Full Length Research Paper

"Toxic effects of 4-nonylphenol on the embryonic development of African catfish *Clarias gariepinus* (Burchell, 1822) "

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The present study investigated effects of environmental relevant concentrations of 4-nonylphenol (0.05 to 0.08 to 0.1 mg/l) on the reproduction and embryonic developmental stages of catfish (*Clarias gariepinus*). To determine the effect of 4-nonylphenol on reproduction; catfish were exposed to three concentrations of 4-nonylphenol in a flow-through-system during spawning period (some for one week and other for two weeks). At an estimated 4-nonylphenol concentration the fertilization rate and hatching rate were significantly decreased with 4-nonylphenol concentrations increasing while the incubation period, the mortality rate and malformed embryos ratio were increased. Also, the development of embryos and larvae was affected by 4-nonylphenol in terms of morphological changes and histopathological alterations.

Key words: 4-nonylphenol, hatching, mortality, embryos, *Clarias gariepinus*.

INTRODUCTION

Endocrine disrupting chemicals (EDCs) are characterized by their influence on endocrine systems resulting in reproductive, developmental, neurological, and immune dysfunction [Cooper and Kavlock, 2001]. Many studies have shown that exposure to EDCs can cause physiological, histological, and behavioral alterations in fish during reproductive period [Jobling et al., 1996; Bjerselius et al., 2001] nonylphenol ethoxylates (NPEs) are alkylphenolics which are EDCs compounds [Harries et al., 1997; Kidd et al., 2007]. Nonylphenol ethoxylates are chemical used widely in the production and formulation of many commercially sold products: as an industrial and commercial detergent, as an emulsifier in wax for fruit and vegetables, as a polymer resin in plastic food packaging and polyethylene plastic, in cosmetic products) such as shin cream, deodorant, makeup, Hair dye, and shampoo) and even in spermicides [Environment Canada, 2002; European Union, 2002; Hano et al., 2009]. The European Union has banned the use of NPEs in detergents, and Canada has set stringent water quality guidelines, as NP compounds are toxic and their use because they may have an immediate or long-term harmful effect on the environment or its biological diversity [Environment Canada, 2002; European Union, 2002]. Bacterial degradation of nonylphenol ethoxylates which are non toxic compounds product is nonylphenol (NP) which is toxic [Hano et al., 2009; Karafakioglu et al., 2011] and estrogenic both on vitro and vivo assays [Folmar et al., 2002]. Exposure to NPEs metabolites causes organisms to develop both male and female sex organs; increases testicular growth and sperm counts in male fish; and disrupts normal male to female sex-ratios, metabolism, development, growth and reproduction [Gray and Metcalfe, 1997; Colborn et al., 1999]. Many studies have been reported that NP has physiological and developmental effects related to its estrogenecitity; those defects as reproduction failure in medaka [Kang et al., 2003] and fathead minnow [Harries et al., 2000] and significant decrease of hatchability in medaka [Yokota et

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al., 2001]. NP can cause developmental toxicity in aquatic organisms by disruption of oestrogen [Kammann et al., 2009]. Also, [16] reported that NP is toxic to the killfish causing lethal and sublethal developmental abnormalities after 96 h of exposure.

Gametes, gonadal tissue, early embryo and larval stage are the most sensitive stages of development to pollutants [Rosenthal, 1976; Holdway et al., 2007]. Yang et al. [2006] has been reported that gametes with either poor quality or decreased number would result in low fertilization success and thus a low survival rate of offspring. Prenatal or postnatal life is a very vulnerable period and exposure to the critical period may cause profound effects during the whole life [Birnbaum, 1994]. It has been reported that the freshly hatched larvae are generally very sensitive to environmental parameters [Blaxter, 1988] and Lahnsteiner et al. [2005] reported that 4-nonylphenol might affect the fish embryogenesis. Embryos and larvae of fish of catfish *Clarias gariepinus* have often been used in toxicity studies of environmentally relevant substances [Osman et al., 2007; Mahmoud et al., 2009; Mekkawy et al., 2010]. Catfish, *Clarias gariepinus* is a suitable test organism in toxicological research due to short spawning intervals and to the transparent eggs [Osman et al., 2007; Mahmoud et al., 2009; Mekkawy et al., 2010].

The influence of environmental relevant concentrations of 4-nonylphenol on the fertilized eggs, the developing embryos and the hatched larvae is unclear [Lahnsteiner et al., 2005]. Therefore, the aims of the present study are investigation the reproductive effects including alterations of reproduction (fecundity, fertilization rate, hatching ability and mortality rate) and morphological and histopathological malformations on catfish, *Clarias gariepinus* exposed to sublethal concentrations of 4-nonylphenol.

MATERIALS AND METHODS

Specimens collection

Specimens of adult Catfish *C. gariepinus* were collected from the River Nile at Assiut and then were transported to Fish Biology Laboratory of Zoology Department, Faculty of Science, Assiut University. The fish (500. 1200 g) were fed on a commercial pellet diet (3% of body weight per day) and kept together in 100 l rectangular tanks containing tap water (conductivity 2000 ls/cm; pH 7.5; oxygen 88 to 95% saturation; temperature 27 to 28°C; photoperiod 12:12 light: dark). After 2 week acclimatization, fishes were used for the experimental setup.

4-nonylphenol

Nonylphenol was obtained from Sigma- Aldrich (Schnelldrof, Germany)**.**

Gamete collection

Mature African catfish, *Clarias gariepinus* (weight of 900 to 1500 g) were collected from the River Nile at Assuit, Egypt and transported to the Fish Lab, Zoology Department, Assuit University. The criteria applied for the selection of spawners were those described by [26]. The catfish were kept in 100 L glass tanks to be acclimatized for two-week period at 27 to 29°C, pH = 7.56, dissolved oxygen 88 to 94% saturation. The photoperiod was a 12 h light to 12 h dark cycle and the catfish were fed on a commercial pellet diet (3% of the body weight/day).

