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The effect of a sudden temperature decrease on selected physiological indices in the common carp

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Abstract: The objective of the study was to evaluate stress invoked by a temperature decrease in the common carp (n = 56). After acclimatisation to laboratory conditions at a temperature of 27.8 ± 1.0 °C, the fish (n = 28) were transferred to 16.8 ± 1.0 °C water (group T2; temperature difference Δ –11 °C); the rest of the fish were kept at the original water temperature (group T1). The examination of individual fish was made after 6, 12, 24 and 48 hours (h). The impact on the haematological and biochemical indices was evaluated. Furthermore, the effect on the food content passage speed was investigated and a histopathological examination was performed. The changed haematological indices in the T2 group returned to the T1 values 48 h after a temperature decrease, except for the neutrophil-lymphocyte ratio which was found to be higher in all the T2 samplings. Most of the monitored biochemical indices decreased in the T2 group and some of them remained that way at 48 hours. On the contrary, the liver enzymes increased in the T2 group at 48 hours. The passage of food through the digestive tract was mostly finished after 48 h for both groups. However, in the warmer water, the intestine was empty in 71.4% cases after 24 h already, while in the T2 group, the food content passage was only completed in 14.3% of the fish at the same time. Local gill and skin necrosis, and mononuclear cell infiltration were found in the T2 group. A sudden change in the temperature can affect various parameters of the common carp, however, these changes can mostly be eliminated within 48 hours.

Keywords: ammonia; cold stress; *Cyprinidae*; food passage; fish; immunity

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Fish are ectothermic organisms and, thus, the surrounding water temperature is critical to their appropriate metabolism. A sudden temperature change leads to the inability of maintaining homeostasis and the physiological levels of haematological and biochemical indices, behaviour changes and even death (Portz et al. 2006; Donaldson et al. 2008; Portner and Peck 2010; Foss et al. 2012). The temperature stress develops when the sudden difference between the water temperatures is > 10–12 °C, and even > 3 °C in the case of juvenile fish (Svobodova et al. 1993). The response to this change is different for every fish, depending on the species, acclimatisation, immune and health status, adaptability etc., as summarised by Donaldson et al. (2008). However, some similarities can be observed among species. Because the metabolic rate depends on the water temperature, in fish transported to colder water, the process of feed digestion is disrupted, slowed or even stopped. Thus, undigested feed with gas creation can be observed in the intestine. Due to the enlarged and gaseous digestive tract, the fish lose swimming balance and mortality rises. Additionally, paralysis of cardiac and respiratory muscles is a common finding. Moreover, the overall fish metabolism is decreased and consequently, the $NH_3 + NH_4^+$ (ammonia) excretion by the gills is slowed and accumulates in the blood plasma, causing autointoxication and death (Svobodova et al. 1993; Smutna et al. 2002; Portz et al. 2006; Svobodova et al. 2007).

Even though cold stress and its effects on fish is the topic of many scientific studies, the present knowledge is still insufficient and needs to undertake further studies with a wider analysis. The aim of our study was to expose the common carp *Cyprinus carpio* (Linnaeus 1758) to short-term cold temperature stress and, subsequently, assess its impact on the physiology and, in case of changes, to observe how long it takes the body to return to the unstressed levels. The water temperatures were selected to be in an optimum life range (27.8 ± 1.0 °C) and to achieve moderate stress conditions (16.8 ± 1.0 °C). This manuscript is focused on one of the most fundamental stressors in fish farming, acute hypothermia.

The hypothesis was that the fish metabolism and food content passage speed would be slowed, as a response to the cold stress, and the ammonia levels would rise. The main focus of this experiment was to study the ammonia excretion and whether the autointoxication with this metabolite can be observed as a reaction to the acute cold stress. Additionally, the negative effect of the sudden temperature decrease could result in the disruption of the energetic metabolism in the cells and possibly affect the function of the internal organs. An additional hypothesis was to consider the effects on the fish immune system, asif the temperature change is drastic, the nonspecific and specific immunity response can be impaired and result in a reduced overall welfare level.

MATERIAL AND METHODS

Ethical statement

The experiment was approved by the Institute's Ethics Committee and carried out in accordance with institutional guidelines and national legislation, specifically Act No. 246/1992 Coll., on the Protection of Animals against Cruelty, as amended.

Fish and experiment conditions

The experiment was carried out in the experimental facility of the Department of Zoology, Fisheries, Hydrobiology and Apiculture, Mendel University in Brno, Czech Republic. A total of 56 one- to two-year-old *C. carpio* (body weight 93.88 ± 29.16 g, total length 18.45 ± 1.83 cm, male to female ratio 50 : 50) were obtained from a local hatchery (Pohořelice, Plc., Czech Republic). The fish strain used was a crossbred Pohořelice mirror carp × Amur mirror carp; artificial spawning was performed in May 2017 and the fish were kept in ponds until being transported to the experimental facility in September 2017 where they were veterinary examined for negative viral, bacterial or parasitic infections and quarantined.

