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Full Length Research Paper

4-Nonylphenol induced morphological and histopathological malformations in *Bufo regularis* tadpoles

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Global decline in frog populations is thought to indicate environmental damage caused by human activity. Pollution especially chemicals are found to contaminate aquatic ecosystems and their animals including fish and amphibians during their adult life and sensitive stages of development. Nonylphenol ethoxylate (NPE) is one of the most dangerous chemicals that are recorded in aquatic environments, bacterial degradation of nonylphenol ethoxylates produces more toxic nonylphenol (NP) which is estrogenic both on vitro and vivo assays. In present work, the exposure of embryos of Egyptian toad *Bufo regularis* to different sublethal doses of 4-nonylphenol (1.5, 2.5, and 3.5 µgl^{-1}) resulted in mortality rate increase and as a result some morphological malformations with histopathological changes in some organs were revealed. This study indicated the destructive effects of 4-Nonylphenol on the tadpoles of Egyptian toad.

Keywords: 4-Nonylphenol; malformations; histopathology, tadpoles; *Bufo regularis*

INTRODUCTION

Global decline in frog populations is thought to indicate environmental damage caused by human activity and it has been reported that amphibians populations are declining globally with a drastic rate (Brunelli et al., 2009, Stuart et al., 2004). Pollution especially chemicals are found to contaminate aquatic ecosystems and their animals including fish and amphibians during their adult life and sensitive stages of development (Radhaiah et al., 1987). Previous studies have been shown that water pollution play an important role in amphibians decline (Feng et al., 2011). Nonylphenol ethoxylate (NPE) is one of the most dangerous chemicals that are recorded in aquatic environments, particulary in river water(Clark et al., 1992, Rivero et al., 2008, Tsuda et al., 2000). Such chemicals are widely used in the production and formulation of many commercially sold products (e.g. industrial and commercial detergent, polymer resin, cosmetic products). Bacterial degradation of nonylphenol ethoxylates produces more toxic nonylphenol (NP) (Hano et al., 2009) which holds a vital position in the environmental contamination. Such compound is also estrogenic both on vitro and vivo assays (Folmar et al., 2002, Sayed et al., 2012a, Servos, 1999). The effects of environmental pollutants on amphibians vary considerably depending on the timing of expouse during the life cycle (Brunelli et al., 2009).

Many studies indicated that the exposure of fishes to 4-nonylphenol caused reproductive toxicity (Gronen et

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al., 1999, Harries et al., 2000, Jobling et al., 1996, Kang et al., 2003, Sayed et al., 2012a, Yokota et al., 2001). Other previous studies reported that NP has an estrogenic effect on some amphibians (Takase et al., 2007) and disrupt the development of the embryos and larvae of other (Chan et al., 2010). (Feng et al., 2011) indicated that 4-nonylphenol affected the sperm dynamic parameters, morphology and fertilization rate of *Bufo raddi.* Because of the amphibians have been utilized in scientific research and in education and due to their sensitivity to climate and habitat changes and environmental contamination many scientists have used amphibian embryos to evaluate the effects of toxins, mutagens, and teratogens (Stuart et al., 2004). We focused our attention on nonylphenol as a chemical factor affecting the survival of embryos and larvae and thus are being one environmental stressor causing the decline of amphibians. The aim of the present study was to investigate effects of chronic larval exposure to ecologically relevant concentrations of 4-Nonylphenol on mortality rate, development and morphology of Egyptian toad tadpoles. Also, In this study we investigated the effects of three doses of 4-Nonylphenol on histology of some organs.

MATERIALS AND METHODS

Specimen collection

Couple of male and female of Egyptian *Bufo regularis* were collected from fresh water pond at 1km from River Nile at Assiut in July 2011 and transported to Fish Biology Laboratory at Zoology Department, Faculty of Science, Assiut University. After spawning the eggs ribbons placed in a separated glass aquarium filled with clean water. Close and continuous observations have been carried out during the early stages of cleavage. After hatching and when tadpoles begin to feed our experiment started. The embryos at beginning of feeding were at stage 44 (4days age and 6.8mm length) (Sedra and Michael, 1961) were fed on brine shrimp (*Artemia franciscana*) three times a day and kept together in 50 l rectangular tanks containing tap water (conductivity 2000 ls/cm; pH 7.5; oxygen 88–95% saturation; temperature 27-28 ºC; photoperiod 12:12 light: dark).