For collection of semen, male fishes were anaesthetized with 200 mg/l Ms_222 (tricaine methane sulphonate, Crescent Research Chemicals, Phoenix, Arizona, USA) buffered with 800 mg/l sodium bicarbonate and one of the testes was removed surgically. Alternatively the fish were killed and the whole gonads were removed. Testes were cleaned from the blood by surgical towels. The sperms from the testes were pressed through a mesh fabric into a sterile dry petridish and used directly for dry fertilization. For collection of eggs, ovulation was artificially induced. Females were injected intra-peritoneal with pellets (gonadotropin-releasing hormone analogue, GnRHa, D-Ala6, Pro9 Net) containing 2.5 to 3.0 mg of water soluble dopamine antagonist metoclopramide (Interfish Ltd, Hungary) dissolved in 0.65% NaCl. One pellet was used per Kg body weight. 10 to 11 h after injection, the fish was stripped and the eggs were collected in clean dry plastic containers; dry fertilization was considered.

Experimental setup

The adapted adult fish classified into four groups (10 fish per each): control, 4-nonylphenol-treated group (for15 day/ for 0.05mg/l day), 4-nonylphenol-treated group (for 15 day/for 0.08 mg/l day), and 4-nonylphenol-treated group (for 15 day/for 0.1 h/ day). In the present study, the range of NP exposures was 0.05-0.1 mg/l and these concentrations were chosen in accordance with environmentally observed values. The conditions of the experiment were as that of acclimatization with changing all the tap water and concentrations of 4-nonylphenol every day.

First experiment

After artificial spawning for mature male and mature female untreated fishes, and dry fertilization occurred, the fertilized egg transported into Peterdishes represent control, 4-nonyphenol exposed (0.05 mg/l), 4-nonylphenol exposed (0.08 mg/l), and 4-nonylphenol (0.1 mg/l) groups, then at 4, 30, 30, 40 h-PFS fertilization rate, incubation period, hatching rate, and mortality rate were calculated and samples were taken for morphological and histological preparations.

Second experiment

After artificial spawning for mature male and mature female untreated fishes, and dry fertilization occurred, the fertilized eggs at age of 18 h-PFS were exposed to different dosed of 4-nonylphenol (0.08; 0.05 and 0.1 mg/l) to calculate the incubation period and hatching rat at 28 h-PFS. Also, at 29 h-PFS other fertilized eggs were exposed to different doses of 4-nonylphenol (0.08; 0.05 and 0.1 mg/l) to calculate the mortality rate at 30, 32, 37 h-PFS.

Third experiment

Mature female of catfish was exposed to 0.1 mg/l 4-nonylphenol for one week then artificial spawning occurred using untreated mature male. The fertilized eggs were used to calculate fertilization rate, incubation period, hatching rate, mortality rate at 4, 30, 30, and 50 h-PFS respectively and also samples taken for morphological and histological malformations studies at different developmental stages.

Morphology

Malformations were documented using a dissececting microscope (NOVEL MEDICALCO., LTD. XSZ-109 B) and a digital colored video camera (Sony, AVT-Horn).

Scanning electron microscope (SEM)

Eggs after fertilization and embryos after hatching were fixed with 5% glutaraldhyde in 100 mM phosphate buffer (pH 7.4, 4°C) for 24 h. They were post fixed with 1.5 osmium tetraoxide for 2 h and washed four times with 100 mM phosphate buffer (pH 7.4). Some eggs were cut into halves with a fine razor. After slowly dehydrating with an ethanol series, the samples were dried at 30 to 40°C, and then glued to stubs coated with 20 nm of gold and viewed with Scanning Electron Microscope (JSM 5400 LV) at 15 Kv at Electron Microscope Center, Assuit University, Egypt.

Hematoxylin-Eosin   (HE)   histopathological preparations

For microscopic preparations, after 15 days, 3 larvae of each group were fixed in 10% neutral buffered formalin. Fixed larvae were processed routinely for paraffin embedding technique. Larvae were sectioned at 5 to 7 in thickness and then stained by Harris's hematoxylin and eosin stain (H & E) according to [Bancroft and Stevens, 1982]. Sections were visualized and studied using OLYMPUS microscope model BX50F4 from Olympus optical Co., LTP. Japan.

Statistical analysis

The basic statistics, means, standard errors and ranges were estimated. The analysis was done using the SPSS package [SPSS, 1998] at the 0.05 significance level.

Ethical statement

All experiments were carried out in accordance with the Egyptian laws and University guidelines for the care of experimental animals. All procedures of the current experiment have been approved by the Committee of the Faculty of Science of Assiut University, Egypt.

RESULTS

Fertilization rate, incubation period, hatching rate and mortality rate

During the first experiment, the fertilization rate at 4h-PFS was 84.67± 7.5 in control group and decreased to 27.67 ± 2.5 % (Table 1) in the group exposed to 0.1 mg/l 4nonylphenol. The decrease in the fertilization rate with 4-nonylphenol concentrations increase was significant at $P<0.05$. Also, in the third experiment the fertilization at 4h-PFS was 10.6 ± 7.149% (Table 3) and this value less than that of control (Tables 1 to 3).

In the first experiment (Table 1), the hatching time at 30 h-PFS was (22 to 25) h after fertilization at 28 to 29°C in the control groups while the hatching time delayed under 4-nonylphenol exposure to (22 to 28), (23 to 29), and (23 to 29) h in groups exposed to 0.05, 0.08, and 0.1 mg/l 4-nonylphenol respectively. In the second experiment (Table 2), the incubation period at 30h-PFS was 22 ± 0.44 in the control group at 28 to 29°C. Significant increase in the incubation period was recorded with increase concentrations of 4-nonylphenol. Also, incubation period was (23 to 26) h in the third experiment (Table 3) where fertilized eggs obtained from fertilization of exposed female to 0.1 mg/l and unexposed male.

