Full length article

Immune response of juvenile common carp (*Cyprinus carpio* L.) exposed to a mixture of sewage chemicals

M. Tarnawska[a,*], M. Augustyniak[a], P. Łaszczyca[a], P Migula[a], I. Irnazarow[b], M. Krzyżowski[a], A. Babczyńska[a]

[a] *Department of Animal Physiology and Ecotoxicology, Faculty of Biology and Environmental Protection, University of Silesia in Katowice, Bankowa 9, 40-007 Katowice, Poland*
[b] *Polish Academy of Sciences, Institute of Ichthyobiology & Aquaculture in Gołysz, Kalinowa 2, 43-520 Chybie, Poland*

A B S T R A C T

Pharmaceuticals and household chemicals are important components of municipal sewage. Many of them are biologically active, disrupting not only hormonal regulation of aquatic animals but also, indirectly, disturbing their immunological protection. In the environment, chemicals rarely act as individual substances, but as elements of mixtures. Therefore, the aim of this study was to check whether the acute laboratory exposure of common carp juveniles to a mixture of ibuprofen, sodium dodecyl sulphate (SDS), dimethyl sulfoxide (DMSO) and 17 α-ethynylestradiol in increasing concentrations, modifies the levels of innate immunity (lysozyme, C-reactive protein) as well as general stress (metallothioneins, heat shock proteins HSP70) markers in brain, liver, gills, spleen and mucus. The levels of the markers were measured by an immunodetection technique. Not only do the pharmaceuticals and household chemicals impair immunological reactions of young carp in various tissues but also do that in a concentration-dependent manner in the liver, gills, spleen and mucus. This has a very important implication, since it may result in higher sensitivity of young fish to pathogens due to energy allocation to defence processes. The comparisons of the pattern of stress reactions in the studied organ samples indicated that mucus appeared to be a good, non-invasive material for monitoring of environmental state and fish conditions.

1. Introduction

Carps and other cyprinids are important fish at a global scale. Their production in 2016 was over 50% of the total freshwater aquaculture production (32 138 921 tonnes) (FAO 2018). Common carp (*Cyprinus carpio*) is one of the most popular species in the European Union. It is farmed in inland freshwaters in 18 Member States. However, over half of total production of this species originated in only two countries: Poland and the Czech Republic [1].

Since this species has high importance in freshwater aquaculture, many aspects of its physiology, nutrition, genetics, and diseases have been studied in past decades [2]. The role of common carp in ecosystems has been examined, but the knowledge about the immune potential of juveniles under sewage chemical exposure is still lacking.

The immune system of fish is physiologically similar to that of other vertebrates, despite certain differences. In contrast to higher vertebrates relying on the adaptive immune system, most fish species are free-living organisms from early embryonic stages and depend predominantly on

their innate immune system [3–6]. Nonspecific immunity is commonly divided into three compartments: the epithelial/mucosal barrier, the humoral parameters and the cellular components. The epithelial and mucosal barrier of the skin, gills and alimentary tract are extremely important in fish, being constantly immersed in media containing potentially harmful agents [7,8]. The immune system and response of fish can be greatly influenced by various factors. Chemical stress inducers present in water are of main interest in relation to this study. Anthropogenic chemicals, present in sewage, may leak to surface and groundwater and thus can also be detected in aquaculture [9]. Many synthetic chemicals are recognised as endocrine disruptors (ED) and once released into the environment are likely to spread and accumulate in wild species. These compounds mimic the effect of natural hormones, interact with their receptors and induce the activation of the same physiological pathways, affecting reproduction and altering neuro-development and metabolic processes also in lower invertebrates [10,11]. Biologically active compounds are also known to modify the immunological response of aquatic organisms. For example, the level of C-

reactive proteins (the main inflammation marker, elevated as the results of exposure to harmful chemicals), is regarded as a biomarker of not only general fish fitness but is also helpful in the assessment of the pollution of the water in their habitat [12]. Even if the specific mechanisms of ED influence on the immune system are still not fully understood, their environmental effects and immunological response correlations have been already described [13]. Rogers et al. [14] in their review analyze among others estrogen receptors, that transduce signals disturbing immunity mechanisms. There is a number of scientific reports that reveal an estrogen-influenced changes in the level of immune parameters in fish. Milla et al. [15] reviewed this influence at various levels of fish immunity defense. Especially interesting are however, the data proving the changes in the antibacterial protein levels in response to exposure to estrogen-like compounds. The data can be found in Fatima et al. [16]. In this study, *Carassius auratus* individuals were exposed to herbicide that has an endocrine disrupting action on fish. In the animals exposed to the herbicide mixture, lysozyme activity was enhanced. Authors concluded the exposed fish were more sensitive to infections than the control ones. This was proved by higher mortality of fish exposed to the pesticides and then infected with bacterial suspension. What is even more alarming, the contact of parents with endocrine disruptors is also reflected in the immune potential of their offspring. Even if the exact mechanism is still unknown, in the experiment of Dong et al. [17] the exposure of *D. rerio* parents to Bisphenol A (an endocrine disrupting chemical) appeared to result in innate immune dysfunction of the offspring.

The exposure of young fish to harmful chemicals may result in their higher sensitivity to pathogens due to energy allocation to detoxication processes, instead of immune defense. In case of prolonged exposure this may cause the elimination of immature individuals from the population and disturbances in the overall age structure. In aquaculture, this may be the cause of not only economic but also cultural losses.

General fitness of the organism relies on the proper functioning of each organ and system. Under environmental stress, this condition appears even more important since a new counterbalance between organismal functions has to be established. In this paper, the choice of the organs studied was dictated by the localisation and potential contact with the external environment (gills and mucus) and metabolic role connected with processing of molecules of both internal and external origin (liver and spleen).

Substances tested in our project widespread and frequent components of municipal sewage are present in microconcentrations in the environment. They belong to the groups of pharmaceuticals (ibuprofen, 17 α-ethynylestradiol), detergents (sodium dodecyl sulphate – SDS) and solvents (dimethyl sulfoxide – DMSO, although it is also used as a medicament itself). The chemicals enter the natural environment as the result of inefficient (or lack of) sewage processing. Their toxicity manifests itself at various levels of biological organisation and, among others, they disrupt endocrine functions in vertebrate and invertebrate aquatic animals. The endocrine disrupting activity of ibuprofen was reported in mussels [18] and in zebrafish [19]. The role of DMSO in endocrine disruption was found in mudsnails [20]. 17 α-ethynylestradiol is a hormonal pharmaceutical with endocrine disrupting action towards non-target animals, especially fish [21] [22–24]. Due to its abundance in freshwater ecosystems together with its reproductive and sex ratio effects on the ecosystem balance, 17 α-ethynylestradiol has been included into a European Commission watch list of 10 substances for which European Union wide monitoring should be performed in the aquatic environment [25]. There are numerous studies conducted in aquatic environments demonstrating either the range of concentrations or toxicity values for aquatic organisms, concerning these chemicals, depending on their nature and application (e.g.: ibuprofen: [26,27], 17 α-ethynylestradiol: [28,29], DMSO [30,31] and SDS [32,33]).

