sustainability

MDPI

Article

Key Performance Indicators of Common Carp (*Cyprinus carpio* L.) Wintering in a Pond and RAS under Different Feeding Schemes

Piotr Eljasik [1], Remigiusz Panicz [1,*], Małgorzata Sobczak [1] and Jacek Sadowski [2]

[1] Department of Meat Sciences, Faculty of Food Sciences and Fisheries, West Pomeranian University of Technology in Szczecin, 4 Kazimierza Królewicza Street, 71-550 Szczecin, Poland; peljasik@zut.edu.pl (P.E.); malgorzata.sobczak@zut.edu.pl (M.S.)

[2] Department of Aquatic Bioengineering and Aquaculture, Faculty of Food Sciences and Fisheries, West Pomeranian University of Technology in Szczecin, 4 Kazimierza Królewicza Street, 71-550 Szczecin, Poland; jsadowski@zut.edu.pl

* Correspondence: rpanicz@zut.edu.pl; Tel.: +48-91-449-66-64

Abstract: Overwintering impacts common carp performance, yet the nature of changes is not known. The aim of the study was to compare the zootechnical and key performance indicators (KPI) of *Cyprinus carpio* wintering in a pond with no supplementary feeding (MCF), in a Recirculating Aquaculture System (RAS) fed typical (30% of protein and 8% of fat) carp diet (AFC), and in a RAS fed high protein (42%) and fat (12%) diet (ABF). The analysis showed that ABF fish had the highest final body weight and the Fulton's condition factor, as well as the lowest food conversion rate compared with AFC and MCF fish. Histomorphological assessment revealed that MCF fish had thinner skin layers, a depleted population of mucous cells in skin, an excessive interlamellar mass in the gills, and no supranuclear vacuoles in the intestine compared to fish from RAS. At the molecular level, higher transcript levels of *il-1β* and *il-6* transcripts were found in the gills of MCF than in fish from RAS. The transcript level of the intestinal *muc5b* was the highest in ABF fish. Relative expression of *il-1β* and *il-6* in gills were presumably the highest due to lamellar fusions in MCF fish. Described KPIs may assist carp production to ensure sustainability and food security in the European Union.

Keywords: gene expression; gills; intestine; *mucin 5b*; performance

Citation: Eljasik, P.; Panicz, R.; Sobczak, M.; Sadowski, J. Key Performance Indicators of Common Carp (*Cyprinus carpio* L.) Wintering in a Pond and RAS under Different Feeding Schemes. *Sustainability* **2022**, *14*, 3724. https://doi.org/10.3390/su14073724

Academic Editor: Mario D'Amico

Received: 17 February 2022
Accepted: 19 March 2022
Published: 22 March 2022

1. Introduction

Aquaculture is a key food production sector globally [1]. In 2018, its total production was 114.5 Mt in live weight, with a total farmgate sale value of USD 263.6 billion. A considerable part of this production was represented by freshwater aquaculture (51.3 Mt, 62.5%), of which 47 Mt (91.5%) consisted of finfish production. The freshwater production of finfish is forecasted to reach 60% of global aquaculture production by 2030, according to the report, State of World Fisheries and Aquaculture (SOFIA) [2]. Among the five most important aquaculture species, four belong to Cyprinidae: the herbivorous grass carp, *Ctenopharyngodon idella* (5.7 Mt, 10.5%); the omnivorous common carp, *Cyprinus carpio* (4.2 Mt, 7.7%); and two planktivorous: silver carp, *Hypophthalmichthys molitrix* (4.8 Mt, 8.8%), and bighead carp, *Hypophthalmichthys nobilis* (3.1 Mt, 5.8%) [2]. The latest report published by the Food and Agriculture Organisation (FAO) on the top 10 species groups in global aquaculture in 2019 showed that "carps, barbels and other cyprinids" are the main species group in freshwater aquaculture, amounting to nearly 24.8% (29.8 Mt) of 120 Mt of world production. The publication also showed that production of this group has increased 1.5% from the 2018 level and, consequently confirming the upward trend predicted in the report SOFIA [3]. However, as emphasized by Belton et al. [4], this trend was possible mainly due to intensification rather than horizontal expansion, i.e., increase in production per unit land and water.

For decades, the main farmed cyprinid species in Europe has been common carp (hereafter referred to as carp) that was the mainstay, both traditionally and commercially, for fisheries production in "land-locked" central European Union (EU) [5]. Total production of *C. carpio* in the EU in 2019 was 65,715 tonnes, and the Czech Republic followed by Poland, Hungary and Germany (ranked in order of production size) provided nearly 80% of carp production in the region [6]. In the EU, carp is produced with the conventional Dubisch method, which involves multiple fish transfers into different stage ponds throughout production. A crucial part of carp farming is wintering (October through April) that happens twice, i.e., after the first and second year of production. In this period, fish do not receive feed and are relatively active; hence, their growth and condition parameters undergo gradual deterioration [7,8]. However, with the existing farming model, carp production has continued its declining trend in the EU (by 10% between 2009 and 2018) due to numerous circumstances, such as predation by protected wildlife (cormorants, great egrets and otters), increasing cost, lower subsidies, and consumer preference for carnivorous finfish such as trout or salmon [6,9]. Carp farmers in the EU are seeking opportunities that would increase the consumption of carp throughout the year, not only in the Christmas period, in which sales reach up to 90% of their annual values [10]. Moreover, consumer awareness of animal welfare causes decreased interest in purchasing live fish and, consequently, a gradual increase in carp processing into a more convenient form, i.e., carcasses, slices, sheets and fillets. These circumstances, but also global indicators, such as climate change (e.g., water shortages, eutrophication), force carp farmers to quickly update their business models accordingly. One way in which the economic profitability and sustainability of carp production can be improved is its eco-intensification, as assessed in the GAIN (Green Aquaculture Intensification) project, which includes shortening the conventional production time (from 33 to 19 months) by running part of the rearing process (first wintering) in a closed recirculating aquaculture system (RAS) decoupled with aquaponics (Figure 1).

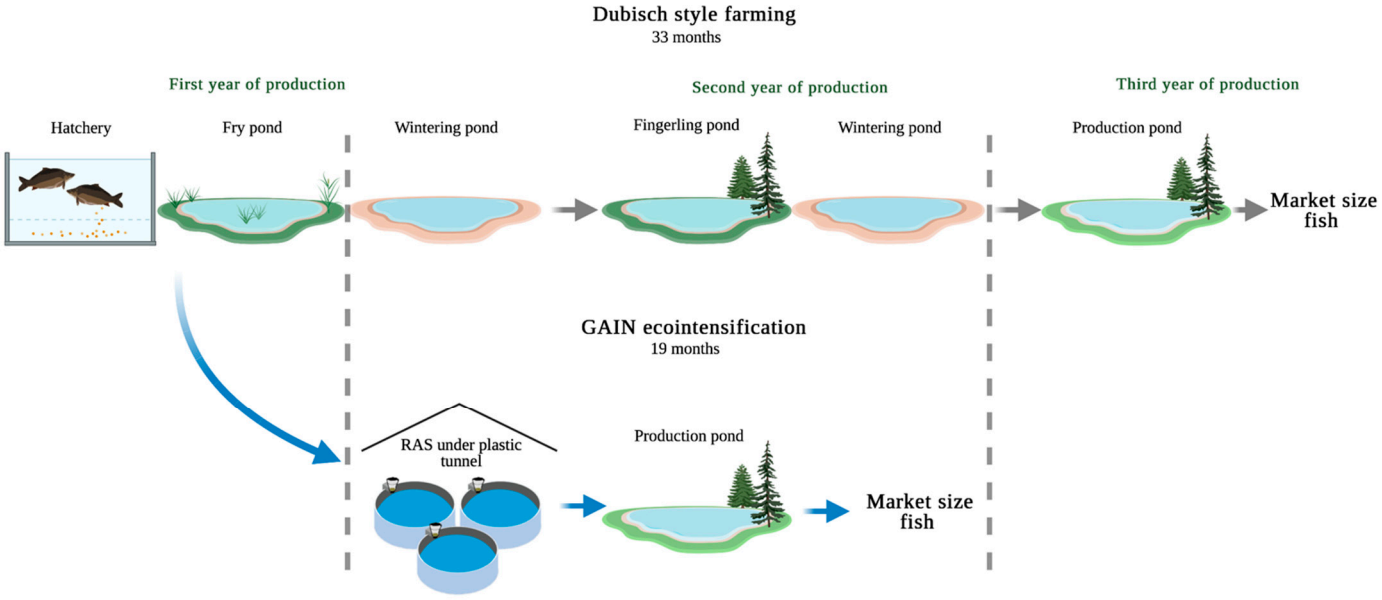

Figure 1. Overview of the traditional (Dubisch style) and eco-intensive (GAIN) culture of common carp.

Eco-intensification, however, requires verification by assessing a set of key performance indicators (KPI), i.e., growth efficiency (weight gain, feed intake), resource utilisation (food conversion ratio), health and welfare issues (survival rate, enteritis), and in market-size fish, quality (fillet yield, taste). For *C. carpio*, various bioindicators in muscle [11], liver and intestine [12], and skin [13] have been identified. Moreover, several performance indicators such as total growth rate, survival rate, protein efficiency ratio and feed conversion ratio were commonly used for common carp farmed in cages [14] and ponds [12] during

production season. However, to the best of our knowledge, the KPIs in common carp overwintering have never been assessed and are currently not available for this species. Therefore, the aims of the study were to compare the histological and molecular KPI of *C. carpio* wintering in a pond with no supplementary feeding (MCF), in a RAS fed typical (30% of protein and 8% of fat) carp diet (AFC), and in a RAS fed high protein (42%) and fat (12%) diet (ABF), and to assess whether wintering common carp in RAS can be a viable option to shorten culture time and contribute to the eco-intensification of common carp production in Europe.

2. Materials and Methods

2.1. The Experimental Trial and Fish

The ethical committee of the Faculty of Food Sciences and Fisheries, West Pomeranian University of Technology in Szczecin approved the fish trial. "Guidelines for the treatment of animals in behavioural research and teaching," published in *Animal Behaviour*, were adhered to during the trial [15].

The experimental setup was designed to tighten the production cycle from the traditional 33 months to 19 months with subsequent welfare improvement. The fish trial was performed at the Fisheries Research Station (FRS), Nowe Czarnowo, Poland (53°120′36″ N 14°270′48″ E) in a RAS system under a plastic tunnel in ambient, i.e., weather-dependent conditions. One week prior to the trial start, 1620 fish (47.18 ± 1.82 g) were obtained from the carp farm in Maliniec and distributed for acclimatisation (n = 270 per tank) in two sets of three tanks (n = 6), with a tank capacity of 2.7 m^3 each (Figure 2).

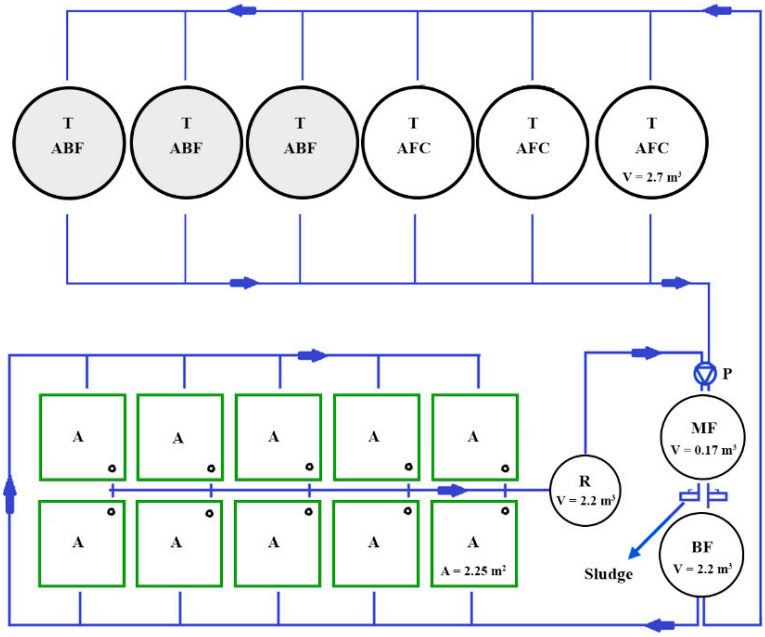

Figure 2. Schematic representation of the recirculating aquaculture system (RAS) used in this study. Abbreviations: T, fish tank; ABF, fish fed ABF diet; AFC, fish fed AFC diet; A, aquaponic tray; R, reservoir; P, pump; MF, mechanical filter; BF, biological filter. Blue arrows indicate water flow direction.

The 203-day trial (October–May 2019/2020) was performed in triplicate (n = 3 $diet^{-1}$). Fish were automatically fed with two commercial feed blends, with different levels of protein and fat, i.e., typical Agro-Fish Carp (AFC, 30% and 8%, respectively) and high energetic Aller Bona Float (ABF, 42% and 12%, respectively). Fish were fed diets at a rate of 1.5% body weight using automatic feeders. The rate of each diet was calculated based on information from biweekly bulk weighing of fish. During the trial, the temperature of the water in the RAS tanks was between 6.0 (January) and 26.2 °C (May), oxygen saturation between 40% and 82%, and pH value between 5.75 and 6.75. Traditional wintering was

performed in the Maliniec carp farm (MCF), (53°42′5″ N 15°21′22″ E) in an approx. 2.5 m deep wintering pond with a total surface area of 3 ha. During that period, the temperature in the wintering pond was between 4 and 8 °C until May, and feeding was not applied.

2.2. Sample Collection

At the end of the wintering trial, fish (n = 6) from each dietary treatment in RAS (n = 2 per tank) were sacrificed using a lethal dose of 2-phenoxyethanol (2 mL L^{-1}), (Sigma-Aldrich, Saint Louis, MO, USA). Simultaneously, fish from the wintering pond (n = 6) were sacrificed with the same procedure. Briefly, fish gills (2nd and 3rd arches), proximal intestine and head kidney samples were collected immediately and secured in DNA/RNA Shield™ (Zymo Research, Irvine, CA, USA) and, until RNA extraction, were stored at −80 °C. Additionally, a piece of proximal intestine bulb (approx. 5 mm), skin (1 × 1 × 0.5 cm) from the mid-dorsal epaxial body and 2nd and 3rd gill arch samples were collected and washed with deionized water and covered with 10% buffered formalin solution in 50 mL glass jars at room temperature for 5 h [16]. To assess the wintering and nutritional effects on carp in RAS, the following zootechnical parameters were calculated: final body weight (FBW) as an average weight of the carp at the end of the wintering period, and feed conversion ratio (FCR), Fulton's condition factor (K) and survival rate (SR) using the Equations (1)–(3):

$$FCR = FC \times WG^{-1} \quad (1)$$

$$SR = FN \times IN^{-1} \times 100 \quad (2)$$

$$K = (W \times 100) \times L^{-3} \quad (3)$$

where:

FC—feed consumed (g);
WG—weight gain (g);
FN—final number of individuals;
IN—initial number of individuals;
W—fish weight (g);
L—fish length (cm).