Hatching rate (hatched larvae/ fertilized egg) at 30h-PFS in control group was 60 ± 7.2%. The decrease in the hatching rate was significantly in groups exposed to sublethal doses of 4-nonylphenol (first experiment; Table 1). In the second experiment (table 2), the hatching rate at 28h-PFS was (80 ± 17.088, 60.33 ± 13.61, 47 ± 6.24 and 20.667 ± 8.32)% at $P<0.05$ in the control, 0.05, 0.08, and 0.1 mg/l 4-nonylphenol groups. The hatching rate decreased significantly at $P<0.05$ with increase 4-nonylphenol concentrations. Also, we recorded 27.27 ± 11.99 % hatching rate in the third experiment.

Table 1 showed increase in the mortality rate at $P<0.05$ where the values were (2.33 ± 2.5, 5.33 ± 3.5, 6.87 ± 1.52 and 10.67 ± 3.05) % in the control, 0.05, 0.08, and 0.1 mg/l 4-nonylphenol respectively. In the second experiment the mortality rate increased significantly at $P< 0.05$ in the exposed groups in comparison with control at 30, 32, and 37 h-PFS. The mortality rate was at 40h-PFS was 23.47 ± 8.79% in the third experiment (Table 3) and the malformed embryos percentage was 14.3 ± 9.4.

Table 1. Fertilization rate, incubation period, hatching rate and mortality rate (mean ± SD)% after exposure to different doses of 4-nonylphenol during early developmental stages of the African catfish *Clarias gariepinus* (First experiment).

Test / Treatments	Control	0.05mg/l 4-nonylphenol	0.08mg/l 4-nonylphenol	0.1mg/l 4-nonylphenol
Fertilization rate at 4h-PFS**	84.67 ± 7.5 (77-92) a	41.67±9.93 (36-45) b	37.67±2.58 (36-45) c	27.67± 2.5 (25-35) d
Incubation period at 30h-PFS	23 ±1.224 (22-25) a	23± 2.35 (22-28) b	25± 2.29 (23-29) c	28± 2.68 (23-29) d
Hatching rate at 30h-PFS	60 ±7.2 (52-66) a	47± 7.2 (41-55) b	37±4.35 (34-42) c	33.67± 1.5 (32-35) d
Mortality rate at 40h-PFS	2.33 ±2.5 (0.0-5) a	5.33±3.5 (2-9) b	6.67± 1.52 (5-8) c	10.67±3.05 (8-14) d

*Different letters indicated significant difference between groups at 0.05 levels. **h-PFS (hour-post fertilization stage).

Table 2. Hatching rate, Incubation period and mortality rate (mean ± SD) % after exposure to different doses of 4-nonylphenol during early developmental stages of the African catfish *Clarias gariepinus* (Second experiment).

Test / Treatments	Control	0.05mg/l 4-nonylphenol	0.08mg/l 4-nonylphenol	0.1mg/l 4-nonylphenol
Hatching rate at 28h-PFS	80 ± 17.088 (62- 96) a	60.33 ± 13.61(45- 71) b	47 ± 6.24 (42-54) c	20.667 ± 8.32 (14- 30) d
Incubation period at 28h-PFS	22 ±0.44 (22-23) a	23± 0.44 (22-23) b	24± 0.84 (24-26) c	25± 0.75 (24-26) d
Mortality rate at 30h-PFS	10 ± 5.56 (4- 15) a	13.67 ± 6.65 (8- 21) b	43.667 ± 8.02 (36- 52) c	59.33 ± 10.69(50-71) d
Mortality rate at 32h-PFS	19 ± 4.58 (14-23) a	36.667 ± 9.07 (30-47) b	55.33 ± 19.34 (33- 67) c	68.33 ± 15.5 (53- 84) d
Mortality rate at 37h-PFS	2 ± 2 (0.0- 4) a	28.667 ± 15.94 (11-42) b	48.33 ± 4.72 (43- 52) c	68.33 ±9.07 (58- 75) d

*Different letters indicated significant difference between groups at 0.05 levels. **h-PFS (hour-post fertilization stage).

Table 3. Fertilization rate, incubation period, hatching rate, mortality rate and malformed embryos (mean ± SD) % during early developmental stages of the African catfish *Clarias gariepinus* after exposure of female to 0.1mg/l of 4-nonylphenol then fertilized with unexposed male (Third experiment).

Test / Treatments	0.1 mg/l 4-nonylphenol
Fertilization rate at 4h-PFS**	10.6 ± 7.149 (0-25)
Incubation period at 30h-PFS	24.5±1.045 (23-26)
Hatching rate at 30h-PFS	27.27 ±11.99 (9-54)
Mortality rate at 40h-PFS	23.47 ± 8.79 (10-40)
Malformed embryos	14.3 ± 9.4 (0-36)

**h-PFS (hour-post fertilization stage.

Morphological malformations in post-fertilization stages

Many gross morphological malformations (yolk sac oedema, body curvature, fin blistering, irregular head shape, dwarfism, pericardial oedema, collapsed tail, egg shrinkage, and egg or embryo invagination) were recorded in groups exposed to different doses of 4-nonylphenol (0.05, 0.08 and 0.1 mg/l) in comparison with control group. Some of the affected fertilized eggs or embryos were recorded with one or more of these malformations (Figures 2 to 7).

