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Sublethal effects of the herbicide thiobencarb on fecundity, histopathological and biochemical changes in the African catfish (*Clarias gariepinus*)

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Abstract

Fishes have been widely documented as useful bioindicator for ecotoxicological studies because of their differential sensitivity to pollution. Thus, the present study was carried out to determine the sub-lethal effects of commercial herbicide thiobencarb (CITRON®, 50EC) on the African catfish (*Clarias gariepinus*). Females fish were exposed to sub-lethal concentration of thiobencarb (½ LC_{50}, 0.72 ppm) for 3, 9 and 15 days. Severe abnormality in the swimming behavior was observed in fish groups exposed for 9 and 15 days. Adverse effects on the ovary and liver weights were observed. The absolute fecundity was significantly decreased by all thiobencarb-treatments, for which the lowest value was observed at 15-day treatment in comparison with that of the control. A significant decline (p<0.01) in the activities of serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) was observed in fish as the exposure period continued as compared to the control. Similarly, glucose and total protein levels were also significantly declined (p<0.01) with the exposure period. Histopathological changes in the liver tissue of fish exposed to thiobencarb were characterized by necrosis, changes in nuclear shape, formation of vacuoles and atrophy of hepatocytes. The ovary of fish exposed to thiobencarb for 15 days showed atretic vitellogenic oocytes and proliferation of follicular cells as well as inflammatory cells infiltration. These results indicate that thiobencarb is toxic and has the potential to impair on the physiological activities in African catfish. Therefore, the use of thiobencarb should be strongly controlled and carefully monitored to minimize its negative impacts on the aquatic ecosystems.

Keywords: Toxicity, Fecundity, Biomarkers, Biochemistry, Histopathology

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Introduction

The use of herbicides to control weeds has been documented as a part of agricultural practices throughout the world (Nwani *et al.*, 2010). These herbicides can enter aquatic ecosystems as a result of the intensive and poorly regulated usage causing harmful effects on fish population, cellular functions, growth and behavior of aquatic organisms (Hanazato, 2001; Zou, 2003; Relyea, 2005). Presently, there is an increasing world concern over the indiscriminate use of the herbicides that result in environmental pollution and toxicity risk to non-target organisms (Velisek *et al*., 2009). Thiobencarb [S-((4-chlorophenyl) methyl) diethyl-carbamothioate] is one of the most commonly used herbicides against grass weeds (Johnson, 1997; Rao *et al.*, 2007). This herbicide belongs to the thiocarbamate herbicides and is classified into group N of the Herbicide Resistance Action Committee (HRAC, 2010). According to the HRAC, the target site of the group N herbicides is described as the lipid synthesis except for acetyl-CoA carboxylase (ACCase). It is a systematic, pre-emergence herbicide that acts by inhibiting shoots of emerging seedlings (USEPA, 1997). The commercial formulation of thiobencarb (Citron® 50%) is being used extensively in Egypt, at 1.5-4.5 kg 10,000 m^{-2}, to control weeds in transplanted rice before seeding and early post-emergence application (Anonymous, 1993).

Fishes, as one of the most representative organism in freshwater, are often on the top of the trophic level. Due to bioaccumulation of herbicides, the concentration of herbicide in fishes may become high enough to induce toxic responses in fishes and also in humans who may use these fishes for consumption. Fishes may also be considered good bioindicators for ecosystem health (Cavas, 2011). Fishes species are the most sensitive to aquatic pollutants during their early life stages (Jiraungkoorksul *et al*., 2002). The African catfish, *Clarias gariepinus* selected in the present study seems to occur nearly in all water bodies inhabited by fishes. It is also one of the most popular fishes cultured in Egypt and other African countries next to the tilapiine fishes (Tesfaye, 1998; Nyamweya *et al*., 2010). This species is an aquaculture candidate that can narrow the gap between the demand and supply of animal protein in developing countries (Odo *et al.*, 2017). It is also considered an attractive model for toxicity studies because of its availability throughout the year, voracious feeding habit, prolific reproduction and general hardness in culture environments (Nwani *et al*., 2015). It can easily acclimatize to laboratory conditions. Thus, it is an excellent model for ecotoxicological studies.

Previous studies have investigated the toxicological effects of several herbicides in fishes (e.g. Nwani *et al*., 2010; El-Sayed *et al.*, 2013; Nwani *et al*., 2015; Sherif *et al*., 2015). However, the sub-lethal effects of thiobencarb on *C. gariepinus* fish have not been completely shown. Thiobencarb is defined as moderate to high toxicity to

fish by Villalobos *et al*. (2000), because it resists hydrolysis in aquatic environments and is bioaccumulated is in fish *in vivo*. However, chronic toxicity effects of thiobencarb on *C. gariepinus*are still not precisely known. To evaluate toxic stress of environmental contaminants, biochemical parameters have been widely used. These parameters are including the levels of plasma proteins, glucose, and other enzymes, like alanine aminotransferase (ALT) and aspartate aminotransferase (AST) (Burtis and Ashwood, 1996; El-Sayed *et al*., 2007; Kumari *et al*., 2011; Nwani *et al*., 2015). Therefore, biochemical analysis of blood plasma are useful in monitoring the physiological status of fish~~,~~ health in the aquatic environment, and considered as an important biological factors in aquaculture (Hochachka and Mommsen, 1995). Biochemical markers such as stress proteins and metabolic enzymes generally reflect stress conditions in fish induced by a broad range of environmental factors (Schwaiger *et al*., 1997; Marchand *et al*., 2008; Velisek *et al*., 2013).

Morphological indices, especially condition factor (CF) and HSI, have been proposed as an "exposure index" to environmental contaminants (Kopecka and Pempkowiak, 2008; Nwani *et al*., 2015). The CF, a somatic biomarker, is indicative of health and reflects feeding conditions as well as energy consumption and metabolism (Schulz and Martins-Junior, 2001; Alberto *et al*., 2005). The hepatosomatic index (HIS) refers to the relative liver size and relates to the hepatic enzyme activity for chemical detoxification (Fang *et al*., 2009). Some reports have demonstrated that CF declined in fish exposed to environmental pollutants (Khan, 2003). The hepatosomatic index (HSI) reflects the relative liver size and is linked to the hepatic enzymes activities for detoxification of compounds, indicating exposure to pollutants (Yeom *et al*., 2007; Fang *et al*., 2009). Toxic substances in the water may affect the fish growth by changing metabolism directly and increasing the energy required to maintain homeostasis, or they can indirectly influences growth by reducing food availability (Sadauskas-Henrique *et al*., 2011).

