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Influence of short- and long-term exposition to Roundup on the spontaneous and hormonally stimulated LH secretion and gills condition in Prussian carp (*Carassius gibelio*)

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Abstract. Roundup is the most extensively used herbicide in world agriculture. In the present study we examined the influence of Roundup at the concentration of 15 mg L^{-1}on luteinizing hormone (LH) secretion and reproduction of Prussian carp females. Experiment was conducted at the University of Agriculture, Department of Ichthyobiology and Fisheries in Kraków, Poland from May to October 2010. Control fish were kept in Roundup–free water. Short-term exposition lasted 10 days, and long-term exposition 6 months. At the end of both experiments samples of blood were collected and all fish were injected with salmon GnRH analogue (D-Arg^6 sGnRH-A, 10 µg kg^{-1} of b.w.) and pimozide (dopamine antagonist, 5 mg kg^{-1} of b.w). After injection blood samples were collected at 6, 12 and 24 hours. LH levels were measured by the ELISA. For histopathological analyses gill samples were stained with H&E method. Analysis of LH concentrations did not show any significant differences between LH levels neither in experimental nor control group at both times of exposition. However the pathological changes in gills in fish from experimental groups in short and long-term exposition were found.
Key Words: gills, LH, Prussian carp, Roundup.

Introduction. Pesticides, herbicides or fertilizers used to control the growth of vegetation are toxic to aquatic organisms. Herbicide Roundup is one of the most widely used in the world because of its efficacy in weed control. It is also used to control algal blooms and aquatic vegetation in ponds, lakes, fishponds, and canals (Cox 1998; Jiraungkoorskul et al 2002). The toxic properties of glyphosate - the main active ingredient of Roundup - have already been well documented (Folmar et al 1979; Duke 1988; Malik et al 1989). This component is relatively safe for fish (96hr LC_{50} for Cy*prinus carpio* is 620 mg L^{-1}) (Neškovic et al 1996). However, the formulation Roundup contains not only glyphosate but also a surfactant - polyethoxylated tallowamine (POEA), which significantly increases the toxicity of the main product. The toxicity of Roundup depends on the species and the physicochemical properties of water. The LC_{50} (lethal concentration) values for 96 h of exposure varies from 2 to 55 mg L^{-1} for fish species (WHO 1994; Jiraungkoorskul et al 2002).

There are several investigations dealing with the negative impact of Roundup and glyphosat on aquatic organisms and fish, among others. For example histopathological studies on *Clarias gariepinus* showed that glyphosat in concentration of 1.9 to 45 mg L^{-1} caused mononuclear infiltration, mononuclear degeneration, and spongiosis in the brain (Ayoola 2008a). In other hand Roundup affected acetylcholine esterase activity in the brain and muscle of fish (Glusczak et al 2011; Modesto & Martinez 2010). Studies on alkaline phosphatase activity in liver and heart of carp showed that Roundup increases the activity of this enzyme at concentrations of 2.5 to 10 mg L^{-1} (Neškovic et al 1996).

Also in gills from Roundup treated fish it was registered histological alterations such as epithelial hyperplasia and subepithelial edema (Neškovic et al 1996; Olurin et al 2006).

However, only few works are dedicated to the problem of breeding. In the literature there is no information on the influence of Roundup on gonadotropins secretion in fish. It is known that sub-lethal concentrations of pollutants in waterways may affect the hormonal regulation of reproduction (Carragher & Sumpter 1990; Pottinger & Pickering 1990; Kime 1995). Roundup can influence some hormone secretion or affect the activities of different enzymes. For example, Soso et al (2007) found that sub-lethal concentrations of Roundup may have deleterious effects on the reproduction of *Rhamdia quelen* expressed by the levels of testosterone (T), 17β estradiol (E_2) and cortisol.

The main object of this study was to verify whether Roundup affects the spontaneous and stimulated by GnRH (gonadotropin-releasing hormone) analogue and pimozide (dopamine antagonist) secretion of LH (luteinizing hormone) in Prussian carp *Carassius gibelio* which is a model species of carp having high resistance to adverse environmental conditions.

Material and Methods. The experiment was conducted at the University of Agriculture, Department of Ichthyobiology and Fisheries in Kraków, Poland, in cooperation with Comparative Anatomy of Vertebrates Department of Jan Kochanowski University in Kielce.