The data for MCF on Fulton's condition factor (n = 20), final body weight (bulk weighting of 100 fish) and survival rate (based on total biomass) were collected as part of the standard Maliniec carp farm screening after wintering, and none of the fish were harmed for the purpose of this study.

2.3. Total RNA Extraction and Synthesis of the cDNA

All preserved samples were homogenised in 750 µL Tri Reagent® (Zymo Research, Irvine, CA, USA) for 60 s using Minilys® homogenizer (Bertin Corp., Rockville, MD, USA). Total RNA was extracted using Direct-zol™ RNA MiniPrep kit (Zymo Research, Irvine, CA, USA), with DNase I treatment, to avoid contamination with genomic DNA. The extracted DNA was quantified and quality checked using NanoDrop 2000 (ThermoFisher Scientific, Waltham, MA, USA) and electrophoresis on 2% agarose gel. The 260/280 ratio of all RNA extracts was 1.8–2.1, and signs of RNA degradation were not observed. Transcriptor First Strand cDNA Synthesis Kit (Roche, Basel, Switzerland) and 1 µg of RNA were used for reverse transcription, following the manufacturer's instructions, using anchored oligo(dT)18 primers.

2.4. Assessment of Gene Expression in Gills, Intestine and Kidney

Real-time PCR was conducted on LightCycler® 480 II (Roche, Switzerland). LightCycler® 480 SYBR Green I Master (Roche, Basel, Switzerland), 0.1 µM of each primer and 5 µL of 10× diluted cDNA templates were used for the reaction in the final volume of 20 µL. All reactions were performed under the following thermal profile: initial activation at 95 °C for 5 min, 45 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 10 s and

extending at 72 °C for 15 s. At the end of each qPCR melting curve analysis (65–95 °C) was conducted to ensure the specificity of amplification. To ensure the absence of genomic DNA contamination, a random RNA sample was tested accordingly. Relative expression of the common carp genes in the 2nd and 3rd gill arches [*immunoglobulin M* (*IgM*), *interleukin 1 beta* (*il-1β*), *interleukin 6* (*il-6*), *interleukin 8* (*il-8*), *tumour necrosis factor alpha* (*tnf-α*), *mucin 5b* (*muc5b*), *lysozyme C* (*lysC*), *superoxide dismutase 1* (*sod1*), *catalase* (*cat*), *glutathione peroxidase* (*gpx*) and *glutathione S-transferase* (*gst*)], in the proximal intestine [*IgM*, *il-1β*, *il-6*, *il-8*, *tnf-α*, *muc5b*, *lysC*, *heat shock protein 70* (*hsp70*) and *heat shock protein 90* (*hsp90*)] and in the head kidney [*il-6*, *il-8*, *il-1β*, *tnf-α*, *IgM*, *muc5b*, *lysC*, *inducible nitric oxide synthase* (*inos*), *nuclear factor-erythroid 2-related factor 2* (*nrf2*)] were measured as well as two reference genes: *60S ribosomal protein L8* (*rpl8*) and *40S ribosomal protein S11* (*40sRNA*) (Table 1).

Decimal dilutions (ranging from 0.92 to 1.10) were performed to evaluate and correct the efficiency of qPCR reactions. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative gene expression using the GeneEx (MultiD Analyzes, Göteborg, Sweden) software [17].

2.5. Histomorphology of Gills, Skin and Intestine

Fixed in 10% buffered formalin samples of 2nd and 3rd gill arches, skin from the mid-dorsal epaxial body and proximal intestine fragments were dehydrated using alcohol and were saturated in intermediate solutions (benzene, benzene: paraffin) [16]. Next, the samples were embedded in paraffin blocks and then trimmed and serial sectioned (6 ± 1 µm, Rotary Microtome MPS-2, Opta-Tech, Warsaw, Poland). Gill and skin samples were stained with alcian blue and periodic acid–Schiff (AB/PAS, pH 2.5) [29]. Intestine samples were stained with haematoxylin and eosin (H&E) and alcian blue (AB) [30]. All samples were mounted with DPX mounting medium and covered by coverslips. Twelve glass slides (3 fish × 4 slides) for both RAS diets and traditional wintering and all tissue types were randomly selected and examined using an Eclipse E600 microscope (Nikon, Tokyo, Japan) with 100× objective and the NIS-Elements Basic Research software (Nikon Instruments Europe B.V, Amsterdam, The Netherlands). Gill samples were examined to assess the thickness of the epithelial tissue, and the number and area of the mucous cells (MC). In the skin samples, the thickness of the epidermis was assessed, and the ratio between the thickness of the epidermis (E) and the outer part (*stratum spongiosum*, SS) and the deeper part (*stratum compactum*, SC) of the dermis was calculated to compare the share of each layer between ABF, AFC and MCF fish. Moreover, the area of MC and the number of MC per 100 µm of epithelium were counted manually. The total number of acidic, neutral and mixed (acidic and neutral) mucin-containing cells per 100 µm of epidermis was counted. Intestine samples were examined to assess the size of supranuclear vacuoles (SNV), width of lamina propria (LP), number and area of goblet cells (GC) per villus, and thickness of sub-epithelial mucosa (SM). Gill, skin, and intestine samples were also checked against the presence of pathological changes. Measurements of histological section characteristics were made according to the methodology described earlier for different fish species [31–33].

2.6. Statistical Analysis and Data Visualisation

Throughout this paper, data are shown as mean ± standard deviation unless otherwise specified. The Shapiro–Wilk test (significance level $p < 0.05$) was used to assess the normal distribution of data. Depending on the normality of distribution, the ANOVA or the Kruskal–Wallis test and subsequently the Dunnett's, Tukey HSD and Dunn's post hoc test were used to assess significance of differences, using Statistica 13.3 software (TIBCO Software Inc., Palo Alto, CA, USA). Visualisation of data was performed using R-package ggplot2 [34] and bio-render.com (access on 6 March 2022).

Table 1. Sequences of *C. carpio* primers used for qPCR analysis.

Gene		Primer Sequence 5′ -> 3′	Tm (°C)	Function	Reference
IgM [1]	F	TCGTATTAGCACCCCCAGAG	53.8	First line of host defence against infections	[18]
	R	TCATCAGCAAGCCAAGACACA	52.4		
il-1β [2]	F	CCTGAAGAAGAGGAGGCTGTCA	56.7	Mediator of inflammatory response	[19]
	R	AAGGAGGCCAGTGGCTCTGT	55.9		
il-6 [3]	F	CCGCACATGAAGACAGTGAT	51.8	Stimulating acute phase protein synthesis	[20]
	R	GGGTATATTTGGCTGCAGGA	51.8		
il-8 [4]	F	TGGAGCTCTTCCCTCCAAG	53.2	Attracting and activating neutrophils	[20]
	R	AGGGTGCAGTAGGGTCCAG	55.4		
tnf-α [5]	F	CCTTGGAAGTGACATTTGCTTTT	51.7	Signalling events within cells	[19]
	R	GCTGTCTGCTTCACGCTCAA	53.8		
muc5b [6]	F	CAGCCCTCTTCCTCTTTCATC	54.4	Ensure normal mucus clearance	[20]
	R	CCACTCATCTTTCCTTTCTCTTC	53.5		
hsp70 [7]	F	TGAGAACATCAACGAGCCCA	51.8	Protein maturation, re-folding and degradation	[21]
	R	TTGTCAAAGTCCTCCCCACC	53.8		
hsp90 [8]	F	AAAGACCAGGTCGCCCACTC	55.9	Protein maturation, re-folding and degradation	[22]
	R	AGTACTCGTCGATGGGCTCG	55.9		
sod1 [9]	F	TGGTCCACCGTGAGCTTTATT	52.4	Antioxidant enzyme	[23]
	R	GACAACACAAACGGCGGCAT	53.8		
gpx [10]	F	TGCAACCAGTTCGGACATCA	51.8	Catalyses the reduction of hydrogen peroxide	[24]
	R	GAAGCCATTTCCAGGACGGA	53.8		
cat [11]	F	CTGGAAGTGGAATCCGTTTG	51.8	Maintaining the cellular redox homeostasis	[25]
	R	CGACCTCAGCGAAATAGTTG	51.8		
gst [12]	F	TACAATACTTTCACGCTTTCCC	51.1	Protect cellular macromolecules	[26]
	R	GGCTCAACACCTCCTTCAC	53.2		
rpl8 [13]	F	CTCCGTCTTCAAAGCCCATGT	54.4	Ribosomal protein coding	[27]
	R	TCCTTCACGATCCCCTTGATG	54.4		
40sRNA [14]	F	CCGTGGGTGACATCGTTACA	53.8	Ribosomal RNA gene	[28]
	R	TCAGGACATTGAACCTCACTGTCT	55.7		

[1] *Immunoglobulin M*; [2] *interleukin 1 beta*; [3] *interleukin 6*; [4] *interleukin 8*; [5] *tumour necrosis factor alpha*; [6] *mucin 5b*; [7] *heat shock protein 70*; [8] *heat shock protein 90*; [9] *superoxide dismutase 1*; [10] *catalase*; [11] *glutathione peroxidase*; [12] *glutathione S-transferase*; [13] *60S ribosomal protein L8*; [14] *40S ribosomal protein S11*.

3. Results

3.1. Basic Performance and Welfare Indices of Carp Wintering in Pond and RAS

Final body weight of MCF fish (56.44 ± 9.80 g) did not differ significantly compared with the initial weight of the fish (47.18 ± 1.82 g). The final body weight of fish from RAS differed significantly ($p = 0.01$) between AFC (91.01 ± 3.05 g) and ABF (113.30 ± 4.71 g). Fulton's condition factor *K* was significantly ($p = 0.03$) lower for MCF fish (1.81 ± 0.20) compared with fish before overwintering (1.92 ± 0.12). Additionally, *K* was significantly higher ($p = 0.003$) for AFC (2.11 ± 0.17) and ABF (2.20 ± 0.19) fish compared with MCF fish and those before experimental RAS. The survival rate did not differ significantly between AFC (99.91 ± 0.19%) and ABF (99.54 ± 0.47%) fish but was higher than in traditional pond wintering (survival rate 70%, pers. comm. M. Gzyl). The feed intake was significantly

higher (p = 0.05) for ABF (129.91 ± 3.04 g) than for AFC (120.54 ± 4.61 g), and consequently FCR was significantly lower (p = 0.01) for ABF (1.98 ± 0.09) than for AFC (2.72 ± 0.07).

3.2. Histomorphology of the Gills, Skin and Intestine of Carp Wintering in Pond and RAS

Gill histology of carp demonstrated that the thickness of the epithelial tissue between the secondary lamellae in MCF was lower than in AFC and ABF (p = 0.01), as the ratio between the length of the secondary lamellae and the interlamellar cell thickness was 1.43, 3.96 and 2.92, respectively (Figure 3A–C).

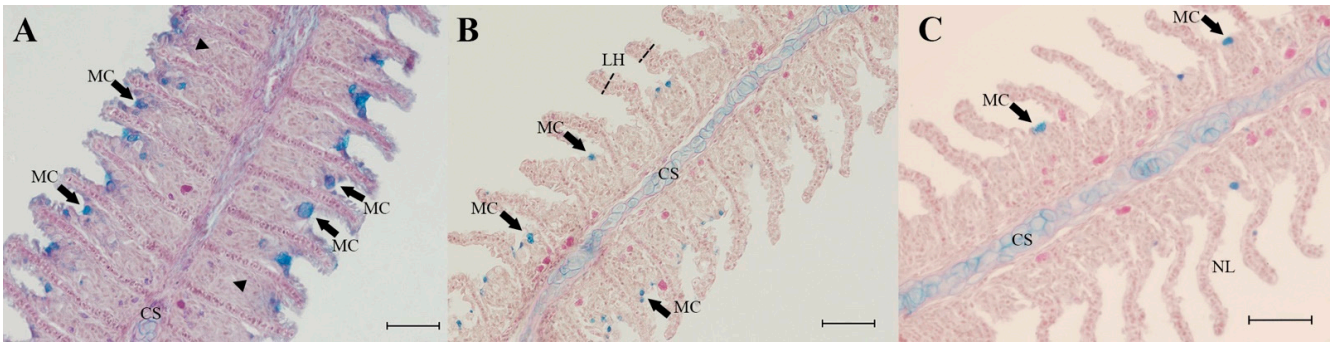

Figure 3. Histological sections of gills from *C. carpio* wintering in different conditions. (**A**) Fish from ponds (MCF); note the epithelial cell hyperplasia and numerous mucous cells (MC) seen in the interlamellar space. (**B**) Fish from RAS fed with typical carp diet (AFC); note less numerous MC and thickened lamellae caused by epithelial cell hyperplasia (LH). (**C**) Fish from RAS fed with high fat and protein diet (ABF); note low number of MC and normal appearance of lamellae (NL). CS, cartilaginous structure. AB-PAS (pH 2.5) reaction. Bar = 50 µm.

In some cases, in the gills of carp wintering in ponds, extensive epithelial cell hyperplasia led to complete lamellar fusions (Figure 4A). In carp wintering in the RAS, the gills of AFC carp had thicker secondary lamellae than in ABF carp due to epithelial cell hyperplasia (Figure 4B,C) and an increased number of mucous cells (Figure 4D). The number of mucous cells (MC) was significantly higher (p = 0.01) in the MCF group (n = 54 ± 3) than in the AFC (n = 17 ± 3) and ABF groups (n = 10 ± 2). Moreover, the size of MC was the largest (p = 0.01) in MCF (112.6 ± 26.96 µm^2) compared with AFC (44.8 ± 9.37 µm^2) and ABF (59.1 ± 28.01 µm^2) fish. Sections of skin were compared between fish wintering in RAS (ABF, AFC) and ponds (MCF) (Figure 5). The epidermis was thicker in AFC fish (64.71 ± 3.89 µm) than the other two groups, which were also different from each other (ABF 53.39 ± 3.32 µm, MCF 48.47 ± 3.05 µm). The measurements of the epidermis, *stratum spongiosum* and *stratum compactum*, and subsequent calculation of the ratio between the thickness of each layer (ABF 1:3.4:4.6, AFC 1:1.2:2.9, MCF 2.3:1:4) showed that, in all fish, SC was the thickest part in the ABF, AFC and MCF fish. The SS was the thickest layer both in ABF and AFC, except the MCF fish, in whom thickness of SS was the least according to the calculated ratio (Figure 5A–C). The overall skin morphology showed higher amounts of subcutaneous fat (adipose tissue) deposits in ABF fish (Figure 5B) than in AFC (Figure 5C). Lack of subcutaneous adipose tissue was observed between SS and muscle tissue in the skin of MCF fish (Figure 5A). The area of MC was the largest in ABF fish (153.5 ± 25.01), (Figure 5D) than in AFC (84.96 ± 19.43) and MCF (46.18 ± 21.43) carp (p < 0.001). The total number of acidic, neutral and mixed (acidic and neutral) mucin-containing cells per 100 µm of epidermis was the lowest in MCF fish (2.8 ± 1.1), (Figure 5E) and between fish from RAS fed typical carp (AFC 4 ± 0.7) and high (ABF 7.2 ± 0.8) fat and protein diets (p < 0.01), (Figure 5F).