Previous studies related to the effects of pesticides on fish reproduction are rare and do not comprise the diverse range of events involved in reproduction such as oocyte maturation, spawning, fecundity and fertilization (Mir *et al*., 2011). Pesticide-induced reproductive failure or dysfunction is evident from the available reports on Indian fishes (Singh and Singh, 1982; Singh *et al*., 1997). Mir *et al.* (2011) investigated the effects of sub-lethal concentrations of the organophosphate pesticide dimethoate on the gonadosomatic index (GSI) of the fish, *Cyprinus carpio communis*. The maximum reduction in GSI values was obtained at the highest concentrations of dimethoatein of the treated fish ovaries showed histomorphological disorders. Furthermore, the reduced GSI was found directly proportional to the pesticide concentration and the

duration of the exposure (Mir *et al*., 2011). The sub-lethal effects of another insecticide, fipronil, on the reproduction dynamics of the zebrafish (*Danio rerio*) were also reported (Boaru *et al*., 2013). The effects on reproductive parameters were evident even at the lowest concentration of fipronil when applied, while exposure to the highest dose decreased the spawning, fecundation and hatching percentages in comparison with the control group (Boaru *et al*., 2013).

Histopathology provides a rapid method to detect effects of irritants, especially chronic ones, in various organs (Johnson *et al*., 1993; Bernet *et al*., 1999). Greenfield *et al*. (2008) explained that the histological alterations in selected target organs are sensitive biomarkers for xenobiotic effects, they occurred earlier and provided a better evaluation of the effects of aquatic pollution than any single biochemical parameter. The histopathological pictures of the organs can corroborate with the biochemical changes accounting for the functional disruptions in the activity of the organs due to cellular damage (Kalaiyarasi *et al*., 2017). The exposure of fish to chemical contaminants likely induces a number of lesions in different organs (Bucke *et al*., 1996). Since fish liver is regarded as a major site of storage, biotransformation and excretion of pesticides, histological changes in the liver were chosen as criteria for the sub-lethal action of thiobencarb. Histological investigations of fish liver repeatedly proved to be an extraordinarily sensitive tool to reveal both adaptive processes and detrimental effects in fish induced by organic pollutants (Cengiz and Unlu, 2006). However, scarce information is available on histopathological changes in fish ovaries due to herbicides' toxicity and particularly thiobencarb. Singh (2015) showed that exposure of common carp (*C. caprio*) to sub-lethal concentrations of dimethoate, for 96 h and 36 days, caused considerable structural damage to ovaries, which included breaking of ovigerous lamellae, lifting and fragmentation of follicular lining and zona radiata of mature oocytes.

Thus, this study investigated the impact of sub-chronic toxic effects of the herbicide thiobencarb on biometric indices, fecundity, serum biochemical analysis, and histopathology in the African catfish (*C. gariepinus*), an economically important freshwater fish worldwide.

Materials and methods

Experimental fish and chemical

The adult African catfish, *Clarias gariepinus,* with 205.0±4.03 g mean weight and total length 34.2±0.16 cm were purchased from a private commercial catfish farm and used as laboratory models. They were transported and placed in large plastic containers to the Biology Unit, Fish Diseases Department at Animal Health Research Institute, Dokki, where they were acclimatized for 3 weeks in plastic tanks (capacity of 300L). Water was renewed daily to help *C. gariepinus* acclimatize to the new environment. The fish were fed daily with a

commercial pellet diet (35% crude protein) at 3% of their body weight twice a day. The water used was analyzed weekly by standard methods (Eaton, 2005) for temperature, dissolved oxygen, hardness and pH, which were recorded as 23.5±1.5°C, 7.5±0.4 mg L^{-1}, 230.5±4.5 mg L^{-1} as $CaCO_3$ and 7.8±0.5, respectively. During the acclimatization period, experimental fish were monitored for disease conditions and mortality (OECD, 1992). In the present study, a commercial formulation of thiobencarb herbicide (CITRON® 50 EC) manufactured by Rhône-Poulenc Ag company, was used as stock solution.

96 hour-acute toxicity test

To determine the LC_{50}-96 h value of thiobencrab for the African catfish, acute toxicity, bioassays were conducted in 40 L glass aquaria (60×30×30 cm size) in a semi-static laboratory system. The study was conducted according to the Organization for Economic Cooperation and Development (OECD, 1992), guideline No. 203. A set of 10 fish specimens were randomly exposed to different concentrations (0, 0.35, 0.70, 1.40, 2.80 and 5.60 mg L^{-1}) obtained by serial dilution of the stock solution. The experiment was set in triplicate to obtain the LC_{50}-96 h value of thiobencarb exposure for African catfish. Feeding was stopped 24 h prior to and during the 96-h exposure period in order to prevent interference with stomach contents and wastes in the fish culture water (Smith *et al.*, 2007; Olufayo, 2009). During the acute toxicity test, the mortality and survival rates of the fish were recorded daily under each test concentration. Fish are considered dead if there is no visible movement (e.g. gill movements) and if touching of the caudal peduncle-making no reaction. Dead fish are removed to avoid pollution of the water. The LC_{50}-96 h value of thiobencarb was determined using the PC-Probit software based on Finney's Probit analysis procedure (Finney, 1971).

Sub-lethal toxicity tests

Based on the 1.44 mg L^{-1} (LC_{50}-96 h-acute toxicity) obtained value, a sub-lethal concentration of 0.72 mg L^{-1} corresponding to ½ LC_{50} was used for this study. A total 120 acclimatized-fish females of mean weight 215.0±3.5 g were used in this experiment. The fish were divided into four groups at which a set of 30 fish were introduced in triplicate treatments (10 fish/ replicate) for each group in a 50 liter- capacity aquarium. Fish in Group-1 served as control which was held in tap water with no pesticide exposure. Fish in treated groups were exposed to the selected concentration of thiobencarb for different periods as follows: 3 days (Group-2), 9 days (Group-3) and 15 days (Group-4). Semi-static system was used in this experiment at which the test solution was renewed every two days (Nwani *et al.*, 2015). During the experiment, fish were fed with commercial pellet feed at 3% of body mass once a day and mortality was recorded. To prevent oxygen depletion, experimental tanks were continuously oxygenated by using an air pump. Dead

fish were immediately removed to avoid possible deterioration of the water quality. During the test period, fish behavior was observed daily for hyperactivity, equilibrium status, swimming rate, fins movement, jerky movements, air gulping and skin discoloration. The water temperature, pH, dissolved oxygen concentration and salinity were measured on each renewal day.

