Endosulfan suppresses growth and reproduction in zebrafish

Alagusundaram Balasubramani* and Thavamani Jegajothivel Pandian

School of Biological Sciences, Madurai Kamaraj University, Madurai 625 021, India

To study the effects of endosulfan, a mild estrogen-mimic, on survival, growth and reproduction of the model zebrafish *Danio rerio*, the hatchlings were discretely immersed at selected doses (44–1400 ng/l) for 14 h. Immersion at the dose of 350 ng/l resulted in 12 and 27% mortality at the end of the treatment and at the age of sexual maturity respectively, as well as in the production of 72% females. The frequency of 'juveniles' increased from 17% at 44 ng/l to 44% at 1400 ng/l. Endosulfan acted as a growth suppressant and the magnitude of its suppression increased in the order of female > male > juveniles. During the 240-day experiment, it also postponed sexual maturity in females from the 120th dph (day post-hatching) in the control to the 181st dph in those treated at 1400 ng/l, reduced the spawning frequency (16–3 times) and cumulative fecundity (1424–159 eggs) by affecting the processes of maturation of eggs and vitellogenesis. In the treated but persisting males, it postponed sexual maturity (120–129th dph) and reduced fertilizability (91–13%) by reducing the motility duration of the sperm.

Keywords: Discrete immersion, disrupted reproduction, ferminization, growth suppression, juveniles.

AS a broad-spectrum cyclodiene pesticide affecting the central nervous system, endosulfan is widely used in the agriculture and horticultural fields of more than 70 countries[1]. Immediately following the application, endosulfan present in the Asian rice fields[2] is as high as 44–56 µg/l, although the approved limit for the presence of endosulfan in natural waters[3] is 0.02 µg/l. It is rapidly accumulated and bio-concentrated about 2700 times in freshwater green algae[4]. The detected levels of endosulfan in the Indian edible fishes range from 2 µg/g muscle (e.g. *Labeo rohita*, *Puntius* spp., *Wallago attu*)[5] to 77 µg/g muscle (e.g. *Catla catla*, *Channa striatus*)[6]. Hence a life-long study on the effects of endosulfan in a model fish like the zebrafish has become necessary. Numerous Indian publications report on the effects of endosulfan in edible fishes, but most of them are limited to haematological and biochemical parameters[7].

Naqvi and Vaishnavi[8] have indicated that persisting endosulfan affects a spectrum of physiological events, including the endocrine cascade leading to disruption of reproduction in fishes. Khillare and Wah[9] have shown that the sub-lethal chronic exposure of *Barbus stigma* to endosulfan inhibited spermatogenesis, and damaged spermatocytes and spermatids. Pandey[10] has reported that the endosulfan-treated females of *Colisa (Trichogaster) fasciatus* suffer from (i) retarded ovarian activity, (ii) thicker ovarian wall, (iii) reduction in oocyte size (stages II and III) and (iv) clumped yolk deposition in oocyte stage III. Obviously, not only males but also females suffer from endosulfan treatment. A computer search (www.google-scholar.com) has shown that there is no publication that describes the toxicological effects of endosulfan on reproduction of the experimental model zebrafish, *Danio rerio*[11]. This article reports on the effects of discrete immersion of hatchlings in mild doses of endosulfan, on the survival, growth and reproduction of the zebrafish.

Materials and methods

Endosulfan

A commercial-grade liquid endosulfan (6,7,8,9,10-hexachloro-1,5,5a,9, 9a-hexahydro-6,9-methano-2,4,3-benzodioxithepin-3-oxide; 35% EC) marketed by Jaipan, India[12], was used throughout the experiment; from a 1.75 µl/l stock solution, the desired nominal concentrations were prepared[13].

Experimental fish

Danio rerio Hamilton–Buchanan 1822, a cyprinid experimental model was chosen[11,14]. Adult individuals were purchased from a local fish farm in spring 2003 and reared in four large circular aquaria (150 dia. × 180 *H*, in cm) containing well-aerated water (5.5 mg O_2/l; 26 ± 1°C; 14L : 10D) and aquatic plants. From these healthy brooders, hatchlings were obtained for the experiments. The hatchlings depend on yolk; following the 2nd or 3rd dph (days post-hatching), they were fed on paramecium and boiled yolk granules of hen's egg, subsequently on *Artemia nauplii* for a week and on synthetic pellet food from the 20 to 25th dph. The feed ensured a supply of 30% protein. Adults (brood stock) were fed on *Chironomus* larvae and/or *Tubifex tubifex ad libitum*, twice a day.