Many studies have focused on the effects induced by the exposure to a specific compound. However, combined effects of mixtures of substances with dissimilar modes of action are much more frequent in the environment [34,35]. The influence of time on the nature of environmental changes should be considered. The difference between acute and chronic environmental impacts will be seen in the immune response. Acute impacts will involve the innate immune system whereas chronic impacts will involve the adaptive immune system. Due to limitations of the adaptive immune system (poikilothermic nature, narrow repertoire of antibodies and the slow proliferation, maturation and memory of their lymphocytes), in fish the nonspecific immunity is an essential component in combating pathogens [4–6]. Innate immune system, comprises a series of physiological and biochemical reactions, including, e.g. acute phase proteins production which may be an indicator for fish health [36–39].

The acute phase proteins, e.g. C-reactive protein (CRP), lysozyme (Lys) and stress proteins: metallothioneins (Mts), heat shock proteins (HSP) are biochemically and functionally unrelated proteins. They are predominantly synthesised in the liver and involved in a variety of defence related activities, e.g. restoration of damaged tissue and healthy condition [39,40].

C-reactive proteins and lysozyme are present in the body fluids of both invertebrates and vertebrates. CRP is commonly associated with the acute phase response. Lysozyme, in turn, is bactericidal, hydrolysing b- [1,4] linked glycoside bonds of bacterial cell wall peptidoglycans [7,8]. Metallothioneins, apart from their role in the regulation of homeostasis of biogenic elements and elimination of xenobiotic metals, contribute to an antioxidative defense and the regulation of apoptosis and cell growth [40]. HSP is a highly conserved family which is the most studied as a prospective biomarker of stress. It plays under favorable conditions an essential role in a cell, by chaperoning proteins during folding, assembly, intracellular trafficking and degradation. It is also involved in the cell regulatory pathways [41,42]. Moreover, for monitoring purposes, HSP70, Mts and Lys are regarded as early warning biomarkers [43–46].

Taking all the information above into account, in this paper we decided to verify the following hypotheses:

(i) The exposure of common carp fry individuals to the mixture of pharmaceuticals and household chemicals modifies the concentration of selected stress and acute phase proteins in various organs. The degree and direction of the modification depends on the mixture concentration.

Assuming tissue specificity we decided to compare only the pattern of acute phase and stress protein response not the absolute values of their concentrations. Therefore the following hypothesis will be tested:

(ii) The pattern of stress and acute phase protein concentration reflects the organ function and specificity.

2. Material and methods

2.1. Experimental fish

Juvenile common carp (*Cyprinus carpio* L.) (9-month-old, average initial weight 26 ± 1 g) were obtained from Institute of Ichthyobiology and Aquaculture in Gołysz, Polish Academy of Sciences. Acclimatisation and rearing were carried out in 60-L aquariums in static conditions with the following water quality parameters: temperature 19.0 ± 2.0 °C; pH range 7.3–7.6, mean aeration 58%. Fish were distributed in 6 aquaria at a density of 50 fish per aquarium and acclimatised for two weeks prior to the experiment. During the pre-experimental period fish were reared under specific pathogen-free conditions at 20 ± 1 °C in UV treated re-circulating systems under a 12:12 h light:dark cycle. Carp were fed daily with pelleted dry food (Aller Aqua, Poland) at 3–5% body weight.

All procedures involving animals were conducted in accordance with Polish laws on animal experimentation and were approved by the

Table 1
Concentrations of the mixture components for each experimental group. The concentration of compounds are multiplied by 1x, 3.2x, 10x, 32x and 100x for group A-E respectively.

Compound [µg/L]	Group A	Group B	Group C	Group D	Group E	Control O
Ibuprofen	0.8	2.6	8	26	80	0
SDS	16.7	53.5	167	535	1670	0
DMSO	8	26	80	260	800	0
17 α-ethynylestradiol	0.0008	0.0026	0.008	0.026	0.08	0

Local Ethic Committee for Experiments on Animals (resolution no 10/2014).

After acclimatisation, the fish were exposed to a mixture of pharmaceutical and household chemicals (Table 1) for 72 h (each aquarium was assigned to one experimental group, randomly). Prior to the application in tanks, chemicals were initially dissolved in distilled water. The mixtures were added in a single dose. The concentration of compounds, obtained by the usage of the following multipliers: 1x, 3.2x, 10x, 32x, and 100x for groups A-E respectively, used in the experiment was environmentally realistic and selected according to literature data and OECD requirements [20,47–51].

All selected compounds were purchased in the highest available purity from Sigma Aldrich.

After this treatment, the fish (6 per each experimental group) were transferred to the laboratory of the Department of Animal Physiology and Ecotoxicology, Faculty of Biology and Environmental Protection, where they were immobilised on ice in plastic bags for 30 s for mucus collection and then decapitated swiftly. After that, the fragments of gills, liver, brain and spleen (approx. 50 mg) were immediately excised, placed in Eppendorf tubes and frozen at −70 °C until further analyses.

Before the analyses, the biological material was defrosted in air. Thawed samples were homogenised on ice in 1 mL of 0.1 M Phosphate-Buffered Saline (PBS) buffer, pH 7.4 with 5 mM sodium azide, 0.1 mM phenylmethylsulphonyl fluoride and 20 mM 2-mercaptoethanol.

Homogenates were then centrifuged at 4 °C, 15 000 g for 10 min. In the supernatants, total protein concentration was measured and detection of lysozyme (Lys), C-reactive protein (CRP), metallothionein (Mts) and heat shock proteins (HSP70) was performed.