Biochemical analysis

At the end of the duration of each exposure, 4-6 fish from each replicate of each group were randomly selected for blood sampling. Blood was collected by puncturing the caudal vein with a 2 mL- plastic heparinized syringe needle. The blood was emptied into 5 mL- heparinized blood bottles without any anticoagulant and centrifuged at 1,000 *xg* for 10 minutes to obtain the serum for analysis. The enzymatic aspartate AST and ALT activities were analyzed according to the method of Reitman and Frankel (1957). Serum protein and glucose levels were analyzed according to the methods of Young (1990) and Schmidt and Schmidt (1963), respectively. All chemical reagent-kits were purchased from BioMed Diagnostics, Giza, Egypt.

Determination of biometric indices

Immediately after blood sampling, body weight (BW) and body length (BL) were recorded. The CF of each fish was calculated according to the formula: CF=BW (g)/ BL $(cm)^3\times$ 100. After measuring the fish biometric characteristics, both ovaries and hepatopancreas were dissected and weighed in order to determine the GSI and HSI for each fish according to the following formulae:

GSI (%)=(Gonad weight/BW×100) and HIS (%)=(Liver weight/BW×100)

Estimation of fecundity

Fecundity has been considered as the number of ripening eggs in the female prior to spawning (Bagenal and Braum, 1978). Gravimetric method was used for estimation of the fish fecundity (Bithy *et al*., 2012).Three matured female samples were randomly selected from each group. Then these matured females were dissected and the whole gonads from each specimen were removed out intact. The matured ovaries were weighed nearest gm by a sensitive scale. These ovaries were then split longitudinally and kept in Petri-dish. Then, weights of right and left gonad were measured. Three samples, each was taken from the anterior, middle and posterior regions of each ovaries and kept in Petri-dish. All the ovaries were kept in Petri-dish with few drops of Gilson's fluid for 10 minutes with periodical shaking. The number of ripen eggs (F1) for the sub-sample was estimated by using the following equation (Yelden and Avsar, 2000):

Fecundity (F1)=[(No. of eggs in sub-sample×gonad weight)/Weight of sub-sample]

Later, by taking the mean number of three sub-samples (F1, F2, F3), the total (absolute) fecundity for each female fish was estimated F= (F1+F2+F3)/3. In addition, the relative fecundity

parameters were also estimated for each fish as absolute fecundity (F) divided by: body length (FBL), body weight (FBW) and gonad weight (FGW).

Tissue histopathological analysis

At the end of each exposure period, fish were randomly selected for histopathological examinations using the routine standard histological technique (Bancroft and Cook, 1994). Liver and ovaries tissues were isolated from the control - and thiobencarb-treated groups. Physiological saline solution (0.75% NaCl) was used to rinse and clean up the tissues. They were fixed in neutral buffered formalin (10% NBF) for 48 hours. The samples fixed in formaldehyde solution were processed through graded series of alcohols, cleared in xylene and embedded in paraffin wax. The tissues were sectioned into thin sections (5-7 µm), dehydrated and stained with Haematoxylin and Eosin (H&E) stain (Bancroft and Cook, 1994). Histopathological lesions were examined using light microscope and photographed.

Statistical analysis

Data obtained were subjected to statistical analysis using one-way analysis of variance (ANOVA) and means separated by Fishers Least Significant difference test at 95% probability. Statistical analyses were performed using a computer program SPSS, version 14 for Windows.

Results

Toxicity and behavioral changes of fish

The 96 h-acute toxicity test of thiobencarb herbicide to *C. gariepinus* adult females showed that LC_{50}-96 h value was found to be 1.44 mg L^{-1}. The sub-lethal concentration (½ LC_{50}) of thiobencarb affected behavioral characteristics of *C. gariepinus*. The control specimens were not hyperactive and showed normal swimming patterns, color and fins movements throughout the exposure period. Several distinct unusual swimming behavior and increased deformities in fish exposed to sub-lethal concentration of thiobencarb were observed during the exposure period including: lack of balance, agitated or jerky swimming, air gulping, sudden quick movement, excessive secretion of mucus. Moreover, the color of fish skin was changed from normal darkly pigmentation in the dorsal and lateral parts to very light pigmentation in the dorsal and lateral part, as well as peeling of the skin was observed. Severe abnormality in swimming behavior was observed in fish groups exposed for 9 and 15 days where faster opercular movement, surfacing and swallowing of air were observed. With increase in duration of the exposure, swimming and body movements were retarded and copious mucus was secreted and deposited in the buccal cavity and on the gills.

Biometric parameters

There was a significant reduction (p<0.01) in the mean body weight of *C. gariepinus* from groups exposed to

selected concentration of thiobencarb for periods of 9 and 15 days in comparison with that of control group (Table 1). Ovarian weight revealed highly significant (p<0.01) decrease in *C. gariepinus* females exposed to thibencarb in all three different groups in comparison with the control group. Mean ovarian weights of fish from group exposed to thiobencarb for 3 and 15 days were highly decreased by 87.1 and 89.5% , respectively, than that of the control. Mean hepatic weight was also significantly decreased (p<0.01) in fish from groups exposed to herbicide for 9 and 15 days as compared to that of the control. Likewise, there was a significant reduction (p<0.01) in the condition coefficient (CF) in the fish exposed to thiobencarb for different exposure periods in comparison with that of the control. The most significant decrease (p<0.01) in the CF (0.4) was in the fish exposed for 15 days in comparison with that of the control (0.8). Also, the results presented in Table 1 showed that mean GSI values were significantly (p<0.01) decreased in fish females from all three groups exposed to thiobencarb as compared to that of the control. The GSI values were 2.8, 3.5 and 2.8 in fish exposed to thiobencarb for periods of 3, 9, and 15 days, respectively, as compared to that of the control (17.0). However, there were no significant differences (p>0.05) in the mean body length, HSI and CF between thiobencarb-treatments and the control group.

