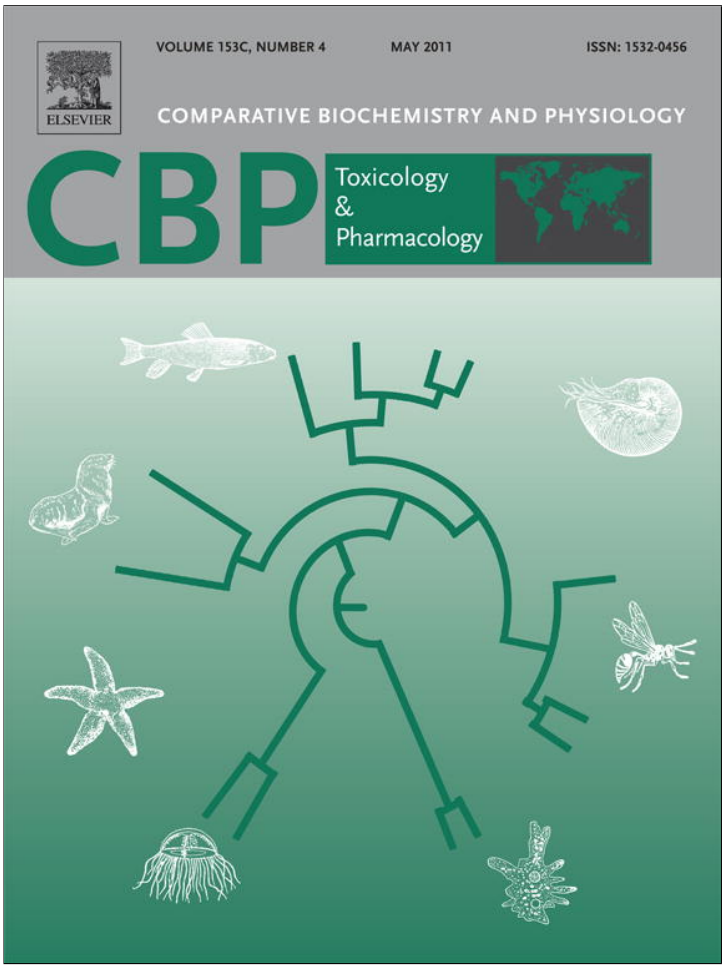

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Endosulfan affects health variables in adult zebrafish (*Danio rerio*) and induces alterations in larvae development

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ABSTRACT

Adult zebrafish (*Danio rerio*) were exposed to 0 (control), 0.16 or 0.48 μg/L of the insecticide, endosulfan, for 28 days. Haematology, whole body ions, thiobarbituric acid reactive substances (TBARS), Na^+K^+–ATPase, organ histology and reproduction were assessed in adults. The resulting offspring were examined for latent effects on development (heart rate and morphometrics). On day 14, adult fish exposed to 0.16 μg/L endosulfan showed significantly lower red blood cell counts than those exposed to 0.48 μg/L endosulfan; adult fish exposed to 0.16 ug/L also showed elevated TBARS compared to controls. Both concentrations of endosulfan caused a 4.0 fold increase in Na^+K^+–ATPase activity compared to controls (ANOVA, $p<0.05$). On day 14, the livers of fish exposed to endosulfan had fewer, enlarged hepatocytes, with cell diameters greater than the controls (ANOVA, $p<0.05$). Morphological alterations in the progeny of fish exposed to endosulfan were observed. Heart beat frequency was significantly lower in larvae from exposed adults to 0.16 μg/L compared to the control (ANOVA, $p<0.05$). These findings show that sublethal exposure to endosulfan causes adverse sublethal effects in adult *D. rerio*, and effects on the development of their offspring.

1. Introduction

Endosulfan (6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,4,3-benzodioxathiepin-3-oxide) is currently the only chlorinated hydrocarbon insecticide used worldwide to treat food crops, as a wood preservative, and in pest control strategies (Capkin et al., 2006; Jonsson and Toledo, 1993a). However owing to its persistence in the environment and toxic effects, endosulfan was classified as a Persistent Organic Pollutant (POPs) during the Stockholm Convention on POPs, supporting the ban on its use and production in several countries (POPRC, 2010). Water contamination can occur through spills, drift, atmospheric transport, field runoff, individual misuse, and improper disposal (Gilliom et al., 2006). Fish are highly sensitive to low (μg/L) concentrations of endosulfan (Jonsson and Toledo, 1993a) and there are several reports of acute toxicity with LC_{50} ranging from 0.5 to 20 μg/L (Capkin et al., 2006; Magesh and Kumaraguru, 2006). Endosulfan is known to bioaccumulate in fish, but is also quickly eliminated from the body. For example, after a 21 day exposure, yellow tetra (*Hyphessobrycon bifasciatus*) and zebrafish (*Danio rerio*) eliminated more than half their body burden in only 5 days (Jonsson and Toledo, 1993b; Toledo and Jonsson, 1992).

The majority of studies of endosulfan toxicity in fish have been carried out using acute exposures. These acute studies have reported histopathological changes in gills leading to respiratory distress and ionoregulatory disturbances (Capkin et al., 2006; Cengiz and Ünlü, 2002; Jonsson and Toledo, 1993a). Liver necrosis (Capkin et al., 2006; Cengiz et al., 2001; Jonsson and Toledo, 1993a; Nowak and Kingsford, 2003), as well as necrosis in haematopoietic tissue and renal tubules of rainbow trout (*Oncorhynchus mykiss*) has also been observed (Capkin et al., 2006). Endosulfan exposure can cause haematological disturbances, although previous work has reported both reductions (Jenkins et al., 2003) and increases in red blood cell count, haemoglobin and haematocrit (Naidu et al., 1987).

Reports on ionic regulation are also varied. In mrigal (*Cirrhinus mrigala*) exposed to 1.1 μg/L of endosulfan for 96 h, increased sodium concentration in gill and liver tissue along with increased potassium concentration in gill, liver, brain and muscle was observed (Swarup et al., 1981). In contrast to these findings, no effects on sodium, potassium or chloride have been reported in Atlantic salmon (*Salmo salar*) fed up to 500 μg/kg dietary endosulfan; suggesting that oral exposure may be less toxic (Petri et al., 2006). However, Atlantic salmon fed with 0.005 and 0.05 mg/kg endosulfan showed changes in spleen morphology suggesting an increased effort of the spleen to

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maintain normal haematology (Berntssen et al., 2010). It has also been suggested that dietary endosulfan is toxic to Nile tilapia, *Oreochromis niloticus* at concentrations up to 0.5 mg/kg (Coimbra et al., 2005, 2007).

