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HISTOPATHOLOGICAL CHANGES INDUCED BY SEVERAL FUNGICIDES UPON GALL-BLADDER STRUCTURE IN *PELOPHYLAX RIDIBUNDUS*

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Abstract
The histopathology of three fungicides on gall-bladder structure in frog Pelophylax ridibundus were investigating by light microscopy. The frogs were experimentally exposed to sub-lethal concentration of the fungicides. The toxic substances were administered by intraperitoneal shots, one shot every two days, in a scheme of 3 weeks. Tissues samples were fixed in 8% neutral formalin and processed using histological method. The epithelial metaplasia was observed as response to the harmful action of the toxic and the presence of leukocyte infiltrations.

Keywords: gall-bladder, frog, fungicides.

1. INTRODUCTION

Fungicides have multiple purposes in industry, agriculture and households including: seed protection during storage, transport and germination, protection of forest fruit, seedlings, field flowers, in warehouses or transport, prevention from mildew that affects damaged surfaces, protection of carpets, wood from homes and factories. With their wide range of uses, fungicides can have side effects in human poisoning. The most severe "epidemic" of pesticide poisoning has been caused by the unconscious consumption of grain seeds treated with organic mercury or hexachlorobenzene. Most currently used fungicides do not cause severe poisoning because they generally have low toxicity in mammals, are poorly absorbed and applied in the form of suspensions which are rapidly absorbed by the plants.

Propiconazole is frequently detected in aquatic ecosystems (Levine et al., 1999; Castillo et al., 2000), due to its increased persistence, while its presence in soil is poorly detected (Kim et al., 2002). Although propiconazole is used to combat fungal infections, its accumulation in water may cause side effects in aquatic organisms (fish, amphibians).

Propiconazole metabolism has been studied in mammals and 5 metabolites have been found and isolated from urine in the first 24 hours. Cytotoxicity of propiconazole is said to be much higher compared to cytotoxicity induced by its metabolites (Chen et al., 2008).

Champion 50 WP is a copper-based contact fungicide, used to prevent and combat bacteria and bacterial burns in vineyards, fruit trees, vegetables, potatoes. It contains 50% Cu in the form of $Cu(OH)_2$.

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It is part of the third group of toxicity, being harmful to earthworms, fish and other aquatic organisms. Copper is an essential trace element for plant and animal life, being involved in many biological processes (Unerwood, 1977). More than 30 enzymes are known to use copper as co-factor (Harris, 1991), among which ferrooxidase, cytochrome oxidase and superoxide-dismutase. The lack of copper in organisms causes the appearance of severe symptoms.
On the other hand, excessive copper becomes toxic, because it is a source of direct or indirect pollution (Atchinson et al., 1987). It reaches aquatic ecosystems, either by using many pesticides based on copper or in industry and it affects aquatic organisms. Unlike organic pollutants, this compound does not undergo changes in aquatic organisms and bioaccumulates in target organs such as liver, kidneys, muscles, intestines, spleen, causing alteration of these structures.
The toxic action of copper is manifested by blocking biochemical reactions, acting on proteins that have the -SH group or removing trace elements that are active sites of certain enzymes. These changes generate reduction of cellular metabolism, stimulation of lipid peroxidation, inhibition of oxidative phosphorylation, disruption of calcium homeostasis, and changes in the structure and permeability of cell membranes (Farkas et al., 2003).
Dithane M-45 is a contact fungicide containing 80% mancozeb, with protection role and a broad spectrum to fight pathogens in field crops, vegetables, flowers, orchards and vines, as well as seed treatment. These substances have low levels of toxicity that can be absorbed by the skin and their metabolites are carbon disulfide and ethylene thiourea. However, these metabolites are known to be responsible for various malfunctions of the nervous system (Edwards et al., 1997).
The present study emphasizes the effect of three fungicides (Tilt 250EC, Champion 50WG, Dithane 45M) on Pelophylax ridibundus species, widely spread in aquatic ecosystems with the histopathological picture of the gall-bladder affection.