Table 1: Growth parameters, condition coefficient (CF), gonadosomatic index (GSI) and hepatosomatic index (HSI) in catfish females (*Clarias gariepinus*) after exposure to thiobencarb herbicide for different periods (mean±SE).

Exposure period	Body weight (g)	Body length (cm)	Ovarian weight (g)	Hepatic weight (g)	GSI	HSI	CF
Control	230.1 ± 30.0	33.0 ± 1.2	42.0 ± 1.1	3.3 ± 0.03	17.0 ± 3.3	1.1 ± 0.0	0.8 ± 0.0
3-days	229.3 ± 3.1	35.0 ± 3.3	5.4 ± 1.2*	2.5 ± 0.6	2.8 ± 1.1*	1.2 ± 0.1	0.5 ± 0.1*
9-days	156.5 ± 5.2*	32.0 ± 2.2	13.0 ± 5.2*	1.6 ± 0.4*	3.5 ± 1.0*	1.1 ± 0.1	0.5 ± 0.1*
15-days	169.0 ± 4.4*	34.0 ± 3.0	4.4 ± 1.2*	1.7 ± 0.6*	2.8 ± 0.9*	1.0 ± 0.2	0.4 ± 0.03*

*Significant differences compared with control value, p<0.01.
GSI, Gonadosomatic index; HSI, Hepatosomatic index; CF, Condition factor.

Fish fecundity

The results of relative and absolute fecundity of *C. garipinus* females in both the control and thiobencarb-treated groups were presented in Table 2. It was obvious that relative fecundity indices (FBW, FBL and FGW) were significantly decreased (p<0.01) in fish from all three groups exposed to thiobencarb for different periods as compared to those of the control. The values of absolute fecundity of females exposed to thiobencarb for different times revealed highly significant decrease (p<0.01), for which the lowest value (181.8) was observed at 15-day treatment followed by 3- and 9-day treatments (532.5 and 1090.9, respectively) as compared to that of the control (33135.0).

Table 2: Relative and absolute fecundity of catfish females (*Clarias gariepinus*) after exposure to thiobencarb herbicide for different periods (mean ±SE).

Exposure period	FBW	FBL	FGW	Absolute fecundity (F)
Control	1969.9 ± 5.3	3814.4 ± 3.5	9899.8 ± 1.9	33135 ± 2.0
3-days	1506.6 ± 2.2*	3541.1 ± 4.4*	1489.8 ± 4.4*	532.5 ± 2.2*
9-days	1263.3 ± 2.3*	3451.7 ± 4.4*	1767.7 ± 6.6*	1090.9 ± 4.0*
15-days	1125.5 ± 2.5*	3474.7 ± 6.6*	2560.6 ± 2.5*	181.8 ± 6.6*

*Significant differences compared with control value, $p<0.01$.
F, Mean number of ripe oocytes per female; FBW, Relative fecundity to body weight (g); FBL, Relative fecundity to body length (cm); FGW, Relative fecundity to gonad weight (g).

Biochemical parameters

Table 3 present the changes in the activities of aspartate AST and ALT in blood serum of *C. gariepinus* fish in response to ½ LC_{50}-96 h of thiobencarb for 3, 9 and 15 days of exposure. The AST and ALT activities were significantly ($p<0.01$) decreased in all treated groups after the exposure periods. Reduction in the enzyme activities increased with exposure period. After 15 days of exposure, the activity of ALT and AST declined to 34.5% (4.0 ±0.1) and 13.9 % (8.2 ±0.9) of the controls (100%), respectively. Likewise, both serum proteins and glucose levels were significantly decreased ($p<0.01$) in the treated groups as compared to the control. The protein levels were found to be 45.2% (2.80±1.0) and 38.7% (2.4±1.0) of controls (100%) after exposure to thiobencarb for 9 and 15 days, respectively. Similar trend was observed for glucose levels, for which after 9 and 15 days of exposure to thiobencarb, glucose levels declined to 60.9% (90.1±2.2) and 50.7% (75.0±3.1) of controls, respectively.

Table 3: Biochemical variables in catfish females (*Clarias gariepinus*) after exposure to thiobencarb herbicide for different periods (mean ±SE).

Exposure period	ALT (IUL^{-1})	AST (IUL^{-1})	Glucose ($mgdL^{-1}$)	Total Protein ($mgdL^{-1}$)
Control	11.6 ± 2.8	58.8 ± 7.5	148.0 ± 3.1	6.2 ± 2.0
3-days	5.5 ± 1.5* (47.4%)	14.2± 0.7* (24.1%)	81.8 ± 3.3* (55.3%)	3.1 ± 0.2* (50.0%)
9-days	4.3 ± 0.4* (37.1%)	14.6 ± 6.1* (24.8%)	90.1 ± 2.2* (60.9%)	2.4 ± 1.0* (38.7%)
15-days	4.0 ± 0.1* (34.5%)	8.2 ± 0.9* (13.9%)	75.0 ± 3.1* (50.7%)	2.8 ± 1.0* (45.2%)

*Significant differences compared with control value, $p<0.01$.
AST, Aspartate aminotransferase activity; ALT, Alanine aminotransferase activity.
Values in the parentheses are percentages of the control.

Histopathological findings

The histology examinations showed severe congestion and hemorrhages in all internal organs especially ovaries and liver in fish exposed to thiobencarb for 9 days. The histological observations of control *Clarias gariepinus* liver tissues (Fig. 1-A)

showed normal typical paranchymatous appearance. The liver was made up of hepatocytes that were polygonal cells with a central spherical nucleus and densely stained nucleolus. The liver of fish exposed to thiobencarb for 3 days showed vacuolar degeneration and necrobiotic changes in hepatocytes and few mononuclear cells infiltration in the portal area (Fig. 1-B). After 9 days of treatment, the liver demonstrated dissociated hepatocytes and few melanomacrophage cells infiltration in the portal area (Fig. 1-C). After 15 days of treatment, there was karyopyknosis of hepatocytes with increased number of melanomacrophages and mononuclear cells infiltration in portal areas of the liver (Fig. 1-D).

