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Influence of long-term exposure to lead on its accumulation and elimination from tissues and on selected reproductive parameters in the Prussian carp (*Carassius gibelio* B.) in pond environment

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ABSTRACT: The bioaccumulation of lead in selected tissues of Prussian carp kept in pond condition during 12 and 24 months of exposure to different doses (8, 13, 24, and 49 mg/kg) of this metal in feed and its elimination from tissues during the following 12-month depuration period was studied. Additionally gonadosomatic index and luteinizing hormone (LH) secretion, as the effect of exposure to Pb, were examined. The concentration of lead in all the studied tissues, except for the gonads, correlated positively with the metal concentration in the diet, and the maximum level was usually achieved after 3 months of the exposure. The highest levels of lead, i.e. 2.1 ± 0.14 mg/kg, were found in the posterior intestine in the 15th month of the exposure, followed by bones, scales and kidneys, in which the level of lead amounted to 1.8 ± 0.20, 1.22 ± 0.07, and 1.17 ± 0.17 mg/kg, respectively. The negative effect of chronic exposure to lead was manifested by a significantly higher spontaneous LH secretion in groups exposed to 24 and 49 mg/kg of lead and a higher LH secretion level 6 h after the stimulating secretion. After 12 months of exposure and 12 months of depuration, as well as after 24 months of exposure, the effects of lead on LH secretion were not observed. Environmental lead can be a potent endocrine disruptor, which may have an adverse impact on fish reproduction. Prussian carps become resistant to the negative effects of lead with age and their organisms cope by reaching a state of homeostasis.

Keywords: Pb; fish; bioaccumulation; depuration; luteinizing hormone

INTRODUCTION

Heavy metals are an important class of pollutants with both lethal and sub-lethal effects on organisms. The latter are a subject of increased attention, as they may produce harmful ecological outcomes (Boyd 2010). An increase in heavy metals toxicity and their bioaccumulation in various tissues of aquatic organisms threatens the biodiversity of ecosystems and the health of consumers (George et al. 2011). Moreover, metal bioaccumulation patterns in fish tissues can be used as an effective indicator of environmental contamination. The tissue-specific accumulation of metals has recently been proposed as a key indicator of chronic exposure (Bergman and Dorward-King 1997).

Environmental pollution with heavy metals may pose a serious risk of physiological and biochemical problems, genetic abnormalities, and behavioural disorders (Scott et al. 2003; Kwon et al. 2015; Munoz et al. 2015). Other observed effects of lead contamination in animals include neurological, gastrointestinal, reproductive, circulatory, immunological, histopathological, and histochemical disorders (Iavicoli et al. 2006; Mobarak and Sharaf 2011; Sharma et al. 2011; Steuerwald et al. 2014). The negative influence of lead on organisms is observed not only in the case of the use of large

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doses of this metal, but also very frequently in the case of chronic exposure to its small doses. For example, Mobarak (2008) reported that low levels of lead exposure during the early development period resulted in long-lasting cognitive and neurobehavioral deficits, persistent immune changes, a delay in sexual maturity, reduced fertility, irregular estrus, and reduced number of corpora lutea in human and experimental animals. Lead can easily penetrate cell membranes and cross the blood-brain barrier (Steuerwald et al. 2014). It is a known endocrine-disrupting chemical in mammals and is believed to produce this effect through the hypothalamic-pituitary axis (Lucchi et al. 1981; Iavicoli et al. 2006). Weber (1993) observed that multiple effects on reproductive behaviour and overall reproductive success in adult fathead minnows, where lead suppressed spermatocyte production and retarded ovarian development, decreased the number of eggs oviposited, increased interspawn periods, and suppressed embryo development. Thomas (1988) demonstrated the decreases in estradiol levels in female Atlantic croaker as the effect of dietary lead. Khan (2000) reported that lead and Aroclor 1254 significantly decreased luteinizing hormone (LH) levels in Atlantic croaker in response to stimulation by luteinizing hormone-releasing hormone (LHRH) analog *in vivo* and reduced gonadal growth when administrated at a dose of 15 mg/kg body weight for 30 days.

As fish are an important source of food and a major component of the aquatic ecosystem, it is important to assess the adverse effects of lead on fish. In 2013, results of the research on the influence of a 24-month-long exposure of female Prussian carps kept in aquariums to different doses of lead in the feed were published (Luszczek-Trojnar et al. 2013, 2014). The research showed a significant accumulation of the element, varying among different tissues, as early as in the first months of the exposure as well as the tendency of tissues to become saturated with the metal to the level specific for each particular tissue, which did not change over the subsequent months of the exposure. It was also found that lead had a significant influence on some reproductive parameters, in particular in the first year of the exposure. Analyzing the elimination of lead from the organisms of the fish, it was observed that it depends on the type of tissue and the dose of lead the fish were previously exposed to. In hard tissues such as bones and scales, lead was present even after 12 months post exposure.

The temperature of water in the aquariums in which the fish were kept was constant. Over the 24 months of the research the fish were fed only feed in the form of pellets containing four different doses. In our climate conditions fish are exposed to changes in the temperature, including the wintering period. The temperature of water drops much below 8°C, causing the metabolism of some types of fish to slow down and the fish stop eating. In the climate of Central Europe, fish belonging to the Cyprinidae family are caught and placed in overwintering ponds as early as at the end of October and are not fed until March. A weight loss of 5–10% is accepted and overwintering is considered successful. On the other hand, when fish do not eat the feed, which may be contaminated, they may purify their organisms from toxic substances accumulated in the vegetation period.

The aim of the research was to analyze the bioaccumulation of lead and its elimination from different tissues in female Prussian carps kept in nature conditions of ponds, resulting from a chronic exposure to different doses of lead in the feed as well as the impact of such exposure on selected reproductive parameters of the fish.

MATERIAL AND METHODS

Fish and diet. Five hundred female Prussian carps *Carassius gibelio* (Bloch, 1782), originating from the "Górki" Fish Farm in Wiślica, Poland, were used in the study, which was conducted in the Fishery Experimental Station in Mydlniki at the Department of Ichtiobiology and Fisheries of the University of Agriculture in Krakow.

