

Exposure to Thiodan[®] results in lipofuscin accumulation in hepatocytes of the freshwater catfish *Tandanus tandanus*

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ABSTRACT: We investigated the effect of time after pulse exposure to 1.0 µg l⁻¹ endosulfan (applied as Thiodan[®]) on endosulfan residues in the liver and ultrastructural changes in the hepatocytes of the freshwater catfish *Tandanus tandanus*. Time after exposure did not affect the mean residue level in the liver. After exposure to endosulfan, residues in the liver were 227.47 µg kg⁻¹ after 1 d and 282.83 µg kg⁻¹ after 28 d; residues in the bile were 313.97 µg kg⁻¹ after 1 d and 334.53 µg kg⁻¹ after 28 d. At the end of 28 d exposure, lipofuscin was present in up to 69% of hepatocytes of fish containing residues of endosulfan, but absent from control fish. There was a statistically significant increase in the percentage of pyknotic nuclei and altered rough endoplasmic reticulum 28 d after exposure. The mean percentage of cells with altered endoplasmic reticulum ranged from 12.93% (Day 1) to 7.50% (Day 28) for control fish, while for exposed fish it increased from 14.30% (Day 1) to 35.00% (Day 28). The mean percentage of cells with pyknotic nuclei increased from 1.1 to 2.1% in control fish and from 3.8 to 9.6% in exposed fish. Other ultrastructural changes included increased ultrastructural heterogeneity, progressive vacuolation and fractionation of rough endoplasmic reticulum, accumulation of lysosomes and residual bodies, intranuclear inclusions and pseudoinclusions, membrane whorls and myelinated bodies. Protracted senescence was one of the main features of endosulfan toxicity to *T. tandanus* hepatocytes.

KEY WORDS: Endosulfan · Lipofuscin · Hepatocyte

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INTRODUCTION

The fish liver has an important function in biotransforming xenobiotics and a unique position within the fish's circulatory system. As a consequence, its physiology and biochemistry are often affected by toxicants. Endosulfan was shown to cause a number of ultrastructural changes in fish liver, including an increase in the number of cells with pyknotic nuclei, dilation and proliferation of rough endoplasmic reticulum (RER), induction of concentric RER, proliferation of smooth endoplasmic reticulum (SER), degeneration of mitochondria and myelin formation (Braunbeck & Völkl 1993, Arnold et al. 1995, 1996, Nowak 1996). However, the effect of time after exposure on the ultra-

structural changes, including regeneration of the tissue, has not been investigated previously.

Thiodan[®] is a commercial formulation of the organochlorine insecticide endosulfan, which has a wide range of applications. For example it has been used to control mosquitoes in malaria regions, to control *Heliothis* spp. beetles in cotton-growing areas and as an insecticide for other agricultural and horticultural crops. Endosulfan (6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,4,3-benzo-dioxathiepin-3-oxide) replaced other organochlorines because of its shorter persistence in the environment. It has been registered as a priority pollutant by the US Environmental Protection Agency and is classified by the World Health Organisation in the category of technical products that are mod-

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erately hazardous (Arnold et al. 1996). Technical-grade endosulfan consists of a mixture of 2 isomers—alpha and beta endosulfan. It is one of the most toxic chemicals to fish, with a lethal concentration (LC_{50}) as low as $0.014 \mu\text{g l}^{-1}$ reported for the harlequin fish *Rasbora heteromorpha* (48 h LC_{50} in a flow-through system: Alabaster 1969). For freshwater fish in Australian waters, the 96 h LC_{50} ranges from $0.1 \mu\text{g l}^{-1}$ for the European carp *Cyprinus carpio* to $5.0 \mu\text{g l}^{-1}$ for the eastern rainbow fish *Melanotaenia duboulayi* (Sunderam et al. 1992).