*For correspondence. (e-mail: absmani@yahoo.com)

From prolonged observations, potential breeders were identified 12 h prior to spawning. The identified male and female were released into a breeding aquarium (10 l). Spawning occurred the next day early morning between 5 and 7 h; however, for experimental purpose the identified individuals were allowed to almost complete courtship behaviour until 5 a.m. and the gametes were stripped for the experiment.

Discrete immersion

As against prolonged or continuous exposure[15], the discrete immersion technique aims at the immersion of the steroid-sensitive embryonic or hatchling stage in minimum water containing the desired chemical[16]. As it is cost-effective, allows limited handling and reduces pollution, the discrete immersion technique was adopted[17]. From the stock solution, 1 l of water containing the environmentally relevant, nominal concentration of endosulfan was prepared on the day of treatment and a batch consisting of 40 fry was immersed. The nominal concentrations of endosulfan (44, 88, 175, 350, 700, 1050, 1400 ng/l), the duration of an individual immersion and the number of days in which the fry were immersed, were chosen on the basis of preliminary observations and the labile period, as known from earlier work[18]. The fry were immersed for a cumulative period of 14 h, i.e. 2 h each on the 18th, 19th, 20th, 22nd, 23rd, 24th and 25th dph. When the treatment on the selected days was completed, each experimental batch was transferred to separate rearing aquaria and the procedure was repeated. Each treatment was replicated five times. On completion of the entire sequence of immersions, the surviving fry in each batch were counted and released into a marked rearing aquarium ($45L \times 30B \times 15H$, in cm) and were allowed to grow with *ad libitum* feeding. Subsequently, monthly growth measurements were made; for this randomly selected individuals were anesthetized and kept in a glass tray, on the bottom of which a transparent graph (mm) sheet was pasted. The measurements were made keeping the tray under a stereomicroscope (10 X, Nikon, Japan).

Sex ratio

From the 90th dph onwards, a male was easily recognized by its slender body shape and a female from its relatively larger size and bigger belly. From the 120th dph onwards, stripping with the resultant milt or eggs readily confirmed the sex of an individual. However, when treated with endosulfan, a few progenies were also obtained, which were dull in colour, relatively lazy in activity and responded neither to a chasing male nor a female. They were separately grown until 240th dph and their identity as partially differentiated individuals was confirmed by stripping, which resulted in oozing out of fluid containing neither sperm cells nor eggs. Subsequently, randomly selected individuals were also subjected to anatomical examination for final confirmation of their status as juvenile. Incidentally, exposure of the zebrafish to either 17α-ethynyl estradiol (EE_2) or 17α-methyltestosterone (MT) resulted in the production of mature females, mature males, immature females, immature males, intersex (gonads occupied with oocytes and testicular tissues) and undefined individuals[19]. On exposure to endosulfan, the zebrafish produced a certain proportion of juveniles, characterized by the presence of partially differentiated testis or ovary; accordingly, they were designated as juvenile males and juvenile females.

Records were maintained for each experimental batch; for the age at which puberty (sexual maturity, as marked by the first spawning) was attained, and the frequency of spawning and milting as a function of age for a period of 240 days.

Motility

On a microscopic slide, 300 µl of tap water was added to 100 µl saline containing milt, and sperm motility duration was estimated using a stopwatch (Shinco, India). The duration of motility was taken in seconds, when about 50% of the sperm cells lacked motility[20].

Fecundity

On attaining sexual maturity, the sex-reversed or persisting female was paired with a normal male and the fertilized eggs were collected. For each experiment, the female was allowed to court three untreated males.

Fertilizability

An individual male was introduced to mate with the selected female and fertilizability (%) of the sperm was calculated by counting the number of dividing eggs as percentage of the total number of eggs laid.

Hatchability

The collected eggs were thoroughly rinsed in clean water before being transferred into a glass bowl (100 ml). The hatchability was calculated by counting the number hatchlings as percentage of the total number of eggs.

Histology

After sexual maturity, the five treated individuals from each series were anesthetized using clove oil in tap water (2 ml/l). From each of these individuals, one of the gonads was dissected out and the dissected gonad was directly

mounted in a tissue-freezing medium (Junk, Leica Instruments GmbH, Germany) at –17°C to obtain 6 μm thin sections in the freezing microtome (Minotome–Microtome Cryostat; IEC, Needham Heights, MA, USA).

These sections on the slide were fixed in methanol (100%) and kept overnight. Subsequently, the slide was rinsed with tap water followed by distilled water for 1 min and stained with haematoxylin and eosin, following the standard procedure described by the manual catalogue card number 74-33828 of the International Equipment Company, MA, USA. Then the sections were permanently mounted on the slide with DPX resin. They were scanned and photographed using a phase contrast microscope (Nikon Optiphot, Nikon Corporation, Tokyo, Japan).