During the 14-day-long acclimatisation to laboratory conditions, the fish were fed twice a day with Skretting C-3 Carpe F commercial pellets (Nutreco N.V., The Netherlands; 33% crude protein, 8% crude fat, 5% fibre, 7% raw ash, 1% calcium, 0.4% sodium, 0.9% phosphor) in the amount of 2% of their body weight. The fish were held in tanks (volume of 1 m^3) connected to are circulation system. The dechlorinated tap water

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parameters were monitored every 12 h (acclimatisation phase) and every 6 h (experimental phase). The water parameters during the experimental phase were as follows: temperature 27.8 ± 1.0 °C, oxygen saturation 97.10 ± 6.87%, pH 8.08 ± 0.19, ammonia 0.85 ± 0.43 mg/l for the T1 experimental group; and temperature 16.8 ± 1.0 °C, oxygen saturation 103.73 ± 2.96%, pH 8.58 ± 0.16, ammonia 0.47 ± 0.46 mg/l for the T2 experimental group. Both experimental tanks were individually aerated and maintained on a12 h light/12 h dark photoperiod during the acclimatisation and whole experimental phase.

The measurement of the O_2 and pH in the water was performed with a WTW Oxi 340i digital sampling system and a WTW pH 340i water quality meter (WTW GmbH, Weilheim, Germany). The ammonia and nitrites in the water were determined using the spectrophotometric methods according to the Animal and Plant Health Agency (APHA 2017) methodology (method No. 4500-NH_3, 4500-NO_2).

Experimental design

The water temperature during the acclimatisation period was maintained at 27.8 ± 1.0 °C. At the beginning of the experimental phase, the fish were fed in the morning and after one hour they were randomly allocated into two groups – one group (28 fish) was moved into a tank with a water temperature of 16.8 ± 1.0 °C (T2) for the cold stress assessment and the second group (T1, n = 28) was moved to a tank with a similar temperature as during the acclimatisation phase and served as a control group.

The fish were not fed anymore during the experimental period and were kept at a stable temperature, respective to the treatment. After 6, 12, 24 and 48 h, the fish (n = 7) in both tested groups – T1 and T2 were sampled.

Haematological analysis

Initially, blood was taken from the caudal vein without anaesthesia and after sampling, the fish were stunned with a blow to the head and killed by spinal transection. The blood was stabilised with sodium heparin (50 IU per 1 ml of blood).

From the whole blood, the red blood cell count (RBC), the white blood cell count (WBC) and the differential leukocyte count were enumerated. The RBC and WBC were counted using a Burker haemocytometer, where heparinised blood, diluted with a Natt-Herick solution (1 : 200 ratio), as applied. The differential leukocyte count was evaluated based on the blood smears stained with a Hemacolor set (Merck Co., Darmstadt, Germany). A total of 100 leukocytes were assigned to categories according to the morphology as follows: lymphocytes, monocytes, myelocytes, metamyelocytes, banded and segmented neutrophils. The neutrophil-lymphocyte ratio (NLR) was calculated as the ratio between the absolute neutrophil count and the absolute lymphocyte count. The haematocrit (PCV) was determined using the microhaematocrit method with centrifugation of the blood in capillary tubes (Bull et al. 2000). The haemoglobin concentration (Hb) was determined by the photometric cyanohaemoglobin method at 540 nm (Drabkin and Austin 1932). The RBC, PCV and Hb were used for the calculation of the mean erythrocyte haemoglobin (MCH), mean erythrocyte volume (MCV) and mean corpuscular haemoglobin concentration (MCHC).

Biochemical analysis

Immediately after sampling, one half of the heparinised blood was centrifuged (800 × g, 4 °C, 10 min) to obtain the plasma. The values of the albumin (ALB), alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), ammonia, total protein (TP), cholesterol (CHOL), phosphorus (PHOS), glucose (GLU), lactate dehydrogenase (LDH), lactate (LACT), triglycerides (TG) and calcium (Ca) were evaluated in the fish plasma samples. The analysis was performed using a commercial kit (BioVendor PCL, Czech Republic) and a Konelab 20i biochemical analyser (Thermo Fisher Scientific, Czech Republic) following the manufacturer's instructions.

Morphometric analysis and digestive tract activity

Every fish was weighed and measured to gain its biometrical data. The monitored data were as

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follows: body and spleen weight (g), standard and total length (cm) and data for the evaluation of the food content passage speed – digestive tract and feed weight (g), intestine length and length of the part of intestine where the feed was present (cm). The length of the intestine containing the food content was measured with a ruler after preparing the intestine of each fish. The body weight in grams and the fish standard length in cm were used to calculate Fulton's condition factor (CF) as follows: CF = (weight/standard length ^ 3) × 100. The spleen-somatic index (SSI) was calculated as follows: SSI = spleen weight (mg)/total body weight (g).

Histopathological analysis

The gill (upper limb of the arch), skin (above the ventral fin), cranial kidney and liver tissues were dissected for the histopathological examination for every fish individually and immediately fixed in buffered 10% neutral formalin. Then, the samples were dehydrated with isopropanol and inserted into paraffin wax. A microtome tool with a thickness of 4 µm was used for the sample slicing and haematoxylin-eosin dye was applied for the sample staining. Each sample was examined with a light microscope; the magnification of the images was 100× to 400×.

Statistical analysis

The statistical analysis for all the indices was performed using the statistical software Unistat for Excel v6.5 (UnistatLtd., England). At first, all the data were tested for normal distribution using the Shapiro-Wilk test. In the case of normality, the data were evaluated with the unpaired *t*-test. The nonparametric Mann-Whitney test was applied to the results with the non-normal distribution. The statistical evaluation was carried out between the T1 and T2 groups at the same time point for all the indices and the difference was considered statistically significant when $P < 0.05$ (*) and $P < 0.01$ (**). The data are reported as the mean ± standard deviation (SD). Box plot graphs were constructed using the median, Q1, Q2 with whiskers of the maximum 1.5 interquartile range and outliers denoted as points.