In addition to toxic effects on ionic regulation and haematology, there are concerns that the neuro-endocrine effects of endosulfan will impact on reproduction or reproductive behaviour (Matthiessen and Roberts, 1982). In some fish species such as *O. mykiss*, *H. bifasciatus* and *D. rerio*, endosulfan has shown acute neurotoxic effects evidenced by erratic swimming, excitation, convulsions and loss of balance prior to death (Capkin et al., 2006; Jonsson and Toledo, 1993a). Swarup et al. (1981) found an increase in irritability and aggressiveness in fish exposed to 1.1 μg/L of endosulfan for 96 h. Brain abnormalities have also been associated with endosulfan exposure. Shukla and Pandey (1986) found more vacuoles in pituitary cells (gonadotrops and thyrotrops) and histological abnormalities in the hypothalamic cells of *Sarotherodon mossambicus* exposed to 1 μg/L of endosulfan; this could affect the function of the hypothalamic–pituitary–gonad axis. Endosulfan has also been classified as a potential endocrine disrupting chemical (GFEA, 2004) and is known to produce reproductive pathologies in fish during acute exposures (Chakravorty et al., 1992; Dutta et al., 2006). There are concerns that gamete quality could lead to alterations in fertilization rate or trans-generational effects in offspring (Yang et al., 2006).

Clearly, studies on the acute toxicity of endosulfan have helped identify target organs and possible toxic mechanisms in fish, but far less is known about chronic toxicity; and there are concerns that some effects may persist after exposure (latent effects). The first aim of the present study was to evaluate the effects of a 28-day sublethal exposure on adult zebrafish with end points relating to osmoregulatory status, oxidative stress, haematological parameters and histopathology of the adults. The second aim was to assess the reproductive success of the adults, and crucially, developmental effects on the next generation of unexposed offspring in clean water.

2. Materials and methods

2.1. Experimental design and endosulfan concentrations

Sexually mature zebrafish (*D. rerio*), were obtained from a brood stock reared and maintained at the University of Plymouth, UK. The females (n = 99) had an initial body mass (BM) of 454 ± 9 mg and 34 ± 0.2 mm fork length (FL) and, the males (n = 198) 345 ± 4 mg BM and 33 ± 0.2 mm FL (mean ± S.E.M). Thirty-three fish were distributed to each of nine (33 fish/tank, triplicated design, 3 tanks per treatment) static glass aquaria containing 10 L of water; we tried to maintain a proportion of two males per female (2:1). Fish were acclimatized to these tanks for 10 days with constant aeration. During the acclimatization period, water quality parameters were measured daily (means ± S.E.M., n = 10; temperature, 26.3 ± 0.1 °C; dissolved oxygen (DO), 6.6 ± 0.04 mg/L; oxygen saturation, 81.1 ± 0.5%; pH, 6.6 ± 0.01 and NH_3–N, 0.7 ± 0.04 mg/L). Fish were fed once per day to *satiation* with flakes (Aquarian® Tropical Fish Flakes) 3 h before a daily 80% water change. *Artemia salina* was offered every other day at the same quantity per aquarium. The photoperiod was maintained at 12 h light:12 h dark with 30 minute dawn:dusk transition periods.

After the acclimation period, 10 fish per aquarium were lightly anaesthetised in a buffered solution of tricaine metasulfonate (MS-222, 80 mg/L) and mass and fork length recorded. Fish were sampled at days 0 (initial fish prior to exposure), 14 and 28 of endosulfan exposure. At each endpoint, fish were sampled (2 fish/tank, n = 6 fish/treatment) for histology, haematology and somatic indices. For biochemistry analysis, 9 fish/treatment were sampled (3 fish/tank) at each endpoint. The condition factor/growth indices were calculated using all the data obtained from the above fish (n = 15 fish/treatment).

Two nominal concentrations of endosulfan (0.16 and 0.48 μg/L) and a control group without endosulfan (water only) were used, with the lower concentration representing a sublethal dose equal to about 10% of the 96 h LC_{50} for *D. rerio* (1.6 μg/L) (Jonsson and Toledo, 1993a). Stock solutions were prepared from technical-grade endosulfan (analytical standard α and β isomers, Pestanal®, Sigma-Aldrich Laboratories, Seelze, Germany). Briefly, 100 mg of endosulfan was initially dissolved in 50 mL of analytical grade acetone (99%, Fisher Scientific, UK). This initial stock solution (2 g/L endosulfan) was diluted to 1:10 with deionized water to 0.2 g/L endosulfan, and then a second dilution (1:200 in deionized water) to give a final concentration of 1 mg/L endosulfan. This final dilution contained only 0.05% acetone, and was used to dose the static aquaria to the selected concentrations. The final acetone concentration per aquarium was negligible (<0.005%). The concentrations of endosulfan were maintained by daily 80% water changes with the appropriate addition of the working stock solution. The entire experiment was subject to ethical approval and was independently monitored by a fish health expert.

2.2. Growth and feeding

From the first day of exposure, activity and mortality were determined each day prior to a water change. The time from first feeding to the last food intake was registered within each tank using a stopwatch from the third day of exposure. At each time point in the experiment, fish were terminally anaesthetised in MS-222 (800 mg/L) and mass and fork length were recorded. Relative condition factor (*Kn*) was calculated according to Craig et al. (2005). With the length and body mass obtained from 200 fish a logarithmic length–mass equation was obtained to calculate the relative condition factor ($Kn = W/aL^b$), where W is the body mass (g), L is the length (cm), *a* is the y-intercept (0.01054144) and *b* is the slope (−2.984). The y-intercept and the slope were used in the exponential form.

In addition, specific growth rate (SGR, $[(\mathrm{Ln(MF)} - \mathrm{Ln(MI)}) \times 100]/t$) was determined at 14 and 28 days of exposure, where MI and MF are the initial and final mass and t is the time expressed in days. Liver and gonads from six fish per treatment were removed and weighed in order to determine the hepatosomatic (HSI) and gonadosomatic indices (GSI). The somatic index was calculated as index = (organ mass/body mass) × 100%.

2.3. Haematology and histological evaluation

The same individuals were used for haematology and histological analysis of the gills and liver. Following terminal anaesthesia, blood samples were collected by caudal severance in heparinised micro-haematocrit tubes, and then diluted 1:50 in Dacie's solution, and the blood cells counted (Handy and Depledge, 1999). After blood sampling, the abdominal cavity was carefully opened and the whole fish was fixed in buffered 3.7% formaldehyde for 48 h. The fish were then dehydrated with increasing concentrations of ethanol, cleared in xylene, and then embedded in paraffin wax and sectioned (8 μm) longitudinally (Reichert Jung 1130 Rotary Microtome, Leica). Sections were stained with Mayer's haematoxylin and eosin (H&E) and photographs of gill and liver tissue were obtained using an Olympus digital camera (C-2020 Z) on a Olympus Vanox-T microscope.

One gill arch of each fish from every treatment was examined to evaluate lamellar fusion (LF), epithelial lamellar hyperplasia (LH), microcirculatory alterations and epithelial lifting (EL) using the severity codes as described in Velasco-Santamaría and Cruz-Casallas (2008). Briefly, a score 0 for LF was defined as lamellae without notable fusion; 1, up to one-third lamellar fusion; 2, up to two-thirds lamellar fusion and, 3, lamellar fusion greater than two-thirds. The severity codes for EL were 0, without evidence of epithelial lifting, 1 with epithelial lifting of up to 30%, 2 with approximately 30 to 60% epithelial lifting or 3, greater than 60%. In terms of LH, a score of 0 meant normal gill epithelium with no hyperplasia detected; 1,

multifocal LH and slight inter-lamellar proliferation; 2, multifocal LH from approximately 30 to 60% with partial inter-lamellar obliteration and, 3, diffuse increases in cellular number above 60% with total inter-lamellar obliteration. Finally, the microcirculatory alterations were classified from 0 when circulatory alterations were not observed to the highest score of 3 when severe generalized circulatory changes like congestion and (or) haemorrhage were observed.