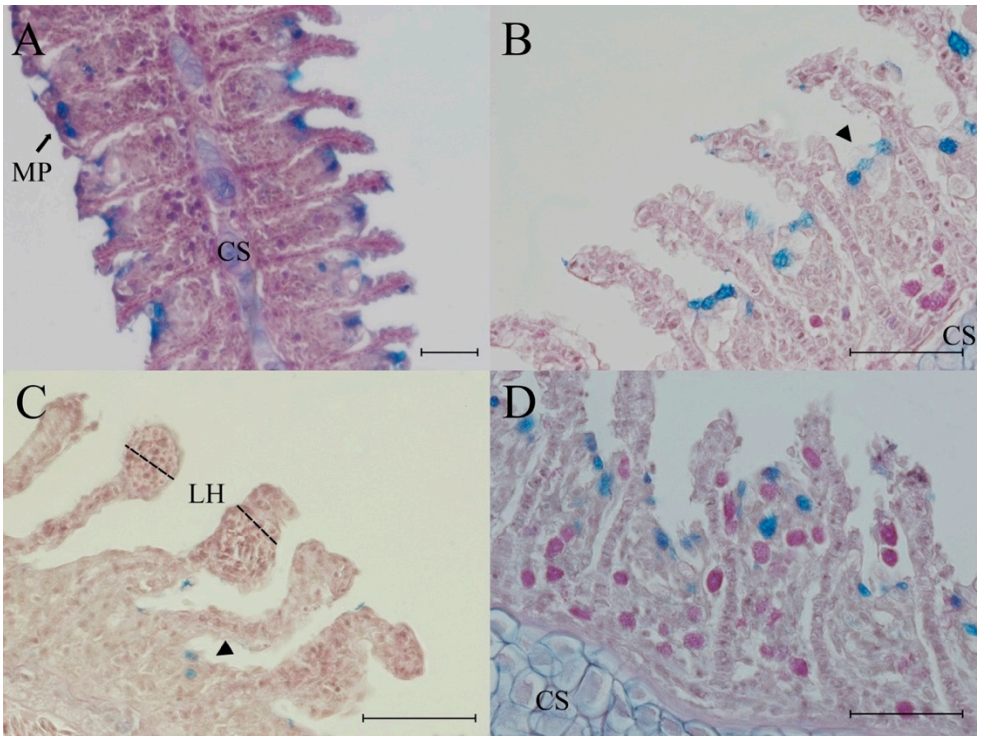

Figure 4. Histological changes in gills from *C. carpio* wintering in different conditions. (**A**) Extensive epithelial cell hyperplasia and metaplasia (MP) in the gills of fish from ponds (MCF). (**B**,**C**) Thicker secondary lamellae due to epithelial cell hyperplasia (arrowheads) and (**D**) numerous MC in the gills of fish from RAS fed with typical carp diet (AFC). CS, cartilaginous structure. AB-PAS (pH 2.5) reaction. Bar = 50 μm.

Results from the histological evaluation of the intestine samples showed that AFC fish had a significantly (p = 0.01) larger size of supranuclear vacuoles, smaller size of lamina propria, an intermediate number of goblet cells and lower thickness of submucosa compared with ABF and MCF. The GC area was significantly (p = 0.01) larger among AFC and MCF fish compared with ABF fish (Figure 6, Table 2).

3.3. Gene Expression in the Gills and Intestine of Carp Wintering in Pond and RAS

Gene expression in the gills showed that fish from traditional wintering (MCF) in the pond had a significantly higher relative mRNA expression of *il-6* and a high level (over 13-fold change) of *il-1ß* compared with fish from RAS (Figure 7). In contrast, the *IgM* and *gpx* activities were significantly lower in MCF compared with AFC and ABF. The expression of *sod1* was similar between both RAS groups and significantly higher in ABF compared with MCF. A difference between AFC and ABF was found in the expression of other oxidative stress-related genes (*cat* and *gst*). Expression in MCF was similar to that in AFC and ABF for cat and similar to that in AFC for *gst*.

The effects of wintering in the pond and RAS on the expression of genes in the intestine of carp are shown in Figure 8. Gene expression analysis in the intestine showed that fish overwintering traditionally in the pond had a significantly lower relative mRNA expression of *IgM*, *il-6*, *il-8* and *hsp90* than fish wintering in the experimental RAS (AFC, ABF). Additionally, the level of *il-6* transcripts in the intestine of fish fed AFC diet was significantly lower than in ABF carp. The transcript level of the mucin gene *muc5b* was the highest in the intestine of ABF fish compared with MCF and AFC carp. Gene expression of the third interleukin *il-1ß* and the chaperon *hsp70* were similar in the intestine of ABF and MCF fish, and significantly higher compared with AFC fish. No difference in the expression of *tnf-α* between fish wintering in the pond and RAS and between fish from RAS fed AFC and ABF diets were observed.

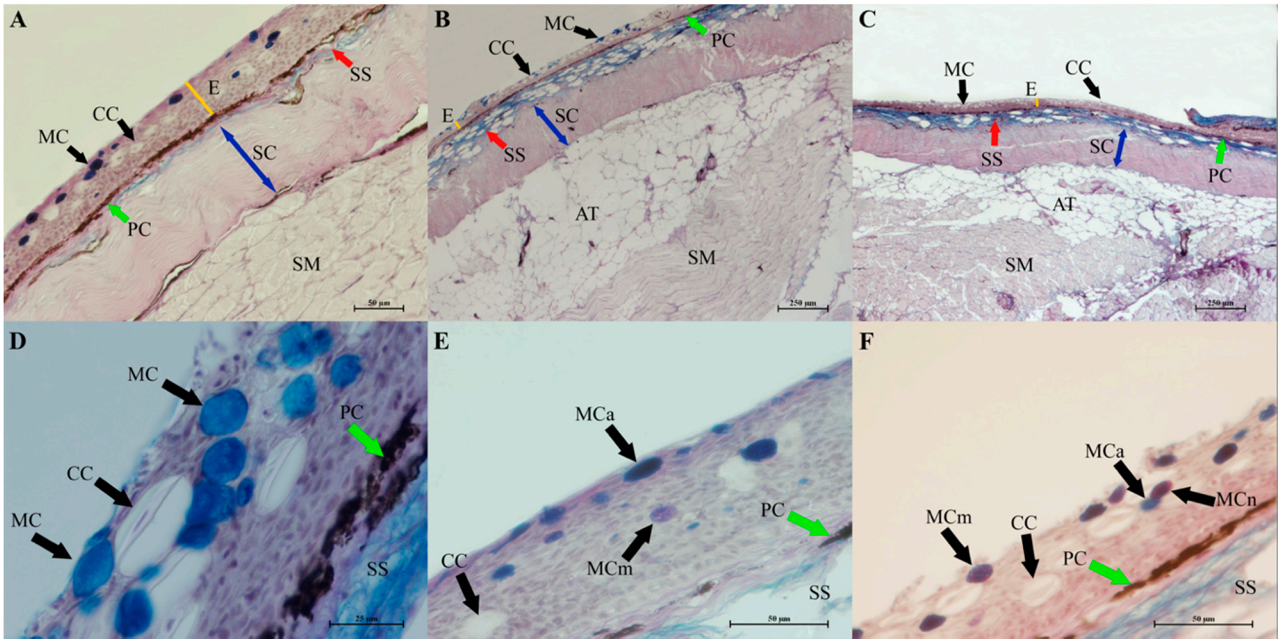

Figure 5. Histological changes in skin from *C. carpio* wintering in different conditions. Differences in skin layer structure of fish from ponds (MCF) (**A**), fish from RAS fed with high fat and protein diet (ABF), (**B**) and fish from RAS fed with typical carp diet (AFC). (**C**) Common carp and changes in abundance and morphology of different mucin containing cells in ABF (**D**), MCF (**E**) and AFC (**F**) fish. Abbreviations: E, epidermis; SS, outer part (stratum spongiosum) of dermis; SC, deeper part (stratum compactum) of dermis; PC, pigment cells; AT, adipose tissue; SM, smooth muscle; MC, mucous cells; MCa, acidic mucous cells; MCn, neutral mucous cells; MCm, mixed mucous cells; CC, club cells. AB-PAS (pH 2.5).

Analysis of relative mRNA gene expression in the kidney samples showed higher expression of *IgM* in the ABF than AFC and MCF carp. The transcript level of *il-1β* was the highest in the MCF fish to the other two groups, which were not different from each other. The expression of the *tnf-α* and *nrf2* genes was the lowest in AFC carp compared to ABF and MCF, which had similar level of transcripts in the kidney samples (Figure 9).

The study also compared relative mRNA expression of *lysC*, *IgM*, *il-1β*, *il-6*, *il-8*, *tnf-α* and *muc5b* between the samples of gills, intestine and kidney individually for the ABF, AFC and MCF carps. In most of the instances, analysed genes in MCF fish had the lowest expression in the intestine (*il-6*, *il-8*, *tnf-α*) or was similar either to the expression level in gills (*IgM*) or kidney (*lysC*). Only in the case of the *il-1β* there was an intermediate level of expression in the intestine found compared to the two other samples (Figure 10A). In the AFC, all the genes had the lowest expression in the intestine samples for the gills and kidney, which were not different from each other (Figure 10B). Fish fed a high fat and protein diet (ABF) had the lowest transcript levels of *lysC*, *IgM*, *il-8* and *tnf-α* in intestine samples. The relative mRNA expression of *il-1β* and *il-6* was higher in gills and intestine than kidney, while the expression of *muc5b* was significantly lower in kidney than gills (Figure 10C).

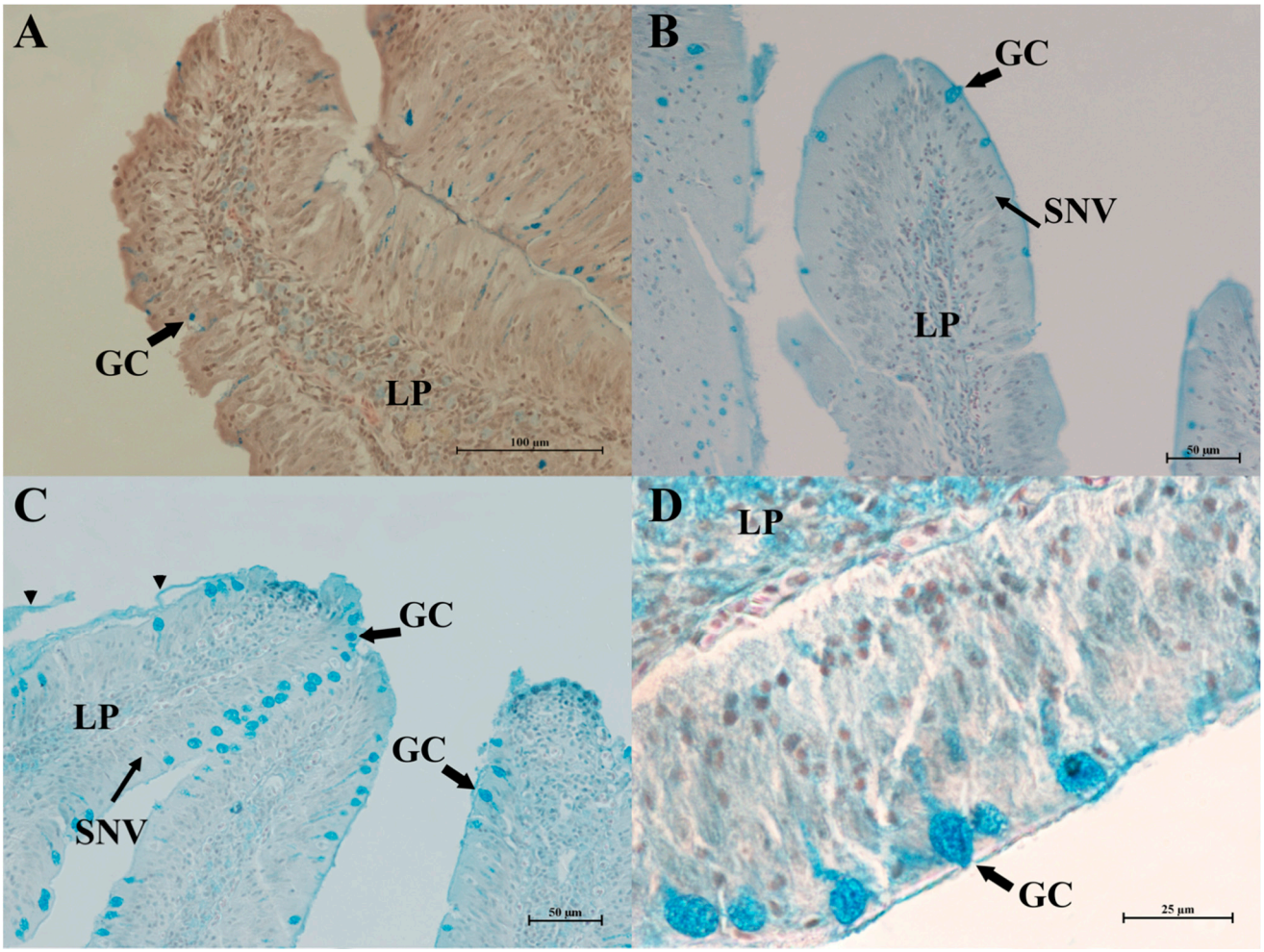

Figure 6. Histological sections of intestinal fold from *C. carpio* wintering in different conditions. (**A**) Fish from ponds (MCF); note low number of goblet cells (GC), lack of supranuclear vacuoles (SNV) and widened lamina propria (LP). (**B**) Fish from RAS fed with typical carp diet (AFC); note increased number of GC and presence of SNV. (**C**) Fish from RAS fed with high fat and protein diet (ABF); note numerous GC, copious amounts of mucin (arrowheads), and presence of SNV. (**D**) ABF fish; note numerous GC.