4-nonylphenol

Nonylphenol was obtained from Sigma- Aldrich (Schnelldrof, Germany) with purity of 99.3%.

Experimental setup

The adapted embryos were subdivided into four groups (50 embryos per each; three replicates): one control and three group exposed to 1.5, 2.5 and 3.5 µg/l for three days with changing the water every day. In the present study, the range of NP exposures was 1.5-3.5 µg/l and these concentrations were chosen in accordance with environmentally relevant concentrations. The conditions of the experiment were as that of acclimatization with changing all the tap water and concentrations of 4-nonylphenol every day. Counting of the dead samples occurred everyday to calculate the mortality rate and at the end of the experiment samples were taken and fixed for morphological and histological preparations.

Morphology

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

Hematoxylin-Eosin (HE) histopathological preparations

For microscopic preparations, 3 embryos were fixed in 10% neutral buffered formalin. Fixed larvae were processed routinely for paraffin emb-edding technique. Larvae were sectioned at 5-7µ 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 divisions and ranges were estimated. The pattern of variation was one-way analysis of variance using the SPSS package (SPSS, 1998) at the 0.05 significance level. Dunnett t-tests treat one group as a control, and compare all other groups against it.

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.

Table 1. Mortality rate and deformed embryos (mean ± SD) % after exposure to different doses of 4-nonylphenol during early developmental stages of the Egyptian tadpoles *Bufo regularis*.

Groups / Test	Control	1.5 µgl^{-1} 4-nonylphenol	2.5 µgl^{-1} 4-nonylphenol	3.5 µgl^{-1} 4-nonylphenol
Mortality rate at 1st day*	0.0 ± 0.0 (0-0) a (A)	2.67±1.0 (1-4) a (B)	4.33±1.53 (3-6) a (C)	9.0± 1.0 (8-10) a (D)
Mortality rate at 2nd day	0.33 ± 0.58 (0-1) b (A)	2.0 ± 1.0 (1-3) a (A)	4.67± 2.08 (3-7) a (B)	17± 4.58(12-18) b (C)
Mortality rate at 3rd day	0.0 ±0.0 (0-0) a (A)	2.33± 1.0 (1-3) a (B)	5.0±1.0 (4-6) b (C)	16.33± 8.39(11-26) c (D)
Total deformed embryos	2.67±1.53(1-4) c (A)	2.17±1.0 (4-6) b (B)	8.0± 3.0 (5-11) b (C)	12.33±1.53(11-14) c (D)

*mortality rate and deformed embryos showing different lower case letters are significant within the test at 0.05 level (vertical comparison) while showing different capital letters are significant within the doses at 0.05 levels (horizontal comparison)