Fish were collected from the ponds of Fisheries Research Station of Agricultural University in Krakow. Experiment was divided into two sets: short-term exposition (10 days in May) and long-term exposition (6 months - from May to October 2010). For the short-term exposure 36 adult females of Prussian carp were used. The mean body weight of fish was 58.88±7.49 g. Fish were acclimated for 10 days to the conditions of the experiment, then they were divided into two groups: control (18 females) and experimental (18 females) and placed in glass tanks (300 L) with aerated water. During the experiment fish were kept in natural day light. The temperature of water was 20±2°C, pH 7.1±0.2, total hardness 428±2.37 $CaCO_3$ mg L^{-1} and ammonia concentration lower than 0.003 mg L^{-1}. Monsanto product - Roundup® 360 SL (41.5% acid equivalent of the isopropylamine salt) at the concentration of 15 mg L^{-1} (sub-lethal concentration according to the Neškovic et al 1996) was added to the tank with experimental fish once, in the first day of exposure. The halflife of this product in water varies from 12 to 60 days (Cox 1998). For the long-term experiment water conditions were similar to the short-term exposition. Fish (36 females of mean body weight 64.8±9.93 g) were acclimatized for 10 days and then were split to two groups: control (18 females) end experimental (18 females). Roundup was added at the concentration of 15 mg L^{-1} at 10 day intervals to experimental tank. In the time of Roundup addition water was exchanged in all tanks. At the end of both experiments samples of blood (200 µl) were collected from anaesthetised fish (Propiscine at 2 ml L^{-1} in immersion) and then all of them were injected with salmon GnRH analogue (D-Arg^6 sGnRH-A at 10 µg kg^{-1} of b.w.) and pimozide – a dopamine receptor antagonist (5 mg kg^{-1} of b.w.). Six, 12 and 24 hours after injection blood samples were taken from all fish. Samples were centrifuged and separated plasma was stored at -20°C. LH levels were measured with the use of ELISA (enzyme-linked immunosorbent assay) method (Kah et al 1989). Effect of Roundup on LH secretion was expressed as a percentage of control secretion.

Twenty-four hours after injection (in both times of exposition to Roundup) fish were checked for ovulation and then they were killed by decapitation. Gonadosomatic index (GSI) was calculated. Also gill samples were taken and fixed in 4% formaldehyde. For histological investigation they were stained with Hematoxyline-Eosine (H&E).

The results were analyzed using statistical program GraphPad Prism (version 5). A nonparametric one-sided Mann-Whitney test (U-test) was performed. Differences between groups were considered significant for $p<0.05$.

Results

LH levels

Short-term exposure to Roundup

LH levels measured in plasma before the GnRH analogue injection did not show any significant differences (p=0.0611) between control and experimental group. Secretion of LH in experimental group at 6 and 12 hours after injection was lower than in control group however these differences were also not significant (p=0.3472 and 0.2806, respectively). The LH levels 24 hours after injection were higher in experimental group than in control, but this difference was not significant (Figure 1A).

Long-term exposure to Roundup

During long-term exposure were not found significant differences between control and experimental group, however in all sampling times LH levels were higher in the experimental than in control group (Figure 1B).

