Alteration in Sperm Release from Zebrafish (*Brachydanio Rerio*) Exposed to DDT

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This study describes a long-term toxicity test within a period of 2 months using zebrafish (*Brachydanio rerio*) as the test species and concentrations of 0.05, 0.5, 5 and 50 μg/l DDT as a model substance. By collecting and counting the number of sperm released during separate mating events we observed that gametes are released asynchronously. Sperm is released in the form of sperm trails laid on the nest surface; subsequently active spermatozoa leave the trails and move in the water for several minutes. Sperm trails consists of bands of viscous material in which sperm is embedded. The water samples for the estimation of sperm presence were collected gradually within 180 min after 24 hr, 2 weeks, 1 month and 2 months of exposure. It was established that the reduction in count, activity of sperm and the average life span of sperm trails were significant ($p < 0.05$) at the tested concentrations 5 μg/l and 50 μg/l DDT after 1 month of exposure. In conclusion, this study demonstrates that long exposure time and higher DDT tested levels accelerate the occurrence of negative effect on the number and activity of sperm released as well as the life span of their trails.

Key words —— DDT, Zebrafish, Sperm, Fertilization, Insemination, Reproductive behaviour

INTRODUCTION

The modes of teleost fish mating are very diverse and include both internal and external insemination., the latter being the most common. Two important shortcomings of external insemination are that the medium is hostile to the survival of gametes, particularly in fresh water where gametes are subject to considerable osmotic shock, and that gametes tend to get diluted beyond the level needed for successful fertilization. As possible way of reducing the disadvantages of these two factors, in most teleost fish with external fertilization, the male and female are in close proximity during mating and release of the sexual products is synchronous.[1)] The simultaneous release of spermatozoa and ova is in part necessitated by the short life of the gametes once they are in contact with the water.[2)] Sperm motility is limited in freshwater fish with external insemination. The majority of teleost fish have acccessory sperm duct glands, usually called seminal vesicles.[3,4)] In a limited number of species these structures are reported to store sperm or to produce androgene-derivates, which serve as pheromones during reproduction,[5)] while in all species they secrete a viscous sialoglycoprotein-rich fluid which mixes with sperm on ejection.[5–7)] As for the role of these secretions, various hypotheses have been suggested, including the attachment of sperm to the egg envelope to facilitate the contact of sperm with eggs,[5,8)] the improvement of the viability of the spermatozoa,[9)] or the protection of the eggs from parasites.[5)]

In three marine teleosts with broadcast spawning and pelagic eggs[10)] it was shown that gametes are released synchronously and disperse in the water, and that the male controls the number of sperm released in each mating. The amount of sperm released is a function of several factors including the size of the female and the number of male daily matings.

There has been a number of reports on the relationship between sublethal treatments with chlorinated hydrocarbon insecticides and behavioural changes in fish. DDT for example is still widely used in tropical countries for pest and malaria control and approximately half of the world production of polychlorinated biphenyls has not yet escaped to the environment from older electrical and other equipment. Thought it is now banned from use

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domestically, Schettler[11] reported that one ton of DDT per day passed through U.S. ports in 1996. Organochlorine distribution in the aquatic environment can be understood by considering the distinctive physiochemical properties of low water but high lipid solubility and relative inertness of these compounds. The literature on organochlorine compounds in the marine environment is very abundant.[12–16] Studies in birds and mammals with chlorinated hydrocarbons of DDT class demonstrated that these compounds exhibit effects which could be interpreted as causing alterations of the hormonal state of the animal.

Fish represent important organisms if the effects of xenobiotics on the fauna of the hydrosphere have to be evaluated in toxicity tests.