RESULTS

Mortality, behaviour and health status

No mortality occurred during the experiment. The fish behaviour was not observed to be influenced in any part of the experiment. The health status of all the experimental fish was without any bacterial, viral or parasitical infection. The CF was not significantly different between the tested time intervals and was maintained between 2.4 and 2.9. The SSI was not changed during the whole trial.

Haematological profile

In general, the fish's exposure to the water with a lower temperature (T2) caused a decrease in almost all the examined haematological indices in comparison with temperature of the T1 group. The PCV was observed to be decreased among all the T2 fish – significantly at 12 h ($P < 0.01$) and 24 h ($P < 0.05$). It was the same as with the Hb, which was significantly decreased at 6 h and 12 h ($P < 0.05$). The RBC was decreased in the colder water at 12 h ($P < 0.01$) and 24 h ($P < 0.05$). The WBC in the T2 group was decreased at 12 h ($P < 0.05$), whereas at 6 h, a significant increase was observed ($P < 0.01$).

With regards to the MCH, MCV and MCHC variables, an increase in the T2 group at 12 h ($P < 0.05$) was observed for the MCHC. The obtained data on the haematological profile are reported in Table 1.

The NLR (Figure 1A) was found to be higher in all the T2 fish, significantly at 12 h ($P < 0.01$) and 24 h ($P < 0.05$). Alterations in the differential leukocyte count were found, mostly in the segmented and banded neutrophils – the banded neutrophils were increased in the T2 group at 6 h ($P < 0.01$) and 12 h ($P < 0.05$); the segmented neutrophils were increased in the T2 group at 6 h ($P < 0.05$) and decreased in the T2 group at 12 h ($P < 0.01$) and 24 h ($P < 0.01$). In the case of the lymphocytes and metamyelocytes, a significant increase was found for the T2 fish at 6 h ($P < 0.01$) and, for the lymphocytes, a decrease was found at 48 h ($P < 0.05$); the monocytes were significantly decreased in the T2 fish at 48 h ($P < 0.05$). To sum up, at 6 h, all the leukocytes were elevated in the cold T2 water.

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Table 1. The results of the haematological analysis in the blood samples of the experimental fish (n = 7) exposed to temperature T1 (27.8 ± 1.0 °C) and T2 (16.8 ± 1.0 °C) at 6, 12, 24 and 48 hours. The data are expressed as the mean ± SD; statistically significant results are in bold (*P < 0.05, **P < 0.01)

Parameter	6 h		12 h		24 h		48 h	
	T1	T2	T1	T2	T1	T2	T1	T2
PCV (l/l)	0.36 ± 0.02	0.35 ± 0.02	0.40 ± 0.03	**0.32 ± 0.02****	0.37 ± 0.02	**0.32 ± 0.05***	0.35 ± 0.03	0.35 ± 0.03
Hb (g/l)	65.67 ± 4.38	**59.74 ± 4.04***	72.17 ± 3.74	**64.94 ± 6.80***	65.26 ± 4.30	58.87 ± 9.14	58.07 ± 5.59	61.61 ± 4.34
RBC (T/l)	1.86 ± 0.16	1.90 ± 0.34	2.25 ± 0.24	**1.85 ± 0.15****	2.22 ± 0.27	**1.81 ± 0.37***	2.12 ± 0.34	1.95 ± 0.28
WBC (G/l)	12.29 ± 5.99	**34.00 ± 9.26****	32.14 ± 9.26	**21.29 ± 5.96***	26.57 ± 12.12	17.57 ± 6.05	28.86 ± 8.53	21.14 ± 7.20
MCH (pg)	35.37 ± 2.55	32.14 ± 4.32	32.48 ± 4.64	35.25 ± 3.67	29.80 ± 3.79	33.10 ± 4.43	27.93 ± 4.53	32.13 ± 4.88
MCV (fl)	195.82 ± 18.75	187.99 ± 28.51	177.93 ± 25.86	173.84 ± 14.49	166.67 ± 18.83	179.56 ± 20.61	169.10 ± 24.69	183.00 ± 25.76
MCHC (l/l)	0.18 ± 0.01	0.17 ± 0.00	0.18 ± 0.01	**0.20 ± 0.01***	0.18 ± 0.01	0.18 ± 0.01	0.17 ± 0.02	0.18 ± 0.01

h = hours; Hb = haemoglobin concentration; MCH = mean erythrocyte haemoglobin; MCHC = mean corpuscular haemoglobin concentration; MCV = mean erythrocyte volume; PCV = haematocrit; RBC = red blood cells; SD = standard deviation; WBC = white blood cells