2. MATERIALS AND METHODS

The biological material is represented by *Pelophylax ridibundus* adults of both sexes, sampled from the lakes near Piteşti. The healthy specimens were found in unpolluted lakes. After sampling, the animals were kept in glass aquariums with clean water which was changed daily to avoid the accumulation of toxins. Acclimatization of animals in the lab lasted 5 days, thus avoiding the sudden death of some specimens.
The following batches were organized for all tested substances:

- **control group** consisting of 18 specimens of untreated *Pelophylax ridibundus*, males and females, kept in tap water that was changed daily; it was divided into 2 sublots: one consisting of specimens of *Pelophylax ridibundus* kept at a temperature of 4-6ºC and the other consisting of specimens kept at a temperature of 22-24ºC. The samples were shot intraperitoneally with physiological serum for poikilotherms (6.5 ‰), a two-day shot for 3 weeks.
- **experimental group** consisting of 18 specimens of male and female *Pelophylax ridibundus*, treated with fungicides administered by intraperitoneal shots, a two-day shot in a 3-week scheme. Animals were kept in similar conditions to those in the control group, and were divided into 2 sublots: one consisting of treated specimens of *Pelophylax ridibundus* kept at a temperature of 4-6ºC and the other consisting of treated specimens kept at a temperature of 22 - 24ºC.

Fungicides used are shown in table 1.

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Table 1. Fungicides used in experiments

Fungicides	Sub-lethal concentrations
Tilt 250EC (250mg/l propiconazole active substance),	$2x10^{-3}$mg propiconazole/g body weight, $0.8x10^{-5}$ml Tilt 250EC/g body weight.
Champion 50WG (50% copper in the form of $Cu(OH)_2$)	$0.25x10^{-3}$mg $Cu(OH)_2$/g body weight, $0.125x10^{-3}$mg Champion 50WG/g body weight
Dithane 45M (mancozeb – active substance).	$1x10^{-3}$mancozeb/g body weight, $0.8x10^{-3}$ mg Dithane M-45/g body weight

Control and experimental groups were made up of specimens with approximately the same weight (60g±5) and the same sex distribution. Throughout the experiment, the animals were fed *ad libitum*. At the end of the treatment, the animals were anesthetized with chloroform and spinalized by a method indicated by Picoș and Năstăsescu (1988). The bile of the treated specimens was sampled, and poikiloterms were fixed in 8% saline formalin. The samples were processed by histological techniques and the sections were studied using the Olympus microscope.

3. RESULTS AND DISCUSSIONS

The gall-bladder is a sacral diverticulum attached to extrahepatic bile ducts (Papilian and Roșca, 1978). The gall-bladder of amphibians has the role of a bile reservoir, as in other vertebrates, but can also alter its composition. It is formed in the postero-ventral region of the liver bud. Its wall is made up of a columnar epithelium doubled by a thin layer of mesenchymal cells. The gall-bladder lumen previously elongates through the cystic duct. The common bile duct is formed by joining this duct with the main hepatic duct. It opens in Anura (tailless amphibians), in the duodenum after joining the two pancreatic channels, which open independently in the duodenum of tailed amphibians. Histologically, it consists of 3 layers: internal tunica formed by epithelium and chorion, fibro-muscular and peritoneal serum tunica.

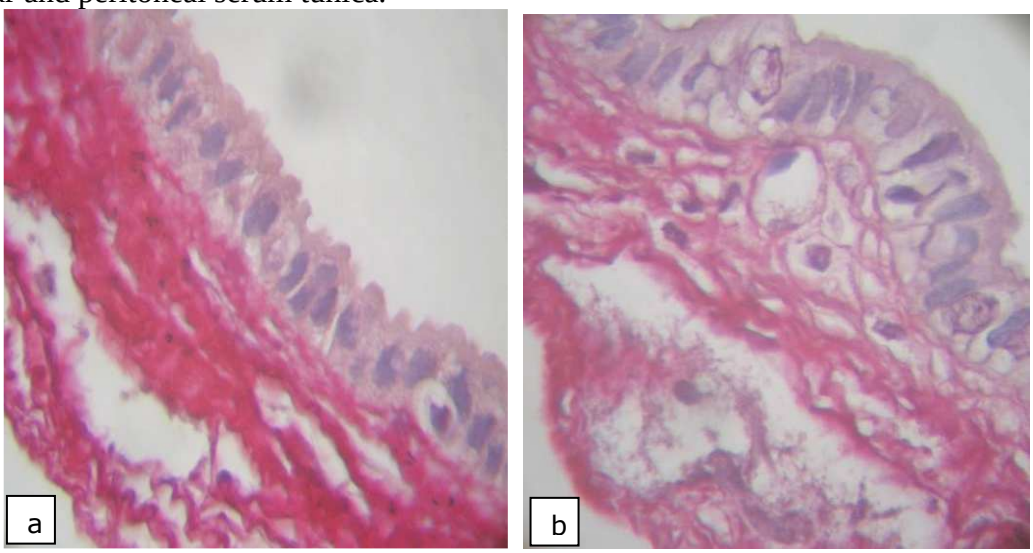

Figure 1. Cross section of Pelophylax ridibundus gall-bladder – control groups kept at a temperature of 4-6°C (a) and 22-24 ° C (b). 400 ×. H-Sirius red