2.2. Total protein concentration

Total protein concentration was measured according to the Bradford method [52]. The method is based on the binding of aromatic amino acids to the Coomassie Brilliant Blue (CBB, G-250, Sigma) dye with the v/v 1 (sample): 50 (CBB solution) ratio. The absorbance was measured at the wavelength of 595 nm, and the colour intensity is proportional to protein concentration. The protein concentration was calculated from the calibration curve prepared from the absorbance measurements of the bovine serum albumin (protein content > 95%, Sigma) solutions of known concentrations [52].

2.3. Innate immunity markers and stress protein immunodetection

For Lys, CRP, Mts and HSP70 concentrations in gill, liver, brain, spleen and mucus samples an indirect ELISA technique was applied, according to standard protocol [53]. Briefly: the wells of 96-well Corning, transparent flat bottom plates (a separate plate for each antigen) were filled with 100 µL of supernatant samples of known protein concentration and then the potentially uncoated areas of the plates were blocked with 1% bovine serum albumin (Sigma; 1 h, 37 °C). Anti-Lys, anti-CRP, anti-Mts and anti-HSP70 primary antibodies (mouse Anti-Lysozyme monoclonal antibody, Abcam, 1:5000; mouse Monoclonal Anti-C-Reactive Protein antibody, Sigma – Aldrich, 1:40 000; mouse metallothionein monoclonal antibody, Stressgen; 1:1000; mouse anti-Heat Shock Protein Monoclonal antibody; Sigma – Aldrich; 1:1000,

respectively; 2.5 h 37 °C). As secondary antibody goat anti-mouse IgG Polyclonal Antibody, AP Conjugate (Stressgen; 1:1000; 2 h 37 °C) were subsequently used. Between each application, the wells were washed 3 times with 0.05% Tween-20 detergent in 0.05 M Soerensen's phosphate buffer, pH 7.4. Finally, the secondary antibody was replaced by 100 µL pNpp (p-nitrophenyl phosphate, Sigma) solution in 10 mM diethanolamine buffer, pH 9.5, 0.5 h, room temperature. Mts concentration was assessed spectrophotometrically at the wavelength of 405 nm by means of Tecan Infinite M200 microplate reader. The contents of the detected proteins were expressed as absorbance values, which are proportional to the antigen contents in the samples. The comparability of the results was guaranteed by the application of the same total protein content per well for each sample and each antigen.

Immunodetection procedures were preceded by 'in silico' analyses of similarities between immunogens for each I-st order antibody and respective carp antigen.

2.4. Statistical analyses

The normality of data for all of the parameters was checked using the Kolmogorov-Smirnov and Shapiro-Wilk tests. The homogeneity of variances was tested using the Levene test. As the results of all parameters fulfilled the criteria for normal distribution and variance homogeneity, parametric tests were used to evaluate the significance of the differences among the experimental groups. Lys, CRP, Mts and HSP levels were expressed as the mean ± SD in the figures (Figs. 1–4). 2-way ANOVA was done (Table 2). Then, since experimental group or organ had a certain effect, Tukey honest significant difference for unequal N (HSD test, ANOVA; p < 0.05) was used within an organ, to identify differences among experimental groups (Figs. 1–4).

Correlation between chemical mixture stress and innate immunity parameters (Lys or CRP; Table 3) in each tissue was separately calculated using Pearson correlation coefficient (r).

Principal Component Analysis (PCA) was performed for evaluating the relations among all parameters for all the tissues analysed together (Fig. 5). Moreover, to find similarity among all parameters in all analysed tissues, Hierarchical Cluster Analysis (HCA) for all combined parameters was carried out (Fig. 6). Statistical analysis was performed using Statistica 13.1.

3. Results

No mortality of fish was recorded for all concentrations tested.

3.1. Lysozyme (Lys) levels

The values of this parameter in gills, mucus and spleen were positively correlated with concentration of mixture of pharmaceuticals and household chemicals tested in the experiment (Table 3). The exposure caused a statistically significant increase in lysozyme levels when compared to the control, in two groups of the highest chemicals concentrations (groups D and E, Fig. 1).

The pattern of the lysozyme response to various concentrations of the chemical mixture was similar in gills, mucus and spleen, irrespectively of the absolute absorbance values.
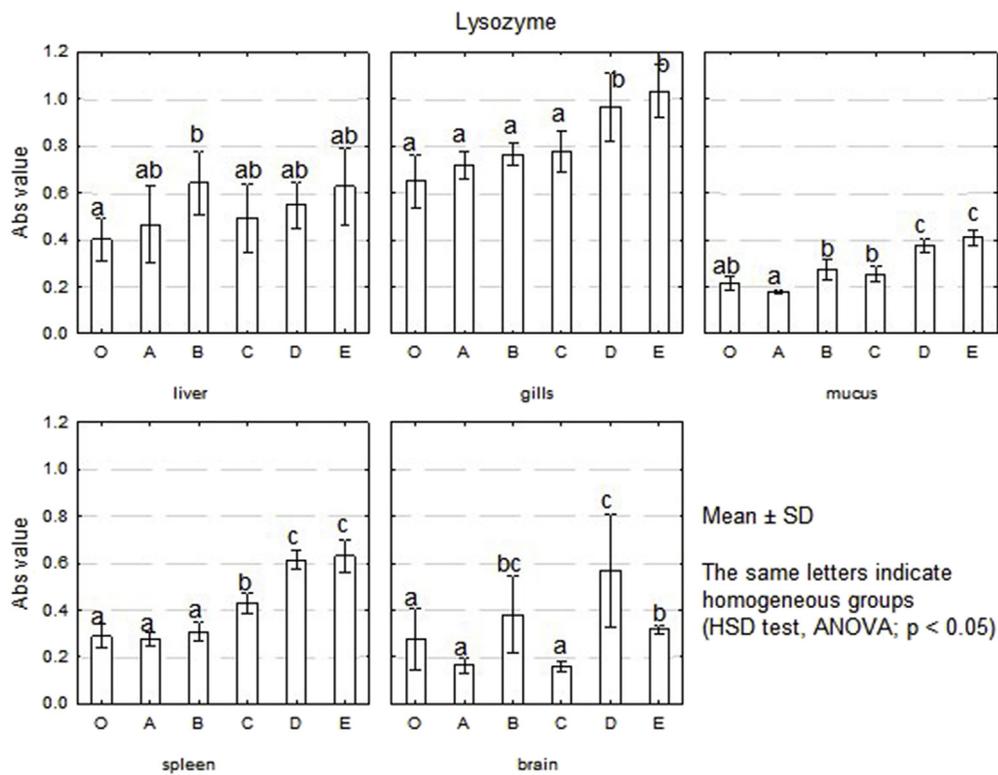
Fig. 1. Lysozyme level (absorbance value, mean ± SD) in different organs and mucus of juvenile common carp (*Cyprinus carpio* L.) from control (O) and experimental (A–E) groups. The same letters indicate homogenous groups (n = 6; HSD test, ANOVA; p < 0.05).