The fish were stocked in the experimental carp pond at a temperature ranging from 10 to 24°C in the vegetation season and from 4 to 10°C in the winter season, at pH of 7.5–8.0, oxygen saturation of about 70%, water hardness of 127 mg $CaCO_3$/l, Pb concentration of 0.000 mg/l.

One-year-old fish with mean body weight of 39.6 g were placed in a one-metre-deep pond with the surface of 500 m^2 divided into 10 equal parts using a steel fencing net. The muddy bottom of the pond was covered with soft macrophytes. In each of the 10 plots, 50 fish were placed. The cages were secured with a net to protect the fish against birds.

The fish were divided into 5 groups (each group in two plots) which were fed control pellets (control group) and pellets contaminated with different concentrations of lead (8, 13, 24, and 49 mg Pb/kg)

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for two exposure periods of 12 and 24 months. The pellets of the complete feed for Prussian carp were produced by the Institute of Ichthyobiology and Aquaculture of the Polish Academy of Sciences in Gołysz using grains, oilseed, fish meal, vitamin and mineral supplements. The feed contained 37% crude protein, 12% crude fat, and 31% carbohydrates. The control and experimental pellets were prepared in the same way, but no lead was added to the control feed. Lead acetate trihydrate (Pb $CH_3COO)_2$ $3H_2O$) (POCH S.A., Gliwice, Poland) was added in proper proportions to standard ingredients before the pelletizing process.

The analysis of lead concentrations in pellet samples showed the following mean levels (in mg/kg): control group – 0.113 ± 0.03; group 2 – 8.07 ± 0.11; group 3 – 13.11 ± 0.14; group 4 – 23.71 ± 0.18; group 5 – 48.62 ± 0.24.

The experiment was preceded by a one-month adaptation period when the fish were kept on a control diet. After 12 months of exposure, each experimental group was divided into two groups. One of them received the same treatment and the second (groups: 2-dep, 3-dep, 4-dep, and 5-dep) was subjected to a 12-month depuration period and received the control feed until the end of the experiment. Throughout the study, fish were fed once a day (3% of their body weight). The share of any additional natural food the fish found at the bottom of the pond in their diet was minimal. The experiment began in June. The research covered two dormancy periods and two vegetative periods.

Tissue sampling. At 3, 6, 12, 15, 18, and 24 months of the experiment, the following tissues were collected from 10–12 randomly harvested fish from each group: kidneys, gills, intestine (divided into anterior and posterior intestine, both without digesta, rinsed with deionized water), muscles, gonads, hepatopancreatic gland (liver), scales, and bone tissue from opercula freed from the skin. These tissues were frozen at –20°C until the analysis of lead concentration.

Prior to the decapitation, the body weight and length of the harvested fish was measured. The data was then used to calculate the Fulton's condition factor using the following formula:

$$K = 100 \times W/l^3$$

where:
W = fish weight
l = body length

GSI analysis. At 12 and 24 months of the experiment, the gonadosomatic index (GSI) was calculated using the formula:

(gonad weight/body weight without viscera) × 100

LH analysis. In order to determine the effect of exposure to different doses of lead on LH blood levels, an experiment was performed after 12 and 24 months of the exposure to analyze spontaneous LH levels as well as LH secretion following hormonal stimulation. Four days before the planned treatment, 15 randomly harvested fish from each group were placed in 300 l aquariums at a temperature raised from 17°C to 20°C. On the day of the treatment, blood was collected from 15 fish from each group, after which intraperitoneal injections of salmon gonadotropin releasing hormone analogue (sGnRHa) (Bachem Feinchemikalien AG, Bubendorf, Switzerland) (10 µg/0.5 ml/1 kg body weight (BW)) and pimozide (Sigma-Aldrich Laborchemikalien GmbH, Seelze, Germany) (5 µg/0.5 ml/1 kg BW) were performed to evaluate the influence of lead on LH secretion (5 µg/0.5 ml/1 kg BW). The blood samples (about 100 µl from each fish) were collected 6, 12, and 24 h after the injection (Figure 1) from the caudal vessels with a 1 ml heparinized syringe (0.4 × 13 mm needle), centrifuged (13 000 *g*, 3 min) in Eppendorf tubes, and the blood plasma was kept at –20°C until the ELISA determination of LH (Kah et al. 1989). Prior to all the activities, the fish were anaesthetized with Propiscin (IRS, Zabieniec, Poland) (0.3 ml/l). The standards, LH hormone, and antibodies were donated by Dr. Bernard Breton (INRA, France). The results of the analysis were expressed as mean ± standard error of the mean (SEM). The results were analyzed using the one-way analysis of variance (ANOVA) and the Mann-Whitney procedure was used to determine significant differences between the means for the control and experimental groups (8, 13, 24, and 48 mg/kg), as well as for those in the experiment performed after 24 months of exposure (8, 13, 24, and 48 mg/kg; 8-dep, 13-dep, 24-dep, and 48-dep). The differences were considered significant at $P \leq 0.05$. To observe the relationships between LH secretion and the lead dose during the exposure, Spearman's correlation coefficients were calculated.

Pb analysis. Prior to the determination, tissues (weight 1–5 g) were subjected to preliminary

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mineralization in the presence of a 3 : 1 v/v mixture of nitric acid (HNO_3 65%) and perchloric acid ($HClO_4$ 70%) (10 ml) for about 20 h. The samples were then heated with a VELP 20/26 digester (VELP Scientifica, Usmate, Italy) by gradually increasing the temperature to 180°C for 6–7 h. The obtained clear liquid was diluted with deionized water to 10 ml and then assayed for the concentration of lead using a Unicam 929 atomic absorption spectrometer (Unicam Ltd, Cambridge, UK) (Agemian et al. 1980). The concentrations were read from the standard curve generated using the standards prepared based on atomic absorption standards made at the Office of Weights and Measures in Warsaw. Each sample was assayed in duplicate (the average values calculated from replicates were used in statistical analysis). Calibration was repeated for every 10 samples. The results were presented in mg Pb per kg wet tissue weight (ww). The minimum level of detection for lead was 0.001 mg/kg.