Table 2. Intestine histological parameters of common carp wintering in ponds (MCF) and in RAS fed with typical carp (AFC) and high (ABF) fat and protein diets.

Parameter/Fish Group	MCF	AFC	ABF
SNV (µm)	ND	23.2 ± 3.13 [a]	18.8 ± 1.67 [b]
LP (µm)	42.4 ± 11.69 [a]	19.3 ± 3.93 [b]	24.5 ± 5.16 [c]
GC (n)	28.0 ± 2.45 [a]	57.6 ± 3.44 [b]	73.5 ± 7.77 [c]
SM (µm)	72.9 ± 9.68 [a]	45.1 ± 8.31 [b]	58.9 ± 4.56 [c]
GC (μm^2)	50.7 ± 13.54 [a]	50.7 ± 9.95 [a]	41.7 ± 10.63 [b]

Abbreviations: SNV, supranuclear vacuoles; LP, lamina propria; GC, number of goblet cell per villus; SM, submucosa; ND, not detected. Different lowercase letters in rows indicate significant differences between values ($p \leq 0.01$). Values were compared with parametric tests.

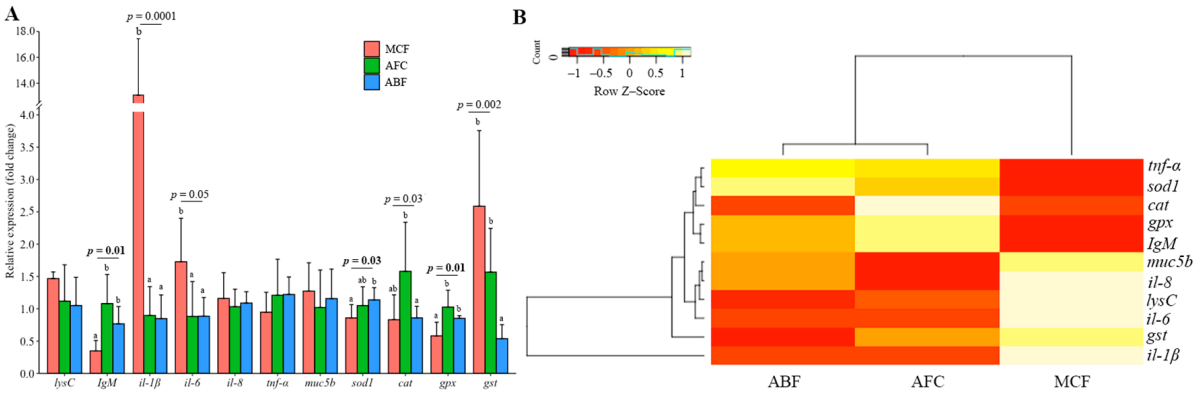

Figure 7. Gene expression profiles (**A**) and heatmap analysis (**B**) for gills of common carp wintering in ponds (MCF) and in RAS fed with typical carp (AFC) and high (ABF) fat and protein diets. Fold changes presented as mean ± SD. Lowercase letters indicate significant differences. *p* values in bold were calculated with parametric tests.

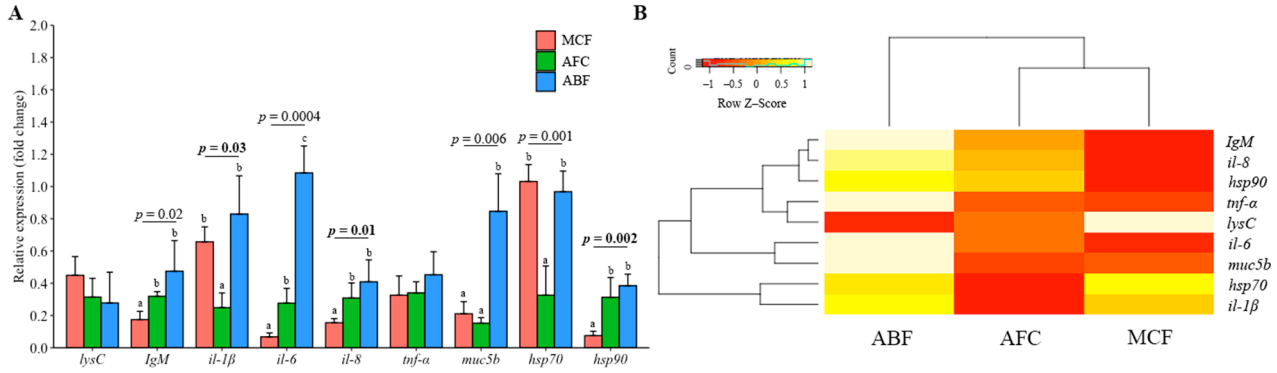

Figure 8. Gene expression profiles (**A**) and heatmap analysis (**B**) for intestine of common carp wintering in ponds (MCF) and in RAS fed with typical carp (AFC) and high (ABF) fat and protein diets. Fold changes presented as mean ± SD. Lowercase letters indicate significant differences. *p* values in bold were calculated with parametric tests.

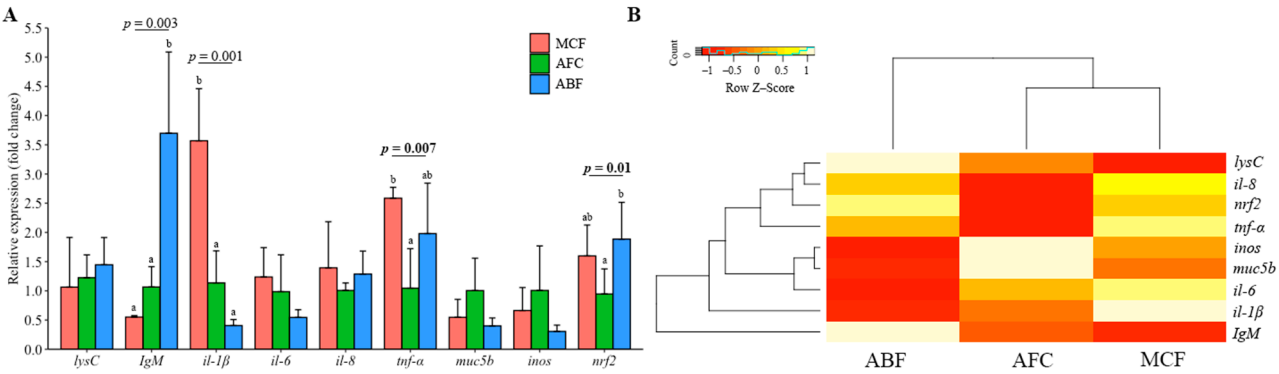

Figure 9. Gene expression profiles (**A**) and heatmap analysis (**B**) for kidney of common carp wintering in ponds (MCF) and in RAS fed with typical carp (AFC) and high (ABF) fat and protein diets. Fold changes presented as mean ± SD. Lowercase letters indicate significant differences. *p* values in bold were calculated with parametric tests.

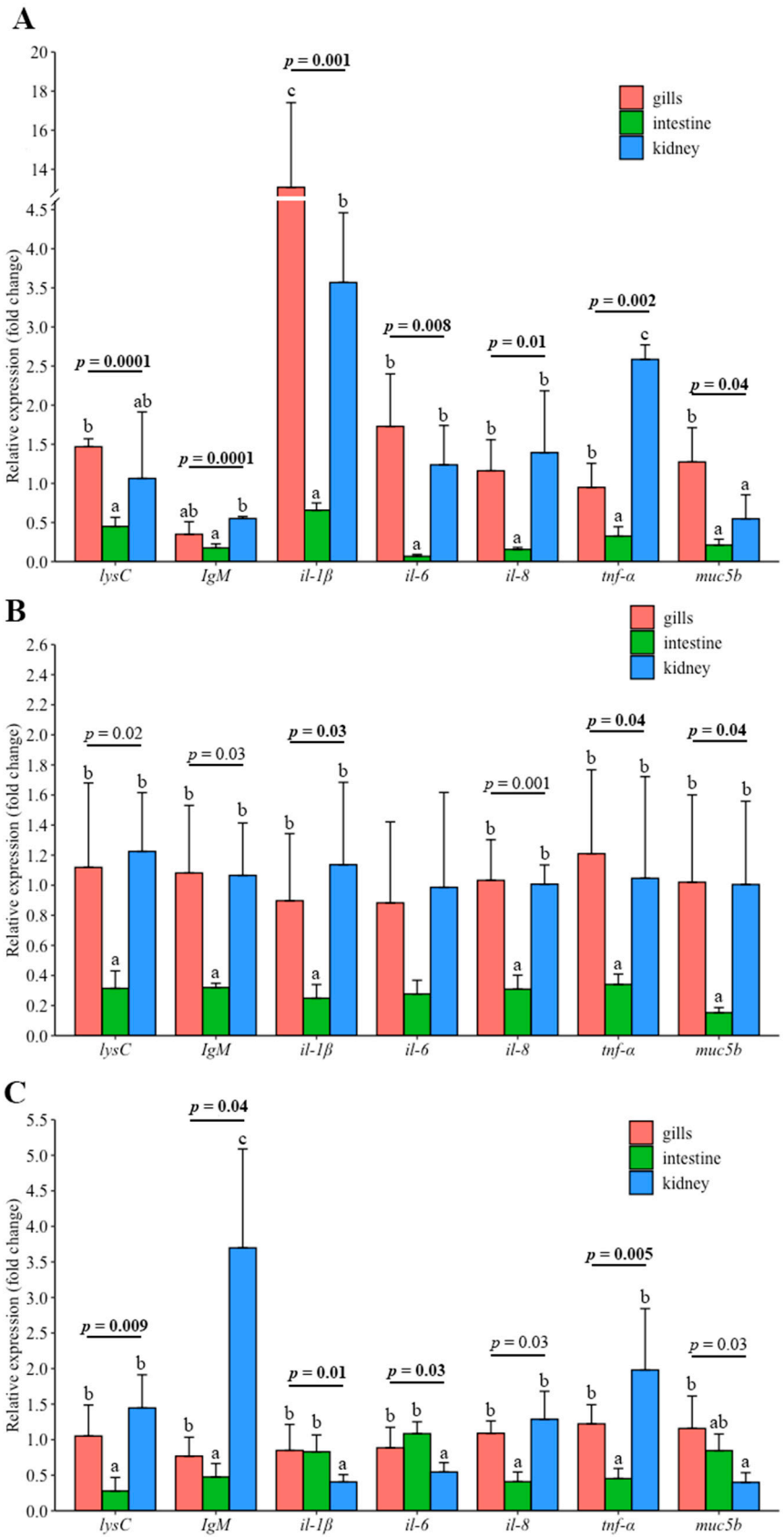

Figure 10. Tissue-specific (gills, intestine, kidney) gene expression profiles of common carp wintering in ponds (**A**, MCF); RAS fed with typical carp diet (**B**, AFC); RAS fed a high fat and protein (**C**, ABF). Fold changes presented as mean ± SD. Lowercase letters indicate significant differences. *p* values in bold were calculated with parametric tests.

4. Discussion

4.1. Differences in Weight Gain, Condition Factor and Mortality of Carp Overwintering in Earthen Ponds and RAS

Multi-stage farming of common carp in Europe, here referred to as the Dubisch style, since the early beginning, has included two wintering periods in >2 m deep earthen ponds until spring when the temperature profile and primary production in ongrowing ponds are favourable to continue production. During wintering that lasts usually from late October to May fish were restricted from feeding and their activity, including metabolic, depending on water temperature [7]. Our study showed minor weight loss and lowered (5.7%) condition in the MCF fish after wintering. According to Lukowicz and Gerstner [35], overwintering of common carp is considered successful if condition factor *K* does not decrease more than 15–20%. Additionally, Geldhauser and Gerstner [36] showed that weight loss of 5–10% is a typical observation for conditions of Central European aquaculture in winter. The fish from RAS (AFC, ABF) had significantly higher weight and better conditions than those wintering in the pond (MCF), which may be due to more favourable thermal conditions and adapted feeding programme. The differences in the final weight between fish wintering in RAS could be attributed to the composition of the feeds, mainly the content of the lipids. In ectothermic common carp the energy is primarily stored in the form of lipids that must be assimilated before the winter period when animals are decoupled from the resource base [37]. In RAS, the ABF carp were fed with the diet containing a higher content of lipids (12%) than AFC (8%), therefore efficiently utilising this compound to meet metabolic demands and continuing to build body mass.

Fish overwintering in earthen ponds may face stress from starvation, cold, and predators which together or individually may disturb physiology and lead to huge economic losses in aquaculture due to elevated mortality rates [38]. Common carp farming includes two wintering stages (W1 and W2) in the colder months (October through April) in subsequent years. Survival rate of fish after W1 and W2, even concerning severity of winter conditions, is significantly lower in one-summer-old carp (50%) than two-summer-old carp (80–95%) [39]. In our study, carp after W1 reached a much higher survival rate of 70% that presumably stems from the efficient accumulation of lipid reserves during warmer, more productive months and low impact of common stressors such as too high stocking density, low water quality and injuries caused by handling, parasites, and diseases [37,40]. The higher survival rate of MCF fish, as well as insignificant weight loss and decreased condition factor, surprise even more, as small-bodied fish have a smaller capacity for energy storage and a higher mass-specific metabolic rate [41]. Thus, they are more susceptible to over-winter starvation and mortality than larger bodied individuals, i.e., two-summer-old carp which can accumulate larger absolute and relative lipid levels, and in the case of the Maliniec carp farm, experience 10% mortality during W2 ([42], pers. comm. M. Gzyl). Survival rate of AFC (99.91 $\pm$ 0.19%) and ABF (99.54 $\pm$ 0.47%) carp wintering in RAS was significantly higher than in the fish from earthen pond but was also better in relation to *C. carpio* cultured in experimental RAS (93.33 $\pm$ 5.70) and comparable to the two biofloc technologies (BFT1 97.57 $\pm$ 3.70, BTF2 96.67 $\pm$ 5.70) and the system with 50% water exchange method (98.67 $\pm$ 1.20) [43]. Such low mortality rate of AFC and ABF fish might be contributed to high quality of water, lack of pathogens and feeding that directly influenced the growth and health state of carp in RAS.