Several authors found influences of xenobiotics on the endocrine system of fish.[17,18] Thomas[19,20] investigated the effects of heavy metals, benzo(a)pyrene (BaP) and a mixture of polychlorinated biphenyl (Arochlor 1254) on endocrine functions of fish (*Micropogonias undulatus*), which influenced reproduction. During the study of long-term toxicity test comprising reproduction and growth of zebrafish with a xenobiotic, Bresh *et al*.[21] stated that the test procedure is not very informative as far as the effect of a substance on spermatogenesis is concerned. Reduced fertilization rates in exposed group as compared to controls may have different causes: direct effect of the substance on spermatogenesis, effect on the released sperm only or on the eggs by change of the egg membrane to the effect that penetration of intact spermatozoa is hindered or prevented. Finally more recently, Ensenbach and Nagel[22] reported that one of the most important effects of xenobiotics on fish is the impairment of reproduction. According to all these reports, why not seeking to establish whether DDT for *e.g*. is involved in falling sperm count in male fish . For this, low DDT concentrations were used according to the level of their accumulated residues in the marine system.The higher tested concentrations were found to be tolerated by the fish for a time sufficient to carry out our investigations within the experimental period. The factor 10 separating the tested concentrations is recommended by the Organization for Economic Cooperation and Development (OECD) guidelines for testing of chemicals.

Because zebrafish produce gametes throughout the year and is very easy to rear, it is assumed to be a suitable model for the sperm count test. The present study aims to assess (a) the number of sperm released during mating events by the male zebrafish, (b) the activity of sperm and life span of the sperm trails released by DDT, treated male zebrafish.

MATERIALS AND METHODS

Test Species —— The experiments were performed with zebrafish (*Brachydanio rerio*, Hamilton Buchanon) supplied by a zoological store in Saarbrücken, Federal Republic of Germany (FRG). Zebrafish thrives in both soft and hard waters, grows quickly at temperatures around 26°C and reaches the sexual maturity within 3 months. This species produces gametes throughout the year and is not difficult to rear under laboratory conditions.[23,24]

Exposure of Male Zebrafish to DDT —— The fish aged between 4–6 months were maintained sexes separate in large aquaria and fed with Tetramin grow-out feed (AZ)25 ad libitum.

Male fish were esposed under flow-through conditions to DDT at different concentrations ranging between 0.05 μg/l and 50 μg/l and separated by a factor 10. An untreated group was kept under the same conditions as control. The flow-through test system consisted of a water tank in which charcoal filtered tap water was aerated and temperature was kept constant at 26°C (± 1°C). Tap water was pumped into the mixing chambers. The toxicant solution was added by a micropipette and a photoperiod of 12 hr was maintained.

Test Procedure and Sperm Count —— After 24 hr, 2 weeks, 1 month and 2 months of exposure to DDT, two males of each group were transferred into glass vessels (20 × 15 × 30 cm, H × W × L, 8 l test solution) and allowed to acclimatize for 3 days (Fig. 1). On day 4, before the introduction of a ripe female 80 ml water samples were removed from the glass vessels. Each aquarium was provided with an artificial nest site consisting on Polyvinylchloride (PVC) pipes builded according to the size of fish. A length of 40 cm tubing was placed under the nest and was held in position close to the bottom. The tubing allowed at any time the collection of water samples without any disturbance of the fish.Additional 80 ml samples were removed from the surface of the water with a beaker. 20 min, 40 min, 60 min, 120 min and 180 min gradually after the introduction of a ripe female (weight = 0.450 g ± 0.2 g), all the water samples collected either at the surface or via the tube under the nest , were analysed for the presence of sperm using the methods of leong[25] and Shapiro[10]