Statistical analysis

Data analyses were based on their mean ± SD. The chi-square test (χ^2) was used to test the null hypothesis of no difference of male : female sex ratio between control and treated groups. Multiple comparison test was performed for survival of fry, inter-milting period, fertilizability, inter-spawning period, fecundity, and hatchability between control and treated series with Tukey test or Dunnet's method after one-way ANOVA. All the statistical analyses were performed using Sigmastat version 2.0.

Results

Survival

At the doses of endosulfan ranging from 44 to 1400 ng/l, the zebrafish fry suffered high (10–47%) mortality at the end of the treatment (Table 1). Notably, even the immersion at 350 ng/l, which ensured the maximum of 72% feminization, led to a mortality of 12% at the end of treatment and 27% at the age when sexual dimorphism was apparent. At any tested treatment dose, no deformed fry, live or dead, was found.

Preliminary experiments, in which the dose was increased above 1400 ng/l resulted in more than 50% mortality at the end of the treatment; hence no effort was made to achieve 100% feminization by increasing the dose of endosulfan.

Growth

On the day of hatching, the zebrafish body length was 3.3 mm in the control and in all the groups in the treated series. The early log phase of accelerated growth was sustained until the 90–120th dph in both the series (Table 2), but from the 120th dph the control female grew significantly faster than the male and attained greater body length of 37 mm on the 240th dph, while the control male attained a length of 32 mm only.

The observed differences in body length between the control and treated series were significant even on the 60th dph, especially in those treated at sub-optimal and super-optimal doses. In all the treated groups, both females and males began to suffer growth suppression prior to puberty on the 120th dph, and the suppression became increasingly significant, as the age advanced to 240th dph. Females treated at the sub-optimal dose suffered relatively stronger suppression than those at the super-optimal dose, but the reverse was true for the males. At the optimal dose of 350 ng/l, growth was also significantly suppressed, as the treated male and female attained a maximum growth of 24 and 28 mm respectively. Hence, even at the optimal dose, endosulfan acted as a catabolic steroid and significantly suppressed growth.

A proportion of the treated individuals remained as juvenile males and juvenile females even on the 150th dph; hence their growth was followed separately until the

Table 1. Effect of different doses of endosulfan on survival and sex reversal of the zebrafish *Danio rerio*, which were previously immersed for 14 h, i.e. 2 h each on the 18th, 19th, 20th, 22nd, 23th, 24th and 25th day after hatching. Each value represents the mean performance of five batches, each consisting of 40 fry

	Survival at the							
	End of treatment[I]		Sexual dimorphism[I]		Sex distribution (no.)			Sex ratio[II]
Dose (ng/l)	(No.)	(%)	(No.)	(%)	Male (♂)	Female (♀)	Juvenile (*J*)	♂ : ♀ : *J*
0	38 ± 1.9	95 ± 2.9[a]	34 ± 1.6	85 ± 3.9[c]	18 ± 0.8	16 ± 1.2	–	0.53 : 0.47 : 0.00
44	36 ± 2.2	90 ± 1.4[a]	30 ± 1.6	75 ± 3.9[d]	14 ± 2.3	11 ± 2.0	5 ± 3.2	0.47 : 0.36 : 0.17
88	36 ± 0.8	90 ± 2.1[b]	31 ± 2.4	78 ± 6.0[c]	15 ± 1.5	12 ± 0.9	4 ± 0.4	0.48 : 0.39 : 0.13
175	36 ± 0.8	90 ± 2.1[b]	30 ± 1.1	75 ± 2.9[d]	14 ± 1.0	11 ± 0.8	5 ± 1.5	0.47 : 0.37* : 0.16
350	35 ± 1.1	88 ± 2.1[b]	29 ± 2.3	73 ± 5.6[d]	4 ± 0.8	21 ± 3.3	4 ± 0.8	0.14 : 0.72* : 0.14
700	35 ± 0.8	88 ± 2.1[b]	26 ± 1.5	65 ± 3.8[d]	5 ± 0.8	15 ± 0.5	6 ± 1.1	0.19 : 0.58* : 0.23
1050	32 ± 1.1	80 ± 2.7[b]	19 ± 1.1	48 ± 2.9[d]	5 ± 0.5	6 ± 0.5	8 ± 1.5	0.26 : 0.32 : 0.42
1400	21 ± 0.8	53 ± 2.1[b]	9 ± 1.1	23 ± 2.9[d]	2 ± 0.4	3 ± 0.5	4 ± 0.8	0.22 : 0.33 : 0.44

[I]All values are mean ± SD; 'b' and 'd' are significantly ($P < 0.001$) different from the respective control 'a' and 'c'.
[II]Chi-square test with Yates correction; *indicates significant deviation ($P < 0.001$) from the expected 0.5 : 0.5 ratio of male to female alone.