Liver histology was quantitatively scored according to both severity of the lesions and distribution (focal, multifocal and diffuse). Necrotic hepatocytes, inflammatory cell infiltration and vacuole hepatocytes were defined as score 0 = non-observed; 1 = mild; 2 = moderate and 3 = severe changes involving significant alteration of appearance or architecture. The number of nuclei, the nuclei and hepatocyte diameter, and the ratio nuclei:hepatocyte were determined in an area of 100 μm^2 per section (in duplicate for each fish) under ×400 magnification. These measurements of cell size were done manually using ImageJ software v. 3.9b (Wayne Rasband, National Institutes of Health NIH, USA) with prior calibration with a graticule of 1 mm length.

2.4. Electrolytes, Na^+K^+–ATPase and oxidative stress

After terminal anaesthesia, whole fish for biochemical evaluation were placed individually into cryotubes (Nunc™, Denmark), snap frozen in liquid nitrogen and stored at −80 °C for later analysis. Frozen whole fish were subsequently ground under liquid nitrogen and 50 mg of tissue was removed for measurement of total ion content. The remainder was homogenised on ice (Cat X520D homogeniser with a T6 shaft, Bennett and Co., Westonsuper-Mare) in five volumes of an ice-cold isotonic solution containing: sucrose (300 mmol/L), ethylenediamine tetra acetic acid (EDTA, 0.1 mmol/L), 4-(2-hydroxyethyl) piperazine-1-ethane sulfonic acid (HEPES, 20 mmol/L) and a few drops of 2-amino-2-hydroxylmethyl-1,3;jsjf-propanediol (Tris 2 M) in order to adjust the pH to 7.8. Aliquots of each whole fish homogenate were then stored at −80 °C until analysed for total protein, Na^+/K^+–ATPase activity and thiobarbituric acid reactive substances (TBARS).

For whole body ion concentrations, homogenised samples were digested in 1N HNO_3 in a proportion 1:10 (tissue mass:volume) for 72 h at 70 °C (Matsuo and Val, 2007). After acid digestion, the samples were centrifuged at 3000×*g* for 5 min and the supernatant analysed for Na^+, K^+, Cl^- and Ca^{2+} concentrations using inductively coupled plasma optical emission spectrophotometry (ICP-OES, Varian 725-ES, Perkin-Elmer Liberty 200 AES with Varian 725 software) against matrix matched standards. Whole body ion concentrations are expressed in μmol/g dry mass.

Na^+/K^+–ATPase activity was measured according to Silva et al. (1977) with modifications. Briefly, 15 μL of each sample and phosphate standard solutions, in triplicate, was added to 400 μL of each of the two assay media (+10 mmol/L K^+, or no added K^+ with 1 mmol/L ouabain) adjusted to pH 7.4. Both media contained 100 mmol/L NaCl, 5 mmol/L $MgCl_2$, 3 mmol/L Na_2ATP and 30 mmol/L HEPES. The samples were incubated at 37 °C for 10 min and the reaction was stopped by adding 1 mL of ice-cold trichloroacetic acid (8.6%, w/v). One mL of colour reagent (9.6% w/v $FeSO_4 \cdot 7H_2O$, 1.15% w/v ammonium heptamolybdate dissolved in 0.66 M H_2SO_4) was added to each tube and developed for 20 min at room temperature. Absorbances were measured at 660 nm (Unicam Heλios ε spectrophotometer) against 0–2.0 mmol/L potassium phosphate standards.

The TBARS assay was performed following the methodology of Camejo et al. (1998) with modifications. Briefly, samples were centrifuged at 15,000×*g* (12,700 rpm) for 5 min and the supernatant collected. To avoid undesired further oxidation of samples, 10 μL of butylated hydroxytoluene (1 mmol/L, BHT, Sigma-Aldrich, Poole, Dorsert, UK) was added to each well of a 96-well plate, with 40 μL of the supernatant or a standard as appropriate. Subsequently, 140 μL of phosphate buffered saline (pH 7.4, Sigma-Aldrich, Poole, Dorsert, UK) and 50 μL of trichloroacetic acid (50%, w/v) were added to each to precipitate protein and reduce turbidity. Seventy-five μL of thiobarbituric acid (1.3% (w/v), Sigma-Aldrich, Poole, Dorsert, UK) was added. The plate was covered and incubated at 60 °C for 60 min. The absorbances were read at 530 nm and 630 nm for turbidity correction (Dynex, MRX microplate reader). A calibration curve was performed using standards of 1,1,3,3-tetraethoxypropane (0 to 250 nmol/mL). Total protein concentrations in homogenates were measured using Bio-Rad protein assay (Bio-Rad Laboratories, United Kingdom) so that Na^+/K^+–ATPase activity and TBARS could be normalised per mg homogenate protein.

2.5. Breeding experiments

At the end of the 28 day exposure period all tanks were carefully cleaned and disinfected. Ten experimental fish were selected randomly in a proportion of 1 female per male (1:1) from each tank in each treatment and maintained in clean water without endosulfan. Fish were fed once per day to *satiation* with flakes (Aquarian® Tropical Fish Flakes) and *A. salina* before a daily water change as before.

Within the tanks, females were kept separate from males by using plastic breeding chambers (20.3×10.2×10.8 cm, Marina Multi-Breeder, Malaysia) in order to avoid uncontrolled breeding. Three natural consecutive breedings were then stimulated 15 days after the end of the endosulfan exposure. For this, a mesh was placed into each aquarium and the females and males were placed together before the end of the light phase. In each breeding trial, the eggs were collected 1 h after the lights came on the following morning. The eggs were examined under a bifocal microscope to assess viability and total fecundity per tank. Three-hundred eggs from each replicate tank were then subdivided and 100 eggs placed into three 250 mL plastic beakers (n = 9 beakers from each treatment) using water from each respective aquarium.

The fertilization rate was determined based on the number of fertilized eggs in relation to the total number per beaker. The criteria for fertilized eggs were defined as translucent eggs after 24 h of fertilization. During the embryo and larval development, morphological observations were recorded twice per day to detect the presence of abnormalities. In order to determine if the morphological abnormalities affected the larval cardiovascular function, the heart beat frequency was determined in larval zebrafish obtained from eggs of adults exposed to 0, 0.16 and 0.48 μg/L. To measure the heart beat frequency, five larvae per beaker at 72 h post-fertilization (hpf) were selected randomly and the beats during 30 s were counted visually using a binocular microscope. During all experiments, dead embryos or larvae (according to the developmental stage) and detritus were removed periodically to avoid water contamination. At 96 hpf, four larvae per beaker were selected randomly, placed in buffered MS-222 (40 mg/L) and fixed in 3% buffered formalin. After no more than 24 h of fixation, the notochord length, and length and height of yolk sac were measured using a microscope adapted with an ocular graticule. The volume of the yolk sac (Vys) expressed in mm^3 was calculated using the formulae:

$$Vys = \frac{\pi}{6} \times L \times H^2$$

where π (Pi) is 3.1416, *L* corresponds to the yolk sac length (expressed in mm) and *H* corresponds to the yolk sac height (expressed in mm).