The results were analyzed using the one-way analysis of variance, and significant differences were determined between the groups at each tissue collection as well as in each group between successive collections using the Mann-Whitney method.

Spearman's correlation coefficients were calculated to examine the relationship between the lead concentration in the tissues and the dose of the exposure.

RESULTS AND DISCUSSION

Fish growth. The negative effects of lead on fish include physiological changes, which may inhibit the growth of their bodies (Woodward et al. 1994). Reports on the inhibited growth of fish resulting from their exposure to lead are inconclusive. For instance, a slower growth caused by extremely high, sub-lethal doses of lead in water was observed in fathead minnows larvae (Mager et al. 2011) and the larvae of the rainbow trout (Mebane et al. 2008). Other research did not prove the influence of lead on the growth rate of fish (Mount 1994; Alves et al. 2006) even after 19 months of the exposure. The results achieved in the present research related to the weight and length of fish exposed to different levels of lead in the feed for 24 months did not show statistically significant differences between the groups in the particular periods in which the samples were collected (Figures 1 and 2). However, while the control fish showed a constant increase in the weight and length of the body in the consecutive months of the research, the weight of the fish from the remaining groups increased minimally or even decreased in the first year (the group exposed to 49 mg Pb/kg), which was later compensated for by an incremental increase in the body weight in the second year of the exposure. In the case of Prussian carps from the group receiving the lowest of the used doses of lead (8 mg/kg), the increase in the body weight (8.9 g) was the most similar to that observed in the case of the control group (11.4 g) over the 24 months of the exposure (Figure 1). It is possible that the statistically insignificant smaller increase in the body weight in the first year of the research resulted from the greater sensitivity of the young, one-year-old, Prussian carps to higher concentrations of lead in the feed. Fish at this age grow at a faster rate, especially in the vegetation period, when they have enough

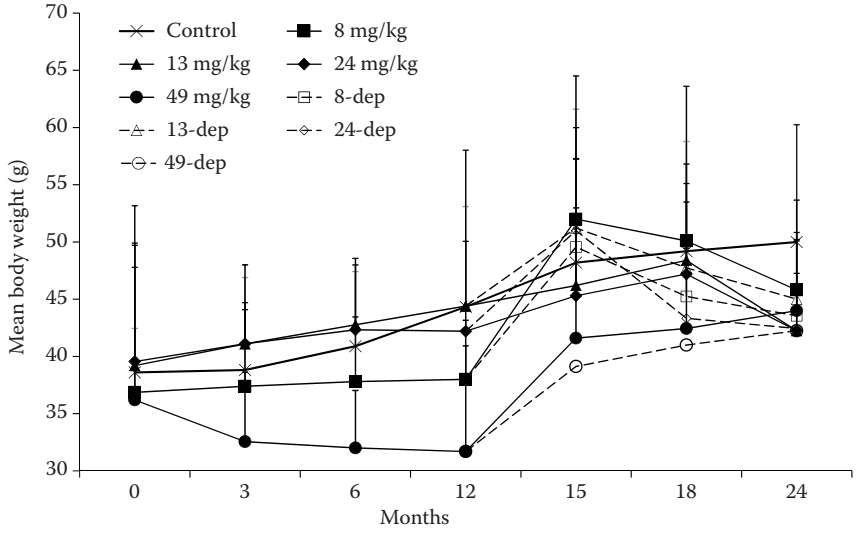

Figure 1. Body weight changes during 24 months of the study period in Prussian carps

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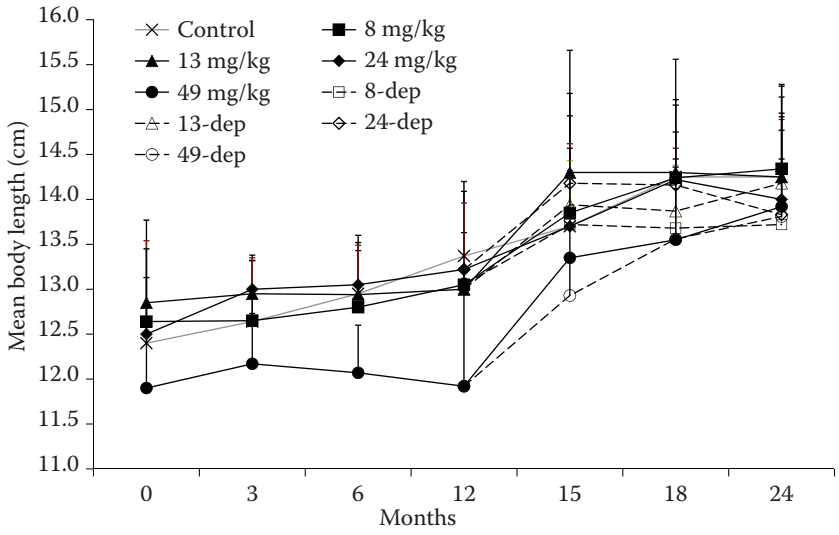

Figure 2. Body length changes during 24 months of the study period in Prussian carps

feed. The observed changes in the body weight probably resulted from the exposure to a toxic agent such as lead. Some authors suggested that the metal may reduce appetite and the effectiveness of food intake by fish (Weis et al. 2003; Mager et al. 2010). However, the lead concentrations used in the present research were small enough for the fish to deal with them and already after a year, in the next vegetation period, the concentrations ceased to cause a decrease in the body weight of the fish. Such differences were not observed when analyzing the increase in the body length. Fish from all the groups grew at a steady rate, and the small, statistically insignificant differences did not depend on the used dose of lead (Figure 2). The good condition of the fish in the entire period of the exposure and elimination was confirmed by Fulton's coefficients ranging from 1.5 to 2.0. No statistically significant differences were found between the groups. The mortality rate of the fish was at about 6% and ranged from 3 to 6 fish during the 24 months of the exposure and from 1 to 3 fish during the 12 months of the purification (Table 1). No correlation between the mortality rate and the dose of lead in the feed was observed.