The mucous membrane has numerous large and small creases in the section (Fig. 1). It is bordered by a simple, cylindrical epithelium with calciform cells apparently devoid of a basal membrane, which appears to be located directly on the chorion. The epithelial cells have a striated border on the

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apical pole, and there is a small number of caliciform cells among them. The nucleus of the epithelial cells is oval, and the cytoplasm is highly eosinophilic. Its mucous membrane secretes an acid mucus (Yamada, 1969).

Hydrolytic enzymes in the gall-bladder were studied on *Rana esculenta* in winter and *Salamandra salamandra* in summer (Oledzka-Slotwinska, 1968). Alkaline phosphatase and lipase recorded a very low activity during summer (zero on Rana during the winter) while acid phosphatase and esterase showed increased activity (less on Rana during the winter)

The action of Tilt 250EC fungicide on *Pelophylax ridibundus* gall-bladder

The gall-bladder, the storage organ of the bile produced by the liver, is slightly affected by the action of Tilt 250EC fungicide. Administration of 2x10-3 mg propiconazole / g body weight concentration in animals kept at a temperature of 4-6°C resulted in increasing the volume of this organ. More than 70% of the specimens accumulated a greater amount of bile in the gall-bladder.

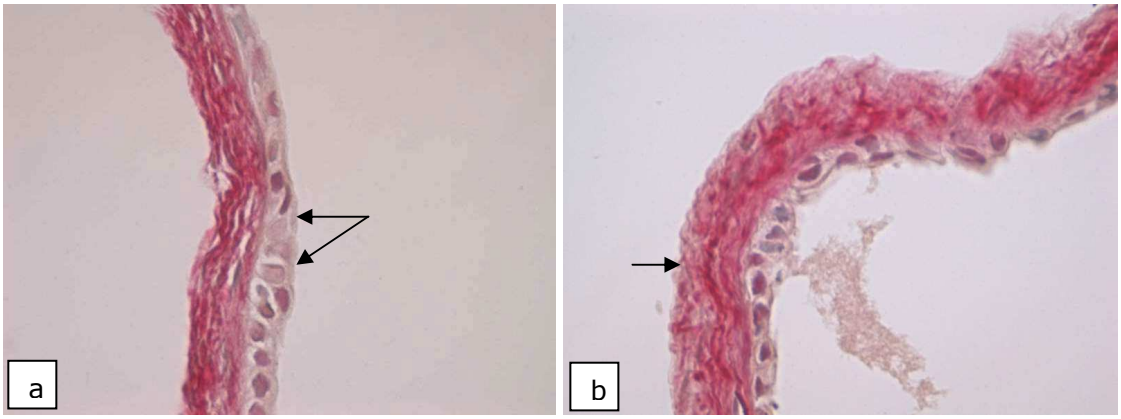

Figure 2. Cross-section of Pelophylax ridibundus gall-bladder (Tilt 250EC intoxication in animals kept at 4-6ºC). a- cubic epithelial cells with striated border. b- thin fibro-muscular tunica with numerous collagen fibers. 100 ×. H-Sirius red

In terms of histology, there is a one-layer coating epithelium consisting of cubic, isodiametric cells with central nucleus. The striated border is well represented in the apical pole of the epithelial cells. There are few mucus-secreting calciform cells among the epithelial cells (Fig. 2a).

The fibro-muscular tunica is thinner and more fibrous, containing many collagen fibers colored in intense red with Sirius red (fig.2b).

The effects of Tilt 250EC fungicide on the gall-bladder in animals kept at a temperature of 22-24°C are similar to those described above.

Thus, the one-layer epithelium consists of elongated, cylindrical cells which have a well individualized striated border at the apical pole. Their nucleus is elongated and placed in the centre. Some epithelial cells have lipid deposits in the cytoplasm that push the nucleus to the periphery. The basal membrane is integral (Fig. 3a). Among the epithelial cells there are leukocyte infiltrations from the blood vessels at the base of the epithelium (Fig. 3b).

The fibro-muscular tunica is thicker and has fewer collagen fibers and numerous elastic fibers.

The histological changes in the gall-bladder following the administration of Tilt 250EC fungicide, are associated with the accumulation of a larger amount of bile, whose role is to store the liver secretion product.