4.2. Influence of Wintering Practice on the Histological and Molecular Indices of Carp Gills

The relative mRNA expression of two pro-inflammatory cytokines, namely *il-1β* and *il-6*, was upregulated in MCF, while the relative mRNA expression of *tnf-α* remained comparable to that in AFC and ABF. A high number of *il-1β* transcripts and, consequently, *il-6* may suggest an ongoing inflammation in MCF fish [44]. Moreover, the epithelial cell hyperplasia visible in the histological picture of MCF gills has been previously linked with inflammation processes caused by different pathogens in Atlantic salmon, *Salmo salar* [45] and rainbow trout, *Oncorhynchus mykiss* [46]. However, no difference in the expression of

il-8 suggests the absence of acute inflammation in favour of chronic inflammation [47]. It is also plausible that changes in cytokine expression are a result of prolonged starvation [48], which in turn creates chronic stress that also influences the immune response of common carp [49]. Moreover, the increased number of MC, and their size, in the gills of MCF fish compared with AFC and ABF suggests more stressful conditions (e.g., water quality) in the wintering pond [50], despite the lack of differences in the transcript level of *muc5b*, encoding a major gel-forming mucin in mucus.

The upregulation of *IgM* in ABF and AFC compared with MCF found in our study may be a result of a higher bacteria load (especially of those pathogenic for carp) present in RAS [51,52]. Such conditions result from higher water temperature in RAS compared with the wintering pond, since both bacterial activity and fish productivity depend on water temperature [53]. In contrast, similar *muc5b* levels in MCF, AFC and ABF may indicate the absence of pathogenic bacteria in both wintering systems, since in channel catfish (*Ictalurus punctatus*), microbial infection has been seen to upregulate the activity of *muc5b* in both the gills and the intestine [54]. To comprehensively understand the influence of wintering on carp, high throughput methods (e.g., RNA-seq) combined with histological observations should be implemented.

We revealed differences in the expression levels of oxidative stress response-related genes (*sod1*, *cat*, *gpx* and *gst*). The higher expression of *gst* in MCF compared with that in ABF could be explained by prolonged starvation of fish in the wintering pond [55]. Moreover, the significant difference between AFC and ABF, and no difference between AFC and MCF, could be related to the lower energy content in the AFC feed, which presumably did not fully cover the needs of fish during the wintering period [56]. The lower expression of *sod1* and *gpx* in MCF compared with that in ABF suggests a more complex response to winter and different winter-related conditions such as starvation and multiple factors influencing the expression level. For instance, differences in the number of *sod1*, *gpx* and *cat* transcripts could be related to the level of minerals in formulated feeds [57,58]. Additionally, the presence of Zn can inhibit the GST activity in vitro [59], which we observed in vivo at a molecular level, since ABF feed contained the highest Zn level (50 mg kg^{-1}). Water flow could be another factor influencing the downregulation of *gpx* in MCF, since there is no water flow in the wintering pond, while in RAS, water flows constantly (1.7 m^3 h^{-1}), thus creating relatively stable conditions in terms of oxygen level [60]. The lack of water flow in the wintering pond may result in hypoxic and high ammonia conditions [61], which also alters oxidative stress response in fish [62]. Suboptimal conditions in the wintering pond could potentially decrease the expression of *sod1* and *gpx* and cause pathological changes in the gills (complete lamellar fusions), as reported for largemouth bass (*Micropterus salmoides*) exposed to hypoxia and high ammonia levels [63]. However, this does not explain the higher expression of cat in AFC compared with that in ABF, and the difference is most probably caused by dietary differences, i.e., lipid and protein levels [64,65].

4.3. Influence of Wintering Practice on the Histological and Molecular Indices of Carp Intestine

A lower number of *IgM* transcripts in the intestine of MCF fish than those from RAS might result from low water temperature (4–8 °C) and ceased feeding during the wintering period. The relatively stable conditions in deep earthen ponds and fish thriving in suboptimal conditions [42] shaped a relatively constant diversity and richness of the gut microbiota [66]. Some of the gut residents are commensal microbiota populations that have been described as recognizable by immunoglobulins [67], thus having a beneficial effect on the immune system of fish [68]. According to Bisht et al. [69], the intestinal bacterial count, including those that maintain gut health, in *C. carpio* was higher in the winter compared with the summer season. In contrast, the increased expression of *IgM* in the intestine of AFC and ABF fish wintering in the experimental RAS was enriched by more complex internal and environmental stimulants (microbiota, feeds, water temperature up to 20 °C). As evidenced by Eichmiller et al. [70], differences in the microbiota of common carp may be due to a combination of the effects of diet, habitat usage, temperature and physiology,

and the populations of gut residents may adapt via a specific and dynamic interplay with immunoglobulins [67].

Analysis of gene expression of pro-inflammatory cytokines to some extent showed a relatively consistent picture and explained observations identified in the MCF, AFC, and ABF intestine slides during histomorphological evaluation. The relative mRNA expression of *il-1β* in the intestine of fish from the wintering pond and from RAS fed the ABF diet was the highest and coincided with the highest severity of enteritis found in these fish compared with AFC carp. However, the source of these observations seems to be different, as MCF fish had enteritis due to prolonged starvation during overwintering in earthen ponds. ABF fish were fed diets with a high amount of energy coming from the increased portion of fat and protein in the administered diet. In the case of fish from the pond, seasonal wintering of fish is a typical procedure in carp farming in Central and Eastern Europe [8], and the species is habituated to tolerate winter temperatures close to 0 °C [71]. Liang et al. [72] reported that adaptation of *C. carpio* to low temperatures is the result of long-term evolution, but differences in the mechanisms of survival at cold temperature have been found among various populations or subspecies. In our study, the high transcript level of the *il-1β* gene in the intestine of MCF fish is directly related to the regulation of the inflammatory processes that developed due to specific conditions during overwintering. As the inflammatory response of the immune system progresses, *il-1β* induces expression of subsequent pro-inflammatory genes, such as *il-6*, *il-8* and *tnf-α* [73]. Apparently, in the case of MCF fish, the severity of physiological changes in the intestine remained at the "typical-for-wintering" level, and further exacerbation of the immune response was unjustified. In contrast, the limited stimulatory relative mRNA expression of the *il-1β* gene in MCF was not observed in RAS fish, as the expression of both *il-6* and *il-8* was ramped up in the latter, specifically in ABF carp fed a high fat and protein diet. The cause of the observed differences in the transcript levels of *il-1β*, *il-6* and *il-8* between AFC and ABF fish arose directly from the feed composition, as fish in the RAS were managed according to the same procedures. ABF fish fed a more energetic diet reached higher final body weight (113.30 ± 4.71 g) compared with AFC carp (91.01 ± 3.05 g). Furthermore, histological evaluation showed symptoms of diet-induced enteritis (thickened lamina propria and sub-epithelial mucosa, altered supranuclear vacuolization, increased number of goblet cells in the epithelium) in the intestine of ABF carp. Despite the presence of "non-infectious sub-acute enteritis", as named by Baeverfjord and Krogdahl [74], Fulton's body condition factor *K* as well as survival rate did not differ significantly between AFC and ABF fish. These results appear promising, as the RAS fish, heavier and in better condition compared with those wintering in the pond, will be transferred to ongrowing earthen ponds in the spring to reach market size (~1.2 kg) within the following 6–7 months (until November) to be harvested before Christmas. The lack of difference in the relative mRNA expression of the *tnf-α* gene between fish groups may suggest that in the intestine, neither acute infection nor ongoing pathogenesis of several chronic diseases were present. The plausible explanation for the comparable number of *tnf-α* transcripts among variants is that this cytokine plays a key role in regulating inflammation, but some of its functions may overlap with *il-1β* [73].

Significant differences, during both the molecular and histomorphological assessments, were noticed regarding mucin production in the intestine of carp. At the molecular level, the transcript level of the *muc5b* gene was the highest in ABF fish, and the histological assessment showed apparently large amounts of mucin found in the intestine of these fish. However, in addition to *muc5b*, the production of mucins is dependent on the expression level of *muc2b*, and according to Marel et al. [75], the latter gene is the main one expressed in the first and second intestinal segments of *C. carpio*. Therefore, the massive amounts of mucins in the intestine of ABF fish could also be due to the high activity of *muc2b*. Nevertheless, our findings show that *muc5b* transcripts were, in addition to the brain, liver, skin and gills [75], detected in the intestine of common carp. The expression of *muc5b* in the gills was significantly higher than in the intestine of AFC ($p = 0.04$) and MCF ($p = 0.04$) carp, but not in ABF. As the relative mRNA expression of *muc5b* in the intestine of MCF and

AFC fish was similar, the upregulation of this gene in ABF fish was stimulated by the feed. Similar results were obtained by Smith et al. [76] who showed that feeds with different inclusion levels of laminarin, a seaweed-derived ß-D-glucan, led to a significant increase in *muc2* and *muc4* expression in the intestine. Further studies in carp should also include the assessment of other mucin genes, as the abundance per mucosal fold of the goblet cells was the highest in the intestine of MCF carp, and surface area was the largest in MCF and AFC fish, while the number of *muc5b* was similarly the lowest.

Tissue damage and the release of danger signals, such as heat shock proteins, may trigger the expression of pro-inflammatory cytokines [77]. According to this, in our study, the high number of *hsp70* transcripts in the intestine of ABF and MCF fish may have influenced the expression of the *il-1β gene*, which subsequently induced the expression of subsequent pro-inflammatory genes, as shown for *il-6* and *il-8*. This mechanism was also described by Li et al. [78] in the various tissues, including the intestine, of common carp after exposure to glyphosate-based herbicide (GBH). Their results also showed that, in addition to *hsp70*, the number of *hsp90* transcripts also increased in response to GBH. In the case of our study, the expression profile of *hsp90* differed from that of *hsp70*, indicating that conditions in the RAS induced higher expression of *hsp90* in the intestine of *C. carpio* compared with fish wintering in the pond. The *hsp70* and *hsp90* genes collaborate in numerous cellular remodelling reactions, but according to Genest et al. [79], each of them carries out some chaperone activities independently.

4.4. Influence of Wintering Practice on the Skin Histology of Carp from Pond and RAS

Lower thickness of the epithelium of the MCF fish than those from RAS resulted from an adaptive response to wintering conditions, allowing this species to withstand 203-day food limitation and lower temperatures. Similarly, Caruso et al. [80] in European eel (*Anguilla anguilla* L.) and Somejo et al. [81] in Nile tilapia found that the average thickness of the epidermis was lower in starved fish than those fed. Additionally, Landeira-Dabarca et al. [82] showed that Atlantic salmon deprived from food for 18 days significantly reduced epithelial tissue turnover and activity. This is in line with our other observations showing that AFC fish fed a less energetic diet had significantly thinner epithelium than ABF due to presumably reduced metabolic rate. The thickness of fish skin is also determined by factors such as species, age, and body region [83]. Therefore, to assess the overall influence of the wintering method on the epidermis, *SS* and *SC* of the MCF, AFC and ABF carp, the ratio between thickness of each layer was calculated. Here, the lowest thickness of the *SS* in the MCF carp showed that overall conditions in the wintering directly reduced activity of this layer. Among all the layers, *SS* is composed of loosely arranged connective tissue and holds fine blood capillaries, nerves, pigment cells and in some species or fish lines, scales [83]. The lower activity and thus the reduced thickness of the *SS* and epidermis may be restored or even increased after exposure to stressors as shown in the studies with common carp and rainbow trout [84,85]. Feeding ABF carp with a diet containing higher levels of fat and protein increased the area of subcutaneous adipose tissue in those fish. Wang et al. [86] demonstrated that feeding the Nile tilapia with high-fat diets increased the deposits of visceral and subcutaneous fat, whereas starved fish increased ß-oxidation of monounsaturated fatty acids in the subcutaneous layer to cover current metabolic demands and reduced the content of adipose tissue in general. The observation is also in line with the results of image analysis that showed a lower fat density in the subcutaneous region in the starved gilthead seabream (*Sparus aurata*) [87].

Limited food availability in the wintering pond presumably reduced both the number and the size of the MC in the skin of the MCF fish. Mucous cell population in the skin shrinks in consequence of limited availability of dietary carbohydrates that are essential to synthetise O-glycosylated glycoproteins, i.e., mucins [82]. During warmer months (May through October), unlike during the winter period, carp utilise a combination of natural food and supplementary feeding with carbohydrate-rich (up to 35–45% of the total diet) cereal grains such as wheat, triticale, maize, barley and rye [88]. Kideys and Hartnoll [89]

described a decline in mucus layer production as an energy-saving mechanism under adverse environmental conditions. In RAS, carp were fed with diets containing different, i.e., 26% (AFC) and 28.4% (ABF), levels of carbohydrates. The difference in the number and size of MC between AFC and ABF carp only to some extent may have resulted from the level of carbohydrate in the feeds itself. Presumably, the ABF feed with higher digestible energy (16.8 MJ kg^{-1}) than AFC (15.29 MJ kg^{-1}) more efficiently covered nutritional requirements of ABF carp and thus had a positive impact on the population of skin MC. In a study on Atlantic salmon fed feeds based on marine- or plant-derived ingredients and characterised by different energy digestibility, two ways by which plant-based diets can alter volumetric density and size of MC were identified [90]. Reduced production and secretion of skin mucins may expose fish to a higher risk from pathogenic microorganisms, viruses or parasites [91]. In our study, no clinical signs of disease caused by ectoparasites were observed; however, in our opinion, approx. 30% mortality of common carp during overwintering to some extent might have been caused by pathogens such as a ciliated protozoan, *Ichthyophthirius multifilis* [92], whereas in RAS, the health and welfare of fish were checked during routine daily maintenance, and almost no common carp mortalities were observed in the feeding groups. Losses of the ABF and AFC fish were minimised as a consequence of a well-adjusted feeding programme, and it is difficult to point out the cause.

4.5. Gene Expression in Kidney under Carp Wintering in Ponds and RAS

The increased expression of *il-1β* in the MCF carp is a commonly observed case of immunometabolism, an interplay between metabolic and immunological processes that was induced by prolonged chronic stress resulting from restricted feeding [93]. According to Liao et al. [94] and Reuter et al. [95] restricted feeding activates the antioxidant system, and once it is insufficient to clear the damage caused by starvation, numerous transcription factors can be activated, such as nuclear factor kappa light chain enhancer of activated B cells (NF-κB). In line with the current biological understanding, NF-κB can induce expression of the *il-1β* gene, leading to induction of inflammation and subsequent expression of the other pro-inflammatory gene *tnf-α* [96,97]. However, upregulation of the *tnf-α* in the kidney of the ABF fish wintering in RAS was presumably triggered by other or additional mechanisms than by the *il-1β* itself. The plausible explanation for the observed difference between the expression level on the *tnf-α* in the kidney of the ABF and AFC carp is the composition of the diets. Tumour necrosis factor-α emerged as a key cytokine that influences intermediary metabolism, and in the study on Swiss mice fed a high-carbohydrate and high-fat diet, only the former was able to increase TNF-α concentration in the liver [98]. Diets rich in various carbohydrates were also found to acutely activate NF-κB and subsequently the synthesis of pro-inflammatory cytokines, namely IL-1, IL-6 and TNF-alpha [99]. In the study on blunt snout bream (*Megalobrama amblycephala*), hepatic NF-κB, TNF-α, IL-1β and IL-6 expression in fish fed diets were all significantly increased, with increasing dietary carbohydrate levels [100]. Our study showed that expression of *nrf2*, similarly to *il-1β*, was higher in MCF and ABF fish compared to AFC. However, upregulation of the *nrf2* in the MCF and ABF may have different backgrounds. In MSC fish, overwintering in pond with limited access to food decreased the energy state of an organism and plasma glucose levels, leading to activation of the AMPK/SIRT1 axis that enhanced the expression of the *nrf2*, an oxidative stress regulator [101,102], whereas feeding ABF fish with a diet containing higher content of carbohydrates than AFC could cause physiological stresses and increased renal transcription of the *nrf2* in the kidney [103].