Lead bioaccumulation and purification. As muscles are the most interesting tissue from the point of view of fish consumers, the accumulation of toxic agents in muscles is usually analyzed (Jumawan et al. 2010; Abdel-Baki et al. 2011). During the chronic exposure to different doses of lead in the feed used in the research, a statistically significant increase in the concentration of lead in the muscles of the analyzed fish was observed already after 3 months of the exposure (Supplementary Figure S1). Such a concentration was observed in the entire period of the exposure, regardless of the season. It was found that the maximum permissible

Table 1. Comparison of mean values of Fulton's condition factor K during the experiment, and total mortality of fish

Months	Experimental groups								
	control	8 mg/kg	13 mg/kg	24 mg/kg	49 mg/kg	8-dep mg/kg	13-dep mg/kg	24-dep mg/kg	49-dep mg/kg
0	2.0 ± 0.18	1.8 ± 0.19	1.9 ± 0.19	2.0 ± 0.17	2.1 ± 0.18	–	–	–	–
3	1.9 ± 0.22	1.8 ± 0.25	1.9 ± 0.22	1.9 ± 0.19	1.8 ± 0.18	–	–	–	–
6	1.9 ± 0.16	1.8 ± 0.23	2.0 ± 0.24	1.9 ± 0.24	1.8 ± 0.23	–	–	–	–
12	1.9 ± 0.23	1.7 ± 0.18	2.0 ± 0.21	1.8 ± 0.21	1.9 ± 0.28	–	–	–	–
15	1.9 ± 0.21	2.0 ± 0.27	1.6 ± 0.20	1.8 ± 0.28	1.8 ± 0.21	1.9 ± 0.23	1.9 ± 0.20	1.8 ± 0.24	1.9 ± 0.24
18	1.7 ± 0.19	1.7 ± 0.28	1.7 ± 0.25	1.6 ± 0.19	1.7 ± 0.21	1.8 ± 0.24	1.8 ± 0.25	1.5 ± 0.23	1.6 ± 0.21
24	1.7 ± 0.28	1.6 ± 0.18	1.5 ± 0.26	1.5 ± 0.21	1.6 ± 0.26	1.7 ± 0.21	1.6 ± 0.19	1.6 ± 0.20	1.6 ± 0.24
Mortality (*n*)	5	6	4	5	4	2	3	1	1

dep = depuration

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level of lead concentration in muscles (0.3 mg/kg), that is safe for consumers according to the Commission Regulation (EC) No. 1881/2006, was exceeded (0.42 mg/kg in the group 24 mg/kg in the 24th month of exposure), which may suggest that the possible consumers of fish subjected to a similar exposure face a risk of contamination. Analogous studies conducted on Prussian carps kept in aquariums and fed for the entire year showed that the concentration of lead in their muscles was almost twice higher (Luszczek-Trojnar et al. 2013). The stay in the natural environment of fish ponds for long dormancy periods resulted in a lower concentration of lead in the muscles of the fish. Compared with the experiment performed on the fish kept in aquariums, fish in ponds did not eat the contaminated feed for a total period of over 10 months (two winter periods). On the other hand, despite the winter breaks in eating the contaminated feed, the concentration of lead in the muscles of the fish exceeded the safe limits, which suggests that the contamination of the environment with lead should not be underestimated, even though the muscles of the fish are not the target tissue of lead bioaccumulation.

In the purification period, following the 12 months of exposure, lead was eliminated from muscles at a faster rate. Already after 3 months, a significantly lower level of lead (0.15–0.21 mg/kg), similar to the level noted in the fish subjected to purification, was observed (Supplementary Figure S2). It is an interesting observation, especially if we compare the results with the results achieved in the dormancy periods, during which the fish did not eat. Even though the fish did not eat the feed contaminated with lead in those periods, the lead concentration in muscles did not decrease. The metal remained in the tissue throughout the entire winter period, and right after the actual purification period began, coinciding with the vegetation period (from the 12th month of the research – June), the metal started to be eliminated from the muscles. It proves that muscles started to be effectively purified from lead as late as in the vegetation period.

The liver of fish is sensitive to environmental pollutants because many contaminants tend to accumulate in the liver at much higher levels than in the other organs (Heath 1995). Analyses of lead concentration in various tissues in fish usually show higher levels of the metal in the liver compared with muscles (Jumawan et al. 2010). However, next to blood cells, to which lead shows the highest affinity, the main target tissues of lead distribution include bones, gills, kidney, spleen, and the intestine. The liver is not considered to be an organ that accumulates lead particularly easily (Mager 2011). In the present research, the tissue of the liver showed a statistically significant accumulation of lead only in the group exposed to 49 mg Pb/kg. However, the maximum lead concentration in the tissue was low and did not exceed 0.25 ± 0.14 mg/kg even in the group of fish exposed to the highest concentrations of lead (Supplementary Figure S3). The tissue of the liver in the Prussian carps kept in aquariums and exposed to lead reacted differently. The level of lead in their livers reached 4.5 mg/kg, i.e. it was 18 times higher (Luszczek-Trojnar et al. 2013). It seems that similarly to muscles, the bioaccumulation of lead in the liver was influenced by the lower general intake of lead as well as by the environment. The constant flow of water and the additional food in the form of benthic vertebrates affected the metabolism in such a way that the ongoing elimination of lead from the liver was much more effective than in the case of the fish kept in aquariums. The achieved results suggest that the liver is not a bioindicator of lead contamination of the environment, as in natural conditions, a lasting increase in lead concentration in the organ was observed only in the case of the highest dose of lead, i.e. 49 mg/kg. The process of eliminating the accumulated lead from the liver in the purification period was as quick as the accumulation process and already after 3 months the level of lead was similar to that observed in the control group. In the later period the level fluctuated, which could result from the release of lead from other tissues to blood and the secondary accumulation of lead in the well vascularized liver (Supplementary Figure S4). Even though the liver is considered to be an organ that is the most susceptible to contamination due to its detoxification function, it does not always show the highest concentrations of lead. In fact, lead concentrations in the liver are often lower than in kidneys and gills (Alves et al. 2006; Mager et al. 2010; Abdel-Baki et al. 2011) and even lower than in muscles (Eyckmans et al. 2013), as was proved in the present research. Other heavy metals, including cadmium and copper, can bind to the metallothionein, creating complexes in hepatocytes, which is a form of detoxification from those substances. As a result, we can observe their high concentrations in this tissue. Lead does

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not show affinity to metallothionein, which is probably why other tissues play a bigger role in eliminating lead from the organism (Mager 2011).

