens (Kruskal–Wallis chi-squared $H = 13.94$, df = 5, $p < 0.001$; Conover test, $p < 0.05$ between June and August, and June and October) from Dimon Lake.

3.4. Histological Analysis and Lipid Content in Bullhead from Dimon Lake and But Stream

Semi-thin liver sections are shown in Figure 4, and hepatocyte ultrastructure in Figure 5. The hepatic parenchyma of a steatotic liver shows evident lipid droplets intensely stained with osmium tetroxide and toluidine blue (Figure 4A) located in the hepatocyte cytoplasm (Figure 5A). Figure 6 presents the changes in lipid area percentage. In the Dimon Lake specimens, the percentage remained constant between June and July before significantly increasing between July and August, followed by a significant decrease from September to October (Kruskal–Wallis chi-squared $H = 10.46$, df = 4, $p < 0.05$; Conover test, $p < 0.05$ for significant comparisons). The percentage in the But Stream specimens was slightly decreased during the study period; the Kruskal–Wallis test detected no significant change. The percentage recorded for June differed significantly between the specimens from the two sites, when the percentage was higher in the But Stream than in the Dimon Lake specimens (Wilcoxon test, $W = 33$, $p < 0.01$) and in September, when it was were higher in the Dimon Lake than in the But Stream specimens (Wilcoxon test, $W = 5$, $p < 0.01$).
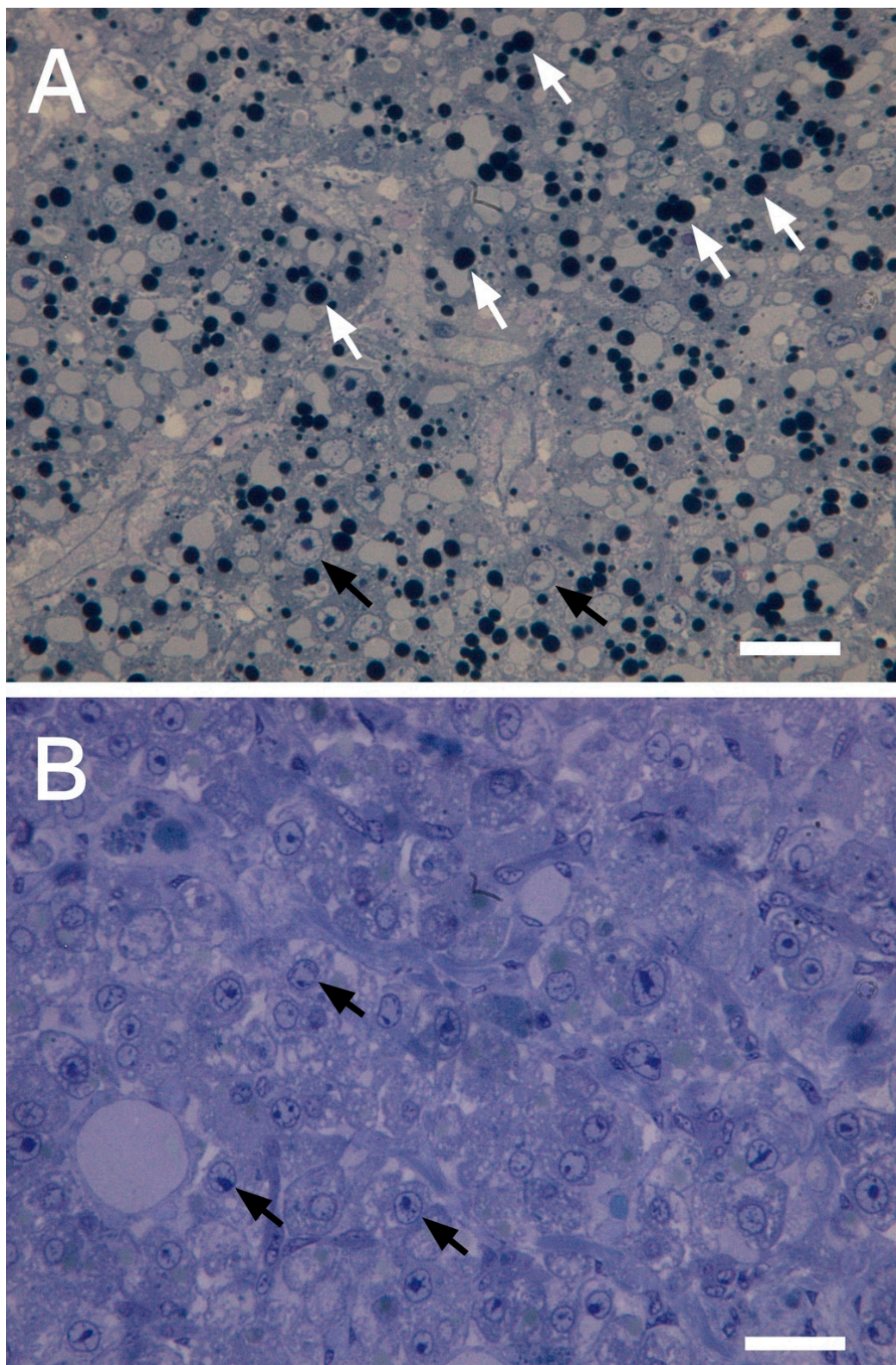
Figure 4. Semi-thin liver sections of *C. gobio*; (**A**) liver parenchyma showing massive lipid droplets (male, June, But Stream).　(**B**) liver parenchyma without lipid droplets (male, June, Dimon Lake). Black arrows: nuclei; white arrows: lipid droplets. Calibration bar = 20 μm.