Irregular head shape, pericardial oedema, and collapsed tail

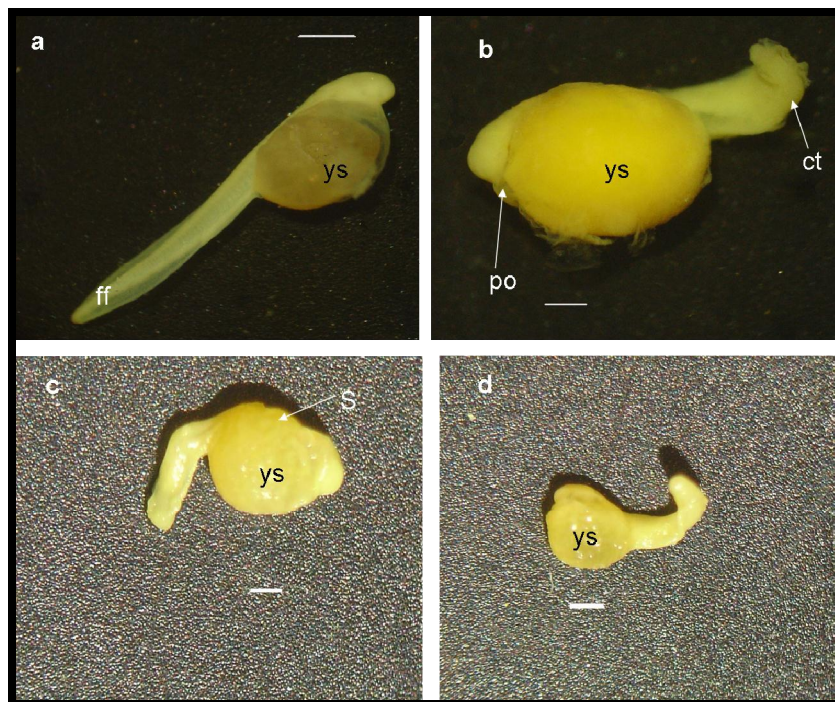Normal newly hatched embryo of *C. gariepinus* was observed in (Figure 1a) and pericardial oedema (po) was observed with collapsed tail (ct) (Figure 1b). Malformed embryos were characterized by irregular head shape (Figures 1c, d, e and 3e) associated with swelling(s).

Body curvature

Notochord curvature was the most frequently observed gross morphological malformation. Different types of notochord curvature were observed; scoliosis (lateral curvature) (Fig 1b, c, d; Fig 2b), lordosis (dorsoventral curvature) (Figures 3b, f; 6d, e and 7a), kyphysis (ventrodorsal curvature) (Figure 3d), C-shaped (Figures 2d and 3e), flat-S-shape (Figure 3c).

Yolk sac oedema

Different shapes of yolk sac oedema were observed

Figure 1. Newly hatched embryos of *Clarias gariepinus*, First experiment. (a) normal embryo 30h-PFS, (b) deformed embryo with collapsed tail and pericardial edoma 30h-PFS exposed to 0.1 mg/ l 4-nonylphenol, (c) deformed embryo with irregular head shape 30h-PFS exposed to 0.1 mg/ l 4-nonylphenol, (d) deformed embryo 30h-PFS exposed 0.08 mg/l 4-nonylphenol. ys, yolk sac; s, swelling; ff, fin fold; po, pericardial edema, and ct, collapsed tail. Scale bar =1 mm.

including; ballon-shape oedema (Figures 1b, c; 3c, d, e, f and 5b, c), bag-shape oedema (Figures 5e and f), oval-shape oedema (Figures 2d; 3b; 5c; 6a and d), and irregular shape yolk sac oedema (Figure 5e). Also, yolk sac oedema was often associated with body curvature (Figures 1b; 2b and d) or fin blistering (Figures 1b and 6c) and irregular head shape (Figure 3e).

Fin blistering

Blistering of fin (the membranous fin was blistered and degenerated) was observed in embryos exposed to 0.08 and 0.1 mg/l 4-nonylphenol (Figures 1c, d; 5e; 6a, b, c and d). Fin blistering was often associated with yolk sac oedema (Figures 1c, d and 5e) and body curvature (Figures 1c, d and 6a).

Dwarfism

Yolk sac malformation caused abnormal growth, so that oedematous embryos were usually shorter than the normal ones. As shown in Figures 5f and 6b, c) the embryos are shorter than unexposed ones in the same developmental stage. Dwarfism always associated with yolk sac oedema (Figure 5f) and often fins blistering (Figures 6b and c).

### Egg or embryo shrinkage and invagination

As shown in (Figure 4a) normal fertilized egg at 17 h-PFS with normal development of embryo while eggs exposed to 0.05 mg/l 4-nonylphenol showed less shrinkage than those exposed to 0.08 mg/l (Figures 5e and b) and those exposed to 0.1 mg/l (Figures 6c and d). Invagination with pores in the fertilized eggs exposed to 0.1 mg/l (Figure 4c) and also in embryos exposed to 0.1 mg/l (Figures 6b and d) were recorded.

Histopathological malformations in post-hatching stages

Many major histopathological categories were appeared in this investigation. Two of these malformations were recorded here in embryos exposed to sublethal doses (0.05, 0.08, 0.1 mg/l) 4-nonylphenol.

Histolopathological changes in the spinal cord.