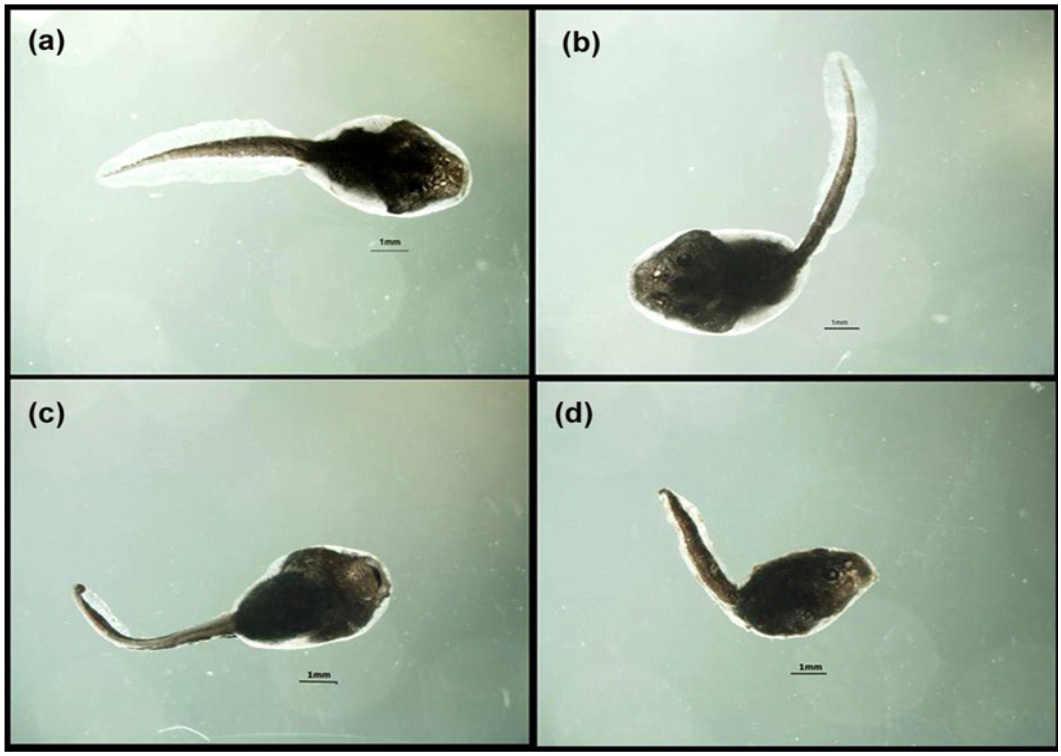

Figure 1. Developmental stages of tadpoles showing (a) control (b) notochordal curvature (kyphosis) after exposed to 1.5 µg/L of 4-nonylphenol (c) kyphosis after exposed to 2.5 µg/L of 4-nonylphenol (d) kyphosis after exposed to 3.5 µg/L of 4-nonylphenol; Scale bar= 1mm.

RESULTS

Variation in mortality rate and deformed embryos count

As shown in Table 1, the mortality rate percentage appears in control group was 0.0 ± 0.0 (R^2=0.996) while this percentage was 9.0± 1.0 (R^2=0.996) in 3.5 µg/l 4-nonylphenol group. Those percentages were increase significantly with 4-nonylphenol doses increase as 2.67±1.0, 4.33±1.53 (R^2=0.996) at 1.5 and 2.5 µg/l 4-nonylphenol groups respectively. Also, the percentage of mortality rate was increased significantly with time of 4-nonylphenol exposure especially in 2.5 and 3.5 µg/l 4-nonylphenol groups where this percentage was 4.33±1.53 and 9.0± 1.0 (R^2=0.994) at the 1st day of 4-nonylphenol exposure and become 5.0±1.0 and 16.33± 8.39 (R^2=0.994) in 2.5 and 3.5 µg/l 4-nonylphenol groups