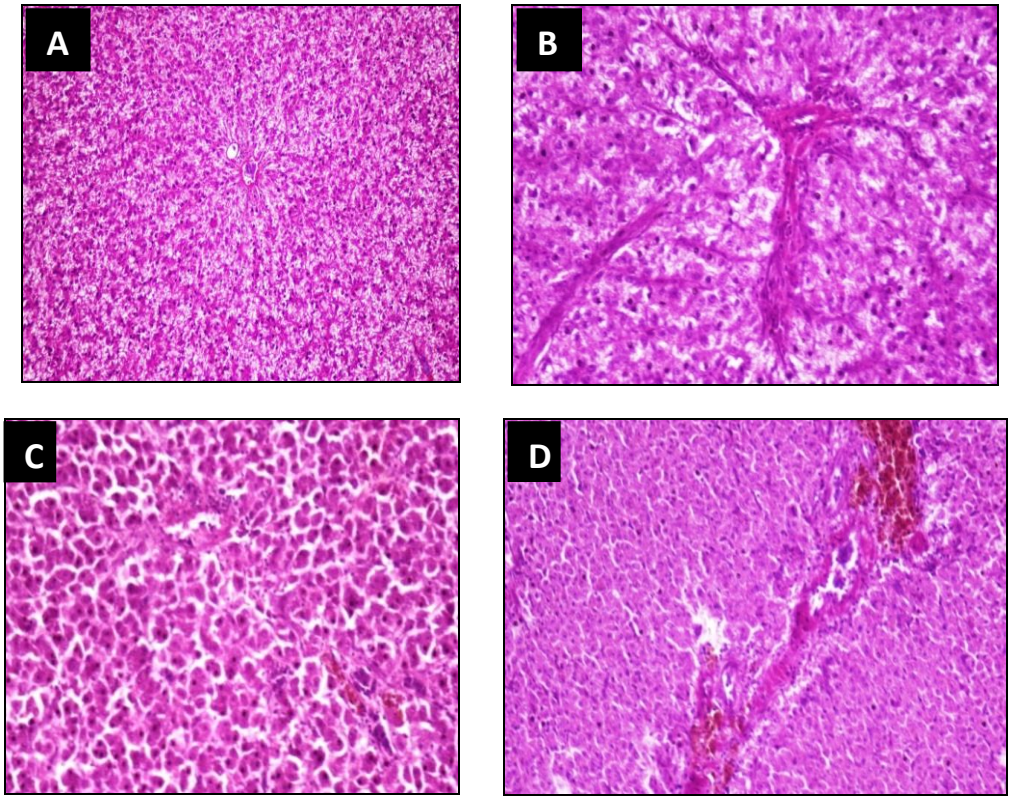

Figure 1: (A) Liver tissue of control fish showing normal histological structure of the portal vein with hepatocytes in the hepatic parenchyma, a quite homogeneous cytoplasm and sinusoids. H&E ×100. (B) Liver tissue of *Clarias gariepinus* exposed to 0.72 mg L^{-1} thiobencarb for 3 days shows vacuolar degeneration, necrosis of the hepatic cells rupture of sinusoids, and few mononuclear cells infiltration in the portal area. H&E, ×100. (C) Liver tissue of *C. gariepinus* exposed to 0.72 mgL^{-1}thiobencarb for 9 days shows dissociation of hepatocytes, few melanomacrophages and mononuclear cells infiltration in the portal area (H and E × 400). (D) Liver tissue of *C. gariepinus* exposed to 0.72 mg L^{-1} thiobencarb for 15 days shows karyopyknosis of hepatocytes, melanomacrophages and mononuclear cells infiltration in the portal area (H and E ×200).

The histological structure of ovaries in the *C. gariepinus* from thiobencarb-exposed and control groups are shown in Fig. 2 (A-E). Normal histological structure of the ovaries in the control group with pre-vitellogenic, vitellogenic and oocyte stages is shown in Fig. 2-A. The ovaries of *C. gariepinus* treated with thiobencarb for 3 days showed many oocytes in the previtellogenic stage and atretic vitellogenic oocytes with an increased

size of follicular cells as well as liquefaction of yolk globules (Fig. 2-B). After 9 days of treatment, an increase in atretic ovarian follicles was shown in addition to presence of mononuclear cells and melanomacrophages in interstituim of the ovary (Fig. 2-C). After 15 days of treatment, atretic vitellogenic oocytes with an increase in size of the follicular cells and liquefaction of yolk globules were observed (Fig. 2-D). In addition, inflammatory cells infiltration were observed in the interstitium of the ovaries (Fig. 2-E).