2.6. Statistical analysis

All values are expressed as mean ± S.E.M. Kolmogorov–Smirnov and Bonferroni tests were done in order to verify the data distribution and homogeneity of variance, respectively. Data transformation was carried out when data were not normally distributed. In cases where data were not normally distributed after transformation, non parametric tests were carried out (Kruskal–Wallis test). There were no tank effects within treatments in any variable; therefore, the data were pooled by treatment for statistical analysis. Data were analysed by two-way analysis of variance (ANOVA) followed by Tukey–Kramer to detect differences between all treatments or pairs, respectively. A rejection level of $p \leq 0.05$ was used. All statistical analyses were conducted using SAS system for Windows software version 8.02 (1999–2001 by SAS Institute, Cary, NC, USA).

3. Results

3.1. General condition of adult fish during endosulfan exposure

Only one mortality was recorded in the experiment, in the 0.16 μg/L endosulfan treatment at day 20. No obvious changes in behaviour were observed in any of the fish which showed normal swimming behaviour and no evidence of respiratory distress or loss of equilibrium during the experiment. Fish continued to feed normally throughout the experiment. In terms of the time taken to eat the food, no significant differences were observed during the whole experimental period. The mean time taken to eat the daily feed was 293.0 ± 5.6 for control, 280.6 ± 6.2 for 0.16 μg/L and 280.7 ± 6.8 s for 0.48 μg/L endosulfan (mean ± S.E.M, n = 72 observations/treatment, ANOVA, $p > 0.05$). After 28 days of exposure to endosulfan no significant differences were observed between treatments in terms of fork length, body mass or condition factor (ANOVA, $p > 0.05$). There were no treatment-dependent differences between the sexes, although as expected mean masses of male fish were about 120 mg less than mature females throughout the experiment. Fish were fed a maintenance ration, so a small and steady growth was expected. The SGR over the whole experiment was positive for all treatments (about 0.7–1.3% growth per day), but with no clear concentration-dependent trend and no significant differences between treatment (Table 1, ANOVA, $p > 0.05$). There were no treatment effects on the relative size of the gonads or liver (Table 1, Kruskal–Wallis, $p > 0.05$). There was no sex effect on these indices (data not shown).

3.2. Haematology in adult fish during endosulfan exposure

Effect of endosulfan exposure on haematology was observed (Fig. 1). There was a transient decrease in red cell counts in the low endosulfan concentration-treated fish at 14 days compared to the high concentration (ANOVA, $p < 0.05$); there was no effect of endosulfan on white blood cell counts. However, red blood cell count had recovered by the end of the experiment with no significant differences (ANOVA and Kruskal–Wallis, for RBC and WBC, respectively, $p > 0.05$) between all treatments.

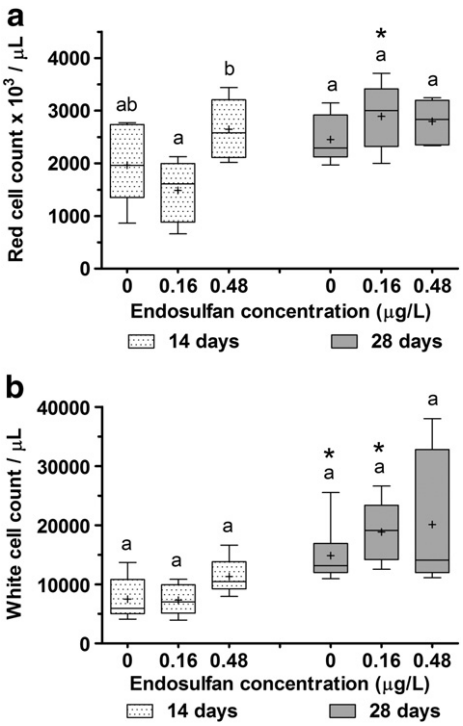

Fig. 1. (a) Red cell and (b) white cell counts in blood of adult zebrafish (*Danio rerio*) exposed to sublethal endosulfan concentrations (control—no added endosulfan, 0.16 and 0.48 μg/L) for 28 days. Values are expressed as median (centre line), mean (+), and the 25th and 75th percentile values. The whiskers show the minimum and maximum values (n = 6). [a,b]Within each exposure time (14 or 28 days), different superscripts indicate significant differences (ANOVA or Kruskal–Wallis, for RBC and WBC, respectively, $p < 0.05$). Asterisk (*) also shows significant time effect at the same concentration of endosulfan ($p < 0.05$).

Table 1
Initial and final mass, fork length, condition factor and somatic index of adult zebrafish (*Danio rerio*) exposed for 28 d to sublethal endosulfan concentrations (0 (control), 0.16 or 0.48 μg/L). Values are expressed as mean ± SEM. Values in parentheses show the number of fish.

Parameter	0 (μg/L)	0.16 (μg/L)	0.48 (μg/L)
Initial mass (mg)	379.8 ± 13.4 (30)	389.1 ± 13.2 (31)	399.3 ± 15.7 (30)
Final mass (mg)	448.7 ± 23.0 (21)	424.8 ± 17.8 (22)	463.6 ± 23.6 (21)
Initial fork length (mm)	33.4 ± 0.3 (30)	33.8 ± 0.3 (31)	33.8 ± 0.3 (30)
Final fork length (mm)	34.7 ± 0.4 (21)	34.1 ± 0.4 (22)	34.5 ± 0.5 (21)
Final condition factor	1.02 ± 0.02 (21)	1.03 ± 0.02 (22)	1.08 ± 0.04 (21)
Final SGR (%/day)	1.02 ± 0.3	0.68 ± 0.3	1.26 ± 0.3
HSI (%)	2.3 ± 0.6	6.1 ± 2.3	3.7 ± 0.9
GSI (%)	7.0 ± 1.9	6.9 ± 2.1	6.9 ± 2.0

There were no significant differences between treatments (ANOVA, $p > 0.05$).

3.3. Liver histology

The livers of control fish showed normal histology with no morphological changes (Fig. 2a). However, necrosis and alterations of integrity in some hepatocytes were diffuse throughout the liver tissue with a severity score between 2 and 3 in both concentrations of endosulfan. Likewise, vacuole formation in hepatocytes and alteration in the cytoplasm structure were observed in both endosulfan concentrations (Fig. 2b, c). Table 2 shows the number of hepatocytes, diameter of nuclei and hepatocytes, and ratio of nuclei:hepatocyte in a hepatic area of 100 μm^2 of adult zebrafish exposed to sublethal endosulfan concentrations. There was a transient decrease in the number of hepatocytes per unit area of liver in the endosulfan-exposed fish compared to controls at day 14 (Kruskal–Wallis, $p < 0.05$). This effect was lost by the end of the experiment (no significant differences in hepatocyte count at day 28 were observed). There were no significant differences in hepatocyte nuclei with an average of about 4 μm (Table 2) at both sampling periods (Kruskal–Wallis, $p > 0.05$). On the other hand, the hepatocyte diameter in fish exposed to endosulfan at 14 and 28 days of exposure was significantly higher (hypertrophy of the hepatocytes) than the control group (Table 2, Kruskal–Wallis, $p < 0.05$). The nuclei: hepatocyte ratio of fish of the control group was significantly higher (Kruskal–Wallis, $p < 0.05$) than the other treatments at both sampling periods. The lower nuclei:hepatocyte ratio of fish exposed to 0.16 and 0.48 μg/L of endosulfan was associated with the increase in hepatocyte diameter.