Table 2. Effect of different doses of endosulfan on the body length (mm) of zebrafish, which were previously immersed for a cumulative period of 14 h, i.e. 2 h each on the 18th, 19th, 20th, 22th, 23th, 24th and 25th day after hatching. Each value represents the mean of growth performance of randomly selected ten fry each from five batches

	Body length (mm) on the						
Dose (ng/l)	1st day	60th day	90th day	120th day ♂	120th day ♀	240th day ♂	240th day ♀
0	3.3 ± 0.3	11.5 ± 0.2[a]	17.7 ± 0.15[c]	21 ± 0.5[e]	26 ± 1.1[g]	32 ± 1.4[i]	37 ± 3.0[k]
44	3.3 ± 0.3	8.0 ± 0.3[b]	13.2 ± 0.20[d]	18 ± 0.6[f]	21 ± 0.8[h]	25 ± 1.0[j]	27 ± 1.4[l]
88	3.3 ± 0.3	8.0 ± 0.5[b]	13.4 ± 0.11[d]	18 ± 0.5[f]	21 ± 1.2[h]	24 ± 1.2[j]	27 ± 1.2[l]
175	3.3 ± 0.3	8.8 ± 0.2[b]	13.1 ± 0.25[d]	19 ± 1.2[e]	21 ± 0.2[h]	24 ± 0.9[j]	27 ± 1.5[l]
350	3.3 ± 0.3	13.4 ± 0.8[b]	16.6 ± 0.78[c]	18 ± 0.7[f]	22 ± 0.6[h]	24 ± 1.0[j]	28 ± 1.8[l]
700	3.3 ± 0.3	11.2 ± 0.5[a]	13.5 ± 0.95[d]	18 ± 0.9[f]	22 ± 1.1[h]	24 ± 0.5[j]	27 ± 1.0[l]
1050	3.3 ± 0.3	9.0 ± 0.8[b]	13.1 ± 0.40[d]	17 ± 0.9[f]	22 ± 0.8[h]	23 ± 1.6[j]	26 ± 0.7[l]
1400	3.3 ± 0.3	8.0 ± 0.2[b]	13.2 ± 0.45[d]	17 ± 0.3[f]	23 ± 0.8[h]	22 ± 1.4[j]	25 ± 0.8[l]

All values are mean ± SD; 'b', 'd', 'f', 'h', 'j' and 'l' are significantly ($P < 0.001$) different from their respective control values, namely 'a', 'c', 'e', 'g', 'i' and 'k'.

240th dph. Briefly, at the optimal dose of 350 ng/l, the growth attained by the juvenile males and juvenile females was 21 and 22 mm respectively. One juvenile male measured 28 mm on the 700th dph, while the control matured male of the same batch grew to 35 mm. Hence, endosulfan suppressed the growth of these juveniles more intensively. The magnitude of growth suppression by endosulfan increased in the following order: female > male > juveniles.

Sex ratio

A few intersexes were present even at the lowest dose of 44 ng/l. With increasing endosulfan dose, the sex ratio was progressively biased towards female and juveniles (Table 1). Treatment at 350 ng/l resulted in the production of 72% females. However, upon doubling the dose to 700 ng/l, feminization was reduced to 58% and the 'juveniles' increased to 23%; redoubling of the dose to 1400 ng/l resulted in the production of 44% juveniles, 33% females and 22% persisting males. Being a mild estrogen mimic, endosulfan failed to induce 100% feminization even at the highest tested dose.

Female

Endosulfan disrupted the following reproductive events and products in a dose-dependent manner: (i) the age of the first spawning was postponed from the 120th dph in the control female to the 181st dph in those treated at 1400 ng/l (Table 3); (ii) the inter-spawning period was also prolonged from 7 to 24 days; (iii) fecundity was reduced from 89 eggs/spawning in the control to 53 eggs/spawning in those treated at 1400 ng/l and (iv) the number of F_1 hatchlings was reduced from 87 to 72% per spawn.

Table 3 also summarizes the cumulative effects of endosulfan on the frequency of spawning and fecundity of F_0 females and production of F_1 fry during the experimental period of 240 days. Briefly, the frequency of spawning was significantly reduced from 16 times in the control to three times in females treated at doses >700 ng/l; correspondingly, the fecundity was also significantly reduced from 1424 to 159 eggs. Likewise, the cumulative hatchability of F_1 fry of the treated F_0 females was significantly reduced to just 114, i.e. 9.2% of the control value.