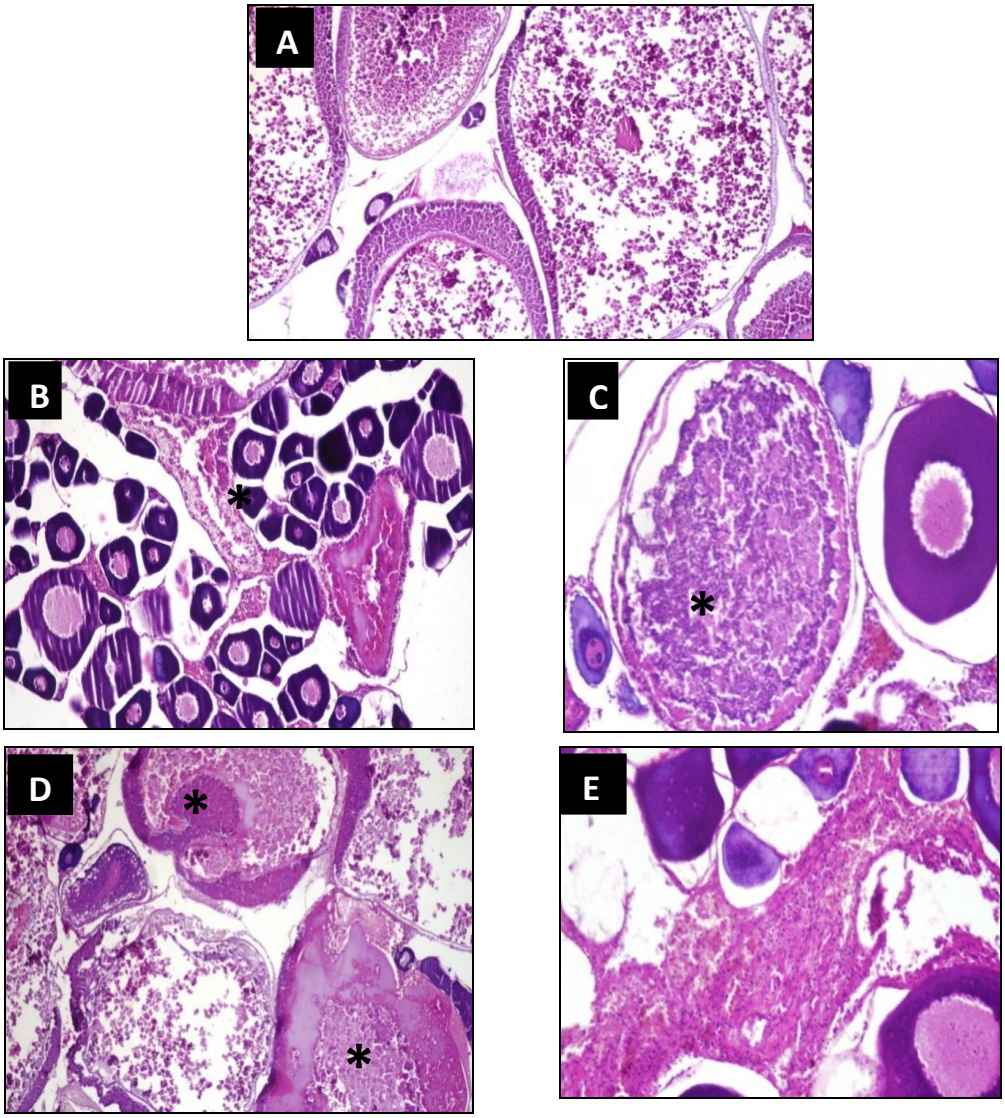

Figure 2: (A) Histological section of ovary in the control group shows vitellogenic oocytes with moderate numbers of vitelline granules, few perinucleolar oocytes, and cortical alveoli oocytes (H and E ×200). (B) Ovarian tissue of *C. gariepinus* exposed to 0.72 mg L^{-1} thiobencarb for 3 days shows chromatin-nucleolus and, perinucleolar oocytes (previtellogenic stage), two atretic vitellogenic oocytes with an increased size of follicular cells and liquefaction of yolk globules (asterisk) (H and E × 200). (C) Ovarian tissue of *C. gariepinus* exposed to 0.72 mg L^{-1} thiobencarb for 9 days shows chromatin-nucleolus and, perinucleolar oocytes, atresia vitellogenic oocytes with follicular cells entering the oocyte to phagocytose degenerating material (asterisk), melano-macrophages, interstitial mononuclear cells (H and E × 400). (D) Ovarian tissue of *C. gariepinus* exposed to 0.72 mg L^{-1} thiobencarb for 15 days shows atresia vitellogenic oocytes, *an* increased ovarian follicle cells, liquefaction of yolk globules and corrugation of vitelline envelope (asterisk) (H and E × 200). (E) Ovarian tissue of *C. gariepinus* exposed to 0.72 mg L^{-1} thiobencarb for 15 days shows inflammatory cell infiltration *in* the interstitium (H and E × 400).

Discussion

Thiobencarb is a systemic pre-emergence herbicide that is used to control broadleaved weeds, annual grasses. It is one of the widely used herbicidesin Egypt to control weeds in rice fields. The evaluation of thiobencarb toxicity to the African catfish, *C. gariepinus,* is important because this species is widely distributed in freshwater bodies in Egypt and they are sensitive to pollutants. The majority of the results indicate that thiobencarb is moderately toxic to fish on an acute basis. Fish are often used as sentinel organisms for ecotoxicological studies because they play number of roles in the trophic web, accumulate toxic substances and respond to low concentration of Mutagens (Cavas and Ergene-Gözükara, 2005). Therefore, the use of fish biomarkers as indices of the effects of pollution are increasing importance and can permit early detection of aquatic environmental problems (Lopez-Barea, 1996; Van Der Oost *et al.*, 2003). Biochemical and physiological biomarkers are frequently used for detecting or diagnosing sub-lethal effects in fish exposed to different toxic substances (De la Torre *et al*., 1999). In ecotoxicology, the LC_{50}-96 h is one of the most valuable parameters for assessing the toxic effects of pollutants. Herein, the LC_{50}-96 h value (i.e. 1.44 mg L^{-1}) was obtained from *C. gariepinus* exposed to thiobencarb, which indicated that this herbicide is highly toxic for fish. The LC_{50} value of thiobencarb obtained for *C. gariepinus* in this study is very close to that reported by USEPA (2008) on different fish species: e.g. bluegill sunfish (0.56-2.6 ppm), channel catfish (2.28 ppm), rainbow trout (1.05-1.5 ppm), depending on percentage of technical grade of active ingredient.

Morphological and behavioral changes are generally indicative of irreversible damage. The results of the present study showed several abnormal swimming behavior and increased deformities in fish exposed to sub-lethal concentration of thiobencarb (½ LC_{50}) during the exposure period. The observed behavioral changes in *C. gariepinus* exposed to thiobencarb in the present study, which indicated internal effects on body physiology, may be attributed to a neurotoxic effect of thiobencarb. Several abnormal behaviors such as incessant jumping and gulping of air, losing their balance, consciousness, rolling movement, surface to bottom movement, sudden quick movement, remaining in vertical position for a few minutes with anterior side or terminal mouth up near the surface of the water, trying to gulp air and tail in a downward position, were similar to the observations of Nwani *et al*. (2010). Such behavioral changes in *C. gariepinus* were also observed after exposure to paraquat herbicide which indicated internal effects on body physiology, might be attributed to a neurotoxic effect of paraquat (Nwani *et al*., 2015; Ladipo *et al.,* 2011). Similarly, respiratory stress, erratic swimming and death of fish were observed in juvenile African catfish, *C. gariepinus,* exposed to glyphosate herbicide for 4 days which varies with

the toxicant concentration (Ayoola, 2008). Pandey *et al*. (2009) indicated that the introduction of toxicant into an aquatic system might decrease the dissolved oxygen concentration which will impair respiration leading to asphyxiation. Therefore, death could occur either by direct poisoning or indirectly by making the medium unconducive for the fish or even by both (Nwani *et al*., 2010).