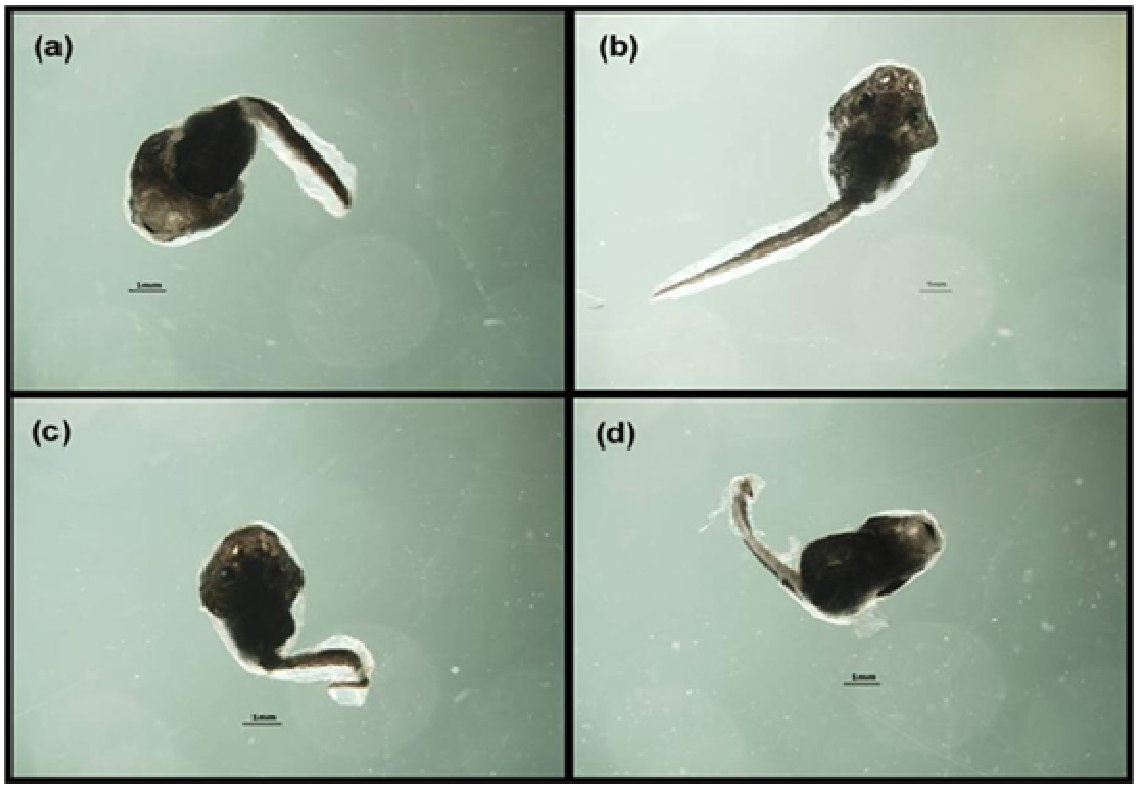

Figure 2. Developmental stages of tadpoles showing different types of malformations (a) Lordosis after exposed to 2.5 µg/L of 4-nonylphenol (b) scoliosis after exposed to 1.5 µg/L of 4-nonylphenol (c) s-shaped after exposed to 3.5 µg/L of 4-nonylphenol (d) fin blistering with yolk sac oedema after exposed to 3.5 µg/L of 4-nonylphenol; Scale bar= 1mm.

respectively at the 3rd day of 4-nonylphenol exposure. The percentage of deformed embryos increased significantly with 4-nonylphenol doses increase were it was 2.67±1.53 (R^2=0.996) in control group and become 12.33±1.53 (R^2=0.994) in 3.5 µg/l 4-nonylphenol group. This means the mortality rate and deformation in tadpoles after 4-nonylphenol exposure is depending on the doses and the time of exposure.

Morphological malformations

Body curvature

The most frequently observed gross morphological deformation was a notochord curvature. Different types of notochord curvature were observed (1) kyphosis (ventrodorsal curvature) (Figure 1b,c,d) (2) Lordosis (dorsoventral curvature) (Figure 2a) (3) Scoliosis (lateral curvature) (Figure 2b) and (4) flat S-shape (Figure 2c).

Yolk sac oedema

Yolk sac oedema was observed in tadpoles exposed to 3.5 µg/L of 4-nonylphenol (Figure 2d) as balloon- shape oedema Malformed embryos were characterized by poorly developed head. Also yolk sac oedema was often associated with notochordal curvature and fin blistering. Yolk sac malformation caused abnormal growth, so that oedematous embryos were usually shorter than the normal ones.

Fin blistering

Blistering of fin was observed in tadpoles exposed to 3.5 µg/L of 4-nonylphenol. The membranous fin was blistered and degenerated (Figure 2d). Fin blistering was often associated with yolk sac oedema and notochord curvature.

Histopathological changes

Nonylphenol was induced histopathological changes includes spinal cord, notochord, liver and eye (Figure 3, 4 and 5). The degree of damage was found to be correlated with nonylphenol doses , organ location and stage of tadpoles.