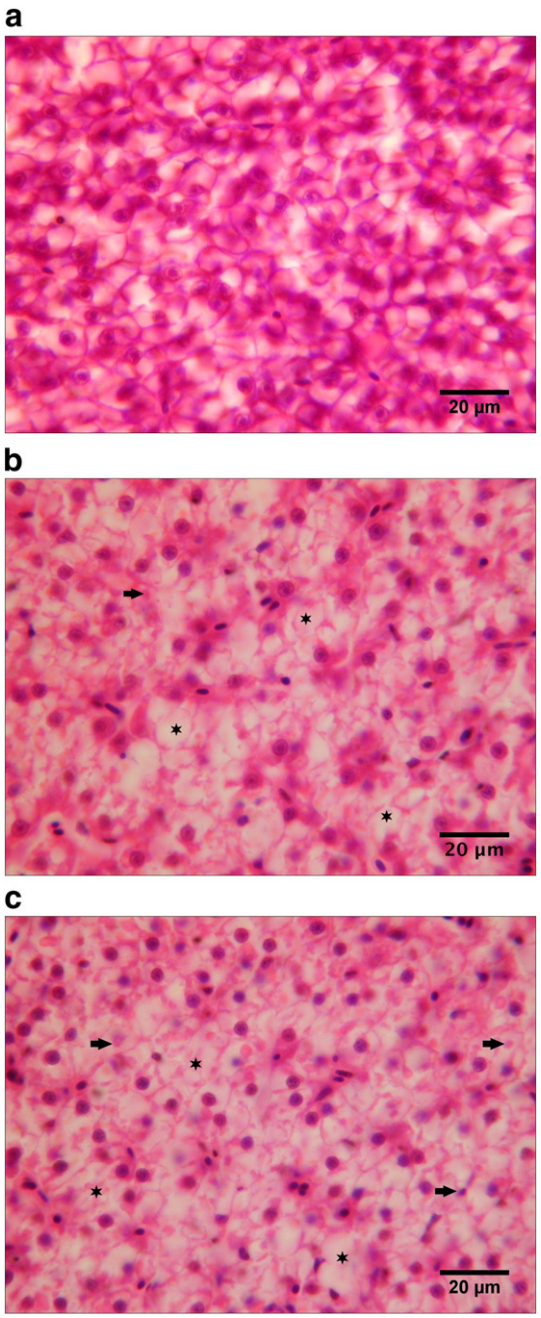

Fig. 2. Liver morphology of adult zebrafish (*Danio rerio*) after 28 days exposure to (a) control (no added endosulfan), (b) 0.16 and (c) 0.48 μg/L of endosulfan. Livers of control fish showed normal histology. Livers from fish exposed to endosulfan showed loss of cytoplasm integrity in some cells. This is indicated by cells with some vacuoles (black stars) and some cells with apparent stages of cellular necrosis (black arrows). Scale bar = 20 μm, and sections were 8 μm thick and stained with H&E.

3.4. Gill histology

The gill also showed histological changes during endosulfan exposure. Histological alterations included lamellar hyperplasia and lamellar fusion with different grades of severity observed in fish exposed to endosulfan (Fig. 3b). In terms of severity, lamellar hyperplasia increased with endosulfan concentration (Fig. 3c). This persisted until the end of the experiment where similar percentages were observed at day 28. Lamellar fusion in the gills did not show a dose-dependent trend but the gills of endosulfan-treated fish showed a lamellar fusion severity of 2. Similarly, moderate lamellar congestion of blood vessels was only observed in 1 and 3 fish exposed to 0.16 and 0.48 μg/L of endosulfan, respectively.

3.5. Electrolytes, Na^+K^+–ATPase and oxidative stress

There were no major effects on whole body electrolyte levels (Table 3). At 14 d of exposure, no significant differences were observed in the whole body Ca^{2+}, Mg^{2+}, K^+ or Na^+ concentrations between the control fish and those exposed to endosulfan, although Na^+K^+–ATPase activity increased at day 14 (see below). At day 28 of exposure, the whole body Na^+ concentration of fish exposed to 0.16 μg/L of endosulfan was significantly higher (Kruskal–Wallis, $p<0.05$) than the control group (Table 3).

Fish exposed to endosulfan showed a transient rise in Na^+K^+–ATPase activity at day 14 compared to controls (ANOVA, $p<0.05$). Fish exposed to 0.16 and 0.48 μg/L of endosulfan showed a 4.0 and 4.5 fold increase of Na^+K^+–ATPase activity compared to the control group (Fig. 4a). However, the Na^+K^+–ATPase activity was not different after 28 d of exposure between any of the treatments (ANOVA, $p>0.05$).

Tissue oxidative stress was assessed using the TBARS assay. Similar to Na^+K^+–ATPase activity, changes in TBARS were only observed at day 14 in the endosulfan-exposed fish. Fish exposed to 0.16 μg/L of endosulfan showed a significant increase (ANOVA, $p<0.05$) in TBARS concentration compared to controls at day 14. No treatment-dependent effect was evident in TBARS by the end of the experiment (ANOVA, $p>0.05$, Fig. 4b).

3.6. Breeding experiments

No differences were observed in the total fecundity or fertility of fish exposed to endosulfan (Fig. 5), however, the lowest concentration of endosulfan produced morphological alterations in the larvae obtained from adult fish exposed to endosulfan. Despite no statistically significant effect on fecundity and fertility, there was a trend towards lower fecundity in the lower endosulfan concentration (214 ± 35 eggs) compared to fish of the control group (391 ± 87 eggs) and 0.48 μg/L treatment (341 ± 84 eggs) (ANOVA, $p>0.05$, Fig. 5).

The notochordal length and the volume of the yolk sac between the three breeding trials and the three concentrations did not show any significant differences (ANOVA, $p>0.05$). The notochord length and yolk sac volume were 2884 ± 15 μm and 94.3 ± 2.9 μm^3 (n = 84), 2951 ±

Table 2
Quantitative analysis of liver cell morphology in adult zebrafish (*Danio rerio*) exposed to 0 (no added endosulfan), 0.16 or 0.48 μg/L of endosulfan for up to 28 days. Values are expressed as mean ± SEM (n = 5 fish per treatment). Data were not collected at time zero from initial stock fish.