Fig. 2. C-reactive protein level (absorbance value, mean ± SD) in different organs and mucus of juvenile common carp (*Cyprinus carpio* L.) from control (O) and experimental (A–E) groups. The same letters indicate homogenous groups (n = 6; HSD test, ANOVA; p < 0.05).
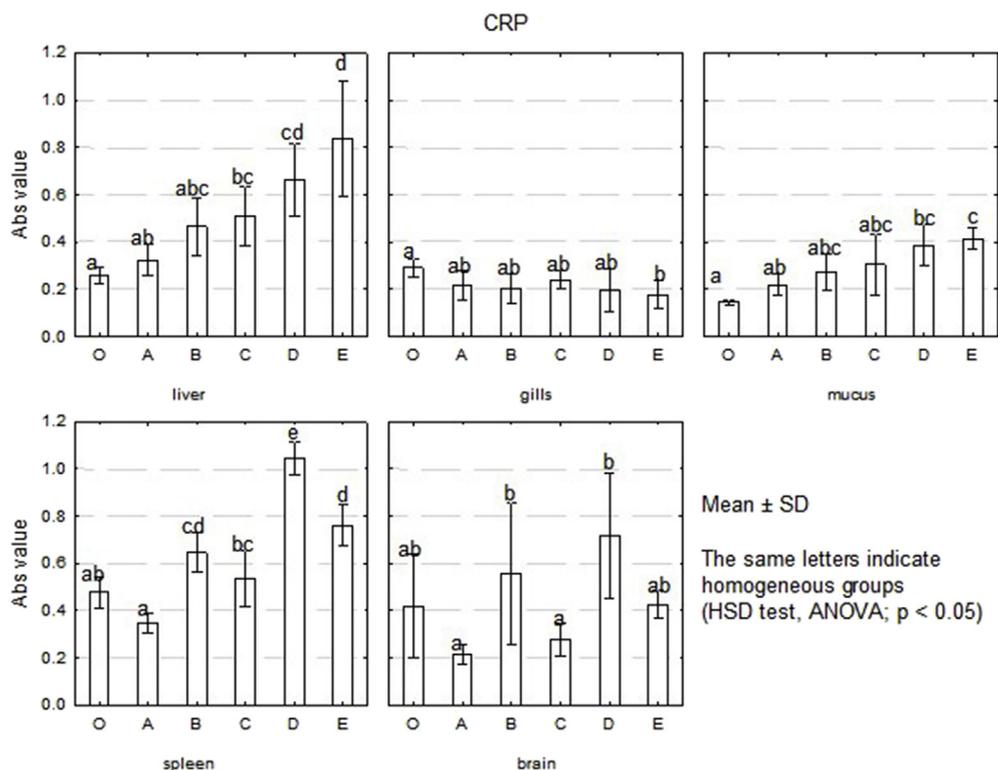
Fig. 3. Metallothionein level (absorbance value, mean ± SD) in different organs and mucus of juvenile common carp (*Cyprinus carpio* L.) from control (O) and experimental (A–E) groups. The same letters indicate homogenous groups (n = 6; HSD test, ANOVA; p < 0.05).
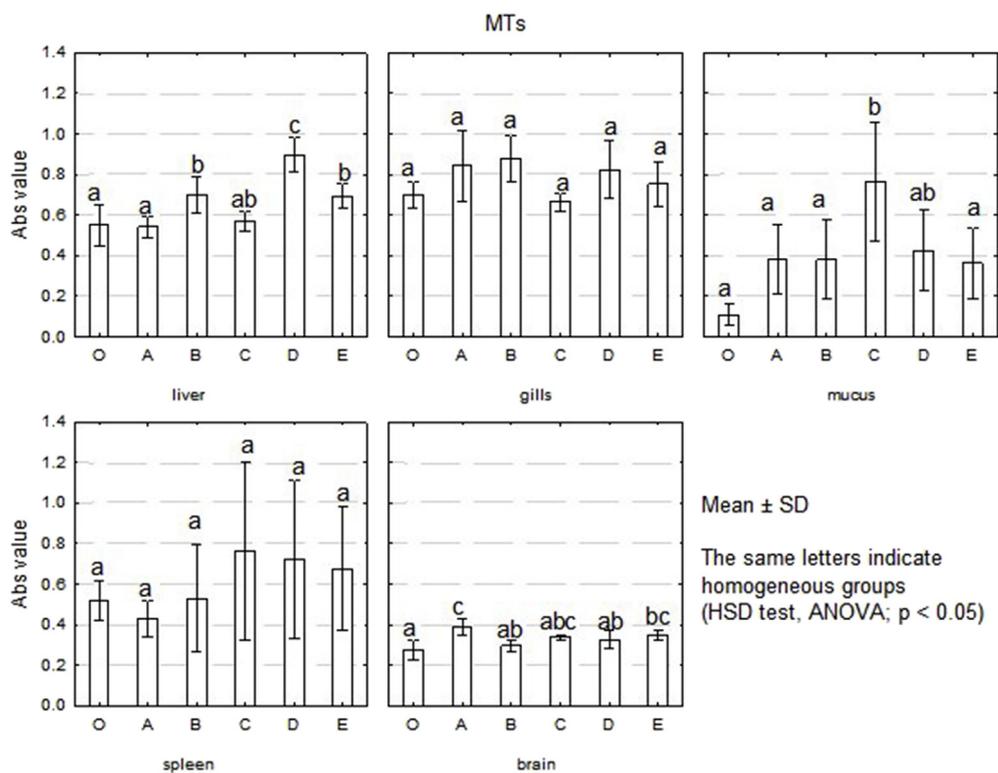
Analysis of PCA revealed significant relationships between lysozyme level and CRP protein concentration in liver, gills, skin mucus and brain, pointing to the importance of innate immunity in eliminating the effects of stress caused by a mixture of chemicals (Fig. 5).