Figure 1 shows the degenerative changes in the oocytes and related structures in the ovary of the treated series. The following changes were recognized: (i) progressive decrease in the number of mature vitellogenic oocytes; (ii) progressive increase in irregular shape of these oocytes; (iii) progressive increase in the number of previtellogenic oocytes, clearly indicating the reduced vitellogenic activity and (iv) appearance of large vacuoles and the degraded products, especially in the ovary of females treated at 350 ng/l (Figure 1 *b*). These degenerative changes resulted in the pronounced loss of oocytes due to loss of cellular contacts in the ovaries of females treated at 350 ng/l. Consequently, generation of a critical minimum number of, say 53–61, matured vitellogenic oocytes 'ready for spawning' required longer intervals between successive spawnings, when the zebrafish were exposed to higher doses of endosulfan.

Male

On the 120th dph, the control males attained sexual maturity and readily yielded milt when stripped. The age at which sexual maturity was attained by the males was progressively postponed to 129th dph, when the dose was increased to 1400 ng/l (Table 4). Further, the inter-milting period, which was just 4 days in the control, was also extended to 24–25 days in the treated series in a dose-dependent manner. Duration of sperm motility decreased drastically from 105 s in the control to about 79 s in the group exposed to 44 ng/l. Subsequently, it gradually decreased to 78 s in the batch exposed to 700 ng/l; it was

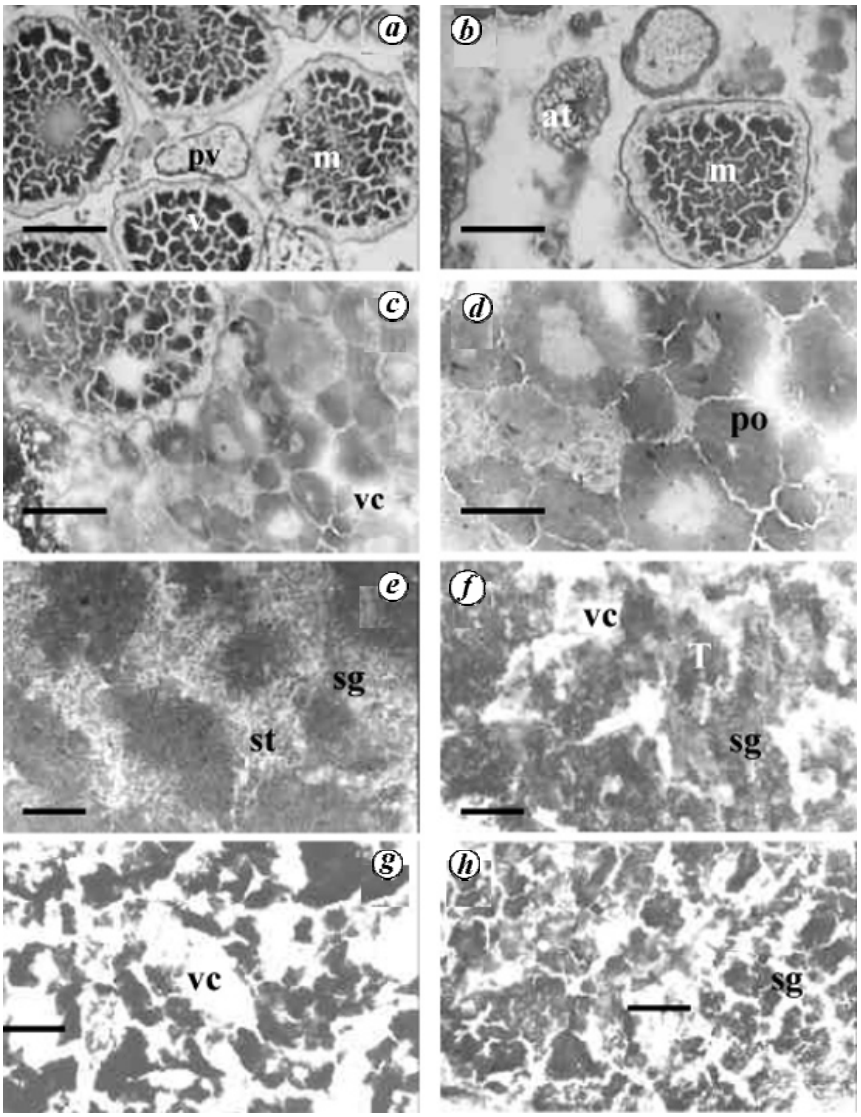

Figure 1. Effect of different doses of endosulfan on the gonad of zebrafish *Danio rerio*. ***a***–***d***, Ovary: ***a***, Control; ***b***, 350 ng/l; ***c***, 1050 ng/l and ***d***, 1400 ng/l. at, Atretic oocyte; m, Matured oocyte; po, Primary oocyte; pv, Pre-vitellogenic oocyte; v, Vitellogenic oocyte; vc, Vacuole. Scale: 300 μm. ***e***–***h***, Testis: ***e***, Control; ***f***, 44 ng/l; ***g***, 700 ng/l and ***h***, 1050 ng/l. sg, Spermatogonium; st, Spermatids; T, Seminiferous tubule and vc, Vacuole. Scale: 25 μm.