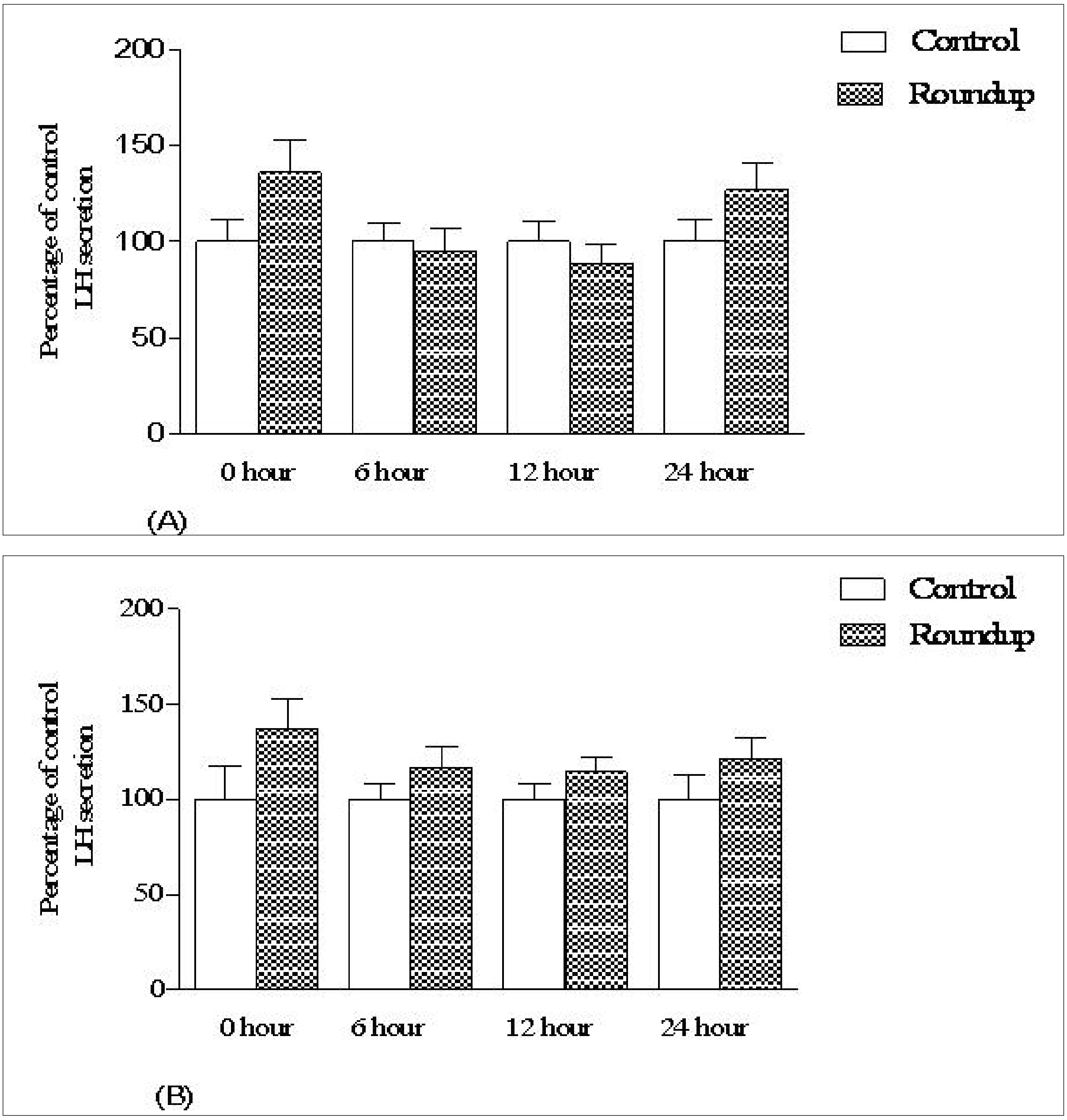

Figure 1. Levels of plasma LH (mean ± S.E.M.) in Prussian carp exposed to sub-lethal concentration of Roundup for 10 days (A) and 6 months (B), expressed as a percentage of control secretion. Time of injection - 0 hour, sampling times after injection - 6, 12, 24 hours.

Ovulation and gonado-somatic index (GSI) determination. In short-term exposure all control and Roundup treated fish ovulated. During long-term exposure to Roundup there was no ovulation in both groups. The mean GSI values were 3.68±0.9% and 4.1±0.56% in control group and Roundup treated fish, respectively. Differences between groups were not significant.

Gill histological studies. There were no pathological changes in gills of fish from the control group in both experiments (Figure 2), however, in gills of Roundup treated fish the pathological changes in the secondary lamellae were observed. In fish from the short-term study alterations like hypertrophy, hyperplasia and edema of the epithelial gill cells were more visible than in fish from the long-term experiment (Figure 3).