Lead accumulates in the intestine of fish, which was confirmed in many studies (Mount et al. 1994; Alves et al. 2006; Ojo and Wood 2007). In the present research the concentration of lead in the intestine of the control fish ranged from 0.07 to 0.5 mg/kg, depending on the season (Supplementary Figures S5 and S6). In the study performed on the fish kept in aquariums, lead concentration in the control group did not show such changes over a year and did not exceed 0.25 mg/kg (Luszczek-Trojnar et al. 2013). This observation itself suggests that in the natural environment of ponds, which contain river waters, whose composition may change over the year, the digestive system of fish is subjected to less stable chemical conditions. The changing levels of Ca and Fe in water may inhibit the absorption of other elements, including lead (Alves et al. 2006; Mager et al. 2011). The accumulation of lead in the intestine as a result of the chronic exposure to the metal increased in a way that was analogous to that observed in the study on the fish kept in aquariums, i.e. it was smaller in the anterior intestine (from 0.9 ± 0.12 mg/kg in the 49 mg/kg group in the 18th month of the exposure) and greater in the posterior intestine (from 2.1 ± 0.13 mg/kg in the 49 mg/kg group in the 15th month of the exposure) (Supplementary Figures S5 and S6). Ojo and Wood (2007) analyzed the absorption of lead in various parts of the digestive system of the rainbow trout and proved that the highest amounts of lead were absorbed by the middle intestine, followed by the posterior intestine, and that the lowest amounts of the metal are absorbed by the anterior intestine and the stomach, which confirms the results of the present research. However, in the case of the fish kept in ponds, the increase in the levels of lead in the intestine could be observed much earlier than in the case of the fish kept in aquariums. As early as after 3 months of the exposure, lead concentration in the group subjected to 49 mg of Pb per kg amounted to 0.8 ± 0.10 mg/kg, and after 6 months the concentration reached 1.6 ± 0.12 mg/kg, while after the same period the concentration of lead in the fish kept in aquariums was twice lower (Luszczek-Trojnar et al. 2013). The concentration of lead in the intestine was different in different seasons. In the vegetation period or right after the vegetation period (3rd–6th month and 15th–18th month of the research) higher lead concentrations were observed in the anterior intestine, while after the dormancy period the lead concentration decreased to the level observed in the control fish (12th and 24th month) (Supplementary Figure S5). In the period when the fish ate the most intensively, the concentration of lead in the posterior intestine increased, while in winter, when the fish did not eat, the posterior intestine underwent purification and the level of lead in all the groups was equal to the level observed in the control group. In the planned purification period (from the 12th to the 24th month of the research), the level of lead decreased in all the groups, but over the remaining period of the research the concentration of lead fluctuated, regardless of the previous dose of lead (Supplementary Figures S7 and S8). The changing levels of lead in the intestine observed in the period of purification could result from the elimination of lead from the organism through that tissue. The role of the intestine in eliminating toxicants was described by Kleinow and James (2001). Even though the experiments performed in ponds and in aquariums were carried out on fish of the same type, origin, age, and size, it was observed that the fish reacted differently to the exposure to lead due to different environmental conditions. While the fish kept in ponds showed a higher concentration of lead in their intestines, the fish from the aquariums accumulated more lead in the hepatopancreas. It is possible that in more favourable natural conditions of fish ponds, containing a small addition of natural food, the intestines of the fish were a better barrier against lead, slowing down or inhibiting its penetration in large amounts to other tissues. The intestine was the only tissue to show higher lead concentrations in the fish from ponds compared with those kept in aquariums. On the other hand, the higher concentration of lead in the intestine clearly suggests that the fish in the pond fed on the pellets that contained lead. The highest concentration of lead from the feed in the intestine was also observed by Alves et al. (2006), who formulated a hypothesis that while gills accumulate most lead contained in water, intestines accumulate most of the metal when it is contained in the feed. It is believed that the high concentrations of lead in the intestine result from the protective role of mucus, whose secretion becomes more intensive as a result of the presence of harmful metals in food (Ojo and Wood 2007). Thus, the high concentrations of lead in the intestine result only

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Table 2. Spearman's correlation coefficients (r) for the relationship of Pb tissue concentration to lead dose during exposure in different groups

Period	Months	Muscle	Liver	Proximal intestine	Distal intestine	Kidney	Gills	Bone	Scales	Gonads
Exposure	3	0.41**	0.51**	0.48**	0.51***	0.81***	0.55***	0.58***	0.92***	–0.26
	6	0.38*	0.45**	0.43*	0.69***	0.68***	0.64***	0.82***	0.90***	0.19
	12	0.49*	0.42*	0.18	0.38*	0.42*	0.62***	0.82***	0.86***	0.18
	15	0.37*	0.71**	0.64***	0.71***	0.59**	0.56***	0.85***	0.92***	–0.12
	18	0.51**	0.35	0.56***	–0.14	0.87***	0.21	0.45*	0.85***	0.33
	24	0.70**	0.53*	0.23	0.52**	0.64***	0.59***	0.47**	0.79***	–0.04
Depuration	3	0.12	0.11	0.27	–0.21	0.12	0.63***	0.64***	0.94***	–0.12
	6	0.25	–0.31	0.01	0.13	0.65***	0.54***	0.68***	0.89***	–0.05
	12	0.16	0.42*	0.17	0.44*	0.53**	0.56***	0.45*	0.80***	0.15

***$P < 0.001$, **$P < 0.01$, *$P < 0.05$

from the adsorbed lead rather than the absorbed one (Alves et al. 2006).