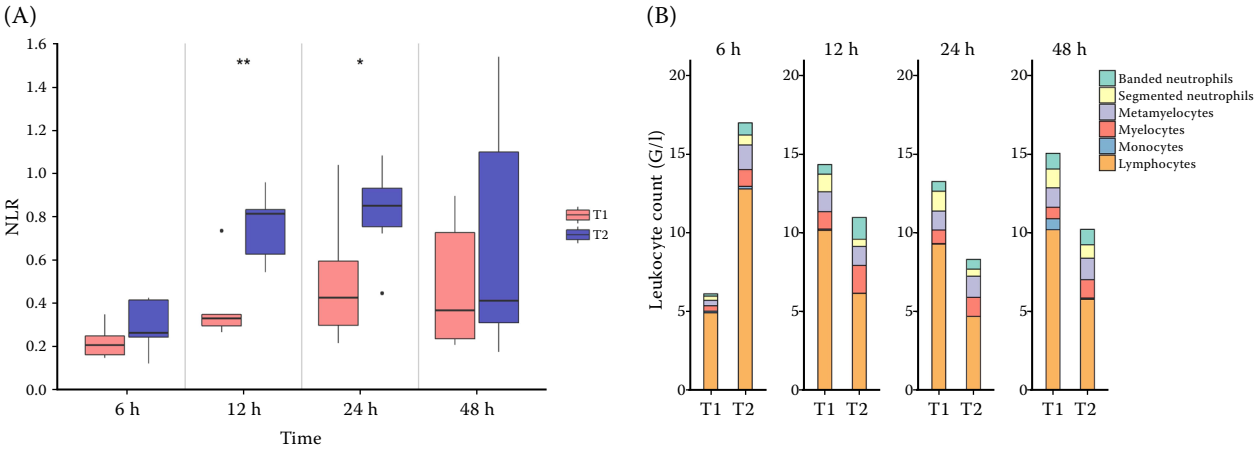

Figure 1. Neutrophil-to-lymphocyte ratio, the NLR (A) and differential leukocyte count (B) in the experimental fish *C. carpio* (n = 7) in the T1 (27.8 ± 1.0 °C) and T2 (16.8 ± 1.0 °C) water temperature at 6, 12, 24 and 48 hours (h). Comparison between the T1 and T2 groups at the same sampling time; *P < 0.05, **P < 0.01

Conversely, from 12 h on, the levels of the lymphocytes, monocytes and segmented neutrophils were lower than in the T1 fish. The increased numbers of metamyelocytes were prevalent at 6 h in the cold water, but at comparable levels with the T1 group in the samplings that followed, while the myelocytes and banded neutrophils stayed at elevated levels in the cold water fish at all the sampling points. The results are shown in Figure 1B.

Biochemical profile

In general, the biochemical indices were decreased in the lower T2 temperature when compared to the T1 temperature. The TP was decreased at 12, 24 and 48 h (P < 0.01), the CHOL and Ca at 12 h (P < 0.05) and 48 h (P < 0.01). The ALB was significantly decreased in all the samplings, the TG in all but one of the samplings (P < 0.01) and the

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ammonia at 24 and 48 h ($P < 0.01$). In contrast, the LACT and PHOS levels increased significantly ($P < 0.01$) at 6 h, which returned to the baseline concentrations in the samplings that followed. Regarding the liver enzymes, an increase and a decrease was observed for the AST ($P < 0.01$) and for ALP ($P < 0.01$), respectively. For the ALT, a decrease was observed at 12 h ($P < 0.05$) at first. However, it was followed by an increase at 48 h ($P < 0.05$). The GLU and LDH did not show any significant changes during the trial. The results of the biochemical analysis are given in Table 2.

When the levels of ammonia excreted into the water were analysed, a slower excretion level was observed in the T2 group. The ammonia in the plasma samples was at a comparable level between the T1 and T2 groups at 6 h and 12 hours. However, at 24 h and 48 h, a significant decrease was observed in the T2 group ($P < 0.01$). In contrast, the ammonia excreted into the water was decreased in the T2 group for all the sampling times (Figure 2).

Digestive tract activity

The results of the digestive tract activity are given in Figure 3. A statistical significance ($P < 0.01$) was found for the weight of the digestive tract at 6, 12 and 24 h and for the feed weight at the same times.

Table 2. Biochemical analysis of the plasma samples of the experimental fish ($n = 7$) exposed to the T1 (27.8 ± 1.0 °C) and T2 (16.8 ± 1.0 °C) water temperature at 6, 12, 24 and 48 hours. The data are expressed as the mean ± SD; statistically significant results are in bold (*$P < 0.05$, **$P < 0.01$)

Parameter	6 h		12 h		24 h		48 h	
	T1	T2	T1	T2	T1	T2	T1	T2
ALB (g/l)	11.97 ± 2.62	**8.53 ± 2.02***	12.38 ± 1.68	**8.06 ± 1.65****	12.64 ± 1.95	**10.28 ± 1.75***	13.39 ± 1.21	**8.87 ± 2.49****
ALP (µkat/l)	0.28 ± 0.22	0.35 ± 0.24	0.33 ± 0.19	0.46 ± 0.85	0.48 ± 0.37	0.17 ± 0.11	0.32 ± 0.13	**0.12 ± 0.07****
ALT (µkat/l)	0.92 ± 0.51	0.98 ± 0.30	0.88 ± 0.32	**0.50 ± 0.16***	0.84 ± 0.13	0.84 ± 0.44	0.62 ± 0.20	**0.96 ± 0.35***
AST (µkat/l)	5.57 ± 3.11	4.84 ± 2.26	6.76 ± 6.50	3.80 ± 2.89	4.41 ± 2.09	5.32 ± 2.58	4.06 ± 2.33	**8.85 ± 3.32****
TP (g/l)	24.77 ± 2.79	22.48 ± 1.83	24.13 ± 2.68	**20.24 ± 1.37****	25.73 ± 1.61	**21.75 ± 1.87****	25.68 ± 1.59	**21.40 ± 1.86****
CHOL (mmol/l)	6.01 ± 0.77	5.82 ± 0.51	6.03 ± 0.86	**5.06 ± 0.43***	6.52 ± 1.23	5.37 ± 0.39	6.62 ± 0.48	**5.01 ± 1.03****
PHOS (mmol/l)	1.39 ± 0.33	**2.09 ± 0.34****	2.10 ± 0.67	2.17 ± 0.50	1.90 ± 0.49	1.75 ± 0.22	2.38 ± 0.82	2.18 ± 0.84
GLU (mmol/l)	9.72 ± 4.59	7.46 ± 2.34	7.33 ± 1.37	6.24 ± 2.72	8.58 ± 2.04	6.56 ± 2.85	6.80 ± 1.61	6.95 ± 2.20
LDH (µkat/l)	13.79 ± 18.16	26.82 ± 17.35	20.48 ± 21.72	15.38 ± 18.84	20.72 ± 17.73	24.72 ± 11.94	15.37 ± 15.45	22.90 ± 19.57
LACT (mmol/l)	0.38 ± 0.25	**0.99 ± 0.45****	0.74 ± 0.66	1.12 ± 1.09	0.60 ± 0.34	0.49 ± 0.19	1.81 ± 1.24	1.71 ± 0.95
TG (mmol/l)	3.61 ± 0.58	**2.75 ± 0.24****	3.34 ± 0.20	**2.39 ± 0.44****	3.62 ± 0.27	**2.66 ± 0.43****	3.06 ± 0.23	2.95 ± 0.45
Ca (mmol/l)	2.35 ± 0.31	2.44 ± 0.12	2.56 ± 0.22	**2.26 ± 0.08****	2.47 ± 0.09	2.41 ± 0.13	2.76 ± 0.32	**2.22 ± 0.29****