Endosulfan (μg/L)	Hepatocyte number/ 100 μm^{2*}		Nuclei diameter (μm)		Hepatocyte diameter (μm)		Ratio N:H (%)*	
	14 days	28 days	14 days	28 days	14 days	28 days	14 days	28 days
0	67 ± 0.9^{a}	67 ± 5.3^{a}	3.99 ± 0.15^{a}	4.00 ± 0.05^{a}	10.63 ± 0.31^{a}	10.21 ± 0.16^{a}	39.59 ± 0.53^{a}	41.23 ± 0.68^{a}
0.16	$58 \pm 4.8^{a,b}$	61 ± 3.9^{a}	3.98 ± 0.10^{a}	3.94 ± 0.10^{a}	$12.50 \pm 0.79^{a,b}$	12.67 ± 0.51^{b}	32.40 ± 1.21^{b}	32.17 ± 1.74^{b}
0.48	53 ± 1.2^{b}	$60 \pm 5.2^{a,\#}$	3.85 ± 0.05^{a}	4.04 ± 0.08^{a}	12.75 ± 0.55^{b}	12.47 ± 0.70^{b}	31.11 ± 1.43^{b}	34.11 ± 2.33^{b}

*Number of hepatocytes per 100 μm^2 are the hepatocytes counts for a standard area of 100 μm^2 on each section. Ratio N:H is the area filled by the nucleus (N) in the area of each hepatocyte (H), in a total area of 100 μm^2 within a randomly selected liver section for each fish.
[a,b]Different superscripts indicate significant differences between treatments within each time point (Kruskal–Wallis, $p<0.05$).
[#]Denote significant time effect within treatment (rows) and parameter (Kruskal–Wallis, $p<0.05$).

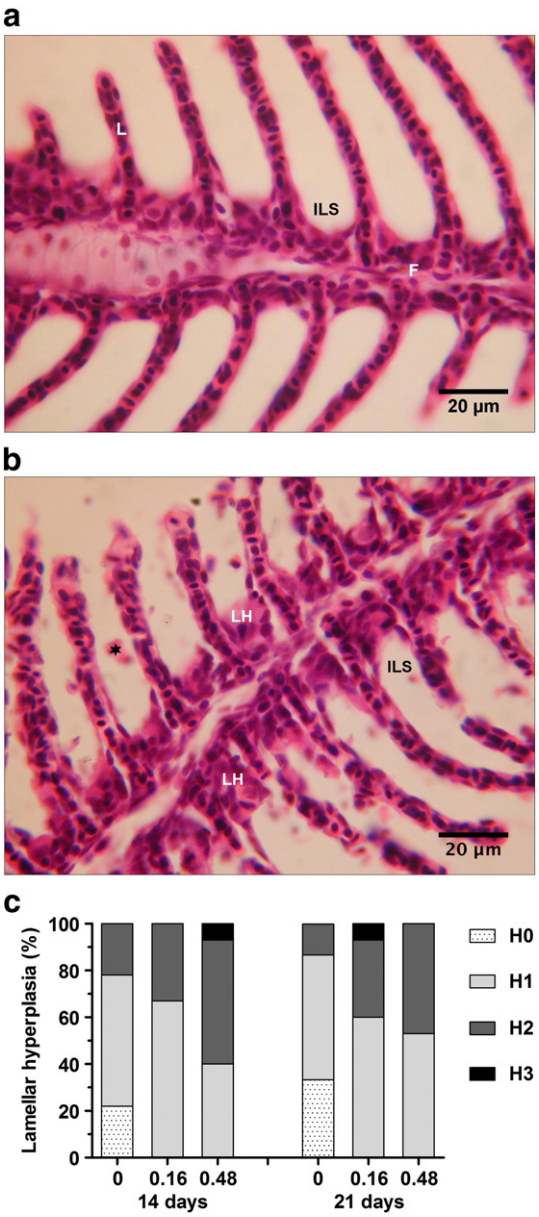

Fig. 3. Gills of adult zebrafish (*Danio rerio*) exposed for 28 days to sublethal endosulfan concentrations. (a) Gills of the control fish show normal histology with small grades of hyperplasia. (b) Gills from fish exposed to 0.48 μg/L of endosulfan showed lamellar hyperplasia scores (LH) and epithelial lifting (black star). Lamellae (L), inter-lamellar space (ILS) and filament (F). Scale bar = 20 μm, and sections were 8 μm thick and stained with H&E. (c) Lamellar hyperplasia from fish of the control group, 0.16 and 0.48 μg/L, sampling at 14 or 28 days of exposure. The hyperplasia was classified as H0: none, H1: mild, H2: moderate and H3: severe hyperplasia. Values are expressed as percentages (n = 5 fish per treatment).

24 μm and 95.3 ± 3.1 μm^3 (n = 55), and 2909 ± 16 μm and 95.1 ± 3.0 μm^3 (n = 68) for control, 0.16 and 0.48 μg/L endosulfan, respectively, with an overall value of 2901 ± 11 μm for notochord length and 94.8 ± 2 μm^3 for yolk sac volume. However, a qualitative analysis showed morphological alterations in the progeny of fish exposed to 0.16 and 0.48 μg/L of endosulfan for 28 days. Larvae and embryos showed occasional spine deformities characterized by severe lordosis and severe ascitis (Fig. 6a, b). In terms of cardiac function, the overall heart beat frequency of larval zebrafish obtained from eggs of adults exposed to 0.16 μg/L was significantly lower (128.3 ± 5.1 beats/min) than the control group (143.7 ± 4.7 beats/min) and the 0.48 μg/L endosulfan treatment (141.0 ± 3.6 beats/min) (ANOVA, $p<0.05$).

4. Discussion

In this study, we show that sublethal endosulfan exposure caused some pathology in the liver and gill of adults with limited effects on osmoregulatory and oxidative stress parameters. The exposed fish remained capable of reproduction, but there were some effects of endosulfan on the resulting offspring; even though they had not themselves been exposed to the pesticide. To our knowledge, this latent effect of endosulfan on the *F1* generation has not been previously reported in fish.

4.1. Sublethal effects of endosulfan on adult zebrafish

There were no effects of endosulfan on body mass, SGR, HSI, or GSI in this study. The absence of growth deterioration confirms that the exposure was sublethal, and some growth was expected given that fish were fed during the experiment. However, there were some histological changes in gill and liver. The histological alterations of hepatocytes including necrosis, hypertrophy, and a high degree of vacuole formation with apparent increase in glycogen storage (Table 2, Fig. 2) in fish exposed to endosulfan, are consistent with changes in lipid levels and the subtle effects of small amounts of lipid peroxidation during endosulfan exposure (Petri et al., 2006). The alterations observed in our study are similar to the findings in *O. mykiss* exposed to 0.6 or 1.3 μg/L endosulfan for 21 d (Altinok and Capkin, 2007), *Gambusia affinis* exposed to 1 to 5 μg/L Thiodan (33.7% endosulfan) for 30 days (Cengiz et al., 2001), *D. rerio* exposed to 0.5 to 5.2 μg/L of endosulfan (Jonsson and Toledo, 1993a), and *O. niloticus* exposed to dietary concentrations from 0.001 to 1 μg endosulfan/g food for 35 days (Coimbra et al., 2007), in which the severity of the lesions increased in proportion to the endosulfan concentration.