further reduced to 46 s in those treated at 1400 ng/l. Consequent to the decreases in sperm motility, the fertilizing ability of the sperm, when allowed to fertilize eggs spawned by a control female, drastically decreased from 91% in the control to 13% in those treated at 1400 ng/l.

Histology

A cause for the observed reduction in the count of sperm cells can be traced to the degenerative changes observed from the sections of testis of males, which were previously exposed to different doses of endosulfan. Among these, most apparent were the following: (i) reduction (Figure 1 *e*) or almost virtual disappearance of the spermatogonia (Figure 1 *h*); (ii) enlargement of the seminiferous tubules (Figure 1 *g*); (iii) decrease in spermatid density and (iv) loss of supporting Sertoli cells.

Juveniles

In all the tested batches, a higher proportion of juveniles was observed after the 150th dph. The testis of the control zebrafish is slender in shape, length about 5 mm and breadth 1.5 mm size, and milky white in colour (Figure 2). In many partially differentiated juvenile males, one arm of the gonads, and in a few others, a part of the arm was thin and shrunken. When squeezed, the oozing fluid contained no recognizable motile sperm.

Table 3. Effect of different doses of endosulfan on spawning and fecundity of the treated F_0 female zebrafish. These treated females were crossed with normal males. Each value represents the mean of five estimates

Dose (ng/l)	Age at first spawning (dph)	Inter-spawning period (day)	Fecundity (no./spawning)	Hatchlings (%)	Spawning frequency in 240 days (no.)	Cumulative fecundity (no.)	Cumulative hatchlings (no.)
0	120 ± 2.3^{a}	7 ± 0.5^{c}	89 ± 5.6^{e}	87 ± 2.3^{g}	16 ± 0.8^{i}	1424 ± 90^{k}	1239 ± 78^{m}
44	144 ± 4.2^{b}	16 ± 2.6^{d}	74 ± 4.8^{f}	77 ± 3.7^{h}	6 ± 1.3^{j}	444 ± 30^{l}	342 ± 23^{n}
88	150 ± 3.2^{b}	16 ± 9.3^{d}	79 ± 5.0^{f}	78 ± 3.0^{h}	5 ± 1.0^{j}	395 ± 15^{l}	308 ± 12^{n}
175	153 ± 2.3^{b}	19 ± 10.7^{d}	69 ± 4.9^{f}	74 ± 3.2^{h}	5 ± 0.5^{j}	345 ± 29^{l}	255 ± 22^{n}
350	152 ± 3.0^{b}	17 ± 9.0^{d}	74 ± 4.6^{f}	76 ± 2.4^{h}	5 ± 0.5^{j}	370 ± 17^{l}	281 ± 13^{n}
700	156 ± 1.7^{b}	19 ± 10.7^{d}	63 ± 4.5^{f}	75 ± 0.8^{h}	5 ± 0.5^{j}	315 ± 29^{l}	236 ± 22^{n}
1050	159 ± 3.9^{b}	23 ± 15.9^{d}	61 ± 7.9^{f}	75 ± 0.8^{h}	4 ± 0.5^{j}	244 ± 43^{l}	183 ± 32^{n}
1400	181 ± 5.8^{b}	24 ± 17.7^{d}	53 ± 7.2^{f}	72 ± 3.2^{h}	3 ± 0.5^{j}	159 ± 29^{l}	114 ± 21^{n}

All values are mean ± SD; 'b', 'd', 'f', 'h', 'j', 'l' and 'n' are significantly ($P < 0.001$) different from their respective control values, namely 'a', 'c', 'e', 'g', 'i' , 'k' and 'm'.