ALB = albumin; ALP = alkaline phosphatase; ALT = alanine aminotransferase; AST = aspartate aminotransferase; Ca = calcium; CHOL = cholesterol; GLU = glucose; h = hours; LACT = lactate; LDH = lactate dehydrogenase; PHOS = phosphorus; SD = standard deviation; TG = triglycerides; TP = total protein

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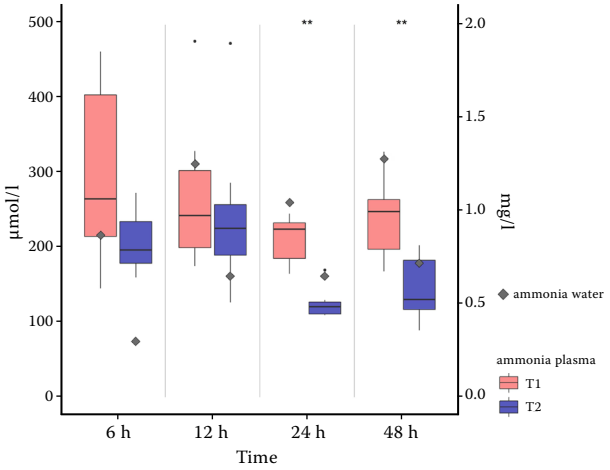

Figure 2. The comparison of the ammonia detected in the plasma (µmol/l) of the experimental fish *C. carpio* (n = 7) – left axis, and ammonia excreted into the water (mg/l) – right axis, in the T1 (27.8 ± 1.0 °C) and T2 (16.8 ± 1.0 °C) water temperature at 6, 12, 24 and 48 hours (h). Comparison between the T1 and T2 groups at the same sampling time; $^{**}P < 0.01$ and outliers denoted as points for the plasma ammonia

The food passage through the digestive tract was slowed down and the weight of the digestive tract and feed was significantly higher for the T2 group. The feed form was more liquid and the intestine was visibly inflated in some fish from the T2 group.

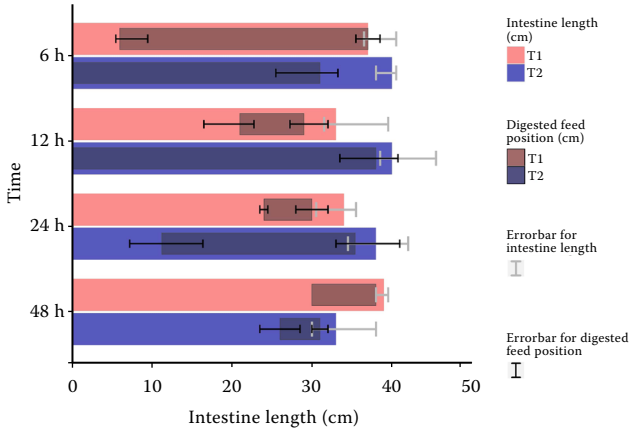

Figure 4. Digestive tract analysis – intestine length (cm) and food content in the intestine (cm) of the experimental fish *C. carpio* (n = 7) in the T1 (27.8 ± 1.0 °C) and T2 (16.8 ± 1.0 °C) water temperature at 6, 12, 24 and 48 hours (h). Comparison between the T1 and T2 groups at the same sampling time

Figure 4 reports the length of the part of the intestine where the food content was present (i.e., where the undigested feed begins and ends) for each fish as compared to the whole intestine length. For 71.4% (n = 5) of the fish in the T1 group, the intestine was empty 24 h after the beginning of the trial, while in the T2 group only for 14.3% (n = 1) of the fish at the same time. However, at