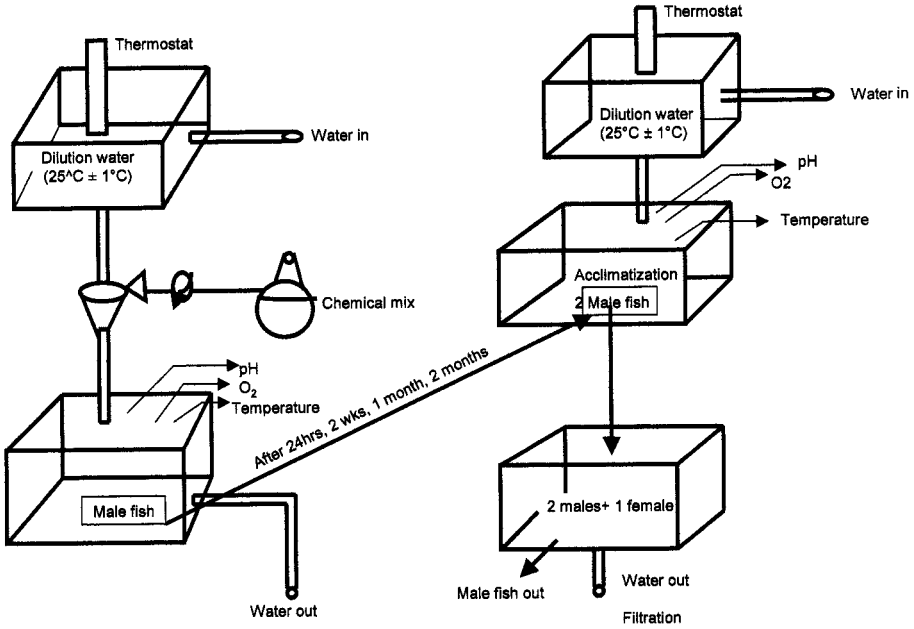

Fig. 1. Flow-through Aquarium Showing the Exposure of Male Zebrafish before the Mating Events

described as follows:

Soon after removal, 6 drops of Rose Bengal were added to the samples for staining the spermatozoa. After 20 min, 16 ml of formaldehyde was added to the sample for preservation, after which the sample was passed through a millipore filter (0.22 μm pore size) under vacuum. The filter paper containing sperm was dried on slide warmer, placed on glass slide, and cleared with immersion oil. The number of sperm was counted under a light microscope at a magnification of 400 $\times$ in an area measuring 0.3×0.3 mm (= 0.09 mm^2). The count was repeated on each 20 randomly selected portions of the filter. The mean value of these readings was used to backcalculate the estimated total number of sperm present in the sample. After have introduced a ripe female holding a male conspecific, male activity was monitored and water samples collected at different time intervals before egg deposition. The first water sample was removed just before the introduction of the female, and served as control, the second was removed as soon as the the female entered the nest, then additional samples were collected gradually within the time interval previously mentioned.

To investigate the sperm which was attached to the nest surface within the sperm trail, the nest ceiling was lined with a transparent acetate sheet which could easily be removed at any time during mating with minimum disturbance to the fish. Following its removal, the sheet was gently rinsed with water, placed in a beaker filled with water and left undisturbed for about 10 min. Subsequently a few drops of Rose Bengal were added to the water which was then processed as described above for no other samples. The sample was then inspected with a dissection microscope for the presence and distribution of sperm.

To estimate the duration of sperm activity, males were anesthetized and after gentle abdominal pressure, sperm samples were collected with a pipette directly from the genital papilla. The sperm were then thoroughly mixed with the water at 21°C, a drop of sperm solution was placed under the microscope and sperm movement monitored until most of the sperm stopped moving.

We found that the artificial sperm trails could be easily obtained by gently sqeezing a male's abdomen while passing his genital papilla over a slide. In this way we could observe the behaviour of sperm laid in a trail. Sperm trails consist of bands of viscous material in which sperm is embedded. Sperm at trail periphery were the first to move and leave the trail. This process continued slowly, up to the complete disappearance of the trail and thereafter the average life span of the sperm trail was determined.

The results are reported according to the exposure time and levels of DDT tested concentrations. Rank test of Mann and Whitney was used to compare the number and the activity of sperm .

Table 1. DDT Concentrations (μg/l) During the Exposure of Male Zebrafish

	DDT	DDT	DDT	DDT
Nominal values	0.050	0.500	5.000	50.000
Real values				
x	0.039	0.425	4.020	43.100
s	0.009	0.115	1.165	14.362

x = mean value; s = standard deviation.

Chemical and Chemical Analysis —— DDT (Chemical Abstract n° : 50-29-3) was supplied by Promochem Gesellschaft mit beschränkter Haftung (GmbH) Wesel, FRG.

The extraction of DDT from water samples was performed using a column type CHROMABOND® C18/6 ml/500 mg described as follows:

The column was conditioned with 2 column volumes ethyl acetate, then 1 column volume methanol, and finally 1 column volume distilled water. The samples were then slowly pressed or sucked through the column. Thereafter, the column was washed with distilled water and dried for 15 min thoroughly under vacuum.