The gills of endosulfan-exposed fish showed proliferative alterations, such as lamellar hyperplasia, which increased with the endosulfan concentration, and lamellar fusion. These results are similar to those reported previously in catfish (Nowak, 1992) and zebrafish (Jonsson and Toledo, 1993a) exposed to endosulfan. Gill morphological changes are a common response to chemical pollutants (Mallat, 1985). However, despite gill injuries, there were only slight haematological and ionoregulatory disturbances in the adult fish. The red blood cell counts in control fish at the end of the present study ($2.36 \pm 0.1 \times 10^6$ cells/μL), were broadly similar to other values reported for adult zebrafish (e.g. Murtha et al., 2003, 3.02×10^6 cells/μL). Endosulfan exposure caused a transient reduction in red blood cell counts at day 14 which had completely recovered by

Table 3
Whole body concentration (μmol/g) of calcium (Ca^{2+}), sodium (Na^+), potassium (K^+) and magnesium (Mg^{2+}) of adult zebrafish (*Danio rerio*) exposed for 28 d to sublethal endosulfan concentrations (control, 0.16 or 0.48 μg/L). Values are expressed as mean ± SEM (n = 6).

Whole body [ion] (μmol/g)	Time (d)	Endosulfan (μg/L)		
		0 (control)	0.16	0.48
Ca^{2+}	0	304.7 ± 18.5		
	14	296.9 ± 29.7	295.7 ± 24.8	301.6 ± 20.2
	28	260.6 ± 8.8	275.0 ± 19.3	252.4 ± 19.2
Na^+	0	46.2 ± 2.5		
	14	45.8 ± 1.4	45.1 ± 2.1	46.0 ± 2.1
	28	41.5 ± 0.4[a]	44.0 ± 0.7[b]	42.9 ± 1.3[a,b]
K^+	0	65.3 ± 2.6		
	14	71.5 ± 1.2	69.9 ± 1.1	70.7 ± 1.2
	28	68.6 ± 0.9	71.0 ± 1.3	69.7 ± 0.9
Mg^{2+}	0	21.1 ± 0.9		
	14	22.0 ± 1.0	21.1 ± 1.0	21.6 ± 0.8
	28	20.6 ± 0.9	20.2 ± 0.9	20.5 ± 0.9

[a,b]Different superscripts indicate significant differences between treatments within each time point (Kruskal–Wallis, $p<0.05$).

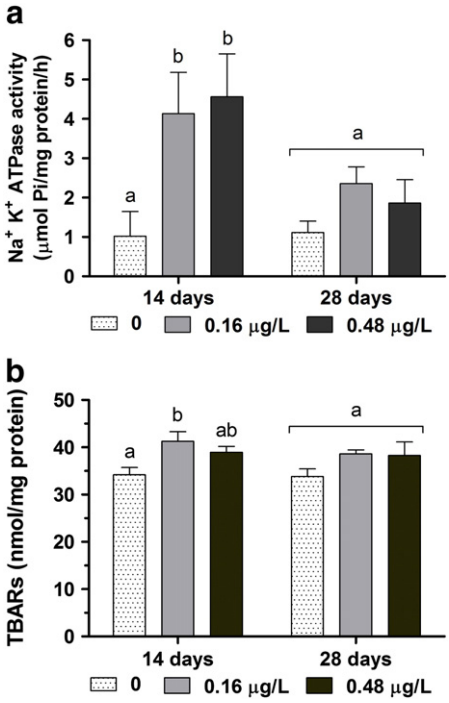

Fig. 4. (a) Whole body Na^+K^+–ATPase activity (µmol Pi/mg protein/h) and, (b) whole body thiobarbituric acid reactive substances (TBARS, nmol/mg protein) of adult zebrafish (*Danio rerio*) exposed for 28 days to sublethal endosulfan concentrations (control, 0.16 and 0.48 µg/L). Values are expressed as mean ± S.E.M (n = 9). [a,b]Within each exposure time (14 or 28 days), bars with different letters indicate significant differences (ANOVA, $p<0.05$).

the end of the experiment (Fig. 1) suggesting that the lowest endosulfan concentration used was not enough to cause a profound anaemia or compromise the oxygen carrying capacity of the blood. However, the decrease in red cell counts at day 14 is consistent with previous reports of sublethal exposure in carp fingerlings *Cyprinus carpio* (Chandrasekar and Jayabalan, 1993). The limited evidence for oxidative stress (TBARS, Fig. 4b) and osmoregulatory disturbance is also consistent with the limited haemolysis of red cells in this experiment. The normal, but increasing trend of white cell counts at the end of the experiment (Fig. 1b) also argues against major haematological disturbances. In our study, the gill injury was modest, and it appears that the lower endosulfan concentration used was not high enough to induce any compensatory haematological response.

In the present study, a transient four-fold or greater increase in Na^+K^+–ATPase activity in the endosulfan treatments compared to controls was observed at day 14, but recovered by day 28 (Fig. 4a). These transient changes in Na^+K^+–ATPase activity in the absence of major losses of ions, suggest the fish were able to maintain osmoregulation. This is a relatively well known phenomena in fish during aqueous exposure to chemicals (e.g. carbon nanotubes, Smith et al., 2007 or copper, Hoyle et al., 2007) and dietary exposure to endosulfan in *S. salar* where despite a reduction in ATPase activity at the beginning, a later recovery at the end of exposure was observed (Glover et al., 2007). The stimulation of Na^+K^+–ATPase with low concentrations of pesticides has also been reported (Duchnowicz et al., 2005). In *Channa gachua* an activation of Na^+K^+ and Mg^{2+} ATPase activity was observed after 15 days of exposure to 2.2 µg/L endosulfan (Sharma, 1988). On the other hand, an inhibition of Na^+K^+–ATPase activity after exposure to endosulfan has also been reported. In the above study, Sharma (1988) also found that higher endosulfan concentration (3.7 µg/L) induced a maximum inhibition in liver, kidney and muscle ATPase activity in *C. gachua* after exposure for 30 days. In *Channa punctatus* an inhibitation in gill Na^+K^+–ATPase activity was observed after oral exposure to 1.2 µg/L endosulfan for 90 days (Sarma et al., 2009).

Endosulfan is known to cause oxidative stress in the tissues of fish and mammals. For example, in *C. punctatus* a significant increase in lipid peroxidation was observed after exposure to 5 µg/L of endosulfan for 24 h (Pandey et al., 2001). Likewise, Hincal et al. (1995) reported an increase in the levels of TBARS and oxidized glutathione in the brain and liver of rats exposed to endosulfan. In this study, the TBARS measurements showed a small transient rise compared to the controls (Fig. 4b) suggesting that some oxidative stress could occur, but not enough to cause overt organ pathology or loss of osmoregulatory functions.

4.2. Reproductive effects of endosulfan in adult zebrafish

The presence of larval abnormalities in the offspring of zebrafish exposed to endosulfan (Fig. 6) may be related to subtle changes in gamete quality, despite no significant alteration in the fecundity, fertility or gonadosomatic index.