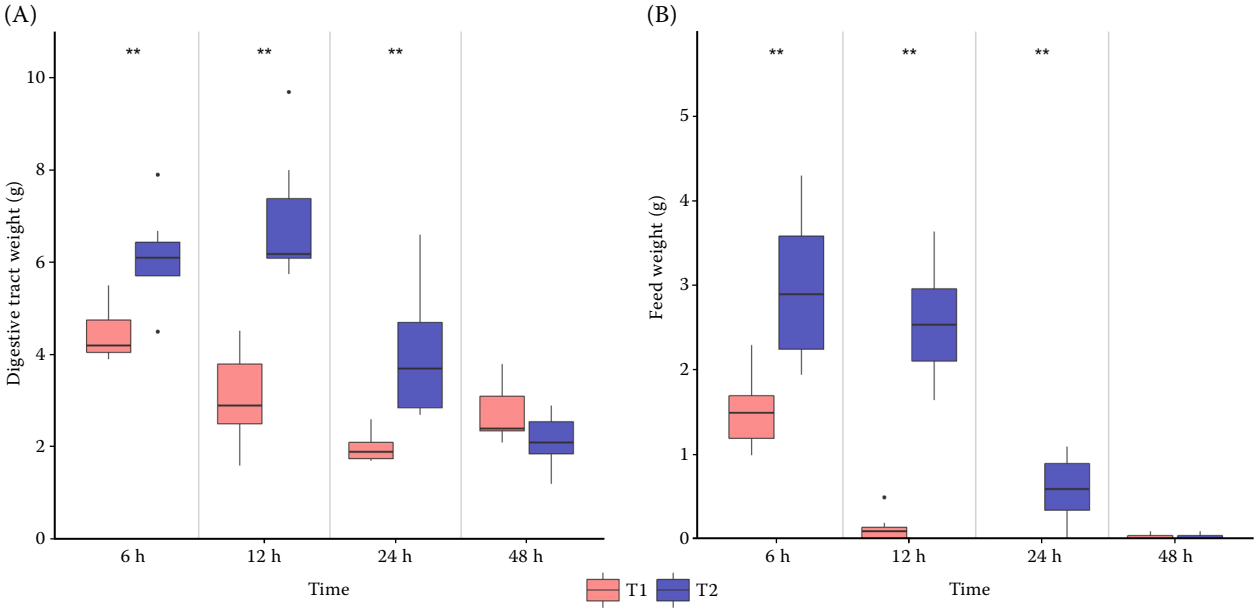

Figure 3. The weight (g) of the digestive tract (A) and undigested feed in the intestine (B) of the experimental fish *C. carpio* (n = 7) in the T1 (27.8 ± 1.0 °C) and T2 (16.8 ± 1.0 °C) water temperature at 6, 12, 24 and 48 hours (h). Comparison between the T1 and T2 groups at the same sampling time; $^{**}P < 0.01$. The outliers are denoted as points

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48 h, the food content passage was finished for 85.7% (n = 6) and for 71.4% (n = 5) of the fish in the T1 and in T2 groups, respectively, which means that equilibration occurred after the subsequent 24 h, probably due to evacuation of the T2 intestine content due to the feed fermentation.

Histopathological analysis

In the gills, damage along with the deformation of the lamellas was observed in the fish exposed to the cold water at 48 h (57.1%, n = 4). In some areas, the lamellas were completely missing and necrotic areas were present (85.7%, n = 6). Vacuolisation and, in some fish, pigmented deposits were also found at 48 h in the T2 group (42.9%, n = 3). The finding of a massive mononuclear infiltration indicates that an inflammation process developed as a response to the cold stress at 48 h (100%, n = 7). The comparison between the histopathological findings in the gills of the fish at 48 h in the T1 and T2 groups is shown in Figure 5.

In the skin samples, the superficial destruction of the epidermis was observed in the T2 group (100%, n = 7) at 48 hours. Moreover, the inflammatory infiltrate was present (85.7%, n = 6) and the mucus mass with exfoliated cells was observed on the skin surface, probably as a consequence of the low temperature (71.4%, n = 5) at 48 hours. The comparison of histopathological findings in the skin of the T1 and T2 groups at 48 h are given in Figure 6.

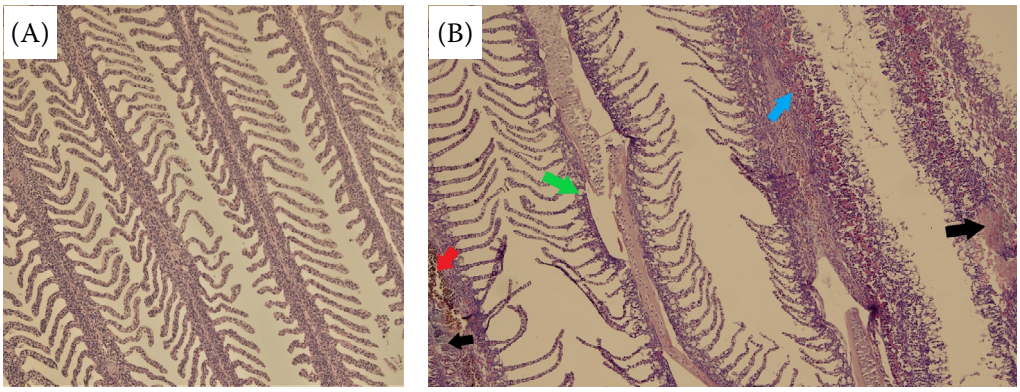

Figure 5. Physiological findings in the T1 (27.8 ± 1.0 °C) *C. carpio* gills (A) at 48 h compared to the histopathological changes (B) in the T2 group (16.8 ± 1.0 °C) at 48 h – devastation and necrosis of lamellas (black), vacuolisation (green), mononuclear infiltration (blue), pigmented deposits (red); magnification 100×