2×500 ml of ethyl acetate were used for the elution. The analysis was performed weekly by gas chromatography using electron capture detector. The results of chemical analysis are presented on Table 1. Dissolved oxygen exceeded 80% of the saturation value, pH ranged from 7.8 to 8.2 and total hardness was 20–24°dH.

RESULTS

Behaviour of Male Fish During Mating Events

The male fish usually reacted to the introduction of a ripe female by courting and leading her to the nest. Early in the courtship sequence, the male turned upside down and stroked or rubbed his anal-urogenital region over the nest surface, repeating this behaviour several times even before the female actually entered the nest. It was observed that during these movements the male kept his genital papilla in contact with the surface of the nest. But after 1 month of exposure to the tested concentrations 5 μg/l and 50 μg/l, high rates of mortality were recorded. The behaviour of male fish changed. Normally healthy male fish react very quickly if a female is introduced into the aquarium, but in these cases, male fish were very lethargic. They did not swim often to the surface and ate mostly when food reached the bottom of the basin. No egg laying by the female was recorded at that time.

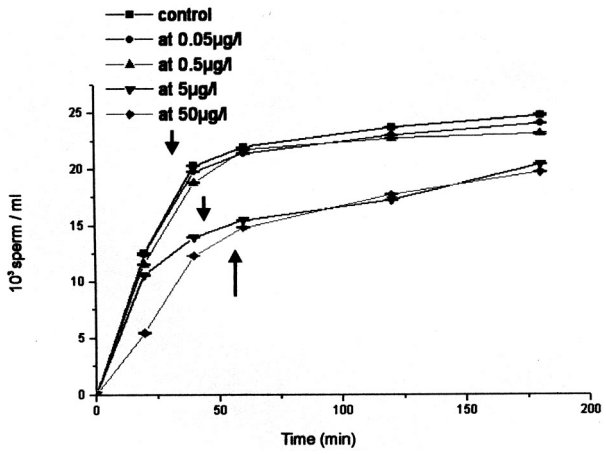

Fig. 2. Mean Number (± S.E.) of Sperm Count Found in Samples of Water, $n = 10$, at the Different DDT Tested Levels After 24 hr of Exposure

The female was introduced at time 0 and a first sample was immediately removed and checked for sperm presence. Arrows indicate the start of egg laying by the female. S.E.: Standard Error.

Sperm Count

In all the control samples collected before the moment that the female was introduced, sperm was not found. In contrast in all trials, sperm was always found in the water after the introduction of the female in the aquarium even before oviposition took place. Sperm numbers with values very close to those observed in samples collected under the nest were also recorded on samples collected from the surface of the aquarium tank. In Fig. 2, sperm concentrations in water samples taken during single mating events are reported for each DDT tested concentration after 24 hr of exposure. Sperm counts tended to increase with time after the introduction of the female, and were much higher in control than in the other treated basins. The values of sperm counted in the test samples ranged from 5.45×10^3 sperm/ml to 19.70×10^3 sperm/ml in 50 μg/l and from 12.51×10^3 sperm/ml to 24.74×10^3 sperm/ml in control within 180 min. The rank test of Mann and Whitney established no significant difference between the samples collected in each treated basin and those of control ($p > 0.05$).

In Fig. 3 sperm concentrations in water samples taken during single mating events for each tested concentration are illustrated but this time after 2 weeks of exposure to the different DDT tested concentrations. The reduction in sperm concentrations

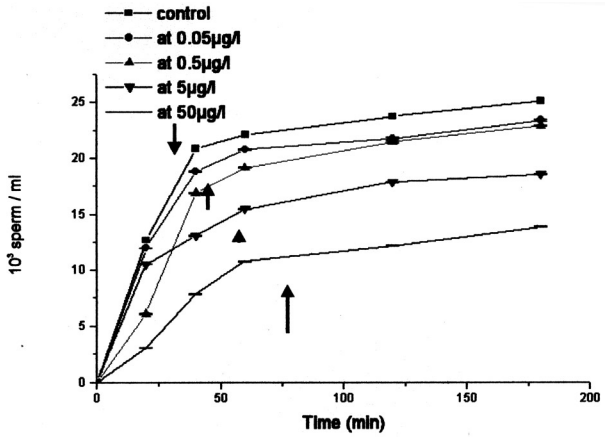

Fig. 3. Mean number (± S.E.) of Sperm Found in Samples of Water, $n = 10$, at the Different DDT Tested Concentrations After 2 weeks of Exposure

The female was introduced at time 0 and a first sample was immediately removed and checked for sperm presence. Arrows indicate the start of egg laying by the female. S.E.: Standard Error.