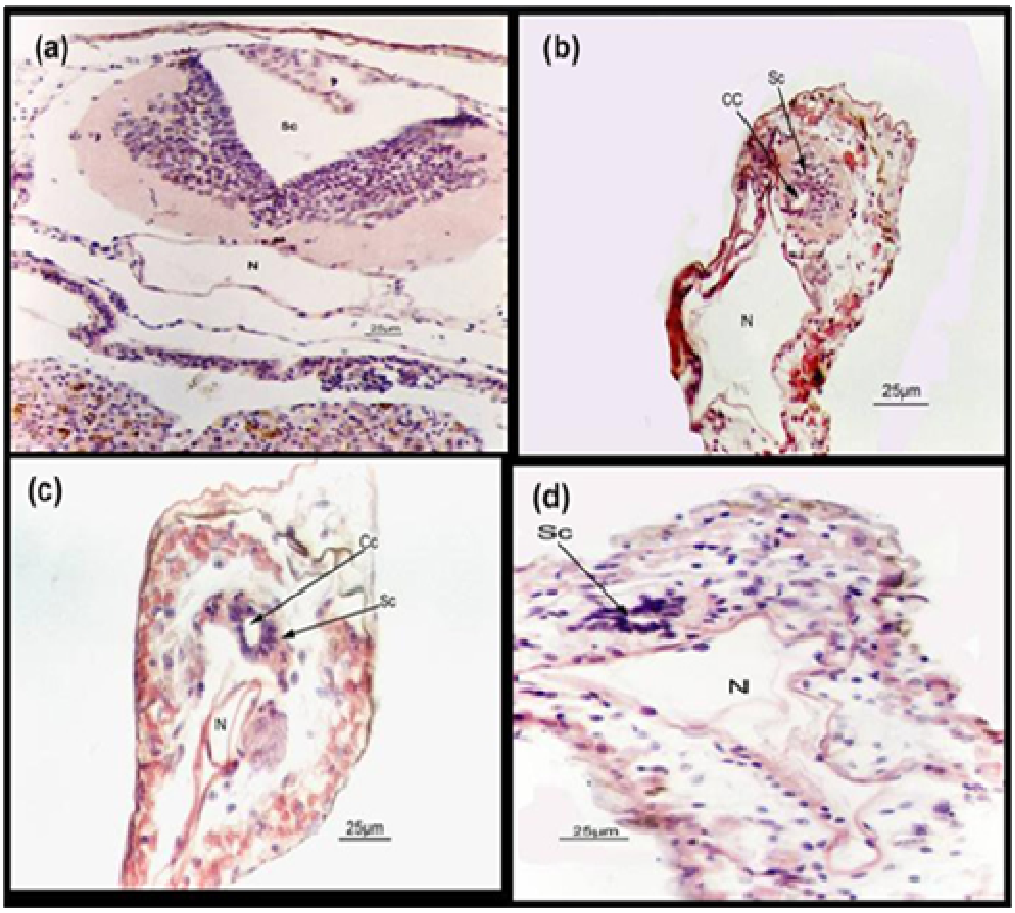

Figure 3. Histological lesions of the spinal cord and notochord in tadpoles after exposure to 4-nonylphenol (a) Transverse section through the spinal cord, control, (b and c) Transverse section through the spinal cord, tadpoles exposed for 2.5 µg/L of 4-nonylphenol (d) Transverse section through the spinal cord, tadpoles exposed for 3.5 µg/L of 4-nonylphenol. N = notochord, CC= central canal, SC=spinal cord. Staining: H and E .

Malformations of the spinal cord

In control tadpoles (Figure 3a), spinal cord is consisted of outer sheath surrounding the white matter which contains nerve fibers and gray matter which contains neurons surrounding the central canal. The tadpoles exposed to 1.5 µg/L of 4-nonylphenol showed less degree of damage (Figure 3b), whereas the spinal cord in tadpoles exposed to high doses of 4-nonylphenol showed severe degree of damage as degeneration of gray matter and central canal (Figures 3c and 3d).

Malformations of the notochord

The tadpoles exposed to different doses of 4-nonylphenol showed variable degree of collapses in the notochord in comparison with the control ones which have a uniform shape (Figure 3). The degree of collapse increased with the increase of dose of 4-nonylphenol. The tadpoles exposed to 1.5 µg/L of 4-nonylphenol had weak collapsed notochord (Figure 3b). In the groups exposed to 2.5 and 3.5 µg/L of 4-nonylphenol severally collapsed notochord were observed (Figure 3c and 3d).

Histopathological changes in liver

4-Nonylphenol treated tadpoles had damaged hepatic tissues and proliferation of the hepatic cells with a decrease in cell size. Accordingly, the hepatocytes lost their normal polygonal shape and boundary between cells become invisible (Figures 4) in comparison with control (Figure 4a). Individal cells were necrotic with condensed granules, some of them were characterized by the absence of nuclei, while the others having pyknotic nuclei were observed (Figure 4d). The hepatic tissues damage increased with the increase dose of 4-nonylphenol. Each hepatocytes has its own nucleus, the number of kuffer increased with a marked decrease in the