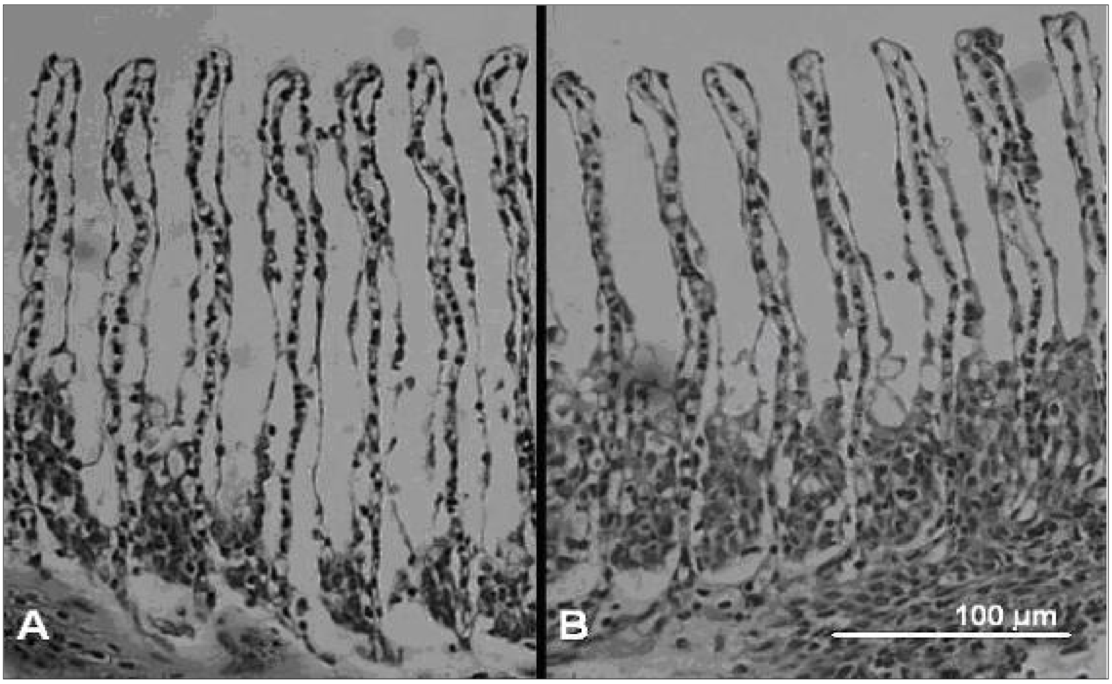

Figure 2. Gill transverse section of control Prussian carp during short-term (A) and long-term (B) exposition.

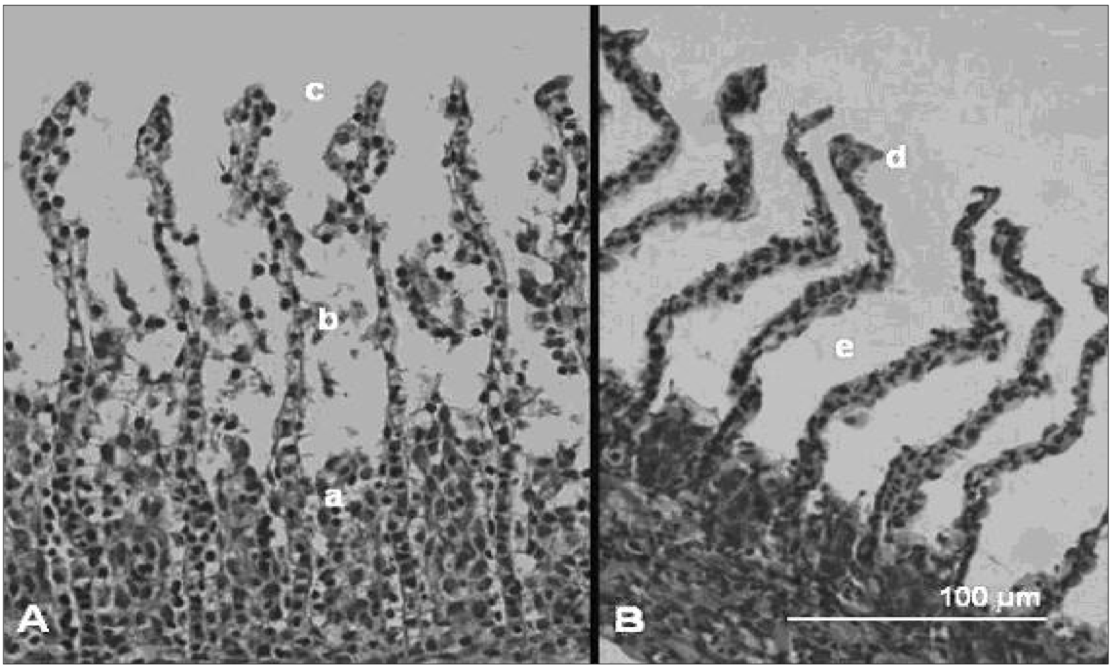

Figure 3. Gills of Roundup treated fish of short-term exposition (A). The hypertophy of the cells along the secondary lamellae (a), as well as laceration of epithelial cells (b) end edema with lifting (c). Long-term exposition (B). Clubbing in epithelial cells (d) and irregular thickening at the base of secondary lamellae (e).