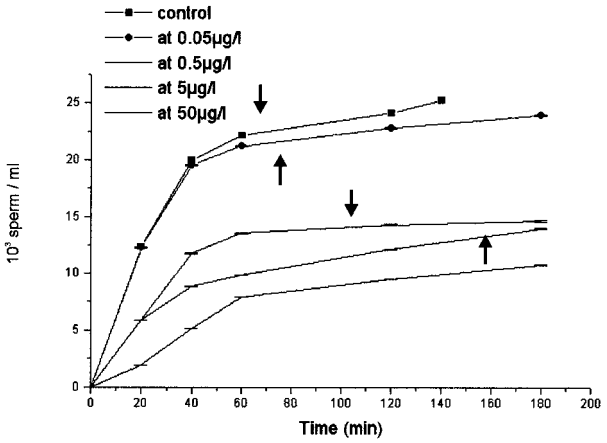

Fig. 4. Mean Number (± S.E.) of Sperm in Water Samples, $n = 10$, at the Different DDT Tested Levels After 1 month of Exposure

The female was introduced at time 0 and a first sample was immediately removed and checked for sperm presence. Arrows indicate the start of egg laying by the female. S.E.: Standard Error.

were still in treated basins not significant when compared to those of control.

Results reported on Fig. 4 and Fig. 5 show the sperm concentrations at different DDT tested levels after 1and 2 months of exposure respectively. The statistical analyses of these results established a significant reduction in sperm concentrations ($p < 0.05$) at the tested concentrations 5 μg/l and 50 μg/l DDT with the values of sperm counted ranging after 2 months of exposure from 3.70×10^3 sperm/ml to 7.72×10^3 sperm/ml and from 1.26×10^3 sperm/ml to 3.74×10^3 sperm/ml respectively. This simply demonstrated that the reduction in sperm release increased with exposure time and the level of DDT.

The sperm counts were found also to increase with time after the introduction of the female. These sperm counts were higher at the lowest tested concentration, while at the highest tested concentration, the increase stopped almost 120 min after the beginning of the mating event.

On the other hand, sperm was always found in the water in which the acetate sheet, removed from the nest at the onset of spawning, had been held for 20 min, suggesting that sperm was attached to the acetate sheet. This was clearly demonstrated for *Brachydanio rerio* in which the presence of sperm, laid in small and thin trails easily visible to the naked eyes, was verified once they were stained. The sperm trails consisted of a flat band of viscous material in which sperm were embedded. Most trails were straight or slightly curved, averaged 7.6 ± 4.4 mm in length (range = 1–25 mm; $n = 41$)

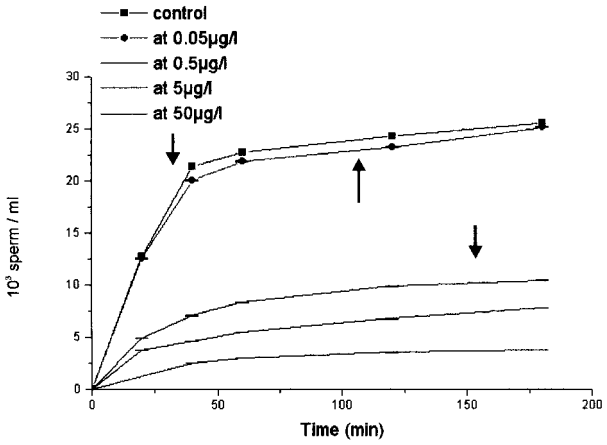

Fig. 5. Mean Number (± S.E.) of Sperm in Water Samples, $n = 10$, at the Different DDT Tested Concentrations After 2 months of Exposure

The female was introduced at time 0 and a first sample was immediately removed and checked for sperm presence. Arrows indicate the start of egg laying by the female. S.E.: Standard Error. No egg laying by the female within 180 min at 5 μg/l and 50 μg/l DDT.

and were 1–2 mm wide. But after 1 month of exposure to the tested concentrations 5 μg/l and 50 μg/l DDT, the sperm trails averaged 4.5 ± 3.36 mm in length (range = 1–18 mm; $n = 37$) and were 1–1.5 mm wide. The sperm trails were found distributed over the whole nest surface.