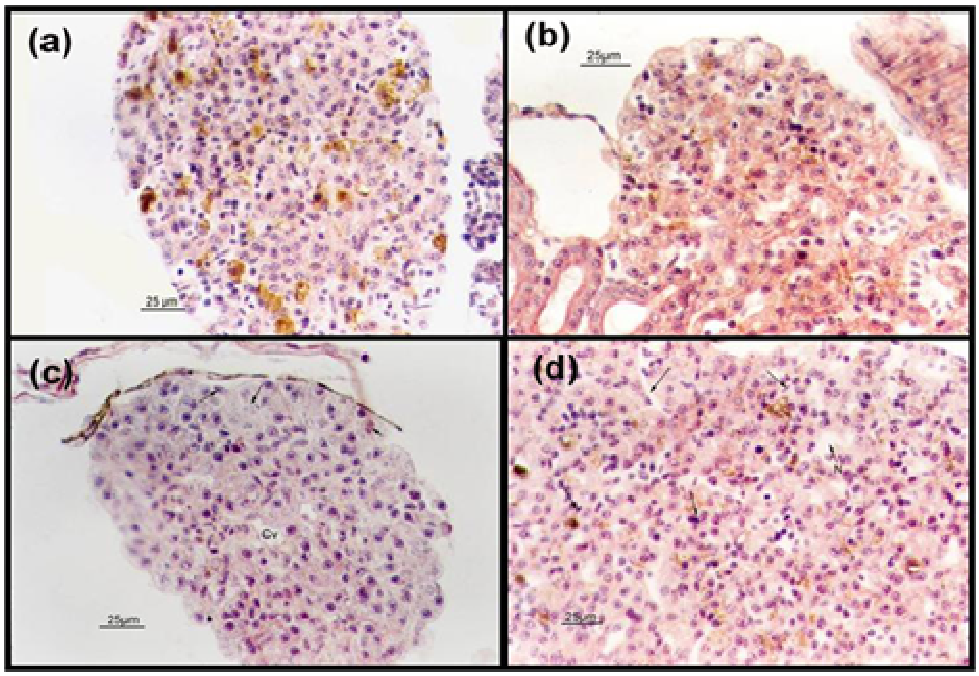

Figure 4. Histological lesions of the liver in tadpoles after exposure to 4-nonylphenol (a) Transverse section through the liver, control, (b) Transverse section through the liver, tadpoles exposed for 1.5 µg/L of 4-nonylphenol (c) Transverse section through the liver, tadpoles exposed for 2.5 µg/L of 4-nonylphenol (d) Transverse section through the liver , tadpoles exposed for 3.5 µg/L of 4-nonylphenol. Staining: H and E .