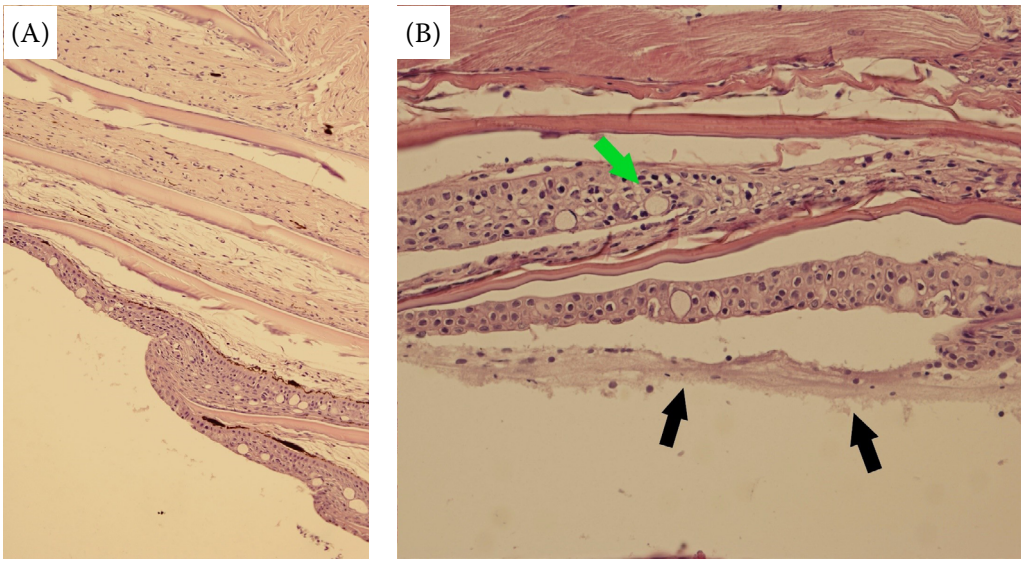

Figure 6. Physiological findings in the T1 (27.8 ± 1.0 °C) *C. carpio* skin (A) at 48 h (magnification 200×) compared to the histopathological changes (B) in the T2 group (16.8 ± 1.0 °C) at 48 h – superficial exfoliated cells (black), inflammatory infiltrate (green); magnification 400×

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The histopathological analysis of the other tissues (cranial kidney and liver) did not reveal any pathological changes.

DISCUSSION

As fish are ectothermic animals, their metabolic rate is much more severely affected by temperature than it is in endothermic organisms. The higher the temperature, the more intensive the body metabolism is (Svobodova et al. 1993). Fish, in general, are able to acclimatise to long-term temperature changes. However, one of the most hazardous stress factors is a sudden change in temperature without acclimatisation. Our study was also focused on the examination of the cold stress effects after the sudden transfer of *C. carpio* to cold water with the aim of simulating the commonly found process in fish farming, where market-ready fish are exposed to a lower water temperature after transport from the rearing ponds to the storage ponds (Svobodova et al. 2007). We endeavoured to investigate the potential changes in the physiological indices and their duration.

During our trial, anaemia developed among the T2 group, probably by a similar mechanism as the vasoconstriction response to cold exposure observed in mammals (Lenis Sanin et al. 2015). In fish, the gill vessels try to reduce the flow of cold blood into the brain while exposed to the cold stress to prevent the sudden heat losses and maintain the body organs in function. Moreover, pathological findings were found in the gill tissue (Figure 5) which are probably related to the lower oxygen exchange between the gill and the external environment (van den Burg et al. 2005; Donaldson et al. 2008; Barros et al. 2009; Lenis Sanin et al. 2015). Regarding the WBC in our study, higher levels were observed at 6 h for the T2 group followed by a decreasing tendency with a subsequent return to the control values. The increase is probably a result of the leukocyte migration from the spleen into the bloodstream, which is a commonly found in an acute response to stress in fish (Ruane et al. 2000; Fazio et al. 2015).

Additionally, neutrophilia was found at 6 h in the cold temperature groups, and from 12 h, the segmented neutrophils (older forms) started to decrease significantly. However, the precursors of the neutrophils, metamyelocytes, were significantly elevated at 6 h in the T2 group. The increase in the NLR – an important stress indicator used in research on mammals, amphibians and fish (Davis and Maney 2018), was found in our study in all the sampling times. In this regard, a sudden temperature change without adaptation is an important stressor for fish, whether the shift is to the upper or the lower limit of the temperature spectrum (Donaldson et al. 2008). All the haematological indices in our study were equal to the control during the 48 hours. Thus, the final temperature did not influence the haematopoiesis and blood cell counts.

Regarding the biochemical profile, the decrease in the plasma ALB, TP and TG in the fish kept in the colder water (T2 group) was caused by lowering their food content passage speed and decreased metabolism. Also, the involved stress could cause osmoregulation disorders, as referred to in the case of various fish species (Liebert et al. 2006; Vargas-Chacoff et al. 2018), and, thus, the relative lowering of the biochemical indices due to the increased blood volume in the vessels could be the reason for these findings. Contrary to the TG, the decrease in the ALB, TP and ammonia induced by decreasing the water temperature in the fish of the T2 group continued 48 h after the beginning of the trial. The effect of the food content passage speed slowdown was the most pronounced in the ALB after 12 h and 48 hours. The ALB concentrations were only at a 65% and 66% level compared to the T1 group.