3.2. C-reactive protein (CRP) levels

The pattern of CRP changes in response to increasing mixture concentration was similar in liver and in mucus. The highest values of the
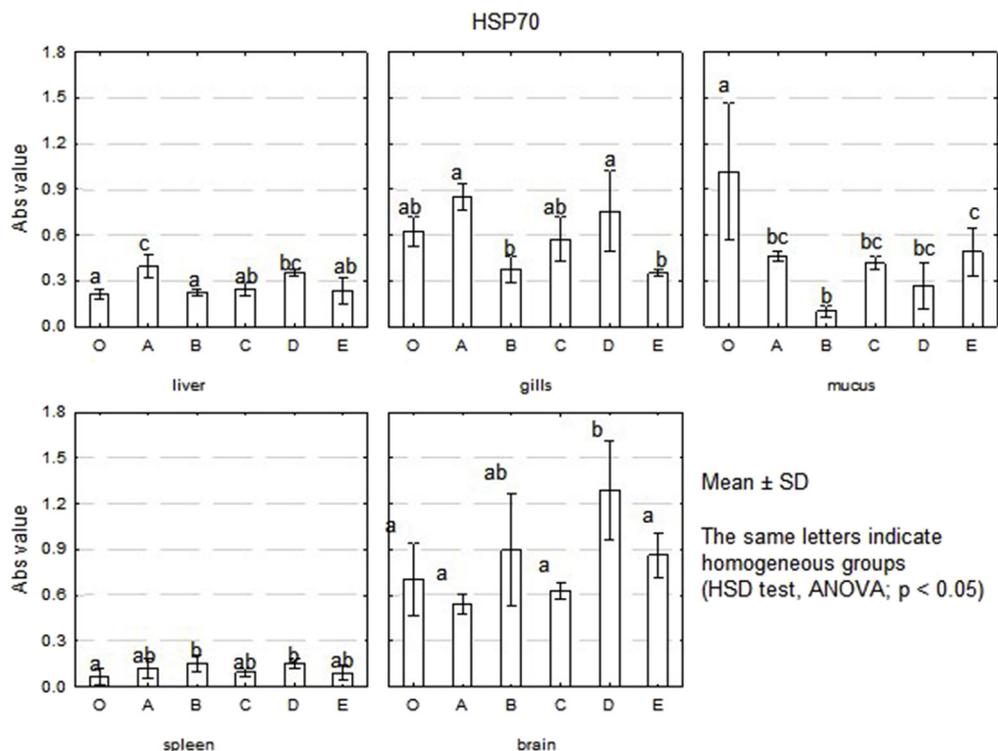

Fig. 4. Heat shock protein HSP70 level (absorbance value, mean ± SD) in different organs and mucus of juvenile common carp (*Cyprinus carpio* L.) from control (O) and experimental (A–E) groups. The same letters indicate homogenous groups (n = 6; HSD test, ANOVA; p < 0.05).

Table 2
Analysis of variance (ANOVA/MANOVA) for concentration of Lysozyme (Lys), C-reactive protein (CRP), metallothioneins (MTs) and heat shock protein 70 (HSP70) of juvenile common carp (*Cyprinus carpio* L.) from different experimental groups (with experimental group and organ as categorical factors).

		Lys		CRP		MTs		HSP70	
	d.f.	F	p	F	p	F	p	F	p
exp. group (1)	5	30.56	< 0.001	28.20	< 0.001	3.93	0.002	7.79	< 0.001
organ (2)	4	143.16	< 0.001	61.50	< 0.001	28.42	< 0.001	110.77	< 0.001
(1) × (2)	20	3.38	< 0.001	6.56	< 0.001	2.40	0.001	9.58	< 0.001

Table 3
Results of correlation analysis between stress and innate immunity parameters (CRP: C-reactive protein [absorbance value]; Lys: lysozyme [absorbance value]) and concentration multiplier of chemical mixture in various tissues of juvenile common carp (*Cyprinus carpio* L.) exposed to the mixture. The Table shows only statistically significant results.

tissue	protein	r	R^2	p
liver	CRP	0.746	0.557	< 0.001
gills	Lys	0.738	0.544	< 0.001
	CRP	− 0.363	0.132	0.032
mucus	Lys	0.808	0.652	< 0.001
	CRP	0.615	0.378	< 0.001
spleen	Lys	0.820	0.673	< 0.001
	CRP	0.495	0.245	0.002

absorbance measured in the individuals exposed to the highest mixture concentrations were about 3-fold higher than the initial values in the control groups in these two organs/tissues. Moreover, the increase of CRP level was proportional to the concentration of chemicals tested in the experiment, indicating that these tissues may be good indices of exposure to the mixture of toxins. In spleen, and especially in the brain, such unequivocal relationships were not found. Interestingly, the level of CRP in the gills of animals exposed to the highest concentration of chemicals in the mixture (group E) was significantly lower when compared to the control (Fig. 2). As in the case of lysozyme, analysis of correlation indicated that values of CRP in liver, mucus and spleen of fish were positively correlated with increasing concentration of chemicals in mixtures tested, while in gills negative correlation was found (Table 3).

3.3. Metallothionein (Mts) content

Independent variable 'experimental group' significantly influenced Mts level, but with a lower p -value than the previous two parameters (p = 0.002 for Mts vs p < 0.001 for CRP and Lys). Biological material (tissues or mucus) also significantly differed in Mts concentration; however, no unambiguous trends have been demonstrated (Table 2, Fig. 3). The exposure of animals to the mixture of chemicals had no effect on the content of Mts in gills and spleen, or it rose moderately in liver (groups B, D and E), mucus (group C) and brain (group A). Analysis of similarities and differences showed that mucus differs in terms of the pattern of response to increasing concentration of experimental mixture when compared to the remaining tissues (Fig. 3).

3.4. Heat shock protein (HSP70) content

Stress proteins reacted in a significant way to the exposure to the mixture of pollutants, as well as showed different concentrations in the tested material (Table 2). The HSP70 level decreased significantly in mucus when the fish were exposed to chemicals (groups A-E). In the brain, however, there was an increase in the concentration of stress proteins in the experimental group D. A similar trend as in the case of Mts was observed – there were no clear relationships between the concentration of heat shock proteins and the concentration of toxins in

the mixture. Additionally, in the spleen, negative correlation between HSP70 and Mts content was found.