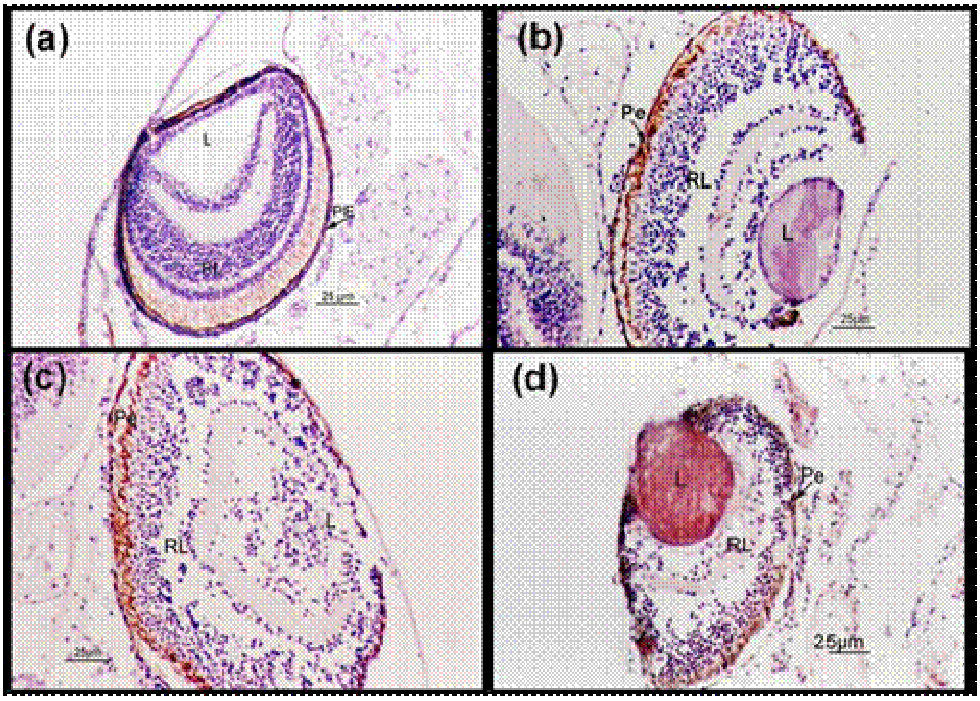

Figure 5. Histological lesions of the eye in tadpoles after exposure to 4-nonylphenol (a) Transverse section through the eye, control, (b) Transverse section through the eye, tadpoles exposed for 1.5 µg/L of 4-nonylphenol (c) Transverse section through the eye, tadpoles exposed for 2.5 µg/L of 4-nonylphenol (d) Transverse section through the eye , tadpoles exposed for 3.5 µg/L of 4-nonylphenol. L=lens; RL=retinal layers; Pe= pigmented epithelium. Staining: H and E.

number of melanomacrophage cells (Figure 4b,c,d).

Malformations of the eyes

The control tadpoles had healthy eyes with well differentiated retinal layers, uniform pigmented epithelium and well differentiated lens (Figure 5a). In contrast the exposed tadpoles exhibited number of malformations depending on the exposure doses. The tadpoles exposed to 1.5 µg/L of 4-nonylphenol showed less degree of malformations. These tadpoles exhibited partially degenerated retinal layers and lens (Figure 5b). The tadpoles exposed to 2.5 µg/L of 4-nonylphenol had irregular and discontinuous pigmented epithelium and degenerated lens and retinal layer with focal areas of necrosis (Figure 5c). This means that the degree of malformations increased with the increasing of the exposure dose of 4-nonylphenol.

DISCUSSION

To our knowledge the present study is the first to examine the effects of 4-nonylphenol on *Bufo regularis* tadpoles (one of the most important amphibian species in Egypt) after exposure during the entire larval development period. Our results showed that exposure to environmentally relevant concentrations of 4-nonyphenol 1.5, 2.5 and 3.5 µg/L resulted in significantly increased mortality in *B. regularis* tadpoles. Our previous study showed that reduced larval growth and a delayed development in African catfish *Clarias gariepinus* occurred also at the lowest 4-nonylphenol concentration 0.05mg/L (Sayed et al., 2012c). 4-Nonylphenol concentrations of 0.08 and 0.1mg/L induced 48 % and 68 % mortality in 37h-PFS *C. gariepinus* embryos of African catfish. The present study showed that *Bufo regularis* tadpoles are sensitive to 4-nonylphenol concentrations likely to occur in their aquatic habitats. Therefore, our results suggest that wild frog populations may be affected by 4-nonylphenol in ecosystems which has profound implications in the light of the amphibian decline.

Our results in the present study are in agreement with other studies showing that inhibition of larval growth is one of the most sensitive indicators of developmental toxicity (Mahmoud et al., 2009, Mekkawy and Lashein, 2003, Osman et al., 2007, Richards and Kendall, 2003, Sayed et al., 2012c). The mortality presented in this study is in agreement with other studies indicating that the survival of embryos decreased after exposure to 4-nonylphenol in a concentration-dependent manner (Bevan et al., 2003, Park et al., 2010, Sone et al., 2004) In this study we assessed the effects of 4-nonylphenol on morphological development in order to explore whether chronic exposure would cause tadpole deformities. A lot of studies have been carried out on the developmental and morphological abnormalities caused by pesticides (Boone et al., 2001, Fordham et al., 2001, Gurkan and Hayretdag, 2012), heavy metals (Kennedy, 1996, Mekkawy and Lashein, 2003, Osman et al., 2007), UVA (Mahmoud et al., 2009) and organic chemicals (Sayed et al., 2012c). 4-Nonylphenol at environmentally realistic concentrations can delay the development of *Crassostrea gigas* larvae and can cause an increase in shell deformities in a population of exposed animals (Nice et al., 2000). It was reported that NP affect the (*Paracentrotus lividus*) during reproduction and embryonic developmental stages (Arslan et al., 2007). Besides, there would be a risk of NP on *B. raddei* reproductive behavior. Moreover, there would be a greater risk for the effects of NP on the reproduction of *B. raddei* considering the longer NP exposure time during the complete life cycle (Feng et al., 2011). (Hano et al., 2009) reported that the number of embryos hatchreased significantly in groups exposed to NP. Also, ≥280 ng/l 4-nonylphenol was toxic and caused a severe decrease in the percentage of larvae of rainbow trout (Lahnsteiner et al., 2005). (Ishibashi et al., 2006) reported that after 21-day exposure significant mortality in 40% males recorded at 100 µg/l 4-NP. The deformities revealed in our study support the hypothesis that environmental chemicals are involved in the increase of malformations observed in wild amphibians (Ankley et al., 2004, Bridges, 2000, Taylor et al., 2005). (Sone et al., 2004) reported that NP induced short body length, microcephaly, flexure, edema, and abnormal gut coiling in X. laevis. Similarly, tail resorption during the metamorphic period was inhibited by NP in Rana catesbeiana tadpoles (Christensen et al., 2005). We have been reported the estrogenity of NP in caffish adults and embryos (Sayed et al., 2012a) also, NP has estrogenic activity in tadpoles as well as in adult amphibians, including B. orientalis (Kang et al., 2003, Kloas et al., 1999, Yang et al., 2005).