A carbohydrate-rich ABF diet possibly also increased synthesis of *IgM* transcripts in the kidney of carp since NF-κB controls the expression of numerous downstream genes that control cell proliferation, survival, stress responses, and immune functions [104]. Furthermore, a study on grass carp head kidney leukocytes showed that the TNF-α-mediated NF-κB pathway is an important signalling involved in protective immune and inflammatory responses [105]. Higher expression of *IgM* in the kidney of the ABF carp was exclusively impacted by feed composition, as both AFC from RAS and MCF overwintering

in the pond showed similar expression of the gene. Krogdahl et al. [106] showed that feeding Atlantic salmon with diets containing soybean molasses, an alcohol extract of soybean meal, causes an inflammatory response in intestinal mucosa as evidenced by the increased level of *IgM*. Other studies showed that the difference in the *IgM* expression in the intestine of gilthead seabream fed a vegetable and fish oil diet further increased when animals were exposed to adverse conditions, e.g., infection with the *Enteromyxum leei* [107]. However, the underlying mechanisms involved in *IgM* activity, especially in the kidney of common carp under different wintering conditions, still remain obscure and warrant further studies.

4.6. Differences in Gene Expression between Gill, Intestine and Kidney in Fish under Different Wintering Conditions

Differences in gene-relative mRNA expression emerge directly from the type of tissue that expresses inherent functions and are additionally challenged by internal and external stimuli. Regardless of the fish group (wintering conditions) the lowest gene expression of the seven analysed genes in most cases was observed in intestine samples. It was especially evident in the samples of AFC fish, whereas in MCF and ABF, the differences were more diverse. All the genes are involved in an array of defence mechanisms, and as evidenced in our study, the gills and the kidney are the primary site for their expression. Gills have a large epithelial surface and are constantly exposed to external milieu; thus, its function goes beyond respiratory gas exchange. To respond to all these challenges actively and accurately, the gills are equipped with their own immune system, called the gill-associated lymphoid tissue (GIALT), thereby substantially contributing to overall fish health and survival [108]. In the case of the kidney, a primary lymphoid organ, elevated expression of the genes in the ABF, AFC and MCF *C. carpio* is a common observation that results from the nature of this organ. Wu et al. [109] showed that comparing other studied organs, including intestine, gene expression of *il-1β* in the kidney and gills was significantly higher, and the observed difference has dramatically increased in the large yellow croaker (*Larimichthys crocea*) upon bacterial infection. Our results were also consistent with some other previous studies on Atlantic halibut, *Hippoglossus hippoglossus* [110], rainbow trout [111] and sea bass, *Dicentrarchus labrax* [112], where the primary role of gills in immunological responses was evidenced. The lower level of expression of most of the genes in the intestine might be related to the concentration and composition of intestinal commensal bacteria [113]. McEntee et al. [114] underlined that homeostasis in the gut depends on the activity of specialised immune cells that are regulated by a cross-talk with symbiotic microbes in the lumen. The overall composition of the microbiota in the intestine of the ABF, AFC and MCF carp is another variable which likely differed due to food/feed quality and availability and the environment (e.g., water temperature) in which the animals were overwintering. For example, the unique influence of multiple and synergic overwintering parameters on gene expression was shown for *il-1β* and *il-6*, which had similar patterns of their transcript level in the gill, kidney and intestine samples from MCF and AFC carps, but not from ABF. The underlying molecular mechanisms in common carp wintering in different conditions still require further and more detailed studies to define the role of symbiotic microbes and environmental factors that determine the performance of fish at the beginning of the next ongrowing stage in spring.

5. Conclusions

Despite being an important stage in a common carp farming, little is known regarding the response of fish to wintering conditions. In this communication, for the first time, we have identified that fish wintering in ponds due to limited access to natural food base and cold-water wintering conditions experienced loss of weight and mortality of 30% compared to common carp from RAS that maintained weight gain and in which only a low number of mortalities was observed during the trial. This observation confirmed the hypothesis that fish overwintering in RAS have better conditions to presumably reach

market size in the second, instead of the third, year of production. The histomorphological examination confirmed that fish adapted physiological responses to the wintering conditions by: (i) adjusting the thickness of layers (epithelium, *stratum spongiosum*, subcutaneous adipose) and changing the population of mucous cells in the skin; (ii) thickening of the interlamellar mass (metaplasia) in the gills; (iii) altering the size of, e.g., supranuclear vacuoles and lamina propria in the intestine. At the molecular level, the relative mRNA expression of the *muc5b* gene was the highest in ABF fish, and the histological assessment showed apparently copious amounts of mucin found in the intestine of these fish, whereas the transcript level of pro-inflammatory cytokines in gills, namely *il-1β* and *il-6*, was up-regulated and correlated with severe lamellar fusions in MCF fish. Here, we described that prolonged chronic stress resulting from restricted feeding increased expression of *il-1β* in the kidney of the MCF common carp, while feeding ABF fish with a diet containing a higher content of carbohydrates than AFC caused physiological stresses and increased renal transcription of the *tnf-α* and *nrf2* in the kidney.

Implementation of eco-intensification can potentially bring economic benefits to carp farmers. Wintering in RAS can save resources required for pond maintenance, fuel and fish for stocking, as well as solve problems with piscivorous predators and water shortages occurring due to climate change. However, investment in RAS facilities can be high, especially for large carp farms; therefore, a true economic analysis should be performed site specific to ensure that eco-intensification of *C. carpio* will bring financial benefits and sustainability to the carp sector.

This study has generated novel findings, insights and ideas which represent an important milestone for the common carp model and paves the way for more focused studies to be performed in the future with regard to the key performance indicators. The histomorphological changes and expression of the genes can be used as biomarkers to further delineate the influence of wintering conditions on fish performance that is crucial to shorten the time to produce market-sized carp, which can help to ensure both sustainability and food security in the EU.

Author Contributions: Conceptualization, P.E., R.P., M.S. and J.S.; methodology, P.E., R.P., M.S. and J.S.; software, P.E. and R.P.; validation, P.E., R.P. and M.S.; formal analysis, R.P., M.S. and P.E.; investigation, R.P., M.S., P.E. and J.S.; resources M.S. and J.S.; data curation, R.P., M.S. and P.E.; writing—original draft preparation, P.E., R.P. and M.S.; writing—review and editing, R.P., M.S. and P.E.; visualization, P.E. and R.P.; supervision, R.P.; project administration, R.P. and M.S.; funding acquisition, R.P., M.S., P.E. and J.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research is part of the EU Horizon 2020 project GAIN. This project has received funding from the European Union Horizon 2020 funding programme, grant agreement no. 773330 (GAIN).

Institutional Review Board Statement: Approval to conduct a fish trial was obtained from the ethical committee of the Faculty of Food Sciences and Fisheries (ZUT in Szczecin, Poland, number 503-08-084-10/4). We adhered to the "Guidelines for the treatment of animals in behavioural research and teaching" published in *Animal Behaviour* [15].

Informed Consent Statement: Not applicable.

Data Availability Statement: The data used to support the findings of this study are included within the article.

Acknowledgments: We wish to thank Marek Bartłomiejczyk and Mateusz Gzyl and his team for providing us with the logistical and technical support which made this study possible.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Costello, C.; Cao, L.; Gelcich, S.; Cisneros-Mata, M.A.; Free, C.M.; Froehlich, H.E.; Golden, C.D.; Ishimura, G.; Maier, J.; Macadam-Somer, I.; et al. The future of food from the sea. *Nature* **2020**, *588*, 95–100. [CrossRef] [PubMed]
2. FAO. The State of World Fisheries and Aquaculture 2020. Sustainability in Action. Rome. 2020. Available online: https://www.fao.org/documents/card/en/c/ca9229en (accessed on 6 September 2021).
3. FAO. Fishery and Aquaculture Statistics. Global Aquaculture Production 1950–2019 (FishStatJ). 2021. Available online: http://www.fao.org/3/cb5186en/cb5186en.pdf (accessed on 6 September 2021).
4. Belton, B.; Little, D.C.; Zhang, W.; Edwards, P.; Skladany, M.; Thilsted, S.H. Farming fish in the sea will not nourish the world. *Nat. Commun.* **2020**, *11*, 5804. [CrossRef] [PubMed]
5. Gál, D.; Kerepeczki, É.; Gyalog, G.; Pekar, F. Changing face of central European aquaculture: Sustainability issues. *Surv. Fish. Sci.* **2015**, *2*, 42–56. [CrossRef]
6. EUMOFA. Freshwater Aquaculture in the EU. 2021. Available online: https://www.eumofa.eu/documents/20178/442176/Freshwater+aquaculture+in+the+EU.pdf (accessed on 6 September 2021).
7. Bauer, C.; Schlott, G. Overwintering of farmed common carp (*Cyprinus carpio* L.) in the ponds of a central European aquaculture facility—Measurement of activity by radio telemetry. *Aquaculture* **2004**, *241*, 301–317. [CrossRef]
8. Woynarovich, A.; Moth-Poulsen, T.; Péteri, A. *Carp Polyculture in Central and Eastern Europe, the Caucasus and Central Asia: A Manual*; FAO Fisheries and Aquaculture Technical Paper; FAO: Rome, Italy, 2010; Volume 554, p. 73.
9. Biermann, G.; Geist, J. Life cycle assessment of common carp (*Cyprinus carpio* L.)—A comparison of the environmental impacts of conventional and organic carp aquaculture in Germany. *Aquaculture* **2020**, *524*, 735273. [CrossRef]
10. Raftowicz, M.; Kalisiak-Mędelska, M.; Struś, M. Redefining the supply chain model on the Milicz carp market. *Sustainability* **2020**, *12*, 2934. [CrossRef]
11. Sobczak, M.; Panicz, R.; Eljasik, P.; Sadowski, J.; Tórz, A.; Żochowska-Kujawska, J.; Barbosa, V.; Dias, J.; Marques, A. Nutritional value and sensory properties of common carp (*Cyprinus carpio* L.) fillets enriched with sustainable and natural feed ingredients. *Food Chem. Toxicol.* **2021**, *152*, 112197. [CrossRef]
12. Eljasik, P.; Panicz, R.; Sobczak, M.; Sadowski, J.; Tórz, A.; Barbosa, V.; Marques, A.; Dias, J. Structural and molecular indices in common carp (*Cyprinus carpio* L.) fed n-3 PUFA enriched diet. *Food Chem. Toxicol.* **2021**, *151*, 112146. [CrossRef]
13. Pietrzak, E.; Mazurkiewicz, J.; Slawinska, A. Innate immune responses of skin mucosa in common carp (*Cyprinus carpio*) fed a diet supplemented with galactooligosaccharides. *Animals* **2020**, *10*, 438. [CrossRef]
14. Eljasik, P.; Panicz, R.; Sobczak, M.; Sadowski, J.; Barbosa, V.; Marques, A.; Dias, J. Plasma biochemistry, gene expression and liver histomorphology in common carp (*Cyprinus carpio*) fed with different dietary fat sources. *Food Chem. Toxicol.* **2020**, *140*, 111300. [CrossRef]
15. Buchanan, K.; Burt de Perera, T.; Carere, C.; Carter, T.; Hailey, A.; Hubrecht, R.; Jennings, D.; Metcalfe, N.; Pitcher, T.; Peron, F.; et al. Guidelines for the treatment of animals in behavioural research and teaching. *Anim. Behav.* **2012**, *83*, 301–309. [CrossRef]
16. Burck, H.C. *Technika Histologiczna*; PZWL: Warszawa, Poland, 1975; pp. 139–140.
17. Livak, K.J.; Schmittgen, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* **2001**, *25*, 402–408. [CrossRef] [PubMed]
18. Korytář, T.; Wiegertjes, G.F.; Zusková, E.; Tomanová, A.; Lisnerová, M.; Patra, S.; Sieranski, V.; Šíma, R.; Born-Torrijos, A.; Wentzel, A.S.; et al. Holzer The kinetics of cellular and humoral immune responses of common carp to presporogonic development of the myxozoan *Sphaerospora molnari*. *Parasites Vectors* **2019**, *12*, 208. [CrossRef] [PubMed]
19. Stolte, E.H.; Nabuurs, S.B.; Bury, N.R.; Sturm, A.; Flik, G.; Savelkoul, H.F.; Verburg-van Kemenade, B.L. Stress and innate immunity in carp: Corticosteroid receptors and pro-inflammatory cytokines. *Mol. Immunol.* **2008**, *46*, 70–79. [CrossRef]
20. Przybylska-Diaz, D.A.; Schmidt, J.G.; Vera-Jimenez, N.I.; Steinhagen, D.; Nielsen, M.E. β-Glucan enriched bath directly stimulates the wound healing process in common carp (*Cyprinus carpio* L.). *Fish Shellfish Immunol.* **2013**, *35*, 998–1006. [CrossRef]
21. Xing, H.; Wang, Z.; Wu, H.; Zhao, X.; Liu, T.; Li, S.; Xu, S. Assessment of pesticide residues and gene expression in common carp exposed to atrazine and chlorpyrifos: Health risk assessments. *Ecotoxicol. Environ. Saf.* **2015**, *113*, 491–498. [CrossRef]
22. Pu, Y.; Zhu, J.; Wang, H.; Zhang, X.; Hao, J.; Wu, Y.; Geng, Y.; Wang, K.; Li, Z.; Zhou, J.; et al. Molecular characterization and expression analysis of Hsp90 in *Schizothorax prenanti*. *Cell Stress Chaperones* **2016**, *21*, 983–991. [CrossRef]
23. Shahi, N.; Ardó, L.; Fazekas, G.; Gócza, E.; Kumar, S.; Rèvèsz, N.; Kumar, S.; Jeney, Z.; Sándor, Z.J.; Molnár, Z.; et al. Immunogene expression in head kidney and spleen of common carp (*Cyprinus carpio* L.) following thermal stress and challenge with Gram-negative bacterium, *Aeromonas hydrophila*. *Aquac. Int.* **2018**, *26*, 727–741. [CrossRef]
24. Agus, H.H.; Sümer, S.; Erkoç, F. Toxicity and molecular effects of di-n-butyl phthalate (DBP) on CYP1A, SOD, and GPx in *Cyprinus carpio* (common carp). *Environ. Monit. Assess.* **2015**, *187*, 423. [CrossRef]
25. Jiang, W.D.; Hu, K.; Zhang, J.X.; Liu, Y.; Jiang, J.; Wu, P.; Zhao, J.; Kuang, S.Y.; Tang, L.; Tang, W.N.; et al. Soyabean glycinin depresses intestinal growth and function in juvenile Jian carp (*Cyprinus carpio* var Jian): Protective effects of glutamine. *Br. J. Nutr.* **2015**, *114*, 1569–1583. [CrossRef]
26. Corcoran, J.; Winter, M.J.; Lange, A.; Cumming, R.; Owen, S.F.; Tyler, C.R. Effects of the lipid regulating drug clofibric acid on PPARα-regulated gene transcript levels in common carp (*Cyprinus carpio*) at pharmacological and environmental exposure levels. *Aquat. Toxicol.* **2015**, *161*, 127–137. [CrossRef] [PubMed]