3.5. Relations among all parameters – Principal Component Analysis (PCA)

Principal Component Analysis was carried out for each tissue separately, and also for all tissues simultaneously. In the liver, the principal component 1 (PC 1) was created by innate immunity parameters and explained 42.96% of all variance. Both innate immunity parameters, and also Mts were correlated well with each other (see angles between vectors). Innate immunity markers and MTs (PC 1) were not correlated with HSP70 (main element of PC 2; Fig. 5). In gills PC 1 explained over 50% of variation, and it is particularly well characterised by parameters Lys and CRP. So, again PC 1 describes innate immunity quite well. However, the values of Lys and CRP are located in the opposite direction, which reflects the negative correlation between these two parameters. Results of PCA analysis stay in compliance with correlation coefficients for CRP or Lys and stress level in gills. As the concentration of chemicals in the solution increases, the level of Lys increases but CRP decreases slightly (Table 3). The results of PCA analysis in mucus also revealed similarities between Lys and CRP; in this case, PC 1 explained 47.66% of variability. Again, PC 1 describe innate immunity very well, and both parameters are correlated well. It means that inflammation indicators increase in the mucus as the environmental stress intensifies. Such a result predisposes mucus as a good material for assessing a risk of exposure to environmental toxins. The second component (explained 27.17% of variability) was associated with Mts content and was not correlated with innate immunity (Fig. 5). Also, in the spleen, PCA analysis showed a similar pattern of Lys and CRP variability and different for HSP and Mts. In the spleen, the results of PCA analysis showed that two principal components (PC 1 and PC 2) explained 88.34% of the total variation of parameter values; however, linking the principal components (PC 1 or PC 2) with innate immunity (Lys and CRP) or stress markers (Mts or HSP70) is more difficult in this case (Fig. 5).

Undoubtedly, the highest value of PC 1 and PC 2 taken together was revealed in the case of the brain, and it amounted to as much as 95.63%. The first component is strongly related with the pattern of variability of Lys, CRP and HSP70 (all parameters are very well correlated). However, for all these parameters, no proportional dependence on the concentration of the substance in the mixture was found, and the pattern of protein content in the subsequent experimental groups showed similar fluctuations. Interestingly, the highest values were found in fish from the experimental group D, while the lowest in the individuals from group A (Figs. 1–2 and 4). The principal component 2 – Mts content – was not correlated with others parameters (Fig. 5).

Analysis of PCA for all tissues revealed PC 1 is created by Lys and Mts (39.93% of variance), and PC 2 is created by HSP70 and CRP (27.01%). Lys and Mts are well correlated with each other, but not with HSP70 or CRP. These two last parameters display negative correlation between each other (Fig. 5). Taking together all results for analysis makes it difficult for generalisation and finding general rules or relations between innate immunity and stress parameters. Therefore, we can claim that such an approach should not be applied.
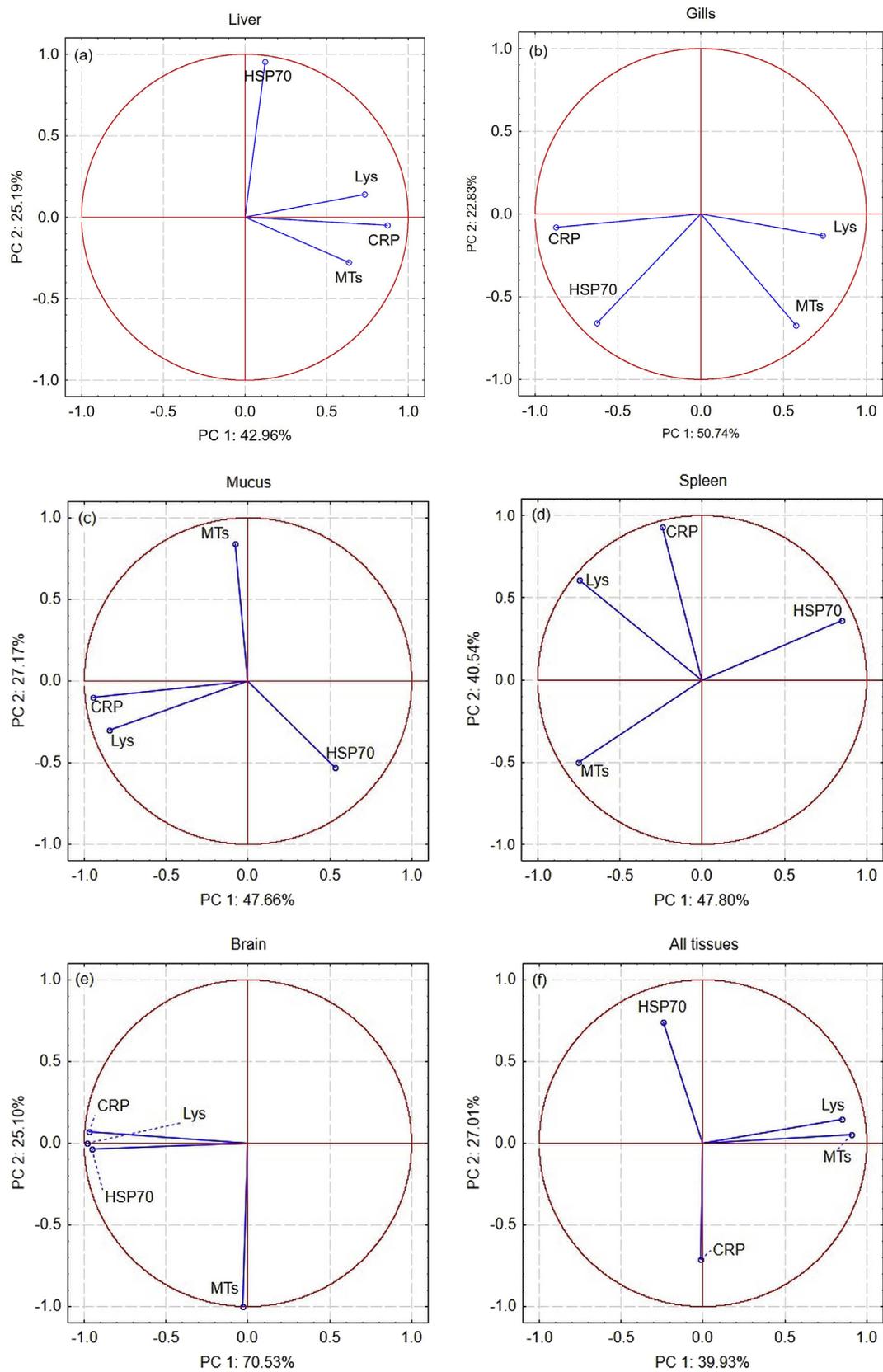
Fig. 5. Principal component analysis (PCA) to evaluate similarities between the absorbance values of the immunity and stress parameters in organs and tissues of juvenile common carp (*Cyprinus carpio* L.) analysed separately (a–e) or jointly (f). Absorbance values of each parameter were pooled from all experimental concentrations.
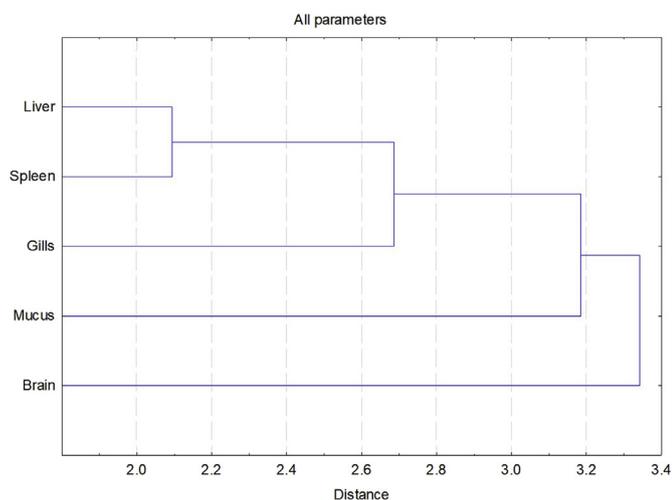
Fig. 6. Dendrogram of a Hierarchical Cluster Analysis (HCA) indicating similarity of tissues of juvenile common carp (*Cyprinus carpio* L.) from all experimental groups, including all measured stress and immunity parameters.