The CF and HSI are indicators of overall health in fish and have been used in toxicological studies as indicators of stress (Lohner *et al.*, 2001). The condition factor shows the degree of wellbeing of the fish in their habitat which it is an index expression for the interaction between biotic and abiotic factors in the physiological condition of fishes (Olurin and Aderibigbe, 2006). In the present study, there was a significant reduction (p<0.01) in CF on day 15, following exposure to 0.72 mg L^{-1}. A similar decrease in CF was reported by Nwani *et al*. (2015) in *C. gariepinus* fish exposed to paraquat herbicide at 1.37 mg L^{-1} by day 15 and to 2.75 mg L^{-1} by day 5 in comparison with the control. Similar results were reported by Khan (2003) in four species of flatfish inhabiting two areas in Placentia Bay, Newfoundland, contaminated either with polycyclic aromatic hydrocarbons (PAHs) or polychlorinated biphenyls (PCBs). Also, Roussel *et al*. (2007) found that condition factor in three-spined stickleback (*Gasterosteus aculeatus*) was affected at medium and high copper concentrations. The condition factor, HSI and GSI are indicators of overall health in fish and have been used in toxicological studies as indicators of stress (Nwani *et al*., 2015). HSI is defined as the ratio of liver weight to body weight. It provides an indication on status of energy reserve in animals. In a poor environmental condition, fish usually have a smaller liver (with less energy reserved in the liver). HSI has been reported the decrease in fish exposed to high concentrations of cadmium and zinc (Alkahemal-Balawi *et al*., 2011). In the present study, there was no significant difference in HSI between the exposed and the control fish on different exposure periods. Previous studies showed that normal HSI values were also observed in different fish species: e.g. *Carassius auratus* exposed to herbicide alachlor (Yi *et al.*, 2007), *C. carpio* exposed to alachlor (Mikula *et al*., 2009; Ensibi *et al*., 2013) and *Oncorhynchus mykiss* exposed to carbamazepine (Li *et al*., 2011). Nwani *et al*. (2015) indicated that normal CF and HSI in fish exposed to paraquat may indicate that herbicide did not affect the liver at the beginning of the exposure, however, with the progression of the experiment, the liver was affected and this has indicated in a decline in CF and HSI. Therefore, the reduction in CF and HSI may indicate a decrease in the overall condition of the fish, which may be due to the effects of paraquat (Nwani *et al*., 2015). GSI is a metric that represents the relative weight of the gonads to the fish weight and considered generally a good indicative of reproductive success (Lowerre-Barbieri *et al*., 2011). It has

been widely used to evaluate reproduction timing because it is inexpensive and easy to compute. Gonadosomatic index of fish increases with maturation being maximum during peak period of maturity and abruptly declines after spawning (Islam *et al*., 2012). In the present study, GSI values were significantly reduced in fish exposed to thiobencarb for different exposure periods as compared to that of the control group. Recently, Silveyra *et al.* (2017) showed that a lower GSI could be observed in the estuarine crab *Neohelice granulate* fish exposed to different concentrations of atrazine with concentration increase. Concerning the absolute fecundity of *C. gariepinus* exposed to thiobencarb, the current results revealed a highly significant reduction in fecundity rates in all treatments. This effect implies a delay in ovarian maturation, caused by several possible factors. Since highly significant decreases in the GSI values were observed by effect of thiobencarb, it seems to be a lower energetic investment in fish reproduction was happened as a result of the exposure to this herbicide (Álvarez *et al*., 2015).The significant reduction in fish fecundity could be also interpreted as a consequence of the possible hormonal disruption exerted by the herbicide, leading to an arrested oocyte growth (Álvarez *et al*., 2015). Additionally, some kind of interference of this herbicide with the endocrine system controlling ovarian growth in fish may become a more plausible hypothesis.

Biochemical and physiological indicators such as enzymes, could be used (as biomarkers) to identify possible environmental contaminations before the health of aquatic organisms is seriously affected (Jiminez and Stegeman, 1990; Barnhoorn, 1996). In toxicological studies of acute exposure, changes in concentrations and activities of some enzymes may reflect cell damage in specific organs (Casillas *et al*., 1983; Heath, 1996). Serum aspartate AST and ALT enzyme activities in fish have been used frequently as bioindicators of toxicant and contamination of marine ecosystems (Philip and Rajasree, 1996; Kim *et al*., 2008). ALT and AST are liver specific enzymes and they are more sensitive measure of hepatotoxicity and histopathologic changes and can be assessed within a shorter time (Balint *et al*., 1997). The changes in the enzyme kinetics of the organs and blood in fish exposed to various pollutants or stressors have been reported by several researchers. e.g. cypermethrin (Velisek *et al*., 2006), nuracron (Gabriel *et al*., 2010), nonylphenol and octylphenol (Kumaran *et al*., 2011) and prometryn (Velisek *et al*., 2013). Goel *et al.* (1982) reported that the results of reduced activities of AST, ALT, alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) in various organs of fish (*Pontius conchonius*) exposed to Malathion in various organs implies destruction in the tissues of the animals. Similar results were observed by Hedayati *et al.* (2010) who reported that exposure of yellowfin sea bream (*Acanthopagrus latus*) to sublethal concentrations of mercury for 3 weeks decreased the

activities of both ALT and AST. Gabriel *et al*. (2012) demonstrated also that exposure of *C. gariepinus* to sub-lethal levels of cypermethrin caused an inhibition in the metabolic enzymes, AST, ALT, ALP and LDH in the organs (gill, kidney, liver) and tissues (muscles plasma). These findings were in agreement with our present study where the activity levels of serum AST and ALT in fish exposed to thiobencarb, at different periods were significantly decreased than those reported in the control fish. This was correlated by the vacuolar degeneration and necrobiotic changes with karyopyknosis found in hepatocytes. Such changes in the enzyme activities disrupt physiological and biochemical processes (De la Torre *et al*., 2005). In the present study, the lower values of serum ALT and AST in fish exposed to thiobencarb as compared to the control suggest a disruption of the transfer of the α-amino acid group of alanine to α-ketoglutarate which results in the formation of pyruvic acid. This implies that there was a disruption of the feeding of amino-acids into energy cycle through alanine-pyruvate pathway representing anaerobic tendency of the tissues (Ghorpade *et al*., 2002). In contrast, increases in AST and ALT activities were reported in freshwater teleost (*Mystus vittatus*) after chronic exposure for 30 days to sub-lethal concentrations of Metasystox (4 ppm) and Sevin (7 ppm) (John, 2007), in juvenile rainbow trout (*O. mykiss*) exposed to 96 h-LC_{50} of carbamazepine (Li *et al*., 2011). Similarly, Nwani *et al*. (2015) reported that the levels AST and ALT significantly increased in *C. gariepinus* after exposure for 15 days to two sub-lethal concentrations (1/20- and 1/10- LC_{50}) of paraquat. The elevated activities of serum AST and ALT indicate liver damage or enhanced transamination. Increased transamination during pesticide challenge has been attributed to the need to meet higher energy demanded by fish (Philip *et al.*, 1995).Proteins are involved in major physiological events, so the evaluation of the protein content can be considered as a diagnostic tool to determine the physiological indices of biota. Blood glucose level in fish is known to be very useful as a criterion for diagnosis of liver and muscle tissue functions (Shakoori *et al*., 1996). In the present study, both protein and glucose levels were significantly decreased after exposure to thiobencarb at different periods as compared to those in the control fish. The reduction in glucose levels after exposure to thiobencarb might be due to rapid utilization of blood glucose during hyper excitability in treated fish, as well as the nervous manifestation which was a characteristic behavior of herbicide toxicity. However, Nwani *et al*. (2015) found that glucose was significantly increased when *C. gariepinus* exposed to two sub-lethal concentrations of paraquat (1.37 and 2.75 mg L^{-1}) for 15 days, while protein levels declined. The increase of glucose levels may be a physiological response to meet the high metabolic demands caused by continued exposure to paraquat (Nwani *et al*., 2015). On the contrary, the reduction in the protein level may be