The kidney is the gateway for heavy metal detoxification in the body (Siscar et al. 2013). Therefore, the highest concentrations of lead were observed in this tissue (Mager et al. 2010; Abdel-Baki et al. 2011). In the present research, already after 3 months of the exposure, the highest level of lead was found: 1.15 ± 0.17 mg/kg in the groups exposed to 24 and 49 mg Pb/kg and 0.8 ± 0.17 mg/kg in the group receiving 13 mg Pb/kg (Supplementary Figure S9). In an analogous experiment performed in aquariums, the observed levels of lead in the kidney after 3 months were similar. However, the level continued to increase until the 18th month of the exposure, when the maximum lead concentration of 7.5 mg/kg was observed in the group exposed to 49 mg Pb/kg (Luszczek-Trojnar et al. 2013). Already after the first dormancy period, in the 12th month of the exposure, the level of lead in the kidney decreased significantly (Supplementary Figure S9), which proves its sensitivity to the break in the consumption of the contaminated feed. The fact that the kidney was not purified completely can be explained by its excretory function, which makes the organ more susceptible to lead from other tissues undergoing purification. Therefore, a decrease in the level of lead right after the actual purification process onset was not expected. Indeed, until the end of the period, the lead concentration in the kidney in groups subjected to lead did not decrease to the level found in the control group. The group receiving 8 mg Pb/kg was the only group in which the effective elimination of lead was observed (Supplementary Figure S10). It can, thus, be concluded that the kidney is a tissue that is susceptible to the presence of lead in the environment and may serve as a bioindicator of lead contamination occurring at the time of an analysis and several months before such an analysis.

The accumulation of lead in the gills during dietary exposures suggests that a branchial role for lead excretion may exist (Alves et al. 2006). The present research confirmed this conclusion, as already after 3 months of the exposure the level of lead in the gills increased significantly in all the groups and continued to be higher than in the control group until the end of the exposure, even though the lead entered the organisms of the fish through their digestive system. The maximum concentration of lead, 0.8 ± 0.07 mg/kg, was found in the group exposed to 13 mg Pb/kg in the feed in the 18th month and in the group of fish receiving 49 mg Pb/kg in the 24th month of the exposure (Supplementary Figure S11). The elimination of lead from the gills was not very effective, as was the case with the kidneys. Throughout the entire purification process, statistically significantly higher levels of lead were observed in the experimental groups, and the only group, in which the purification process was effective, was the group receiving 8 mg Pb/kg in the 12th month of the purification (Supplementary Figure S12).

Bones are a target tissue of lead accumulation. It has been found that about 80–95% of lead in the organism accumulates in bones (Mager et al. 2010) and may remain there for many years following the exposure (Rabinovitz 1991). It is often assumed

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Table 3. Comparison of the gonadosomatic index (GSI) values between experimental groups after 12 and 24 months of the study. Results of the Mann-Whitney test between each two groups and the multiply comparisons Kruskal-Wallis test between all groups have not shown any significant differences

Months	Experimental groups									Significance
	Control	8 mg/kg	13 mg/kg	24 mg/kg	49 mg/kg	8-dep mg/kg	13-dep mg/kg	24-dep mg/kg	49-dep mg/kg	
12	12 ± 1.1	11 ± 1.2	14 ± 1.0	10 ± 1.1	12 ± 1.5	–	–	–	–	ns
24	22 ± 2.1	17 ± 1.6	25 ± 1.9	19 ± 2.1	19 ± 1.9	27 ± 2.3	22 ± 2.2	24 ± 2.1	20 ± 2.0	ns

dep = depuration, ns = not significant

that the bone tissue represents a large reservoir for sequestering lead in an inert capacity, thereby serving as a model of detoxification. In the present research, a significant increase in the accumulation of lead was observed after 3 months of the exposure (0.53 ± 0.04 mg Pb/kg) in the group receiving 49 mg Pb/kg. The maximum level of lead in bones, 1.85 ± 0.20 mg/kg, was found in the 15th month of the exposure in the group subjected to the highest dose of lead (Supplementary Figure S13). Several lines of evidence suggest that lead affects various aspects of bone physiology, including formation, resorption, and fracture healing, and may cause necrosis (Pounds et al. 1991; Carmouche et al. 2005). In mammals, skeletal Pb may be mobilized in response to Ca^{2+} status (Pounds et al. 1991), and a release of the accumulated lead from bones and scales may similarly occur in fish (Persson et al. 1998). The effectiveness of the elimination of lead from bones in Prussian carps used in this research was low and despite the observed small decrease in the concentration of lead in all the groups after the first three months of purification, no decrease in the level of lead was observed in the remaining period of the research (Supplementary Figure S14). It is possible that at the beginning of the elimination process the bone tissue underwent mobilization and began to release lead, but after three months the processes of the secondary accumulation of lead released to blood from other tissues balanced the purification of the bones. The analysis of the purification process in Coho salmons and Longjaw mudskippers, too, showed that lead continued to be accumulated in bones as well as in the spleen after the previous chronic exposure to lead (Varanasi and Gmur 1978), which confirms that the secondary accumulation of lead resulted from the role of erythrocytes in the transport of lead released from other tissues. Our results indicate that bones are a good bioindicator of lead contamination, showing an increased level of lead even after 12 months following the exposure (Supplementary Figure S14).