As Figure 7a shown, normal structure of spinal cord (sc) which consists of outer sheath surrounding the white matter (contains nerve fibers) and gray matter (contains neurons) all surrounds the central canal (cc). The embryos exposed to 0.05 mg/l 4-nonylphenol (Figure 7b)
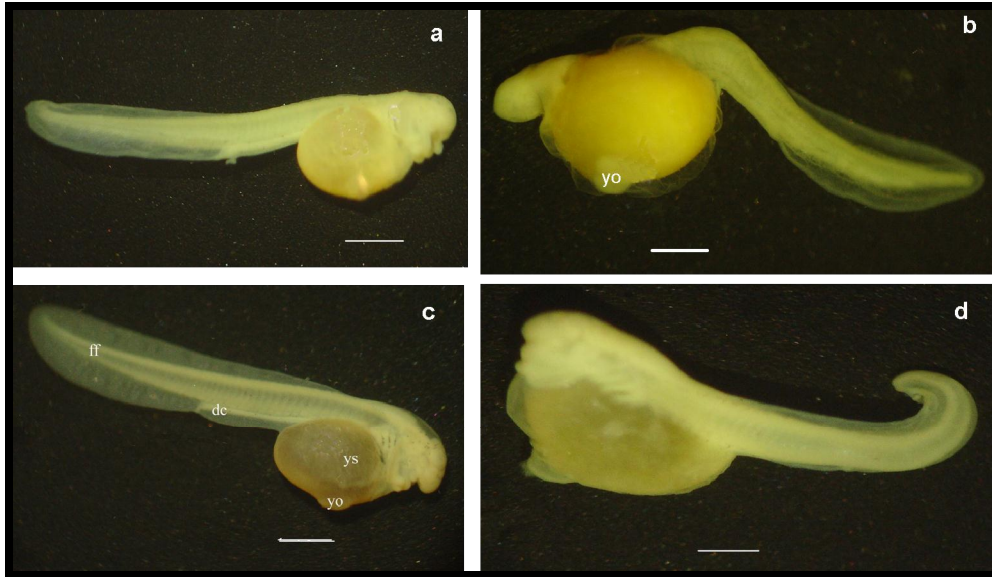
Figure 2. Newly hatched embryos of *Clarias gariepinus*, First experiment. (a) normal embryo 40 h-PFS, (b) deformed embryo with Scoliosis notochord and Ballon-shaped yolk sac odema 40h-PFS exposed to 0.1 mg/ l 4-nonylphenol, (c) deformed embryo with Ballon-shaped yolk sac odema 40h-PFS exposed to 0.08 mg/ l 4-nonylphenol, (d) deformed embryo with C-shaped notochord and Oval-shaped yolk sac odema 40h-PFS exposed 0.08 mg/l 4-nonylphenol. ys, yolk sac; yo, yolk sac odema; ff, fin fold; dc, digestive canal. Scale bar = 1 mm.

showed less degree of damage while severe damage (as degeneration of gray matter and central canal were recorded in the spinal cord of embryos exposed to 0.08 mg/l 4-nonylphenol (Figure 7,c) and 0.1 mg/l 4-nonylphenol (Figure 7d). Also, the embryos obtained from exposed female (0.1 mg/l 4-nonylphenol for one a week) and unexposed male showed severe degree of spinal cord damage (Figures 7e and f).

Histolopathological changes in the notochord

The main histological malformation recorded was abnormal shaped notochord (Figures 7b, c, d, e and f) in comparison with control in which the notochord (n) of the embryos consisted of notochord epithelium (ne) and the vacuolated cells (vc), (Figure 7a). In the embryos exposed to 0.05 and 0.08 mg/l 4-nonylphenol less degree of notochord damage was recorded (Figures 7b and c) while in the groups exposed to 0.1 mg/l 4-nonylphenol, a collapsed-notochord was recorded (Figure 7d). Also, the damage in the notochord was observed in the embryos obtained from exposed female (0.1 mg/l 4-nonylphenol for one week) and unexposed male fertilization (Figures 7e and f).

Notochord collapse (observed in trunk and head regions) is the major defect was recorded in the notochord malformation (Figure 7d and f). Also, degeneration of vacuolated cells was observed and this may be due to the collapse in the notochord which led to a reduction in the size of vacuolated cells.

DISCUSSION

Our results clearly showed that NP exposure concentrations (0.05, 0.08, and 0.1 mg/l according to [Esteavez et al., 2006] of catfish embryos adversely affected embryonic development (Tables 1 to 3). The gametes, the gonadal tissue, the early embryo, and the larval stage are the most sensitive stages of development to pollutants [Rosenthal, 1976] and the early development can be affected by indirect exposure of the parents [Von Westernhagen, 1988]. It has been reported that exposure to EDCs prior to spawning will cause alterations in reproductive behavior, reduced fecundity, reduced fertility, reduced survival and secretion of hormones [Holdway et al., 2007].

Many studies have used longer term exposures (10 to 14 days) to show reproductive effects and eggs collected from 24 h at the rate of 100 µg/l 4-nonylphenol showed reduced hatchability [Holdway et al., 2007]. Folmar et al. [2002] Reported that NP is estrogenic and also lethal to fish [Yokota et al., 2001; Kang et al., 2003; Seki et al., 2003]. [Hano et al., 2009] Reported that NP affected survival, hatchability and swim-up failure and they also stated that the number of embryos hatched decreased significantly in groups exposed to NP. [Yokota et al., 2001] Reported that reduced embryo survival and swim-up success in medaka treated with 183 µg/l 4-nonylphenol.