associated with liver and kidney damages caused by toxicant stress and the consequent utilization of available protein for metabolic activities (Nwani *et al*., 2015). The highly significant hypoproteinemia found in fish exposed to thiobencarb was in agreement with Abbas *et al.* (2007) who indicated that the existence of thiobencarb residues in liver tissue due to its lipophilic nature causing its dysfunction.

The literature on histopathological effects of thiobencarb on *C. gariepinus* fish is somewhat rare. Ayoola (2008) reported that the liver of *C. gariepinus* exposed to glyphosate concentration showed an infiltration of leukocytes, increasing hepatocyte size with pykrotic nuclei, fatty infiltration, congested central vein, severe necrotic, hemorrhage and vacuolization . In the study ~~of~~ done by Risbourg and Bastide (1995), the exposure of fish to atrazine herbicide increased in the size of lipid droplets, vacuolization in the liver. The most frequent encountered types of degenerative changes are those of hydropic degeneration, cloudy swelling, vacuolization and focal necrosis. The liver of the exposed fish had slightly vacuolated cells showing evidence of fatty degeneration. In the present study, the results of histopathological responses of liver and ovary in *C. gariepinus* exposed to sub-lethal concentration of thiobencarb showed different levels of damage with increasing of exposure period. The liver histology showed important alterations including necrosis, changes in nuclear shape, formation of vacuoles and the atrophy of hepatocyte cells. The structure destruction of liver in thiobencarb- treated fish clearly shows the effect of pesticide in destroying the cellular membrane and so necrosis of liver cells. Similar histopathogical symptoms were reported by Banaee *et al.* (2013) in gourami fish (*Trichogaster trichopterus*) when exposed to sub-lethal concentrations of parquet. Due to disturbance in cellular and osmotic regulation power of cellular and biological membranes, the volume of the nuclei and nucleoli increase and it leads to necrosis of liver cells (Ahmad *et al*., 2000). Necrosis of some portions of the liver tissue that were observed probably resulted from the excessive work required by the fish to get rid of the toxicant from its body during the process of detoxification by the liver (Banaee *et al*., 2013). The inability of fish to regenerate new liver cells may also have led to necrosis of hepatic cells of sinusoids (Mostakim *et al*., 2015).

Histological sections of ovaries of females *C. gariepinus* exposed to sub-lethal concentration of thiobencarb showed different levels of alterations with exposure period increase including an increase of oocytes in the previtellogenic stage with an increased size of follicular cells and liquefaction of yolk globules and inflammatory cells infiltration in the interstitium of ovary. In the study of Avigliano *et al*. (2014), the exposure of estuarine crab, *Neohelice granulate*, females to pure glyphosate at 2.5 mg L^{-1} stimulated ovarian maturation over control levels, mainly in terms of a higher GSI and a higher percentage of vitellogenic oocytes, suggesting that exposure to

glyphosate disrupts the hormonal system controlling reproduction. Moreover, the lower degree of maturation shown by control ovaries in comparison with ovaries belonging to females exposed to glyphosate (at 2.5 mg L^{-1}) was in close correlation with the augmented GSI and HIS, suggesting a possible effect of glyphosate as an endocrine disruptor (Avigliano *et al.*, 2014). The results of another recent study by Álvarez *et al*. (2015) showed that exposure of *N. granulate* females to atrazine (2.5, 5 and 15 mg L^{-1}) for 32-days produced a significant reabsorption of previtellogenic oocytes in all experimental groups. Since in those groups most of oocytes were primary, the augmented reabsorption was only observed in this type of oocyte. This result could be interpreted as a consequence of the possible hormonal disruption exerted by atrazine, leading to an arrested oocyte growth, and/or as a non-specific response to the stress induced by the chronic exposure to atrazine, as reported by several previous studies concerning multiple stress factors (Power, 2002).

This study has proved convincingly the sub-lethal effects of commercial formulation of thiobencarb (CITRON®) herbicide to the African catfish, *C. gariepinus*. Our results provide a description of serum biochemical and histopathological alterations in the liver and ovaries of the African catfish, *C. gariepinus*, that can be used as baseline information for further studies and suggest that *C. gariepinus* is a useful bioindicator animal for monitoring the effects of pollutants in water bodies. More detailed laboratory studies with validated assays are needed before they can be established as specific biomarkers. There are several endpoint measures that can be used to assess sub-lethal effects (Mensah *et al*., 2014). For example, at the 'physical' level, measures of growth, morphological changes, and behavioral changes exposed fish are used as endpoint indicators. Also, measures of reproductive performance that are often used to assess sub-lethal response include sexual maturity, fecundity, gonad histopathology, and alterations in reproductive characteristics. Additionally, biochemical measures used as possible endpoints to assess exposed fish include metabolic disruption and lipid peroxidation ((Mensah *et al*., 2014). Moreover, organosomatic indices (e.g, CF, HSI and GSI) are common approaches for assessing fish health and may provide information for evaluating environmental stress (Adams *et al*., 1993). Thus, further studies on the chronic effects of thiobencarb, and the parameters examined herein, are still needed for a greater insight into the mechanism of thiobencarb toxicity.

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