In rats, endosulfan affects spermatogenesis, decreasing sperm concentration and producing spermatozoa abnormalities (Choudhary and Joshi, 2003; Sinha et al., 1995, 1997); suggesting the potential for alterations in zebrafish sperm during endosulfan exposure. Cellular pathology in the ovary and testis of bluegill (*Lepomis macrochirus*) exposed to 1.0 µg/L of endosulfan for 96 h has been reported (Dutta and Dalal, 2008; Dutta et al., 2006). A decrease in plasma vitellogenin in walking catfish (*Clarias batrachus*) (Chakravorty et al., 1992) has also been reported. However, Park et al. (2004) suggest that the response in mosquitofish (*G. affinis*) females to endosulfan was related to the seasonal reproductive condition, with non-breeding females having the most deleterious alterations. Notably, Atlantic salmon exposed to up to 1 mg/kg dietary endosulfan for 16 weeks showed no effects on either vitellogenin, or sex steroid binding protein levels (Berntssen et al., 2010). Overall the weight of evidence is that low microgram levels of endosulfan appear to have limited effects on the ovary or testis.

No behavioural observations were carried out on reproductively active adults, so the possibility of reproductive behavioural alterations remains in this study. In swamp eels (*Monopterus albus*) exposed to endosulfan from 0.05 to 10 µg/L (Hii et al., 2007) and in rainbow trout exposed to 1.3 µg/L or higher concentrations of endosulfan (Capkin et al., 2006), a series of abnormal behavioural responses were observed (Capkin et al., 2006). The behavioural alterations at different degrees could also affect the basal metabolism as observed in other fish species (Velasco-Santamaría and Cruz-Casallas, 2008) and probably the reproductive behaviour necessary for mating.

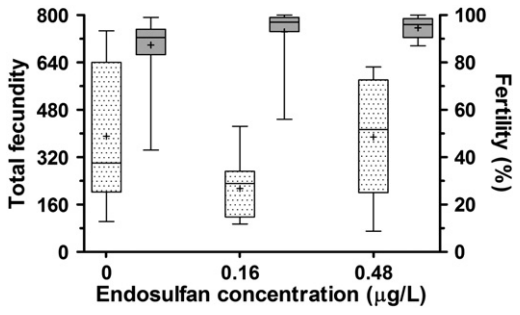

Fig. 5. Total fecundity (dotted bars) and fertility of eggs (gray bars) of zebrafish (*Danio rerio*) obtained after 15 days of finishing the exposure to sublethal endosulfan concentrations (0 (control), 0.16 and 0.48 µg/L). Values are expressed as median (centre line), mean (+), and the 25th and 75th percentile values. The whiskers show the minimum and maximum values (n = 9 beakers from each treatment). There were no significant differences (ANOVA, $p>0.05$).

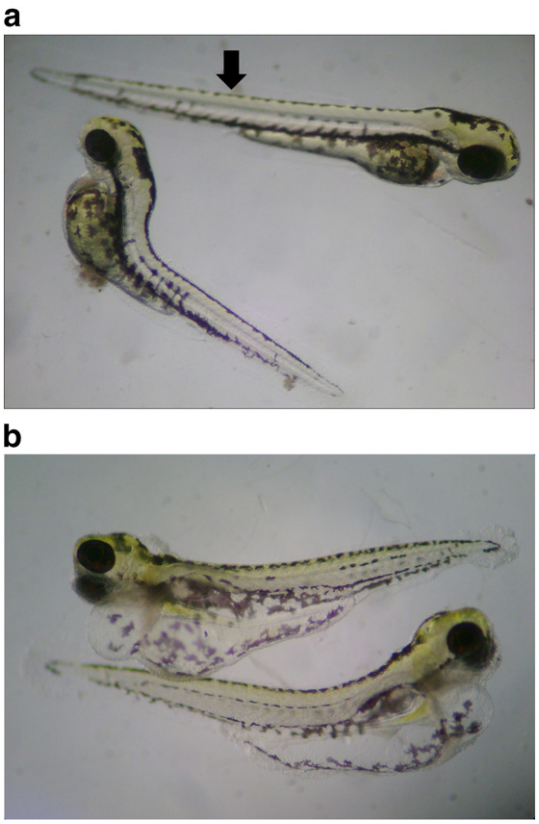

Fig. 6. Abnormalities of larvae of zebrafish (*Danio rerio*) obtained from eggs of adults exposed for 28 d to sublethal endosulfan concentrations (0.16 and 0.48 μg L^{-1}). A, The arrow points to a larva obtained from eggs of the control group. The second larva (lower side) corresponds to a larva from eggs obtained from fish exposed to 0.16 μg L^{-1} showing severe lordosis. B, Larvae from eggs of adults exposed to 0.48 μg L^{-1} of endosulfan showing severe ascitis. 10×.

4.3. Latent effects of endosulfan on offspring

The offspring were never exposed to endosulfan, and although there were no treatment effects in the morphometrics of larvae (yolk sac volume and notochord length), some offspring from exposed adults showed severe lordosis which could compromise the mechanical function of the heart; resulting in reduced heart rates of the larvae from exposed animals. Given, the absence of direct endosulfan exposure in the offspring, these effects can only be explained by gamete quality, or bioaccumulation of endosulfan in the gametes that is carried over to the resulting embryos. The latter is unlikely given the rapid clearance of endosulfan from tropical fish (Jonsson and Toledo, 1993b; Toledo and Jonsson, 1992).

There were no effects on fecundity and fertility in this study, but the quality of the cells in the gametes, or the cell-signalling during development may have altered to cause the observed lordosis. Evidence suggests that defective signalling from the primordial germ cells in the embryo leads to lordosis. Willey and Krone (2001) found that embryos exposed to endosulfan for more than 24 h showed changes in communication between primordial germ cells and the mesodermal signalling centre in the embryo; producing trunk and spine curvature as well as abnormal larval behaviour. The defective embryogenesis would also contribute to the observed defective heart function in this study. Clearly, fish embryos and larvae with lordosis and defective hearts are unlikely to survive, and population level effects may be expected. Prenatal exposure to 17α-ethinylestradiol and nonylphenol has been reported to cause a latent effect on the *F1* generation (Van den Belt et al., 2003; Yang et al., 2006), and this is to be expected for an oestrogen mimic, given the importance of oestrogen in cell proliferation during development. In our study, the mechanism of endosulfan on unexposed zebrafish offspring has yet to be elucidated but neurotoxicity remains possible, given the recent evidence that endosulfan compromises embryonic neurological development in the zebrafish embryo (Stanley et al., 2009).

In conclusion, adult zebrafish exposed to low concentrations of endosulfan show transient haematological and osmoregulatory disturbances, as well as evidence of oxidative stress. These biochemical disturbances were reflected in some subtle histological alterations in the liver and gill, but despite this sublethal toxicity, the adult fish were able to successfully produce gametes. However, the resulting unexposed offspring showed evidence of abnormal development that could affect the long-term survival of the animals. This study has identified a trans-generational hazard of endosulfan to fish, and these latent effects should be considered in future environmental risk assessments.

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