Our study showed decrease in fertilization rate and similar to these results, [Lahnsteiner et al., 2005] reported
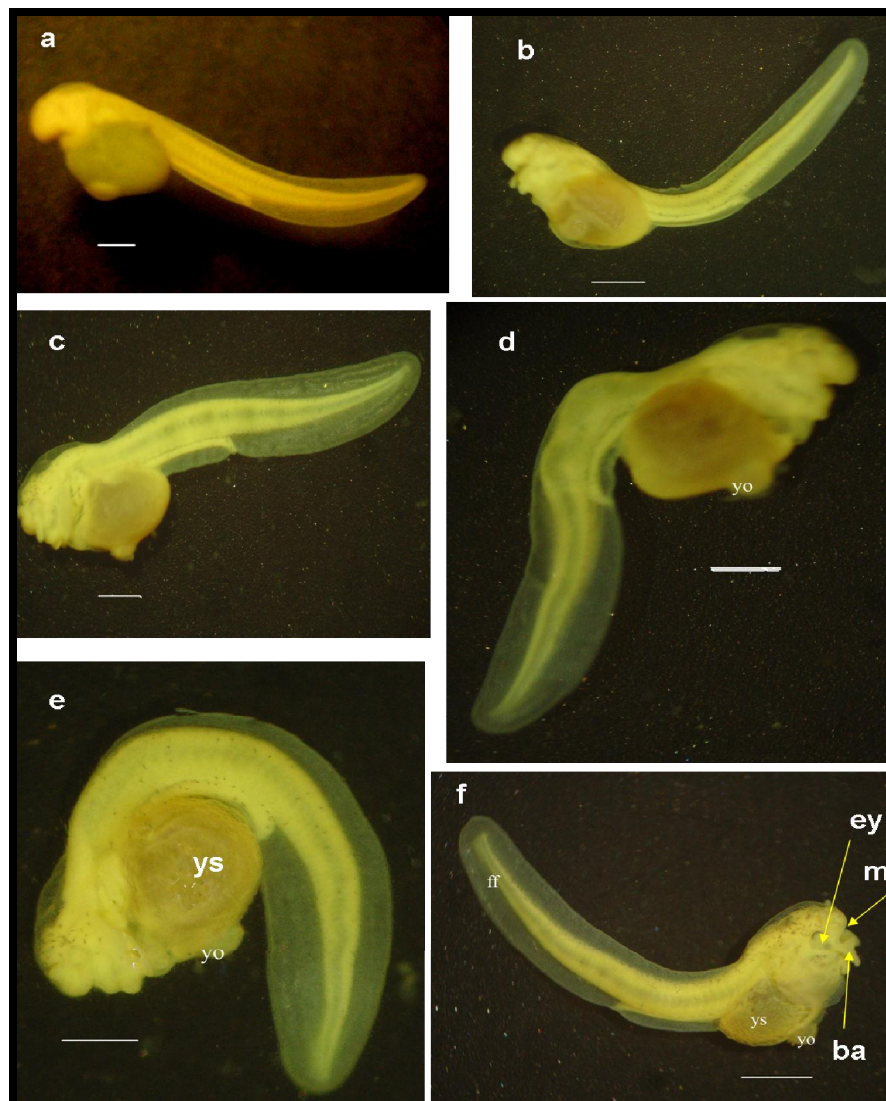
Figure 3. Malformed hatched embryos of *Clarias gariepinus*, Third experiment. (a) 30 h-PFS deformed embryo with yolk sac odema (b) 40 h-PFS deformed embryo with lordosis notochord and yolk sac odema (c) 50 h-PFS deformed embryo with flat-S-shape notochord and yolk sac odema (d) 72 h-PFS deformed embryo with kyphosis notochord and yolk sac odema (e) 72 h-PFS deformed embryo with C-shaped notochord , irregular head shape and   yolk sac odema (f) 96 h-PFS deformed embryo with lordosis notochord and yolk sac odema. ys, yolk sac; yo, yolk sac odema; ff, fin fold; m, mouth; ey, eye and ba, barbels. Scale bar = 1 mm.

that ⁻ 130 ng/l 4-nonylphenol exposures decreased the semen quantity and exposure levels of ⁻ 280 ng/l 4-nonylphenol affected the percentage of eggs surviving to the eye stage and to the yolk sac larvae in rainbow trout while sperm density, sperm motility, sperm fertility, egg viability, and fertilization process were not affected. These results may be due to that 4-nonylphenol could not enter the egg internal during water hardening and during embryonic development because of the presence of the water-hardened eggs shell, the privitelline space, and the oolemma [Alderdice, 1988; Lahnsteiner, 2000]. The

protection by the three permeability barriers was lost at the hatching, so that it is obvious that ⁻ 280 ng/l 4-nonylphenol was toxic and caused a severe decrease in the percentage of larvae of rainbow trout [Lahnsteiner et al., 2005]. Yang et al. [2006] indicated that little effect on fertilization after exposure of female zebrafish to 50 µg/l NP and also Shioda and Wakabayashi [2000] observed no significant difference in the number of hatchings between exposure and controls groups. Ishibashi et al. [2006] reported that F_0 generation medaka showed reduced egg production and fertility after a 21-day
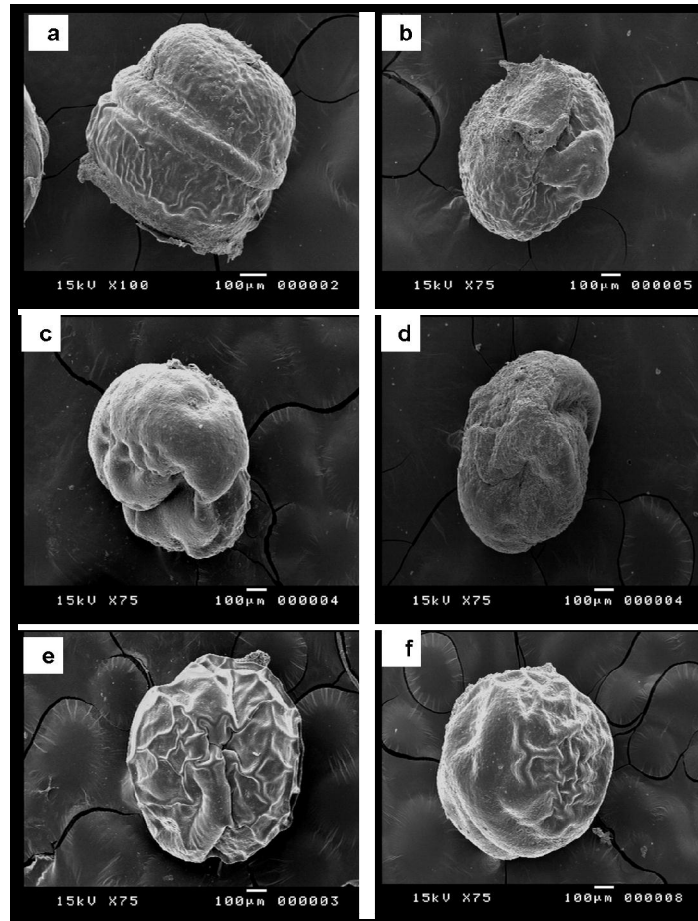
Figure 4. Fertilized eggs of *Clarias gariepinus* 17 h- PFS, (a) unexposed fertilized eggs, (e, b) exposed eggs to 0.08 mg/l 4-nonylphenol, (c,d) exposed eggs to 0.1 mg/l 4-nonylphenol., (f) exposed eggs to 0.05 mg/l 4-nonylphenol.