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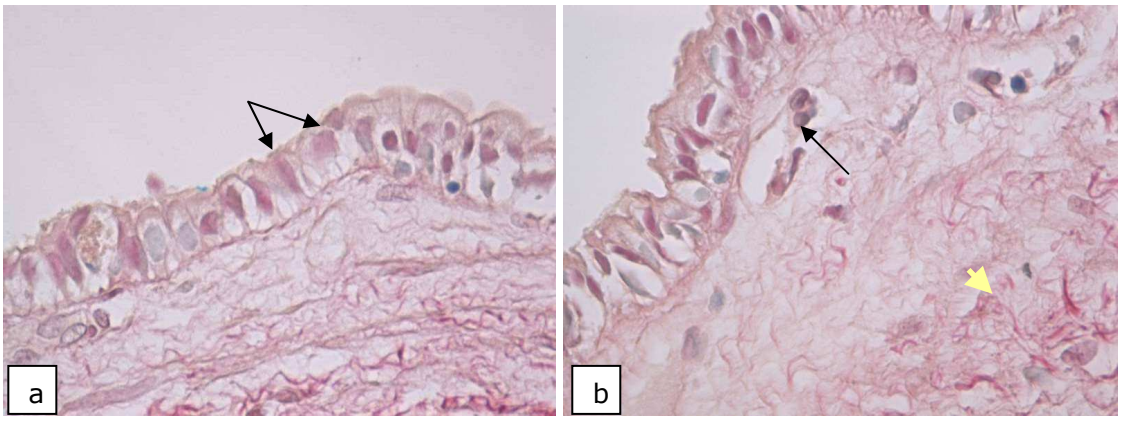

Figure 3. Cross-section of Pelophylax ridibundus gall-bladder (Tilt 250EC in animals kept at 22-24ºC). a - cylindrical cells with striated border. b- leukocyte infiltration (black arrow); collagen fibers (yellow arrow). 400 ×. H-Sirius red.

Champion 50WG fungicide action on *Pelophylax ridibundus* gall-bladder

Since copper can be eliminated through the bile, the gall-bladder also undergoes changes under the action of Champion 50WG fungicide at the 2 thermal levels studied.

The gall-bladder of animals kept at a temperature of 4-6˚C which were administered Champion 50WG fungicide has a one-layer epithelium consisting of elongated cells with elongated nuclei. Some epithelial cells accumulate lipid granules in the cytoplasm that push the nucleus to the periphery (fig. 4). The striated border of these cells is slightly dispersed. The basal membrane is integral. The fibro-muscular tunica is under the epithelium and consists of few collagen fibers and numerous elastic fibers along with flat muscle fibers.

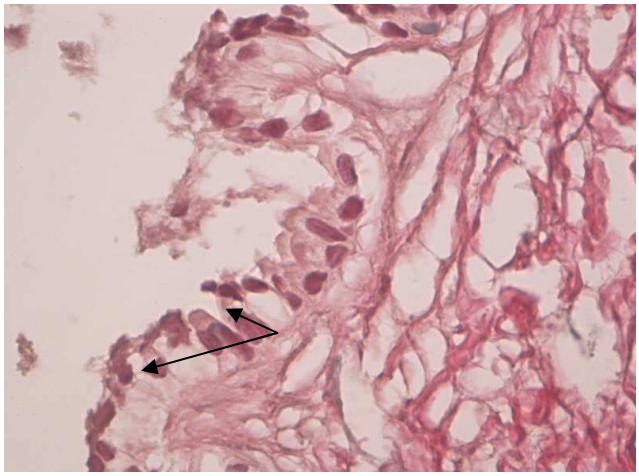

Figure 4. Cross section of Pelophylax ridibundus gall-bladder under the action of Champion 50WG fungicide. Animals kept at a temperature of 4-6˚C. One-layer epithelium consisting of elongated cells with lipid granules in the cytoplasm (black arrows). 400×. H-Sirius red

The administration of the same fungicide concentration in animals kept at 22-24 ° C leads to the layering of the gall-bladder epithelium (Figure 5b). The epithelial cells are tall, cylindrical and have nuclei in the shape of the cell. The phenomenon of lipid granules accumulation in the epithelial cells can also be observed in Fig. 5a.

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There are leukocyte infiltrations at the base of this epithelium as a result of the inflammation generated by the action of the toxic.
The fibro-muscular tunica is thicker, elastic, with fewer collagen fibers.