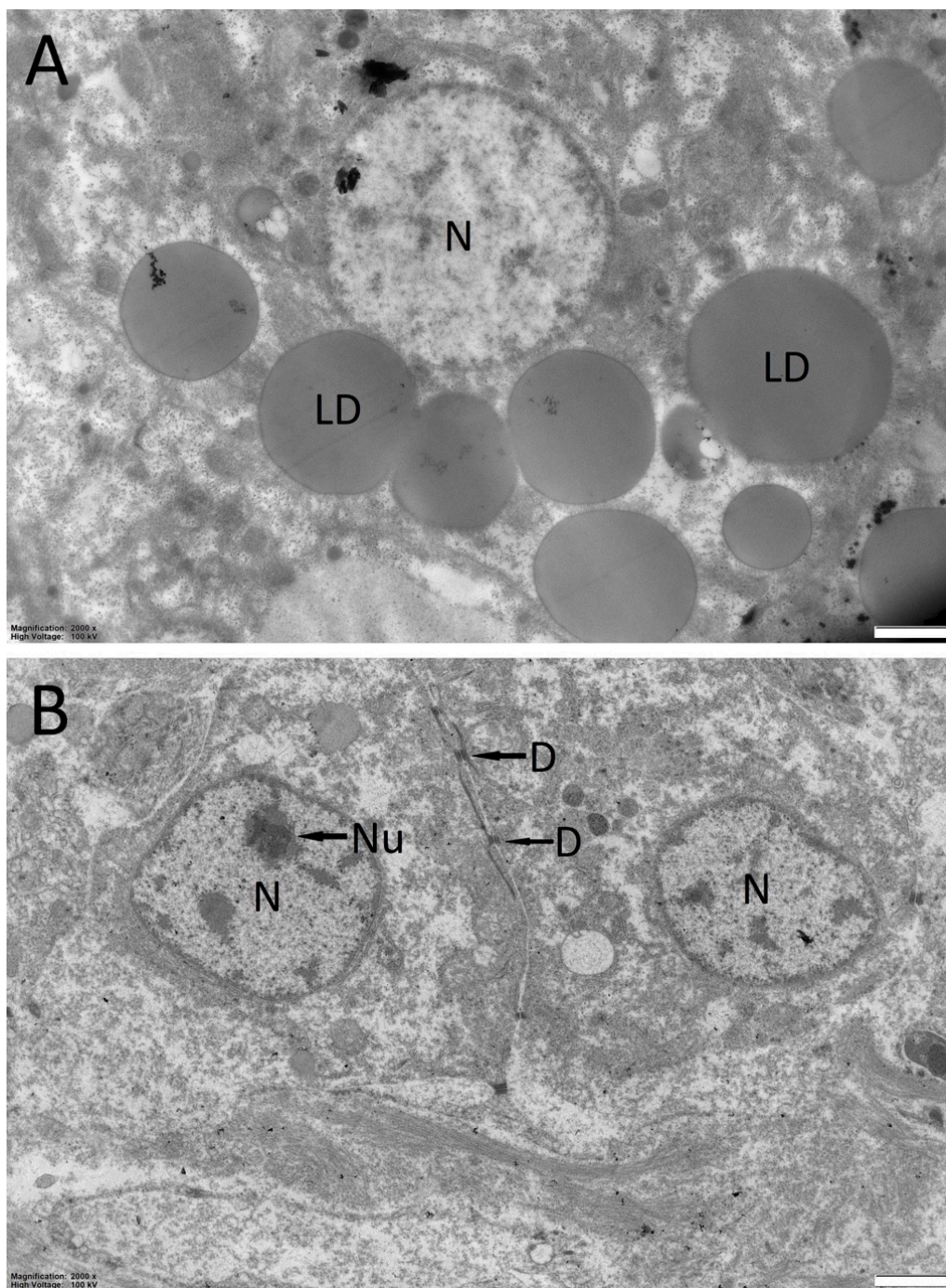
Figure 5. Hepatocyte ultrastructure in *C. gobio*; (**A**) cytoplasm filled with large lipid droplets in a steatotic liver (male, June, But Stream). (**B**) hepatocytes without lipid droplets (male, June, Dimon Lake). D: desmosomes; N: nucleus; Nu: nucleolus; LD: lipid droplets. Calibration bars = 2 μm.
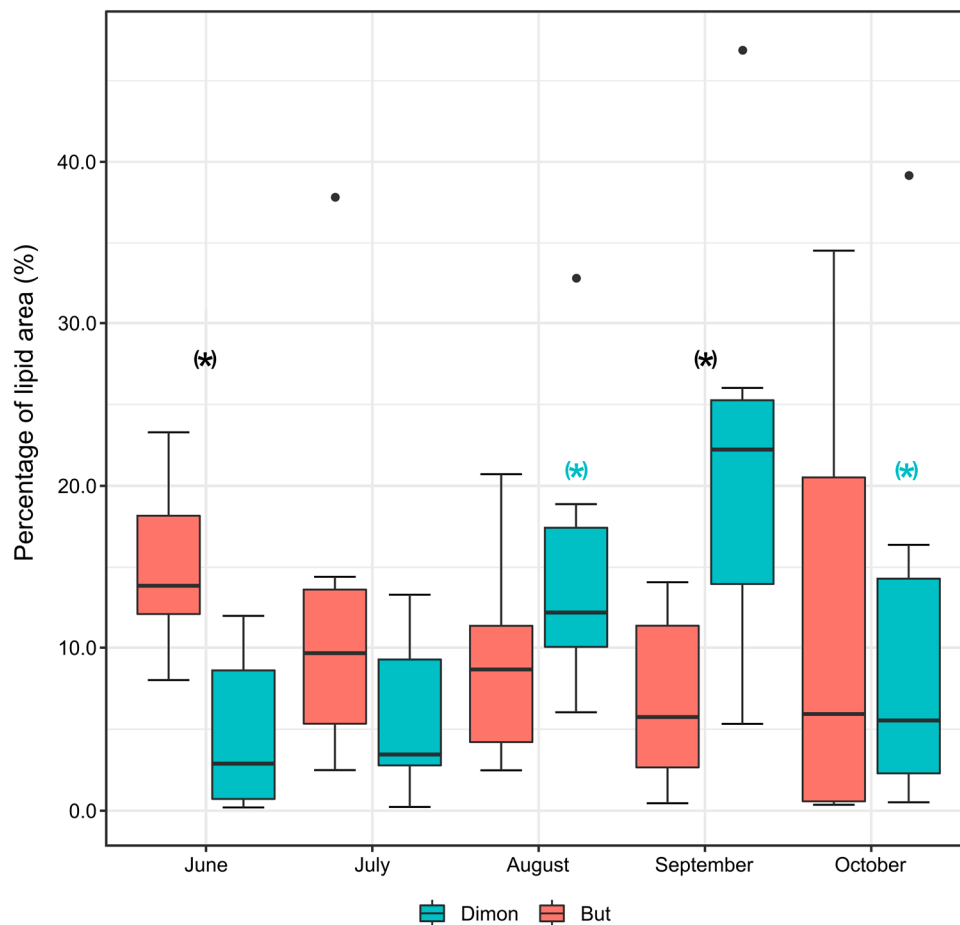
Figure 6. Trends in liver lipid area percentage in the *C. gobio* from Dimon Lake and But Stream (*n* = 6 per month at both sites, including three males and three females). Blue asterisks (* = *p*-level < 0.05) indicate significant differences in Dimon Lake with the previous monthly value. Black asterisks (* = *p*-level < 0.05) indicate significant differences between But and Dimon monthly values.)