exposure to 100 µg/l 4-NP and significant mortality in 40% males recorded at the same concentration. Also, in the F_1 generations the hatchability and hatching time (incubation period) of embryos in the 100 µg/l 4-NP treatment group were adversely affected and this is due to accumulation of 4-NP in eggs after maternal transfer. Also, Shurin and Dodson [1997] found that production of resting eggs and female offspring was affected and a characteristic developmental abnormality was observed in *Daphina*. Decrease in survival and fertilized eggs deformation were recorded in our study and this is similar to results of [Kahl et al., ????] Where they reported that reductions in survival of 20 day old larvae of *Chironomus tentans* and an increase in egg mass deformities were recorded after exposure to 4-nonylphenol and the growth of *Mytilus edulis* and larval development were affected in presence of 4-nonylphenol [Granmo et al., 1989]. Also, Nice et al. [2000] reported that the presence 4-nonylphenol caused a delay in the embryonic development of *Crassostrea gigas* at relevant environmental concentrations. Most authors reported retardation or inhibition of embryonic development at various developmental stages and extension of the entire development time under the effects of pollutants [Mekkawy and Lashein, 2003; Osman et al., 2007; Mahmoud et al., 2009]. Jezierska et al. [2009] stated that the rate of fish (*Cyprinus carpio*) embryonic development (hatching rate, organogenesis, and incubation period) were affected after exposure to copper or lead [ýugowska and Jezierska, 2000; ýugowska, 2005] and cadmium [Witeska et al., 1995].

The malformation rate of larvae increased in the groups with the female exposed to 50 µg/l NP [Yang et al., 2006]. The malformation of vertebral column flexure in fish that resulted from the exposure to pollutants has been documented [Zhong, 2004; Mekkawy and Lashein, 2003; Osman et al., 2007; Mekkawy et al., 2010]. Also, the possible role of pollutants in the aetiology of spinal cord deviations in fish has been discussed [Haendel et al., 2004; Mekkawy and Lashein, 2003; Osman et al., 2007;
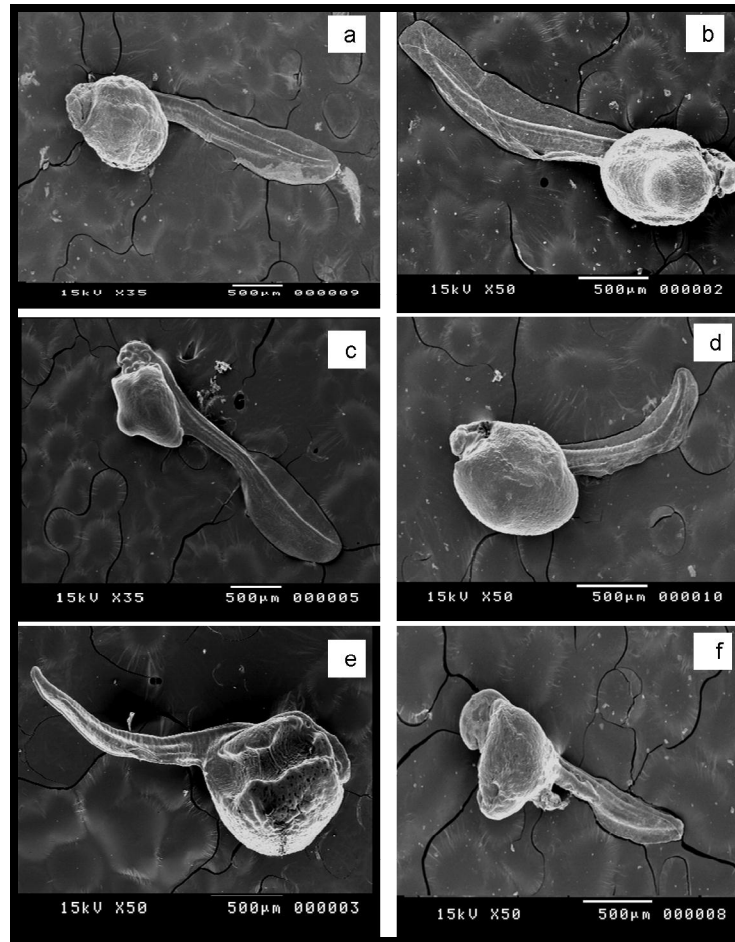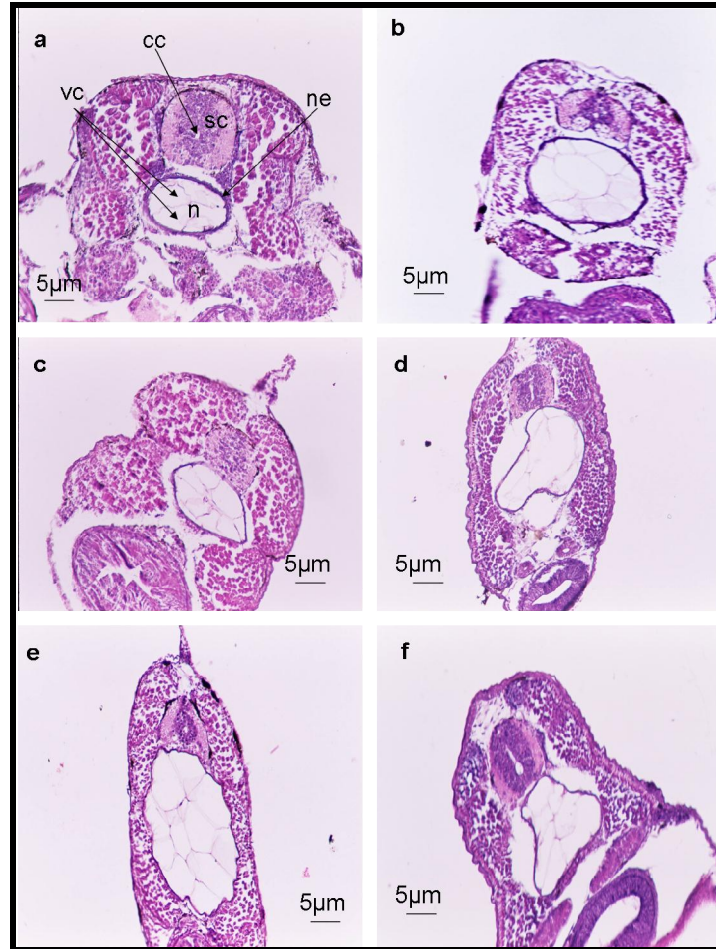
Figure 5. Embryos of *Clarias gariepinus* 37 h- PFS (a) unexposed embryos (b, c, d) embryos exposed to 0.05 mg/l 4-nonylphenol, (e, f) embryos exposed to 0.08 mg/l 4-nonylphenol.
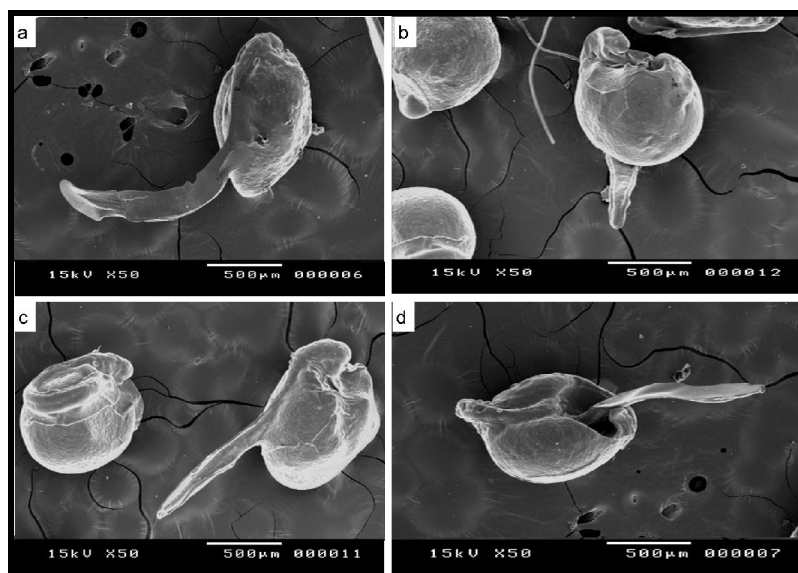
Figure 6. Deformed embryos of *Clarias gariepinus* 37 h- PFS (a, b, c, d) embryos exposed to 0.1 mg/l 4-nonylphenol.