The Hierarchical Cluster Analysis predominately grouped organs/tissues from internal to external locations except for brain (an organ well protected from the influence of external factors) which was a clear outlier. Liver and spleen (two internal organs that have no direct contact with the polluted water) formed the tightest and most homogeneous cluster followed by hierarchical groupings (longer branches = less similar) to gills and mucus (Fig. 6). Then the skin mucus is the material that remains in significant distance from the internal tissues.

4. Discussion

One of the greatest problems in identifying the causes of alterations in immune function in fish is the wide variety of possible causes [54], especially in wild populations, where the anthropogenic impact is uncontrolled. Chemicals, as other stressors, disturb the immunological potential of the animals and may cause their increased sensitivity to pathogens. In this paper, two groups of stress markers have been compared in relation to environmental stressors, acting both directly (as endocrine disruptors) and indirectly, causing general stress.

Environmental stressors rarely act as individual compounds. Anthropogenic pressure usually results in the introduction of a mixture of chemicals and their environmental effects should be considered as the resultant of interacting substances rather than individual components. In the case of fish reproductive or developmental parameters the mixture effects are taken into account [55,56]. Rarely the mixture effects are considered in relation to the immune response. On the other hand, although, contamination of the aquatic environment with ED may violate not only the delicate and precise allostatic interactions between the endogenous estrogen system but also the immune system [57]. Moreover, xenoestrogens have the potential to exert these two effects described above at extremely low concentrations [58].

In experiment designed by Thilagam et al. [59] fingerlings and juveniles of *Lateolabrax japonicus* were exposed to two sublethal concentrations (200 and 2000 ng/L) of 17β-estradiol, for 30 days under laboratory conditions. Alterations in immune parameters were investigated. This exposure induced immunomodulation and the changes caused by oestrogen might affect the function of the immune system in fish.

The mixture of household sewage chemicals tested in this paper caused a significant increase in innate immunity parameters of the common carp juveniles. These changes were statistically significant in all kinds of biological material. Moreover, in mucus, liver, gills and

spleen, the levels of CRP and Lys were significantly correlated with the concentration of the chemicals in the mixture (Table 3). The PCA analysis also revealed the significant contribution of CRP and Lys in explanation of the variability of all data (Fig. 5). Principal component 1, associated with CRP and Lys, can therefore be generalised as innate immunity. Our results therefore confirm that the stress caused by the mixture of chemicals stimulates innate immunity, especially in liver and spleen, but also in mucus. The mechanism underlying the changes still remains unrecognised. However, some suggestions can be found in the study of Jin et al. [60], on the embryos of *Danio rerio* exposed to the ED mixture. The mRNA levels of *D. rerio* innate immunity markers were significantly affected by the chemicals (17β-estradiol, 17α-ethynylestradiol, permethrin, atrazine and nonylphenol at various concentrations). The response was stronger in comparison with those resulting from the exposure to single components. According to the authors, the over-expression of mRNA of the genes, which are closely related to the innate immune system, is possible. This might result in decreasing anti-infection effects of the immune-related cell [60].

Mts and HSP are non-specific general stress markers. Their induction starts immediately, and significant response is detectable within 24 h. Osborne et al. (2007) [61] showed that the HSP90 chaperone function is critical for estrogen receptor signaling and endocrine disruption in fish, providing insight into the molecular mechanisms underpinning the response of fish to exogenous estrogens. The role of HSP90 in these processes has implications for endocrine disruption when the animal is also experiencing chronic or acute stress that results in enhanced expression of HSP. In this paper, the fish were exposed to the chemicals for 72 h and after that time the level of Mts and HSP70 increased significantly, indicating the activation of defence mechanisms. However, the increase, unlike in the case of the immunity markers, was not concentration-dependent. PCA analysis revealed lack of correlation of HSP70 with innate immunity, especially visible in liver, spleen, mucus as well as after analysing all the tissues together (Fig. 5). Having in mind the role of HSP70, the mechanism is not simple to explain. However, as mentioned above, it is highly probable that the time of exposure to toxins is very important in formation of differences between HSP70 and innate immunity stimulation. Extensive biological functions of HSP70, especially during intensive growth of fish, should not be neglected either. Pharmaceuticals and household chemicals have been previously reported to induce HSP70 synthesis in fish. Hallare et al. [62] found a concentration-dependent increase in HSP70 level in the zebrafish embryos exposed to diclofenac and its solvent, DMSO.