Table 4. Effect of different doses of endosulfan on the age of maturity, inter-milting period and fertilizability of sperm drawn from the treated and persisting male zebrafish, which were immersed for 14 h, i.e. 2 h each on the 18th, 19th, 20th, 22nd, 23rd, 24th and 25th day after hatching. These treated persisting males were crossed with normal females. Each value represents the mean of five estimates

Dose (ng/l)	Age at sexual maturity (day)	Inter-milting period (day)	Motility duration (s)	Fertilizability (%)
Control	120 ± 2.9^{a}	4 ± 0.4^{c}	105.0 ± 7.9^{e}	91.0 ± 2.3^{g}
44	120 ± 1.7^{a}	12 ± 1.7^{d}	79.2 ± 5.6^{f}	39.8 ± 4.8^{h}
88	121 ± 2.6^{a}	12 ± 1.6^{d}	81.4 ± 4.9^{f}	44.0 ± 3.2^{h}
175	124 ± 1.1^{a}	12 ± 1.8^{d}	78.8 ± 4.2^{f}	35.2 ± 1.9^{h}
350	124 ± 1.5^{a}	12 ± 1.5^{d}	83.4 ± 11.4^{f}	28.0 ± 5.8^{h}
700	126 ± 2.4^{b}	16 ± 1.5^{d}	77.8 ± 2.3^{f}	27.6 ± 6.0^{h}
1050	128 ± 2.4^{b}	25 ± 2.2^{d}	56.0 ± 9.3^{f}	15.4 ± 3.3^{h}
1400	129 ± 2.3^{b}	24 ± 2.3^{d}	45.8 ± 3.8^{f}	13.2 ± 2.2^{h}

All values are mean ± SD; 'b', 'd', 'f' and 'h' are significantly ($P < 0.001$) different from their respective control values, namely 'a', 'c', 'e', and 'g'.

The ovary of a normal female is paired and pale yellow in colour; each lobe is globular in shape, length about 9 mm and breadth 3.5–4 mm, and contains grape-like bulging mature oocytes (Figure 2). The gonads of the partially differentiated juvenile females were relatively long, slender sacs with no signs of bulging mature oocytes. In fact, a part or entire arm of one ovary appeared like a tube; these gonads consisted of mostly oocytes in atretic stage.

Discussion

Endosulfan readily adheres to particles and its mean duration of persistence is 1½ years in waters and 2 years in sediments and soils[21]. From a life-long study of the zebrafish, this article reports on the effects of discrete immersion of the hatchlings in a not readily biodegradable endosulfan, on survival, growth and reproduction. A brief spell of 14 h immersion at 350 ng/l led to 12 and 27% mortality at the end of the treatment and at the age of sexual maturity respectively. A computer search (googlescholar: zebrafish + endosulfan) indicated the non-availability of publications concerning the effect of endosulfan on survival, growth and reproduction of zebrafish (see also Spitzbergen and Kent[22]). Hence, it was chosen to compare our values with those reported for the zebrafish fed on the not readily biodegradable polychlorinated biphenyls (PCBs)-supplemented feed containing 8 μg/g diet[23]. However, the mortality values reported for zebrafish following discrete immersion in norethindrone acetate, a relatively more readily biodegradable endocrine disrupting chemical (EDC), at 1000 μg/l were 3% only even on the day of sexual maturity (pers. obs.). Therefore, the rate at which endosulfan and its residues are bio-accumulated in the muscles and liver, may serve as an important factor to decide the level of mortality. For instance, the catfish *Tandanus tandanus* tolerated accumulation of endosulfan up to 8.2 ng/g in the liver and suffered no mortality on 24 h continuous exposure to sub-lethal concentrations of endosulfan[24].

The accumulation of an EDC in different organs may be a cause for the observed higher mortality reported for fishes treated with readily and not readily bio-degradable EDCs[25]. As the muscles contribute the largest proportion of the body of a fish, a major fraction of the accumulated EDC in the muscle alters one or more of the physiological processes. For instance, the motility and hence foraging behaviour of the short-term endosulfan-exposed zebrafish were significantly affected[26]. Consequently, the processes

of feeding and absorption are also affected. For instance, the intestinal absorption of glucose by the endosulfan-treated freshwater murrel, *Channa punctatus* is reduced[27]. The reported values for accumulation of endosulfan in the muscle tissues range from 2 to 77 μg/g for the edible fishes collected from the markets of Gujarat[6] and Calcutta[7] city. Therefore, one route through which EDCs like endosulfan suppress the growth appears to be: accumulation of EDC in the muscle → reduced swimming speed → impaired foraging efficiency → lowered food intake → slowed digestion and absorption → stunted growth. Hence it is likely that the accumulated endosulfan in the zebrafish muscle may be a root cause for the observed stunted growth. However, it is not yet known how an EDC like endosulfan, a mild estrogen-mimic[28], disrupts the cascade of endocrine events of somatic growth[29]. Another route through which endosulfan-treated fishes suffer stunted growth, may be through its effect on the central nervous system of the treated fish during the critical stages of life[30].