Activity of Sperm and Life Span of Sperm Trails

We found that in artificial sperm trails, sperm at the trail periphery were the first to move and leave the trail. This process continued slowly until the

Table 2. Activity in Minutes of Sperm Released During Mating Events at All the DDT Tested Concentrations after 24 hr, 2 weeks, 1 month and 2 months of Exposure

	24 hr	s	2 weeks	s	1 month	s	2 months	s
Control	28.7	4.6	29.8	4.6	29.7	4.3	30.3	4.3
0.05 μg/l	28.4	4.6	28.7	4.5	29.2	4.5	29.5	4.4
0.5 μg/l	28.2	4.7	27.6	4.7	28.1	4.4	26.1	4.6
5 μg/l	26.3	4.6	25.1	4.4	22.8*	4.1	21.3*	4.2
50 μg/l	26.1	4.4	22.2	4.5	21.7*	3.9	20.1*	4.0

s = Standard deviation. *Significant difference to control ($p < 0.05$).

complete disappearance of the trail.

When collected directly from the genital papilla, placed on a microscope slide and diluted in water at a temperature of 21°C, sperm remained active for some minutes. The average duration of these activities at the different tested concentrations after 24 hr, 2 weeks, 1month and 2 months are represented on Table 2. These durations were found to decrease with increasing DDT levels and exposure time. Similar observations were made during the evaluation of the life span of sperm trail (Table 3) and the motility of sperm released by the contaminated male zebrafish during mating events.

Table 3. Mean Average Life Apan (in minutes ± S.E.) of Sperm Trails Released by Male Ffish Exposed to the Different DDT Tested Concentrations During Mating Events After 24 hr, 2 weeks, 1 month, and 2 months

	24 hr	2 weeks	1 month	2 months
Control	138 ± 14	140 ± 13	142 ± 15	139 ± 16
0.05 μg/l	138 ± 14	136 ± 14	130 ± 9	135 ± 10
0.5 μg/l	135 ± 15	120 ± 12	115 ± 7	105 ± 8
5 μg/l	128 ± 13	106 ± 8	90 ± 6*	86 ± 5*
50 μg/l	125 ± 11	90 ± 9	81 ± 7*	70 ± 4*

S.E. = Standard Error. *Significant difference to control ($p < 0.05$).

DISCUSSION

Our observations show that the mechanisms of sperm release and egg insemination in zebrafish (*Brachydanio rerio*) is very different from that described in other fishes with external insemination. Sperm is not released freely into the water but in the form of sperm trails laid on the nest surface. Moreover, sperm and eggs are not released synchronously. Rather, the male starts to release sperm before the female starts depositing her eggs. We also showed that the sperm are able to leave the trail and move in the water for several minutes.

The male zebrafish usually reacted to the introduction of a ripe female. It was also observed that during the mating events, the sperm released by male zebrafish increased with time after the introduction of a ripe female. No sperm was found in water samples before the introduction of a ripe female. It could then be established that there is no risk of prematured sperm release by DDT treated male zebrafish.