Data concerning the response of metallothioneins to the chemical stressors, especially those that act as endocrine disruptors, are rather scarce. The available investigations indicate the influence of ED to Mts synthesis, but the synthesis is ceased at the stage of gene expression [63,64]. This effect seems to be tissue (organ) dependent since Werner et al. [65] noticed the increase and decrease in Mts expression in, respectively, the kidney and liver of the fish *Salvelinus namaycush* exposed to ED. This analysis enables us to state that not only do the pharmaceuticals and household chemicals impair immunological reactions of common carp juveniles in various tissues but also do that in a concentration-dependent manner, thus confirming our hypothesis. This has a very important ecological implication since 9-month-old carp used in this study have a strong growth intensity, and most of their energy is consumed by anabolic processes. Moreover, the liver and spleen are engaged in an immunological defence of the organism [66]. Finally, the brain was chosen as an organ which should be under special protection and whose role should be played irrespectively of the external conditions. Hierarchical Cluster Analysis has revealed a high similarity of the internal organs: liver and spleen as well as gills that should be treated as internal organs but having direct contact with water-borne pollutants (Fig. 6). This may indicate that these organs were equally exposed to the pollutants and equally increased the synthesis of protective proteins.

A similar pattern of lysozyme contents in response to a

hormonomimetic substance between liver and spleen was also found in the studies on *Perca fluviatilis* [67]. Also, relatively similar sensitivities of the liver, spleen and gills in common carp exposed to paraquat were found – the lysozyme activity increased in all tested organs after 72 h [68] and atrazine and chlorpyrifos [69]. In the former paper immunological parameters as well as tissue damage were studied while in the latter one – HSP70 and HSC70 levels were compared.

In Xing et al. [69], the level of the brain exposure was similar to the one of liver, kidney and gill. In the present paper, brain responded in a different manner to the pharmaceuticals and household chemicals mixture. This organ was characterised by a specific reaction pattern with the highest values of the parameters in the fish from groups B and D, while in the individuals from A and C groups the level of the studied parameters was significantly lower. This may mean that the response found in the brain does not reflect mixture concentration effect and the efficiency of blood-brain barrier in carp, towards these kinds of chemicals (or, at least, one of them), is concentration-dependent. This question requires analyses since there are substances which cross the barrier and, cause behavioural disorders in fish. Such phenomenon was found for catecholamines in eels [70] and, more recently, for organophosphate pesticides (OP) in the largemouth bass [71] or aflatoxin B1 administered in silver catfish [72]. Dang et al. [71] stressed that although OP pesticides crossed the barrier, it needed more time than in other tissues and suggested that intoxication indices measured in the brain would be a good marker of chronic exposure. In the present study, the experiment was based on acute exposure; therefore, possibly, the effect would be more pronounced after longer exposure.

In nature, most stressors can be considered as acute stressors – over the short term and with high intensity. Chronic situations in which the intensity of the stressor is low but persistent are less common in nature. One of the rare situations appears when fish are subjected to anthropogenic pollutants and ED [73]. Fish represent the animal group most affected by ED exposure since they are continuously and directly exposed to these contaminants [74]. Unfortunately, there are no data on potential effects of pharmaceuticals or household chemicals, or their mixture, on the fish brain. On the other hand, the stress response is driven by a complex network in which the three regulatory systems– neural, endocrine and immune – are deeply involved [73].

Based on the general understanding of endocrine networks, there are numerous potential mechanisms and pathways available for ED, to disrupt the body homeostasis. Perhaps the best-studied mechanism of endocrine disruption is hormone mimicry [57,75]. As potentially biologically active, hormonomimetics used in this study might be harmful and may even impair fish behavior [76]. This requires further analysis due to the abundance of these kinds of substances in aquatic ecosystems.

Special attention in this study to the skin mucus was paid. Mucus in fish creates an innate immunity barrier against pathogens in fish [77]. It contains, among others, antimicrobial and stress proteins that protect fish against harmful environmental factors. Also in this paper, all of the markers of exposure to potentially toxic chemicals have been detected in mucus. Moreover, the levels of immunity markers, lysozyme, and CRP, appeared to increase in a concentration-dependent manner. This greatly predisposes mucus as the source of biomarkers in screening or monitoring studies considering restoration, assessment and revitalisation of anthropogenically changed ecosystems. This secretion may be collected in a minimally invasive way, undoubtedly less invasive than blood or scales samples. This attempt has been made previously also in other studies, comparing potential biomarkers in fish skin mucus in response to various physical, chemical and biological stressors, e.g. nanoparticles, crowding, air exposure or anaesthetic factors [78,79]. According to many reports, mucus is a source of various biomarkers, including antioxidant parameters, enzymes (e.g. esterases, proteases), non-enzymatic proteins (vitellogenins, *zona radiata* proteins), hormones (cortisol), as well as immunity markers (IgM) [78–80]. Surprisingly, the data on CRP, unlike lysozyme, are in literature depauperate, although

this protein has been proposed previously as a biomarker for health status in cultured carp [81].

From among general stress biomarkers, metallothioneins were detected in the fish mucus in response to metals [82]. However, the data about the usage of mucus to study metallothionein concentration (a very useful non-specific stress marker) are very scarce. The results of the present study confirm mucus can be collected when the monitoring study requires the measurement of this parameter concentration, not only in response to metal pollution. On the other hand, mucus HSP concentration was modified by chemicals used in the experiment. However, the changes did not reveal any pattern, just signalled the effects of exposure. In general, papers examining both mucus borne biomarkers and ED are less numerous and concentrate mainly on the vitellogenin level in response to individual hormonomimetics. The application of mucus as a source of non-invasively collected biological material is highly recommended (this study; also [83]). These analyses indicate significant specificity to the kind of biological material (i.e. organs and mucus) in immunological and stress response, according to our hypothesis.

In summary, innate immunity parameters better describe the response of fry carp to potentially biologically active sewage components than general stress markers. Lysozyme and CRP levels in most tissues studied in this project were significantly correlated with the mixture concentration, and, moreover, this is especially true for mucus samples. This enables us to confirm the other hypothesis that the degree and direction of modification in the level of immunity markers (but not stress markers) is concentration-dependent. A possible explanation of these differences between general stress and immunity markers may lay in the fact that chemicals used in this test may mimic the action of natural hormones. They are not recognised as xenobiotics when their concentration is below the tolerance level. However, they are still biologically active and may modify biological processes, including immunological ones.

Therefore, the immunity markers measured in mucus enable the scientists to monitor aquatic ecosystem state and fish fitness in a non-invasive way, at an early pollution phase, even before pollutant pressure starts causing more serious, possibly irreversible harm to fish individuals (and possibly other aquatic animals) individuals and populations. This appears especially important in the case of aquaculture where fish live in high density and the decrease in immune potential of individual animals may cause the disease outbreak in the whole population, as well as in the case of wild populations, being under anthropogenic pressure.

Acknowledgements

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