Figure 7. Histological lesions of the embryos of *Clarias gariepinus* after exposure to 4-nonyphenol (a) transverse section through the anterior region, 96 h-PFS embryo control, (b) transverse section through the anterior region, 96 h-PFS embryo exposed to 0.05 mg/l 4-nonylphenol (c) transverse section through the trunk region, 96 h-PFS embryo exposed to 0.05 mg/l 4-nonylphenol (d) transverse section through the trunk region, 120 h-PFS embryo exposed to 0.1 mg/l 4-nonylphenol (e) transverse section through the trunk region, 96h-PFS embryo obtained from female exposed to 0.1 mg/l 4-nonylphenol for a week and unexposed male (f) transverse section through the anterior region, 120 h-PFS embryo obtained from female exposed to 0.1 mg/l 4-nonylphenol for a week and unexposed male. h-PFS = hour-post-fertilization stage, n = notochord, ne= notochord epithelium, cc = central canal, and sc = spinal cord, vc= vacuolated cells. Staining:H & E.

Mahmoud et al., 2009]. Most larvae with vertebral column flexure died after hatching or survived few days or developed into normal individuals and this may be one contributor causing population decrease in fish after exposure to EDCs [Mahmoud et al., 2009]. The embryonic cathepsin D (CAT D) plays an important role in the process of development and any disruptions of this enzyme will lead to adverse effects on the development and reproductions of fish [Carnevali et al., 2001; Yang et al., 2006]. It has been reported that zebrafish females exposed to 50 µg/l NP showed inhibition of CAT D activities [Yang et al., 2006]. Therefore, the malformation of larvae was believed to be a consequence of or relative to the inhibition of CAT D activities. Our results showed many malformations ranged from fertilized egg damage to embryos and larvae. Similar results were obtained by Metin [2001] who observed a significant decrease in swelling of *Cyprinus carpio* eggs at different concentration of cadmium. Also, it has been reported that zinc treated *Pimephales promelas* eggs became sticky and easily breakable soon after spawning [Benoit and Holcombe, 1978]. The level of swelling may result in

hatching of abnormal larvae [Korwin-Kossakowski, 1996]. The results in this investigation indicate that 4-nonylphenol exposure caused embryonic body malformations. Most commonly observed malformations included collapsed tail, pericardial oedema, irregular head shape, yolk sac oedema, vertebral curvature, dwarfism, damage to entire structure of the spinal cord and notochord. Similar results observed by various authors under the effects of metals on fish embryos [Chow and Cheng, 2003; Mekkawy and Lashein, 2003; Fraysse et al., 2006; ýugowska, 2005; Osman et al., 2007] and also under the effects of ultraviolet [Mahmoud et al., 2009]. Increase in the mortality rate of *Clarias gariepinus* embryos exposed to 4-nonylphenol and this is similar to the results of other authors for various fish species where they indicated that heavy metals intoxication and other pollutants increases the mortality of fish embryos [Sarnowska et al., 1997; Osman et al., 2007; Mahmoud et al., 2009].

In conclusion, the changes induced by 4-nonylphenol in catfish (*Clarias gariepinus*) embryonic development may be related to intoxication of spawners and accumulation of 4-nonylphenol in eggs or a direct effect of 4-nonylphenol on the fertilization process and on developing embryos. 4-nonylphenol intoxication of embryos of *Clarias gariepinus* results in disturbances of developmental process and causes embryonic and larval malformation and mortality.

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