It is known that fish respond to stress by the activation of catecholamines and corticosteroids, followed by increased heart and metabolic rates and increased LACT (Portz et al. 2006). In our study, the LACT levels were significantly ($P < 0.01$) elevated in the colder water at 6 h, while the LACT elevation was probably a response to the vasoconstriction induced by the cold shock and lower oxygen levels resulting from it. Thus, the muscles were probably forced to work under anaerobic conditions and raised the LACT (Guderley 2004). However, the levels returned to the T1 ones after 24 hours. Additionally, Foss et al. (2012) reported the cold stress (Δ –8 °C to –16 °C) impact on Atlantic salmon *Salmo salar* (Linnaeus 1758) with similar results. This experiment induced the raising of the LACT after 1 h, which returned to the control levels after 6 h. In the same study by Foss et al. (2012), the ALT and AST enzymes were significantly increased at 48 h, while the ALP one was significantly de-

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creased at the same time. The ALP enzyme is an indicator of the bile duct function while its activity decreases when fish are fasting and, additionally, could also relate to damage to the RBCs, (Peyghan and Takamy 2002; Hoseini et al. 2018). Its decreasing tendency after 24 h could be a consequence of the slowed food content passage speed and decreased bile production. The RBC fall was most significant after 12 h to 24 h and the sudden decrease in the ALP is probably the response to this fall. AST is a non-specific enzyme and indicator of tissue health, whereas ALT is an enzyme which is most specific for the fish liver and kidney function (Hoseini et al. 2018). Additionally, the AST increases are also connected with the RBC levels and gill tissue damages (Mirghaed et al. 2018). Based on our histopathology results, the liver and kidney tissue did not show any observable changes and, therefore, their effect on the enzyme's activity is negligible. However, as described below, there were some pathological findings observed in the gill tissue which could be relative to the AST changes. The ammonia autointoxication of *C. carpio* by a sudden temperature decrease is a commonly known issue in Czech fish hatcheries (Svobodova et al. 2007), when fish are intoxicated by their own metabolites due to the stopped metabolism and their slowed excretion into the external environment. Therefore, an ammonia analysis was included in our experiment. Even though the concentration of ammonia in the fish plasma was at the physiological levels, i.e., 200–500 μmol/l (Svobodova et al. 2007) in all the examined groups, the ammonia concentrations were decreased in the T2 group compared to the T1 one during the whole experiment, with a significance ($P < 0.01$) at 24 h and 48 hours. In addition, almost double the average ammonia concentrations were excreted into the water in warmer T1 group than in the colder temperature. Thus, as a consequence of the slowed metabolism in the cold water, lower amounts of nitrogenous substances, such as ammonia, are formed and also excreted. However, no common signs of autointoxication such as a gill and body discolouration or behavioural changes were observed. However, the necrosis of the gill's lamellas, with mononuclear infiltration and pigmented deposits were found, as referred to in Figure 5. This damage is probably the reason for the lowered excreted ammonia levels. We hypothesise that the metabolism and the Krebs cycle were decelerated in the colder water, but not to hazardous levels.

The food content passage speed was slowed down when the fish were exposed to the cold stress. In the T1 group at 24 h, the food content passage speed was faster by about 57.1% than in the T2 group at the same time. However, at 48 h, the difference between the T1 and T2 groups was 14.3%, which means that the digestive tract is able to balance the temperature changes after 24 hours. The study of Lopez-Luna et al. (2016) showed similar results. In their study, slaughter fish were fasted for various time-temperature combinations and their gut emptied more quickly in the water with higher temperatures (Δ +11.6 °C).

The histopathological findings in the gills of fish exposed to the cold stress revealed the deformation of the lamellas with elevated infiltration of mononuclear cells. Moreover, haemorrhagic or even necrotic areas were observed. As a response to ammonia autointoxication, toxic necrosis and a darker gill colour along with haemorrhages and oedemas are commonly found (Svobodova et al. 1993; Svobodova et al. 2007; Harper and Wolf 2009), but based on our ammonia analysis, autointoxication was not detected. Also, fish, in general, respond to various stressful conditions by the higher production of skin mucous (Sanahuja et al. 2019). In our experiment, the mucous coagulation was observed on the skin surface probably due to increased activity of mucous cells aiming to support the innate fish immunity in cold water. Fish mucous has a bacteriostatic function and is part of the innate immunity (Saurabh and Sahoo 2008). Moreover, a sudden decrease in the temperature is known to decelerate the production of antibodies, impair the function of natural killer cells and, in consequence, invoke immunosuppression in fish (Harper and Wolf 2009). Thus, the disruption of the physiological mucous barrier could negatively affect the immunity of fish and could increase the presence of parasites in fisheries. For example, Svobodova et al. (2007) observed an increasing parasite invasion due to the cold stress and ammonia intoxication.

In summary, based on the haematological and biochemical indices, the digestive tract analysis and the histopathological indices in our experiment, the deceleration of the fish metabolism after their transfer to the colder water was observed. However, the haematological and digestive tract

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indices were able to balance the cold stress impact approximately after 24 hours. In the case of the biochemistry, the changes in the physiological levels were more permanent. These factors acting together could impact the metabolism, homeostasis and innate immunity of the fish and, thus, have a positive correlation with the occurrence of the diseases in fisheries and hatcheries. Moreover, the changes caused by the cold stress could be, in some cases, lethal and, therefore, a gradual drop in temperature is highly encouraged.

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Conflict of interest

The authors declare no conflict of interest.

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