27. Bickley, L.K.; Lange, A.; Winter, M.J.; Tyler, C.R. Evaluation of a carp primary hepatocyte culture system for screening chemicals for oestrogenic activity. *Aquat. Toxicol.* **2009**, *94*, 195–203. [CrossRef] [PubMed]
28. Gonzalez, S.F.; Huising, M.O.; Stakauskas, R.; Forlenza, M.; Verburg-van Kemenade, B.L.; Buchmann, K.; Nielsen, M.E.; Wiegertjes, G.F. Real-time gene expression analysis in carp (*Cyprinus carpio* L.) skin: Inflammatory responses to injury mimicking infection with ectoparasites. *Dev. Comp. Immunol.* **2007**, *31*, 244–254. [CrossRef] [PubMed]
29. Mowry, R.W. Alcian blue techniques for the histochemical study of acidic carbohydrates. *J. Histochem. Cytochem.* **1956**, *4*, 403–407.
30. Wang, Z.; Du, J.; Lam, S.H.; Mathavan, S.; Matsudaira, P.; Gong, Z. Morphological and molecular evidence for functional organization along the rostrocaudal axis of the adult zebrafish intestine. *BMC Genom.* **2010**, *11*, 392. [CrossRef] [PubMed]
31. Karlsen, C.; Ytteborg, E.; Timmerhaus, G.; Høst, V.; Handeland, S.; Jørgensen, S.M.; Krasnov, A. Atlantic salmon skin barrier functions gradually enhance after seawater transfer. *Sci. Rep.* **2018**, *8*, 9510. [CrossRef] [PubMed]
32. Dang, M.; Pittman, K.; Sonne, C.; Hansson, S.; Bach, L.; Søndergaard, J.; Stride, M.; Nowak, B. Histological mucous cell quantification and mucosal mapping reveal different aspects of mucous cell responses in gills and skin of shorthorn sculpins (*Myoxocephalus scorpius*). *Fish Shellfish Immunol.* **2020**, *100*, 334–344. [CrossRef]
33. Verdile, N.; Pasquariello, R.; Scolari, M.; Scirè, G.; Brevini, T.A.; Gandolfi, F. A detailed study of rainbow trout (*Onchorhynchus mykiss*) intestine revealed that digestive and absorptive functions are not linearly distributed along its length. *Animals* **2020**, *10*, 745. [CrossRef]
34. Wickham, H. *ggplot2: Elegant Graphics for Data Analysis*; Springer: New York, NY, USA, 2016.
35. Lukowicz, M.; Gerstner, P. Hältern und Wintern. In *Lehrbuch der Teichwirtschaft*, 1st ed.; Schäperclaus, W., Lukowicz, M., Eds.; Parey: Berlin, Germany, 1998; pp. 495–503.
36. Geldhauser, F.; Gerstner, P. *Der Teichwirt*, 8th ed.; Ulmer: Stuttgart, Germany, 2003; pp. 1–288.
37. Fernandes, T.; McMeans, B.C. Coping with the cold: Energy storage strategies for surviving winter in freshwater fish. *Ecography* **2019**, *42*, 2037–2052. [CrossRef]
38. Ibarz, A.; Padrós, F.; Gallardo, M.Á.; Fernández-Borràs, J.; Blasco, J.; Tort, L. Low-temperature challenges to gilthead sea bream culture: Review of cold-induced alterations and 'Winter Syndrome'. *Rev. Fish Biol. Fish.* **2010**, *20*, 539–556. [CrossRef]
39. Horváth, L.; Tamás, G.; Seagrave, C. *Carp and Pond Fish Culture*, 2nd ed.; Fishing News Books; Blackwell Scientific Publications Ltd.: Oxford, UK, 1992; pp. 1–154.
40. Pietsch, C.; Hirsch, P. *Biology and Ecology of Carp*, 1st ed.; CRC Press: Boca Raton, FL, USA, 2015; pp. 1–394.
41. Secor, S.M.; Carey, H.V. Integrative Physiology of Fasting. *Compr. Physiol.* **2016**, *6*, 773–825. [CrossRef] [PubMed]
42. Hurst, T.P. Causes and consequences of winter mortality in fishes. *J. Fish Biol.* **2007**, *71*, 315–345. [CrossRef]
43. Tabarrok, M.; Seyfabadi, J.; Salehi Jouzani, G.; Younesi, H. Comparison between recirculating aquaculture and biofloc systems for rearing juvenile common carp (*Cyprinus carpio*): Growth performance, haemato-immunological indices, water quality and microbial communities. *Aquac. Res.* **2020**, *51*, 4881–4892. [CrossRef]
44. Hoseinifar, S.H.; Zou, H.K.; Van Doan, H.; Kolangi Miandare, H.; Hoseini, S.M. Evaluation of some intestinal cytokines genes expression and serum innate immune parameters in common carp (*Cyprinus carpio*) fed dietary loquat (*Eriobotrya japonica*) leaf extract. *Aquac. Res.* **2018**, *49*, 120–127. [CrossRef]
45. Gjessing, M.C.; Steinum, T.; Olsen, A.B.; Lie, K.I.; Tavornpanich, S.; Colquhoun, D.J.; Gjevre, A.G. Histopathological investigation of complex gill disease in sea farmed Atlantic salmon. *PLoS ONE* **2019**, *14*, e0222926. [CrossRef]
46. Powell, M.D.; Yousaf, M.N.; Rasmussen, K.J.; Köllner, B.; Zou, J.; Secombes, C.; Speare, D.J. Immunohistochemical localization of inflammatory cells and cell cycle proteins in the gills of *Loma salmonae* infected rainbow trout (*Oncorhynchus mykiss*). *Fish Shellfish Immunol.* **2014**, *40*, 91–98. [CrossRef]
47. Li, C.; Yao, C.L. Molecular and expression characterizations of interleukin-8 gene in large yellow croaker (*Larimichthys crocea*). *Fish Shellfish Immunol.* **2013**, *34*, 799–809. [CrossRef]
48. Püschel, F.; Favaro, F.; Redondo-Pedraza, J.; Lucendo, E.; Iurlaro, R.; Marchetti, S.; Majem, B.; Eldering, E.; Nadal, E.; Ricci, J.E.; et al. Starvation and antimetabolic therapy promote cytokine release and recruitment of immune cells. *Proc. Natl. Acad. Sci. USA* **2020**, *117*, 9932–9941. [CrossRef]
49. Shimon-Hophy, M.; Avtalion, R.R. Influence of chronic stress on the mechanism of the cytotoxic system in common carp (*Cyprinus carpio*). *Immunology* **2021**, *164*, 211–222. [CrossRef]
50. Hoole, D.; Bucke, D.; Burgess, P.; Wellby, I. *Diseases of Carp and Other Cyprinid Fishes*; Wiley-Blackwell: Honoken, NJ, USA, 2001; p. 280.
51. Yin, X.; Mu, L.; Fu, S.; Wu, L.; Han, K.; Wu, H.; Han, K.; Wu, H.; Bian, X.; Wei, X.; et al. Expression and characterization of Nile tilapia (*Oreochromis niloticus*) secretory and membrane-bound IgM in response to bacterial infection. *Aquaculture* **2019**, *508*, 214–222. [CrossRef]
52. Leya, T.; Ahmad, I.; Valappil, R.K.; Kurcheti, P.P.; Tripathi, G.; Sharma, R.; Bedekar, M.K. Development of species-specific IgM antibodies and elevation of mucosal immune response in *Labeo rohita* using recombinant bicistronic nano DNA vaccine priming. *Fish Shellfish Immunol.* **2021**, *113*, 185–195. [CrossRef] [PubMed]
53. Adams, H.E.; Crump, B.C.; Kling, G.W. Temperature controls on aquatic bacterial production and community dynamics in arctic lakes and streams. *Environ. Microbiol.* **2010**, *12*, 1319–1333. [CrossRef] [PubMed]
54. Liu, H.; Xu, H.; Shangguan, X.; Wang, L.; Liu, X. Identification, annotation of Mucin genes in channel catfish (*Ictalurus punctatus*) and their expression after bacterial infections revealed by RNA-Seq analysis. *Aquac. Res.* **2020**, *51*, 2020–2028. [CrossRef]

55. Bayir, A.; Sirkecioglu, A.N.; Bayir, M.; Haliloglu, H.I.; Kocaman, E.M.; Aras, N.M. Metabolic responses to prolonged starvation, food restriction, and refeeding in the brown trout, Salmo trutta: Oxidative stress and antioxidant defenses. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **2011**, *159*, 191–196. [CrossRef]
56. Chen, L.; Feng, L.; Jiang, W.D.; Jiang, J.; Wu, P.; Zhao, J.; Kuang, S.Y.; Tang, L.; Tang, W.N.; Zhang, Y.A.; et al. Dietary riboflavin deficiency decreases immunity and antioxidant capacity, and changes tight junction proteins and related signaling molecules mRNA expression in the gills of young grass carp (*Ctenopharyngodon idella*). *Fish Shellfish Immunol.* **2015**, *45*, 307–320. [CrossRef]
57. Wang, B.; Feng, L.; Jiang, W.-D.; Wu, P.; Kuang, S.-Y.; Jiang, J.; Tang, L.; Tang, W.-N.; Zhang, Y.-A.; Liu, Y.; et al. Copper-induced tight junction mRNA expression changes, apoptosis and antioxidant responses via NF-rmkappaB, TOR and Nrf2 signaling molecules in the gills of fish: Preventive role of arginine. *Aquat. Toxicol.* **2014**, *158*, 125–137. [CrossRef]
58. Castaldo, G.; Delahaut, V.; Slootmaekers, B.; Bervoets, L.; Town, R.M.; Blust, R.; De Boeck, G. A comparative study on the effects of three different metals (Cu, Zn and Cd) at similar toxicity levels in common carp, *Cyprinus carpio*. *J. Appl. Toxicol.* **2021**, *41*, 1400–1413. [CrossRef]
59. Özaslan, M.S.; Demir, Y.; Küfrevioğlu, O.I.; Çiftci, M. Some metals inhibit the glutathione S-transferase from Van Lake fish gills. *J. Biochem. Mol. Toxicol.* **2017**, *31*, e21967. [CrossRef]
60. Prabhu, P.A.J.; Kaushik, S.J.; Geurden, I.; Stouten, T.; Fontagne-Dicharry, S.; Veron, V.; Mariojouls, C.; Verreth, J.A.J.; Eding, E.H.; Schrama, J.W. Water exchange rate in RAS and dietary inclusion of micro-minerals influence growth, body composition and mineral metabolism in common carp. *Aquaculture* **2017**, *471*, 8–18. [CrossRef]
61. Birnie-Gauvin, K.; Costantini, D.; Cooke, S.J.; Willmore, W.G. A comparative and evolutionary approach to oxidative stress in fish: A review. *Fish Fish.* **2017**, *18*, 928–942. [CrossRef]
62. Svobodová, Z.; Máchová, J.; Kroupová, H.; Smutná, M.; Groch, L. Ammonia autointoxication of common carp: Case studies. *Aquacult. Int.* **2007**, *15*, 277–286. [CrossRef]
63. Zhao, L.; Cui, C.; Liu, Q.; Sun, J.; He, K.; Adam, A.A.; Luo, J.; Li, Z.; Wang, Y.; Yang, S. Combined exposure to hypoxia and ammonia aggravated biological effects on glucose metabolism, oxidative stress, inflammation and apoptosis in largemouth bass (*Micropterus salmoides*). *Aquat. Toxicol.* **2020**, *224*, 105514. [CrossRef] [PubMed]
64. Feng, L.; Chen, Y.P.; Jiang, W.D.; Liu, Y.; Jiang, J.; Wu, P.; Zhao, J.; Kuang, S.Y.; Tang, L.; Tang, W.N.; et al. Modulation of immune response, physical barrier and related signaling factors in the gills of juvenile grass carp (*Ctenopharyngodon idella*) fed supplemented diet with phospholipids. *Fish Shellfish Immunol.* **2016**, *48*, 79–93. [CrossRef] [PubMed]
65. Xu, J.; Feng, L.; Jiang, W.D.; Wu, P.; Liu, Y.; Jiang, J.; Kuang, S.Y.; Tang, L.; Tang, W.N.; Zhang, Y.A.; et al. Effects of dietary protein levels on the disease resistance, immune function and physical barrier function in the gill of grass carp (*Ctenopharyngodon idella*) after challenged with *Flavobacterium columnare*. *Fish Shellfish Immunol.* **2016**, *57*, 1–16. [CrossRef]
66. Element, G.; Engel, K.; Neufeld, J.D.; Casselman, J.M.; van Coeverden de Groot, P.; Greer, C.W.; Walker, V.K. Seasonal habitat drives intestinal microbiome composition in anadromous Arctic char (*Salvelinus alpinus*). *Environ. Microbiol.* **2020**, *22*, 3112–3125. [CrossRef]
67. Xu, Z.; Takizawa, F.; Casadei, E.; Shibasaki, Y.; Ding, Y.; Sauters, T.; Yongyao, Y.U.; Salinas, J.; Sunyer, O. Specialization of mucosal immunoglobulins in pathogen control and microbiota homeostasis occurred early in vertebrate evolution. *Sci. Immunol.* **2020**, *5*, 3254. [CrossRef]
68. Yu, Y.; Wang, Q.; Huang, Z.; Ding, L.; Xu, Z. Immunoglobulins, mucosal immunity and vaccination in teleost fish. *Front. Immunol.* **2020**, *11*, 567941. [CrossRef]
69. Bisht, A.; Singh, U.P.; Pandey, N.N. Comparative study of seasonal variation in bacterial flora concomitant with farm raised fingerlings of *Cyprinus carpio* at tarai region of Uttarakhand. *J. Environ. Biol. Mar.* **2014**, *35*, 363–367.
70. Eichmiller, J.J.; Hamilton, M.J.; Staley, C.; Sadowsky, M.J.; Sorensen, P.W. Environment shapes the fecal microbiome of invasive carp species. *Microbiome* **2016**, *4*, 44. [CrossRef]
71. Tanck, M.W.T.; Booms, G.H.R.; Eding, E.H.; Wendellar Bonga, S.E.; Komen, J. Cold shocks: A stressor for common carp. *J. Fish Biol.* **2000**, *57*, 881–894. [CrossRef]
72. Liang, L.; Chang, Y.; He, X.; Tang, R. Transcriptome analysis to identify cold-responsive genes in Amur carp (*Cyprinus carpio haematopterus*). *PLoS ONE* **2015**, *10*, e0130526. [CrossRef] [PubMed]
73. Zou, J.; Secombes, C.J. The function of fish cytokines. *Biology* **2016**, *5*, 23. [CrossRef] [PubMed]
74. Baeverfjord, G.; Krogdahl, Å. Development and regression of soybean meal induced enteritis in Atlantic salmon, *Salmo salar* L., distal intestine: A comparison with the intestines of fasted fish. *J. Fish Dis.* **1996**, *19*, 375–387. [CrossRef]
75. van der Marel, M.; Adamek, M.; Gonzalez, S.F.; Frost, P.; Rombout, J.H.; Wiegertjes, G.F.; Savelkoul, H.F.; Steinhagen, D. Molecular cloning and expression of two β-defensin and two mucin genes in common carp (*Cyprinus carpio* L.) and their up-regulation after β-glucan feeding. *Fish Shellfish Immunol.* **2012**, *32*, 494–501. [CrossRef] [PubMed]
76. Smith, A.; O'Doherty, J.; Reilly, P.; Ryan, M.; Bahar, B.; Sweeney, T. The effects of laminarin derived from Laminaria digitata on measurements of gut health: Selected bacterial populations, intestinal fermentation, mucin gene expression and cytokine gene expression in the pig. *Br. J. Nutr.* **2011**, *105*, 669–677. [CrossRef] [PubMed]
77. Wedemeyer, G.A.; Barton, B.A.; McLeay, D.J. Stress and acclimation. In *Methods for Fish Biology*; Schreck, C.B., Moyle, P.B., Eds.; American Fisheries Society: Bethesda, MD, USA, 1990; pp. 451–489.
78. Li, Y.; Ding, W.; Li, X. Acute exposure of glyphosate-based herbicide induced damages on common carp organs via heat shock proteins-related immune response and oxidative stress. *Toxin Rev.* **2019**, *40*, 1071–1083. [CrossRef]