As was expected, scales were a tissue in which large amounts of lead accumulated, depending on its dose. Scales also showed the most significant coefficients of correlation between the level of lead and the dose of lead the fish were exposed to (Table 2). The maximum level of lead in scales was observed already after 6 months in groups receiving 24 and 493 mg Pb/kg, and the fluctuations found over the remaining period of the exposure were not statistically significant (0.84–1.23 mg/kg) (Supplementary Figure S15). Unlike the tissues related to the digestive system, scales and bones did not show changes in the accumulation of lead in dormancy periods. The break in feeding did not result in a decrease in the level of lead in hard tissues. The fact that the level of lead in scales

Table 4. Spearman's correlation coefficients (r) for the relationship of spontaneous or stimulated LH release to lead dose during exposure in different groups

Months of exposure/ depuration	Time of blood collection after the LHRH/pimozide stimulation (h)			
	0	6	12	24
12	0.35*	0.34*	–0.08	0.05
24	–0.14	0.11	0.25	0.24
12/12	0.14	0.13	0.05	0.28

LH = luteinizing hormone, LHRH = luteinizing hormone-releasing hormone

*$P < 0.05$

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remained at a level similar to that observed in the kidney suggests a great sensitivity of scales to lead in the environment. The elimination of lead from scales is a long and ineffective process. Similarly to bones, the level of lead did not decrease significantly in the entire elimination period. The group exposed to 8 mg Pb/kg-dep was the only one in which the level of lead in scales was similar to that found in the control group (Supplementary Figure S16). Scales seem to be an excellent bio-indicator of lead contamination in the environment of fish. After a relatively short period, i.e. already after 3 months, the lead concentration in scales increased and was more strongly dependent on the dose of lead than the concentration of lead in other tissues. In turn, the process of purification from lead in scales is incredibly slow. As a result, the traces of the contamination are not "erased", even if the contamination occurred 12 months earlier. In criminology, hair and nails are often tested in order to detect the possible presence of toxic substances that a given person could be exposed to in the past. In the same way we can test scales, which can be collected from live fish.

Effects of lead on reproductive parameters. It has been known for a long time that lead accumulating in different tissues in the organism enters the cells, where it accumulates mainly in mitochondria and the endoplasmic reticulum (Reichert et al. 1979), which may affect the basic life functions of cells and their functioning. As lead inhibits the secretory functions of cells, its presence may affect negatively the functioning of the entire organism. Weber (1993) observed lead-induced alternations to the reproductive behaviour of fathead minnows, including a reduction in time spent on nest preparation and maintenance by males, reduced oviposition by females, and longer interspawning periods. As these effects may have important ecological implications, the analysis of the influence of lead on the reproductive function of fish is important.

In the studies carried out in aquariums, Prussian carps showed statistically significant changes in the lead concentration in gonads even when they were exposed to the lowest dose of lead, i.e. 8 mg/kg (Luszczek-Trojnar et al. 2014). The accumulation of lead in the fish kept in ponds was completely different. The changing levels of lead, ranging from 0.05 ± 0.01 to 0.17 ± 0.03 mg/kg, were observed both in the control group and the groups exposed to lead (Supplementary Figures S17 and S18). Among all the analyzed tissues, gonads are the least sensitive to lead exposure and do not react in a significant way to changes in the concentration of lead in fish kept in ponds. What is very interesting is the fact that in fish kept in aquariums, which did not have breaks in feeding on the contaminated lead, the level of lead accumulation in gonads was high, reaching 0.8 mg/kg, and decreased only after the spawning period due to the ejaculation of spawn, after which the level of lead once again increased. The situation of the fish in ponds was significantly better. Thanks to the slower metabolism in the winter period, the exposure period was significantly shorter, as a result of which gonads did not accumulate lead.

The exposure of the fish to lead had a different effect on some reproductive parameters analyzed during

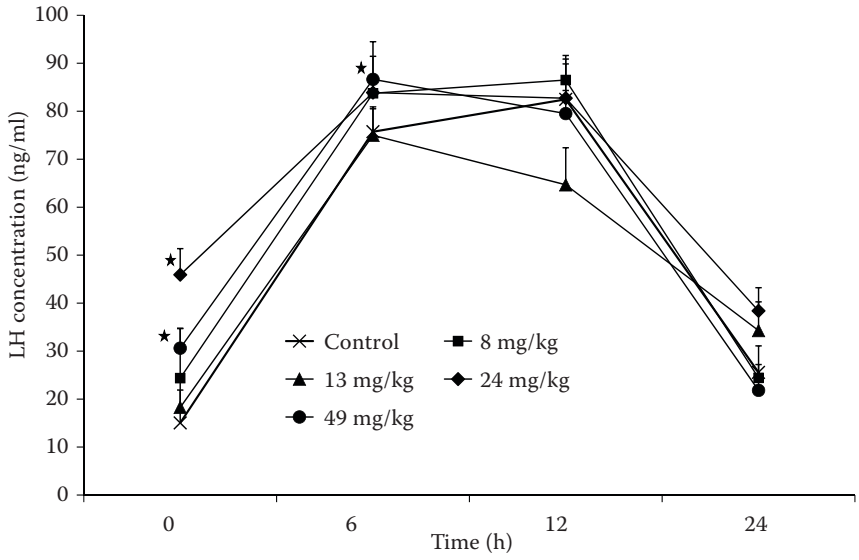

Figure 3. Comparison of plasma luteinizing hormone (LH) concentrations in successive blood collections following the ovulation-stimulating injection after 12 months of female Prussian carp exposure to different doses of lead (groups 8, 13, 24, and 49 mg Pb/kg)

*concentrations significantly different from control ($P < 0.05$); error bars represent standard errors

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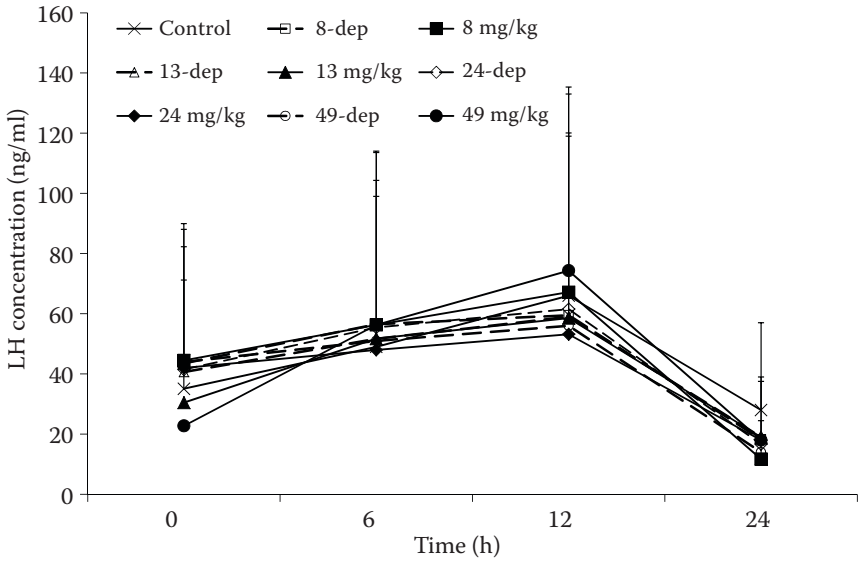