4. Discussion

The freshwater fish species European bullhead (*Cottus gobio*) is widely distributed throughout Europe, from Greenland and Scandinavia in the north to Italy in the south. It is also commonly found in England and Wales, excluding northwest Wales [28]. Generally, *C. gobio* inhabits lotic ecosystems [29]; the species was also released into Dimon Lake as forage fish for salmonids years ago [11].

Hepatic vacuolization is a common finding in farmed fish due to artificial feeding that promotes disequilibrium in the influx of fatty acids into the hepatocytes, altering their metabolism and function [30]. On the other hand, the presence of steatosis in wild fish is not well documented in the literature. Our findings suggested that liver steatosis is rather common in European bullhead since specimens from 14 out of 15 watercourses showed hepatic vacuolization. On the other hand, the deep insights into trends in lipid accumulation varied between the two study sites (Dimon Lake and But Stream).

We observed that the reproductive period of *Cottus gobio* in the But Stream coincides with its reproductive biology [19,31]. In addition, the trend for the GSI and the HSI paralleled the reproductive cycle of this species at both sites. Gonad growth appears to be fueled by liver stores; the gonad growth period is accompanied by a decrease in liver size [32]. Based on these indexes, the reproductive cycle in the bullhead from But Stream can be divided into a pre-spawning period (March), a spawning period in April–May, and an extended post-spawning season starting in June. Differently, the reproductive period in the bullhead from Dimon Lake does not occur until May–June, due to the higher elevation

of the lake compared to the stream, which affects the water temperature. In fact, in Dimon Lake, the optimum water temperature for bullhead reproduction was observed in June.

Generally, in our survey, the HSI was often higher in females than in males from both sites, especially during the spawning season. This may be explained by the dual function (lipid metabolism and yolk precursor synthesis) of the liver during this period in females. Sexual dimorphism of the liver is particularly obvious in oviparous animals since the liver in females produces many proteins involved in the oocyte envelope and vitellogenin reserves, whereas the male liver hepatocytes do not [33]. In addition, we noted a higher GSI in the females because of the heavier gonad weight due to the presence of eggs contained in the enlarged ovary when it reaches maturity. A low GSI for the males was due to the very low energy investment during gamete production, as reported elsewhere [34]. In the But Stream specimens, the percentage of lipid area started to decrease (albeit not significantly) starting in June, whereas, in the Dimon Lake specimens, it increased from June to September, with a significant rise between July and August, at which time there was also an increase in water temperature compared to June and in the abundance of prey (mainly Diptera Chironomidae larvae), as previously reported [35]. These observations support our hypothesis for a rapid increase in food ingestion accompanied by an increase in liver lipid storage (and greater metabolic activity) as fuel for the adverse winter season. Because the lake is covered by ice for several months during winter, the bullhead population had to develop a strategy to survive: the fish are spatially limited and separated from the surrounding terrestrial habitat by the ice cover [36]. The rapid drop in temperature recorded for October (9.7 °C) probably reduced the metabolic rate since metabolic activity slows as the water temperature falls. This decrease may have enhanced lipid metabolization and utilization, explaining the lower percentage in liver lipid area compared to the summer months. During the winter season, the fish inhabiting mountain lakes undergo physiological and metabolic changes that deplete their energy reserves [37]. This is also seen in the K factor, which was decreased in the Dimon Lake specimens caught in October.

Fish body size explains the differences in the magnitude of lipid accumulation across species [3]. Smaller fish, like the European bullhead, have a smaller capacity (i.e., body volume) for energy storage and a higher specific metabolic rate compared to larger fish [10]. Hence, small body size accentuates the need to accumulate large lipid deposits before the food supply diminishes in winter. Furthermore, liver lipid storage can play an important role also in the bulk of gonadal tissues, which are generated during the winter and under the ice, when food sources are limited.

While there was a slight decrease in the K index starting in April in the But Stream specimens, we noted a significant decrease in the K index for the Dimon Lake specimens starting in June, during the post-spawning period, as reported previously for *Cottus cognatus* [32]. In addition, the K index in the males appeared lower, especially after the spawn, which is probably linked to the cessation or reduction in feeding during nest guarding by the males [32]. The slight decrease in the lipid area percentage we observed in the But Stream specimens from June to October indicates that they do not activate an accumulation strategy. A plausible explanation is that, unlike Dimon Lake, the flowing water in But Stream does not allow an ice cover to form, and so the fish remain in contact with the surrounding terrestrial habitat. The fish can forage and capture prey throughout the year, without the need to store lipids to survive the winter.

5. Conclusions

In conclusion, our findings suggest that liver steatosis is rather common in wild *C. gobio*. Furthermore, the trend in lipid accumulation suggests that the *C. gobio* population from Dimon Lake has adapted to the winter conditions at altitude. This strategy ensures that energy can be stored for survival and for fueling the energy demands for reproduction under the ice cover. Further studies are necessary to explore this strategy, for example, by analyzing the types of lipids involved and comparisons with other watercourses.

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