79. Genest, O.; Wickner, S.; Doyle, S.M. Hsp90 and Hsp70 chaperones: Collaborators in protein remodelling. *J. Biol. Chem.* **2019**, *29*, 2109–2120. [CrossRef]
80. Caruso, G.; Maricchiolo, G.; Micale, V.; Genovese, L.; Caruso, R.; Denaro, M.G. Physiological responses to starvation in the European eel (*Anguilla anguilla*): Effects on haematological, biochemical, non-specific immune parameters and skin structures. *Fish Physiol. Biochem.* **2010**, *36*, 71–83. [CrossRef]
81. Somejo, M.B.; Herrera, A.A.; Fabillo, M.D.; Abucay, J.S. The development of integumentary and skeletal systems of starved Nile Tilapia, *Oreochromis niloticus* L. In *New Dimensions in Farmed Tilapia, Proceedings of the Sixth International Symposium on Tilapia in Aquaculture, Manila, Philippines, 12–16 September 2004*; Bolivar, R., Mair, G., Fitzsimmons, K., Eds.; American Tilapia Association, Aquaculture CRSP, and Ministry of Agriculture: Manila, Philipines, 2004; pp. 733–740.
82. Landeira-Dabarca, A.; Álvarez, M.; Molist, P. Food deprivation causes rapid changes in the abundance and glucidic composition of the cutaneous mucous cells of Atlantic salmon *Salmo salar* L. *J. Fish Dis.* **2014**, *37*, 899–909. [CrossRef]
83. Elliott, D.G. Integumentary system. In *The Laboratory Fish*; Otrander, G.K., Ed.; Academic Press: New York, CA, USA, 2000; pp. 271–306.
84. Iger, Y.; Jenner, H.A.; Bonga, S.W. Cellular responses of the skin of carp (*Cyprinus carpio*) exposed to acidified water. *Cell Tissue Res.* **1994**, *275*, 481–492. [CrossRef]
85. Iger, Y.; Jenner, H.A.; Bonga, S.W. Cellular responses in the skin of the trout (*Oncorhynchus mykiss*) exposed to temperature elevation. *J. Fish Biol.* **1994**, *44*, 921–935. [CrossRef]
86. Wang, Y.W.; Zhang, J.L.; Jiao, J.G.; Du, X.X.; Limbu, S.M.; Qiao, F.; Zhang, M.L.; Li, D.L.; Du, Z.Y. Physiological and metabolic differences between visceral and subcutaneous adipose tissues in Nile tilapia (*Oreochromis niloticus*). *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **2017**, *313*, R608–R619. [CrossRef] [PubMed]
87. Ceballos-Francisco, D.; García-Carrillo, N.; Cuesta, A.; Esteban, M.Á. Radiological characterization of gilthead seabream (*Sparus aurata*) fat by X-ray micro-computed tomography. *Sci. Rep.* **2020**, *10*, 10527. [CrossRef] [PubMed]
88. Edwards, P. Pilgrimage to traditional carp pond culture in Central Europe. *Aquac. Asia* **2007**, *12*, 28–34.
89. Kideys, A.E.; Hartnoll, G.H. Energetics of mucus production in the common whelk *Buccinum undatum* L. *J. Exp. Mar. Biol. Ecol.* **1991**, *150*, 91–105. [CrossRef]
90. Sørensen, S.L.; Park, Y.; Gong, Y.; Vasanth, G.K.; Dahle, D.; Korsnes, K.; Phuong, T.H.; Kiron, V.; Øyen, S.; Pittman, K.; et al. Nutrient digestibility, growth, mucosal barrier status, and activity of leucocytes from head kidney of Atlantic salmon fed marine- or plant- derived protein and lipid Sources. *Front. Immunol.* **2021**, *11*, 623726. [CrossRef]
91. Shephard, K.L. Functions for fish mucus. *Rev. Fish Biol. Fish.* **1994**, *4*, 401–429. [CrossRef]
92. Ćirković, M.; Novakov, N.; Kartalović, B.; Pelić, M.; Jovanić, S.; Božić, B.; Đorđević, V. Ichthyophthiriosis–cause of significant losses of carp fingerlings. *Arch. Vet. Med.* **2015**, *8*, 3–12. [CrossRef]
93. Mathis, D.; Shoelson, S.E. Immunometabolism: An emerging frontier. *Nat. Rev. Immunol.* **2011**, *11*, 81. [CrossRef]
94. Liao, Z.; Lin, D.; Jia, J.; Cai, R.; Yu, Y.; Li, W. Innate immune response to fasting and refeeding in the zebrafish kidney. *Biomolecules* **2021**, *11*, 825. [CrossRef]
95. Reuter, S.; Gupta, S.C.; Chaturvedi, M.M.; Aggarwal, B.B. Oxidative stress, inflammation, and cancer: How are they linked? *Free Radic. Biol. Med.* **2010**, *49*, 1603–1616. [CrossRef] [PubMed]
96. Bo, Y.X.; Song, X.H.; Wu, K.; Hu, B.; Sun, B.Y.; Liu, Z.J.; Fu, J.G. Characterization of interleukin-1β as a proinflammatory cytokine in grass carp (*Ctenopharyngodon idella*). *Fish Shellfish Immunol.* **2015**, *46*, 584–595. [CrossRef] [PubMed]
97. Kalb, D.; Vo, H.D.; Adikari, S.; Hong-Geller, E.; Munsky, B.; Werner, J. Visualization and modeling of inhibition of IL-1β and TNFα mRNA transcription at the single-cell level. *Sci. Rep.* **2021**, *11*, 13692. [CrossRef] [PubMed]
98. Ferreira, A.V.M.; Mario, É.G.; Porto, L.C.J.; Andrade, S.P.; Botion, L.M. High-carbohydrate diet selectively induces tumor necrosis factor-α production in mice liver. *Inflammation* **2011**, *34*, 139–145. [CrossRef]
99. Baker, R.G.; Hayden, M.S.; Ghosh, S. NF-κB, inflammation, and metabolic disease. *Cell Metab.* **2011**, *13*, 11–22. [CrossRef]
100. Chen, F.; Zhao, C.Y.; Guan, J.F.; Liu, X.C.; Li, X.F.; Xie, D.Z.; Xu, C. High-carbohydrate diet alleviates the oxidative stress, inflammation and apoptosis of *Megalobrama amblycephala* following dietary exposure to silver nanoparticles. *Antioxidants* **2021**, *10*, 1343. [CrossRef]
101. Salminen, A.; Hyttinen, J.M.; Kaarniranta, K. AMP-activated protein kinase inhibits NF-κB signaling and inflammation: Impact on healthspan and lifespan. *J. Mol. Med.* **2011**, *89*, 667–676. [CrossRef]
102. Xu, C.; Liu, W.B.; Remø, S.C.; Wang, B.K.; Shi, H.J.; Zhang, L.; Liu, J.D.; Li, X.F. Feeding restriction alleviates high carbohydrate diet-induced oxidative stress and inflammation of *Megalobrama amblycephala* by activating the AMPK-SIRT1 pathway. *Fish Shellfish Immunol.* **2019**, *92*, 637–648. [CrossRef]
103. Zhou, C.P.; Ge, X.P.; Liu, B.; Xie, J.; Miao, L.H. Effect of high dietary carbohydrate on the growth performance and physiological responses of juvenile wuchang bream, *Megalobrama Amblycephala*. *Asian-Australas. J. Anim. Sci.* **2013**, *26*, 1598. [CrossRef]
104. Yuan, Z.H.; Feng, L.; Jiang, W.D.; Wu, P.; Liu, Y.; Jiang, J.; Kuang, S.Y.; Tang, L.; Zhou, X.Q. Dietary choline-enhanced skin immune response of juvenile grass carp might be related to the STAT3 and NF-kB signaling pathway (*Ctenopharyngodon idella*). *Front. Nutr.* **2021**, *8*, 652767. [CrossRef]

105. Liu, X.W.; Zhang, J.X.; Feng, L.; Jiang, W.D.; Wu, P.; Kuang, S.Y.; Tang, L.; Shi, H.Q.; Zhou, X.Q.; Liu, Y. Protective effects and potential mechanisms of (2-Carboxyethyl) dimethylsulfonium Bromide (Br-DMPT) on gill health status of on-growing grass carp (*Ctenopharyngodon idella*) after infection with Flavobacterium columnare. *Fish Shellfish Immunol.* **2020**, *106*, 228–240. [CrossRef] [PubMed]
106. Krogdahl, Å.; Bakke-McKellep, A.M.; Røed, K.H.; Bæverfjord, G. Feeding Atlantic salmon *Salmo salar* L. soybean products: Effects on disease resistance (furunculosis), and lysozyme and IgM levels in the intestinal mucosa. *Aquac. Nutr.* **2000**, *6*, 77–84. [CrossRef]
107. Estensoro, I.; Calduch-Giner, J.A.; Kaushik, S.; Pérez-Sánchez, J.; Sitjà-Bobadilla, A. Modulation of the IgM gene expression and IgM immunoreactive cell distribution by the nutritional background in gilthead sea bream (*Sparus aurata*) challenged with *Enteromyxum leei* (Myxozoa). *Fish Shellfish Immunol.* **2012**, *33*, 401–410. [CrossRef] [PubMed]
108. Koppang, E.O.; Kvellestad, A.; Fischer, U. Fish mucosal immunity: Gill. In *Mucosal Health in Aquaculture*, 1st ed.; Beck, B., Peatman, E., Eds.; Academic Press: Cambridge, MA, USA, 2015; pp. 93–133.
109. Wu, J.; Shi, Y.H.; Zhang, X.H.; Li, C.H.; Li, M.Y.; Chen, J. Molecular characterization of an IL-1β gene from the large yellow croaker (*Larimichthys crocea*) and its effect on fish defense against *Vibrio alginolyticus* infection. *Dong Wu Xue Yan Jiu = Zool. Res.* **2015**, *36*, 133–141.
110. Øvergård, A.C.; Nepstad, I.; Nerland, A.H.; Patel, S. Characterisation and expression analysis of the Atlantic halibut (*Hippoglossus hippoglossus* L.) cytokines: IL-1β, IL-6, IL-11, IL-12β and IFNγ. *Mol. Biol. Rep.* **2012**, *39*, 2201–2213. [CrossRef] [PubMed]
111. Syahputra, K.; Kania, P.W.; Al-Jubury, A.; Marnis, H.; Setyawan, A.C.; Buchmann, K. Differential immune gene response in gills, skin, and spleen of rainbow trout *Oncorhynchus mykiss* infected by *Ichthyophthirius multifiliis*. *PLoS ONE* **2019**, *14*, e0218630. [CrossRef] [PubMed]
112. Alix, M.; Bloundeau-Bidet, E.; Grousset, E.; Shirangui, A.; Vergnet, A.; Guinand, B.; Chatain, B.; Boulo, V.; Lignot, J.-H. Effects of fasting and re-alimentation on gill and intestine morphology and indicators of osmoregulatory capacity in genetically selected sea bass (*Dicentrarchus labrax*) populations with contrasting tolerance to fasting. *Aquaculture* **2017**, *468*, 314–325. [CrossRef]
113. Atarashi, K.; Tanoue, T.; Umesaki, Y.; Honda, K. Regulation of Th17 cell differentiation by intestinal commensal bacteria. *Benef. Microbes* **2010**, *1*, 327–334. [CrossRef]
114. McEntee, C.P.; Finlay, C.M.; Lavelle, E.C. Divergent roles for the IL-1 family in gastrointestinal homeostasis and inflammation. *Front. Immunol.* **2019**, *10*, 1266. [CrossRef]