During courtship and spawning, male zebrafish were often observed to move along the test vessels with characteristic movements of their body while keeping their fins spread. This behavior might have the function of accelerating sperm dispersal and mixing with the water, thus promoting egg insemination. But after 1 month of exposure to 5 μg/l and 50 μg/l DDT, the behavior of male fish changed totally. Their frequency and capacity to react after the introduction of a ripe female reduced obviously. They did not swim to the surface and ate only when food reached the bottom of the basin. Therefore the mechanism that the production by the male of sperm trails that continue to release active sperm over prolonged periods of time, would allow the male to spend most of the spawning time defending the nest and the female from potential intruders.was not more observable. Per consequent it could be remarked that the start of egg laying by the female mated with male exposed to tested concentrations after 1 month was retarded. This retardation was found increasing with higher DDT tested levels and time of exposition. The observation that eggs are not necessarily laid directly on the sperm trails indicates that sperm could reach the eggs via the surrounding water. Therefore, the change in male fish behavior affecting its function of accelerating sperm dispersal could be one of the cause of the retardation of egg laying by the female. Per consequent, it could be proposed that DDT levels and exposure time could have a negative effect during mating events on male zebrafish by reducing

their capacity to react in the presence of a ripe female and on female zebrafish by delaying the start of their egg laying.

Sperm trails have already been described in another fish *Hypseleotris compressus*. They consist of a band of adhesive material in which the sperm tails are embedded and are laid by the male in his territory.

How are sperm trails produced in zebrafish? Since the sperm appear to be embedded in a viscous secretion, we suggest that substances produced by the seminal vesicles are added to the sperm to produce the trails. It is known that the seminal vesicles of teleost produce mucoprotein[5,7] and although we cannot rule out their involvement in other aspects of reproduction, our observations suggest that a major role of the semianal vesicles may be the production of material for the sperm trails. In the case of the present study it could be possible that the reduction in length and width of sperm trails after one month of exposure to 5 μg/l and 50 μg/l leads also to the reduction in the secretion of the substance which is added to the sperm to produce the trails. Other eventuality is that the tested concentrations 5 μg/l and 50 μg/l after 1 month of exposure could have affected the male fish by reducing their ability to release mucins, produced either by accessory structures to the gonad[26,27] or by hypertrophied kidney.[28] But the role of these substances in the reproduction is often unclear or unknown. Our results with zebrafish suggest that the production of mucosubstances might be linked to diverse mechanisms of sperm release as well as level of chemicals and the duration of exposure.

The question that remains is to know how the sperm release could have variated if exposure time lasted more than 2 months. However, the results obtained during the experiment period could be already of great help to some authors. For example Sharpe and Skakkebaek[29] who were seeking to know whether estrogens are involved in falling sperm count and disorders of the male reproductive tract.

The reduction in the number of sperm released found in the present study also brought us to question about their quality. Our findings have demonstrated that the level of DDT and the exposure time reduced also the duration of the activity and the average life span of sperm trails released by treated fish during mating events. After 2 months of exposure, a difference of 10.2 min was recorded between the duration of activity of sperm treated with 50 μg/l and that of control. Taking also into consideration the retardation of the start of egg laying by female fish mated with male exposed to DDT after 1 and 2 months, it can be established that the motility of sperm could compromise their fertility. Therefore, the present study can help us also to make some suggestions on the reduction in number of sperm released with regard to the effects of xenobiotics on the reproduction of fish reported by some authors. This reduction in sperm released could therefore be due to the influences of the xenobiotic on the endocrine system as stated Mattison and Thomson,[17] Donaldson and Scherer[18] More recently, Schettler[11] reported that in general wildlife studies demonstrated a relationship between exposures to endocrine disrupting substances and abnormal thyroid function, sex alterations, poor hatching success, decreased fertility and growth, and altered behavior. On the other hand, the reduction in egg production and decreased fertility reported by Ensenbach and Nagel[22] during the investigation of the effect of Dichloroaniline and Lindane on the reproduction of zebrafish could have been due to the negative effects of the chemicals on the sperm released by the male fish as found in the present experiment. How far these modifications in hormone concentrations lead to the decrease of sperm released by the male zebrafish is not known. A strong mechanistic case can be made to explain how exposure to estrogenic chemicals in adult life stage could lead to the alterations in function of the reproductive system of zebrafish. In the actual case, these alterations effect the number , the motility and life span of sperm released. The question remains how will variate these parameters assessed when male fish are exposed for more than 2 months to the different DDT tested concentrations. The suggested mechanisms whereby exposure to DDT could induce sperm count, motility and life span of the trails within a prolonged period are offerred as hypothesis on which to focus discussions and research.

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