In this study, a partially differentiated juvenile is distinguished as different from intersex[19]. When treated with endosulfan, the zebrafish generated more juveniles at higher doses (Table 1); the highest value obtained was 44%. The exposure of zebrafish to EE_2 resulted in the production of 60–90% immature females, i.e. partially differentiated juvenile females[19]. In the absence of relevant information, a comparison of the present data is made with those reported for other fishes previously exposed EDCs. Teather *et al.*[31] exposed medaka to 0.06 μg endosulfan/l from fertilization to 7 dph and reported the presence of almost 10% intersex. Equally high values (>10%) for the production of intersex have been reported for zebrafish treated with MT. In fact, the incidence of 100% intersex in roach *Rutilus rutilus*, has been reported from few English rivers[32]. It is also known that exposure to EDCs like MT and EE_2 generated intersexes in zebrafish. These were characterized by abnormal gonads, which were different from individual to individual[33]. Comparable abnormal gonads were also obtained in the zebrafish exposed to endosulfan. The present study shows that these intersexes also suffered intense growth suppression than the normal males and females.

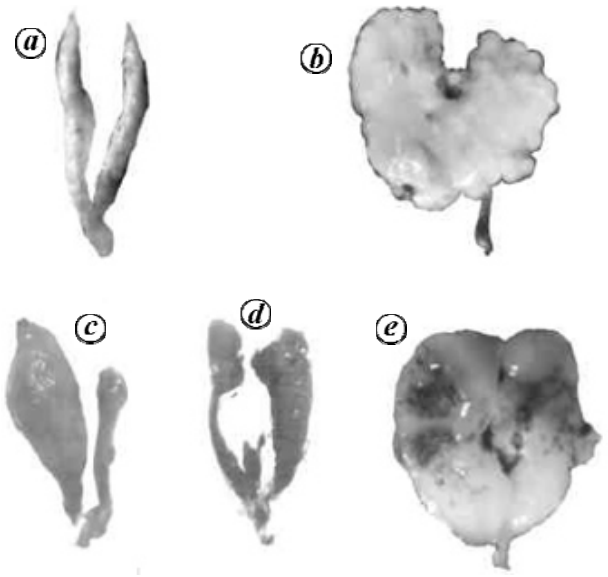

Figure 2. Matured testis (***a***) and ovary (***b***) representative partially differentiated testis (***c*** and ***d***) and ovary (***e***) of juvenile gonads of the zebra fish, which were previously treated with endosulfan.

Our observations that the depuration for a period of over 200 days did not eliminate the negative effects of the brief exposure to endosulfan for a period of 14 h during the early post-hatching stage of the zebrafish, confirm the similar findings of Gormley and Teather[26], who have exposed the Japanese medaka (*Oryzias latipes*) hatchlings to endosulfan at 0.01–10.0 μg/l for 4 and 6 h. Andersen *et al.*[33] have summarized the available information on the dose-dependent reduction in egg production by zebrafish treated with EDCs like lindane, toxaphene, PCBs, EE_2 or MT. Njiwa *et al.*[34] have shown that the fertilizability of zebrafish eggs is reduced, as the DDT-treated males were unable to disperse the sperm. Our findings on the reduced fecundity (i.e. fertilized eggs) of zebrafish that were exposed to endosulfan as fry, are based on observations for periods longer than 60–100 days after the occurrence of the first spawning. Our values are also comparable to those reported by Nash *et al.*[15], who have also made life-long observations on zebrafish treated with EE_2.

From a life-long study, Diekmann *et al.*[35] reported that zebrafish continuously exposed to a model genotoxicant nitroquinoline-1-oxide (NQO) at 2.9 μg/l from fertilization through sexual maturity produced about 40% eggs compared to that of the control. Using mathematical computation, Diekmann *et al*. predicted that 50% females of the zebrafish will become extinct after 3 years, when exposed to 2.9 μg NQO/l, as their fecundity was significantly reduced. In the present study, zebrafish subjected to discrete immersion for a cumulative period of 14 h at optimal dose of 350 ng endosulfan/l, produced 26% eggs compared to that of the respective control zebrafish. Hence, even with a short period of exposure to an EDC like endosulfan, the zebrafish is likely to become extinct earlier than 3 years. Ensenbach and Nagel[13] also predicted the imminent extinction of zebrafish, which after life-long exposure to the other commonly used pesticides 3,4-dichloroaniline or lindane at a dose of 200 or 40 μg/l respectively, completely ceased to produce eggs. Nash *et al.*[15], who also subjected the zebrafish to life-long exposure of 5 ng EE_2/l, reported that the zebrafish produced eggs, but none was viable. These reports and predictions emphasize the need to contain the unlimited use of pesticides in developing countries.

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