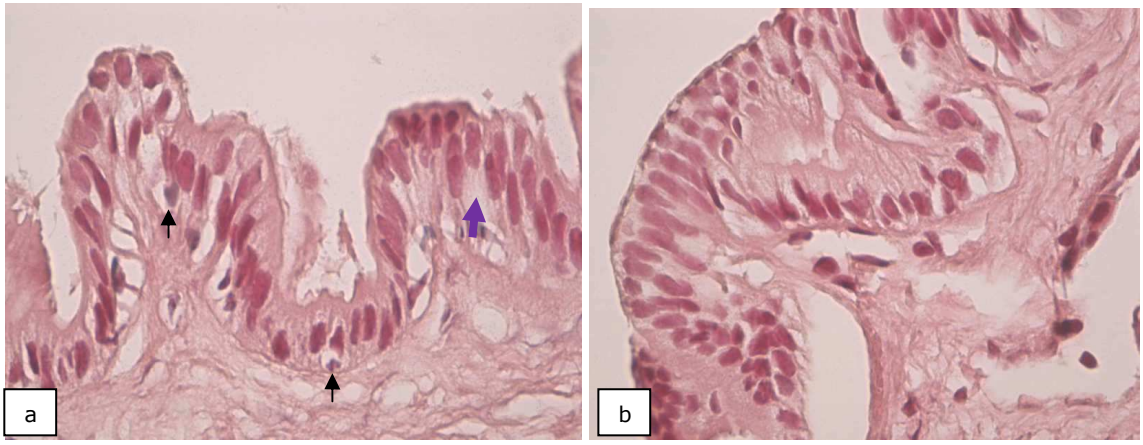

Figure 5. Cross section of Pelophylax ridibundus gall-bladder under the action of Champion 50WG and kept at a temperature of 22-24˚C. a- epithelial cells with lipid granules in the cytoplasm (blue arrow); leukocyte infiltration (black arrows). b- epithelium layering tendency. 400 ×. H-Sirius red

The action of Dithane M-45 fungicide on *Pelophylax ridibundus* gall-bladder

Dithane M-45 fungicide also becomes toxic to the gall-bladder. Therefore, the animals kept at a temperature of 4-6˚C and under the action of this toxic have a tendency of hyperplasia of the epithelium. The epithelial cells are tall with elongated nuclei. They have a striated border at the apical pole (fig. 6). There are leukocyte infiltrations among the epithelial cells as a defense action of the tissue against the action of the toxic. The base membrane of the epithelium is integral. The fibro-muscular tunica is well represented and consists of numerous collagen fibers, which results in higher rigidity of the gall-bladder walls.

Figure 6. Gall-bladder of Pelophylax ridibundus specimens under the action of Dithane M-45 fungicide - temperature of 4-6˚C. Elongated epithelial cells with elongated nuclei. The presence of cell infiltrations (white arrow). Fibro-muscular tunica with collagen fibers. 400 ×. HE and Sirius red

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Figure 7. Gall-bladder of Pelophylax ridibundus specimens under the action of Dithane M-45 fungicide - temperature of 4-6˚C. Hyperplasia of the epithelium. The presence of cell infiltrations (arrows). Fibro-muscular tunica with collagen fibers. 400 ×. HE and Sirius red colour

Administration of the same toxic concentration on animals kept at a temperature of 22-24˚C causes similar changes (Fig.7). The gall-bladder epithelium has the same tendency of hyperplasia. The epithelial cells are tall and have a striated border at the apical pole. They include simple, cubic cells, as well as lymphocyte infiltrations. Fibrosis of the fibro-muscular tunica is more increased, resulting in higher rigidity of the gall-bladder wall.

All these histological changes in the gall-bladder under the action of Dithane M-45 fungicide may be the result of toxic elimination via bile due to the fact that the mancozeb contains zinc and manganese ions, heavy metals that are eliminated via bile.

4. CONCLUSIONS

Propiconazole, the active substance of Tilt 250EC fungicide does not cause clear histological changes in the gall-bladder of intoxicated animals kept at the 2 thermal levels.

The histological changes under the action of Champion 50WG fungicide consist of the presence of lipid granules in the epithelial cells, with the tendency of the striated border to be dispensed, for animals kept at 4-6°C. The animals intoxicated with Champion 50WG and kept at 22-24^{0}C showed hyperplasia of the gall-bladder epithelium, and the presence of leukocyte infiltrations at its base. By acting on the gall-bladder, Dithane M-45 fungicide causes epithelial hyperplasia with the presence of leukocyte infiltrations in animals kept at 4-6^{0}C. Similar changes were also highlighted in animals kept at 22-24^{0}C.

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