It has been reported that endosulfan exposure in short-term laboratory experiments on anurans caused developmental deformities such as lateral flexure of the tail or eye and limb deformities (Brunelli et al., 2009, Harries et al., 2000). (Gurkan and Hayretdag, 2012) has been stated that decreasing of survival percentages of Bufo viridis after copper sulfate exposure. Likewise, it was reported that developmental anomalies and deformations were observed in larvae toad species Xenopus laevis (Fort and Stover, 1996). We recently demonstrated that exposure to 4-nonylphenol caused hematological damage (Mekkawy et al., 2011), biochemical changes (Sayed et al., 2011), endocrine disruption (Sayed et al., 2012a), histopathological alterations (Sayed et al., 2012b) and embryo toxicity (Sayed et al., 2012c) in C. gariepinus adults and embryos.

Morphological and hsitopathologicals changed occurred in the present work after exposure to 4-nonylphenol are similar to the results of (Sayed et al., 2012c) after exposure to embryos of catfish to 4-NP and (Sayed et al., 2012b) after exposure adults catfish to 4-NP and (Chandrasekar et al., 2011) after exposure zebrfish embryos to 4-NP. The notochord plays a vital role in axis formation, somite patterning and in the differentiation of muscle and neural cells (Chandrasekar et al., 2011, Smith, 1993, Yamada et al., 1991) so that the malformations reported in our results may interrupt proper formation of the vertebral body and cause body organs alterations. Our data are consistent with the previously established results that deformities induced in the notochord during early development also produce vertebral defects later in development (Sayed et al., 2012c). As proposed by (Chandrasekar et al., 2011) two possible ways by which 4-NP could elicit its deleterious effects on the notochord. 4-NP might directly inhibit the chordamesoderm differentiation at localized regions and alternatively, 4-NP might act specifically on the basement membrane at the onset of the chordamesoderm differentiation. The malformation of vertebral column flexure in fish that resulted from the exposure to pollutants has been documented (Mekkawy and Lashein, 2003, Osman et al., 2007, Sayed et al., 2012a, Zhong, 2004). The results in this study indicate that 4-nonylphenol exposure caused embryonic body malformations as oedema, vertebral curvature, dwarfism, damage to entire structure of the spinal cord, eye, liver and notochord. Similar results observed by various authors under the effects of metals on fish embryos (Fraysse et al., 2006, Ługowska and Jezierska, 2000, Mekkawy and Lashein, 2003, Osman et al., 2007), under the effects of ultraviolet (El-Bakary and Sayed, 2011, Mahmoud et al., 2009) and nonylphenol (Sayed et al., 2012c, Sayed et al., 2012b).In conclusion,we have shown that chronic exposure to environmentally relevant concentrations of 4-nonylphenpl during the sensitive tadpole period caused reduced larval growth, morphological malfoermations and histological alterations in some organs in *Bufo regularis*. Our results suggest that wild frog populations may be affected by 4-nonylphenol in ecosystems.

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