Figure 4. Comparison of plasma luteinizing hormone (LH) concentrations in successive blood collections following the ovulation-stimulating injection after 24 months of female Prussian carp exposure to different doses of lead (groups 8, 13, 24, and 49 mg Pb/kg) and in females after 12 months of exposure and 12 months of depuration (groups 8-dep, 13-dep, 24-dep, and 49-dep)

no significant differences between groups after the 24-month exposure period as well as after the 12-month exposure and 12-month depuration periods; error bars represent standard errors

the spawning period after 12 and 24 months of the exposure. The GSI is a general indicator of the normal ovarian growth and reproductive development. While some authors observed a significant influence of the exposure to lead on the GSI (Ruby et al. 2000), others did not notice such effects (Weber 1993). While the fish kept in the ponds and exposed to lead did not show any statistically significant differences in the GSI value after 12 and after 24 months of the exposure (Table 3), Prussian carps kept in aquariums showed statistically significant differences in the values of the GSI after 12 months of the exposure (Luszczek-Trojnar et al. 2014).

The influence of lead on the endocrinological changes in mammals (Iavicoli et al. 2006) and fish (Spieler et al. 1995; Rademacher et al. 2003; Sloman et al. 2005) was analyzed on many occasions. In the present research, after 12 months of the exposure spontaneous secretion of LH ranged from 15 ± 6.8 to 45 ± 5.4 ng/ml, and after 24 months the secretion ranged from 23 ± 5.3 to 45 ± 7.7 ng/ml. Statistically significantly higher LH levels in experimental groups (exposed to 24 and 49 mg Pb/kg) compared with the control group were only observed in the experiment performed after the 12th month of the exposure (Figures 3 and 4), as well as statistically significant Spearman's correlation coefficients reflecting the dependency of LH concentration to Pb dose in the feed (Table 4). After the stimulation using the LHRH analogue and a concomitant administration of a dopamine receptor blocker, pimozide, the secretion of LH increased in all the groups. In the sixth hour after the injection, a statistically significant increase in the LH level was observed in the group of fish exposed to 49 mg Pb/kg compared with the control group. The increased level of LH persisted for about 12 h. After 24 h, in turn, it decreased to the level of spontaneous secretion (Figure 3). In turn, the experiment conducted after 24 months of chronic exposure to lead and following the stimulation with the LHRH analogue showed that the LH level increased, too, in all the groups. The highest LH level was observed 12 h post injection, and after 24 h the concentration of LH in blood of fish from all the groups decreased to the spontaneous secretion level. No statistically significant differences were found in this period between the groups (Figure 4). Older, mature fish coped better with the exposure to lead, which did not produce such clear effects as those observed after 12 months of the exposure. It is also possible that the constant exposure to the action of the same toxicant caused the development of defence mechanisms in fish, which coped with its access. The present research on fish kept in the ponds showed that in the first 12 months of the exposure the fish received significantly less lead due to the winter break, which ended shortly before the spawning. The period after the beginning of the vegetative period was too short for the fish to accumulate an amount of lead that could cause effects similar to those observed in the fish kept in aquariums (Luszczek-Trojnar 2014).

Considering that some of the major effects of lead toxicity are attributed to ionoregulatory

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and haematological alternations, several lines of evidence seem to indicate that fish are able to acclimate, at least to some extent, to lead during chronic exposures (Mager 2011).

CONCLUSION

The results obtained in this study indicate that the chronic dietary exposure to lead ranging from 8 to 49 mg Pb/kg had no significant effects on the growth or survival of female Prussian carps kept in the natural condition and that these parameters do not appear to be effective indicators of dietary lead toxicity in this species.

The concentration of lead in all the studied tissues, except for the gonads, correlated positively with the metal concentration in the diet, and the maximum level is usually achieved after 3 months of the exposure.

Even though the doses of lead the fish in the ponds were exposed to caused a relatively low accumulation of lead in muscles, the observed concentrations exceeded the permitted maximum levels for muscles, as a result of which possible consumers may face a risk of contamination.

The experiment on the fish kept in ponds confirmed the results achieved in the experiment on the fish in aquariums with regard to the usefulness of the kidneys, gills, bones, and scales as bioindicators of lead contamination. The research confirmed the previous conclusions that bones and scales, in which the purification process is delayed, may show lead contamination that occurred in the past 12 months or even earlier. It is extremely important, as it may enable the use of scales, which can be collected from live fish, without the need to kill them.

The fish kept in the ponds showed a relatively low sensitivity to lead accumulation in gonads, which resulted in the lack of change of the GSI after 12 and 24 months of the exposure.

The chronic exposure to lead was not without an influence on the reproductive system of female Prussian carp kept in ponds. The negative effect was manifested by a significantly higher spontaneous LH secretion in groups exposed to 24 and 49 mg/kg of lead and a higher LH secretion level 6 h after the stimulating secretion.

After 12 months of exposure and 12 months of depuration, as well as after 24 months of exposure, the effects of lead on LH secretion were not observed.

The present results confirm the earlier conclusions that environmental lead can be a potent endocrine disruptor, which may have an adverse impact on fish reproduction and that Prussian carps become resistant to the negative effects of lead with age and their organisms cope by reaching a state of homeostasis.

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