Discussion. Until recently Roundup was considered as safe for aquatic organisms and classified as non-toxic (WHO 1994). However, the more recent publications have shown that Roundup at concentrations of 2.5-20 mg L^{-1} decrease the activity of superoxide dismutase and glutathion reductase in goldfish brain (Lushchak et al 2009). These changes may suggest that Roundup induces mild oxidative stress in this fish. The toxic effects of Roundup on the brain were also observed in *Oreochromis niloticus* and *Clarias gariepinus* (Ayoola 2008 a,b). In these species different doses of the herbicide (1.9-45 mg L^{-1}) caused certain changes in the brain, like mononuclear infiltration or vascular congestion. Also in other tissues, changes in morphology or enzymatic activities were shown. Alkaline phosphatase activity measured in carp liver and heart was elevated. The changes observed in enzyme activities depend on fish species, doses and the time of exposition (Neškovic et al 1996). Hepatocytes in liver of *O. niloticus* showed extensive pyknotic nuclei, large vacuoles and several leukocyte infiltration (Jiraungkoorskul et al 2002; Ayoola 2008 a,b).

Pathological changes in tissues or differences in enzyme activities are not the only effects observed in fish exposed to Roundup. Also hormonal profiles could by unsettled by Roundup (Walsh et al 2000; Soso et al 2007; Salbego et al 2010). Most information about hormones concerns steroidogenesis. Soso et al (2007) showed that the concentration of cortisol in *R. quelen* females treated with a sub-lethal dose of glyphosate (3.6 mg L^{-1}) increased during 40 days of exposure to the herbicide. The profile of cortisol levels reflect the typical response of fish to stress. In herbicide-exposed females in the last day of experiment estradiol levels were significantly lower than in control fish. This observation shows that cortisol inhibits synthesis and/or secretion of estradiol, as it was demonstrated previously in rainbow trout (*Oncorhynchus mykiss*) (Carragher & Sumpter 1990) and suggests that cortisol may also act directly on the oocyte steroidogenesis. Testosterone levels in *R. qualen* females did not differ from the control fish.

Based on the above mentioned results of Soso et al (2007) and from other investigations (Carragher & Sumpter 1990; Neskovic et al 1996; Ayola 2008 a,b) showing alterations in enzyme activity or hormone secretion it could be expected that sub-lethal concentration of Roundup (15 mg L^{-1}) may also affect the spontaneous or hormonally stimulated LH secretion in Prussian carp. The results obtained from the present work for both short and long–term exposure to Roundup did not show any significant influence on the spontaneous or stimulated (by GnRH analogue combined with dopamine antagonist) LH secretion (Figures 1A and 1B). In May, after 10 days of the experiment all fish (control and experimental) stimulated with hormones, ovulated. This result may suggest that short exposure to sub-lethal concentration of Roundup is not detrimental for the final maturation of oocytes and the ovulation in Prussian carp. Gonadotropin levels after hormonal stimulation were relatively low in individuals from both experiments, despite of the reliable preparation, which is widely used in aquaculture as an ovulation-inducing agent. Such results may be caused by the stress affecting control as well as Roundup-exposed fish kept for 6 month in experimental conditions. To prove this fact the measurements of cortisol levels would be necessary.

During long-term exposure to Roundup GSI values were similar between control and herbicide-exposed group. Since the end of experiment fell on October, ovulation was not observed and fish were entering the phase of gonadal regression and vitellogenic oocyte resorption. Similar results concerning GSI were observed by Soso et al (2007) in *R. qualen* where the index values were similar in Roundup-treated and control fish.

Despite of the fact that negative effects of Roundup on ovarian maturation and ovulation were not found, some detrimental effects of this herbicide was noticed in gills. Histological analysis has shown pathological changes in gill tissue, paradoxically the weaker influence of Roundup on gills was observed during the long-term exposure. It can be related to the fact of the adaptation of gills to chronic exposure to this herbicide. Intensive changes in the epithelium of the gills during short exposition manifested by cell hypertrophy are a defense mechanism to protect the internal tissues as well as they constitute a beginning of a process of adaptation (Meissner & Diamandopoulos 1977). The confirmation of this fact are based on the results from long-term exposure to Roundup presented in this work showing that negative changes were less severe. It should be noted that the increase in thickness of branchial epithelial cells reduces the gas exchange surface, which has a negative impact on the life processes of fish organism (Olurin et al 2006).

Conclusions. Taken together, the results obtained in the present work indicate that Roundup at the concentration of 15 mg L^{-1} did not influence the spontaneous and hormonally stimulated secretion of LH in Prussian carp during short- as well as long-term exposure. Roundup was more toxic for the gills during short- than long-term exposition. The lack of significant influence of Roundup on LH levels and the last stages of ovarian maturity as well as the lower damage of gill epithelium during the long-term exposition show the adaptive ability of this species to the sub-lethal concentration of Roundup use in the present work.

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