The aim of the present study was to determine the effects of endosulfan on the liver structure of the Australian freshwater catfish *Tandanus tandanus* for up to 28 d after pulse exposure. This species is relatively common in the water bodies in cotton-growing areas in New South Wales and Queensland. The experiment conditions were chosen to replicate a situation in a water body contaminated with the pesticide on 1 occasion. Since endosulfan is used in the field as Thiodan® and not as a pure compound, the commercial formulation Thiodan® was used in the experimental exposure.

MATERIALS AND METHODS

Fish collection. Adult *Tandanus tandanus* (males and females, sex ratio 1:1), ranging in size from 380 to 532 mm, were collected by overnight gill-netting from Glenbawn Dam on Hunter River, New South Wales, an area where endosulfan has not been used. They were transported to Sydney in plastic bags filled with water from the dam and oxygen (2:1) that were kept in insulated containers. Ice was added to the containers to avoid an increase in water temperature during transport. Acriflavine (2 to 4 mg l^{-1}) and salt (0.5%) were added to the water as a prophylactic treatment against external parasites.

Laboratory experiment. The fish tanks were filled with Sydney tap water from which chlorine and chloramines had been removed (filtration through sand and activated-carbon filters followed by holding for at least 1 wk in 5000 l epoxy-lined tanks). The water in the fish tanks was filtered through filter wool, activated-carbon and clay pipes contained in an external plastic filter (Sacem Marathon 700, S. Giorgio).

The fish were acclimated to laboratory conditions for at least 2 wk before the experiment started. They were fed every second day ad libitum with frozen chicken liver, live freshwater shrimps and earthworms. The only organochlorine that could be detected in the food was DDE. No difference was noted in feeding behaviour between treated and control fish. Water flow was maintained at the rate of complete replacement of the volume in each tank each day.

The experiment consisted of a static 24 h exposure with a single application of endosulfan at the beginning of the experiment. One fish was allocated to each tank by random selection. The tanks were randomly assigned to control and treated fish. Thiodan® was applied once as a pulse exposure at the beginning of the experiment at a nominal concentration of the active ingredient endosulfan of $1.0 \mu\text{g l}^{-1}$. The concentration of endosulfan was calculated on the basis of the content of active ingredient given by the manufacturer (350 g l^{-1} : Hoechst Australia). The formulation of Thiodan® we used also contained 620 g l^{-1} xylene as a solvent. The measured mean concentration of endosulfan was $0.995 \mu\text{g l}^{-1}$ (SE = $0.04 \mu\text{g l}^{-1}$). The exposure concentrations were chosen on the basis of concentrations reported in natural water bodies in cotton-growing areas in Australia (e.g. Gwydir River and farm dams in New South Wales), existing information on the LC_{50} range for other fish species (0.1 to $5.0 \mu\text{g l}^{-1}$) under static conditions (Sunderam et al. 1992), the size of the fish tested, and on previous experiments (Nowak 1996). After 24 h, the flow-through system was reactivated, the water being replaced at the rate of complete renewal of the tank volume in each tank each day until the end of the experiment. There was no mortality during the experiment; 3 control fish and 3 treated fish were killed 1 d and 3 control fish and 3 treated fish 28 d after the application of endosulfan.

Sample collection. The spinal cord of each fish was cut and the liver and gall bladder immediately dissected out. The time between dissection and fixation was as short as possible (less than 1 min) and there was no difference in the time spent on dissection of control and treated fish. Small pieces of the caudal part of the liver were fixed by immersion in ice-cold 1% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 24 h (Saito & Tanaka 1980).

Collection of samples of the same organs for chemical analysis precluded the use of fixation by perfusion, which otherwise would have been the preferred method. The rest of the liver and gall bladder was kept frozen for endosulfan analysis.

Endosulfan analysis. Samples were analysed individually from each fish. Endosulfan residues in the fish liver were analysed as described previously (Nowak & Julli 1991). Briefly, tissue was emulsified with trisodium citrate and disodium hydrogen orthophosphate and mixed with sodium sulphate. The dry mixture was eluted through silicic acid and alumina with a mixture of dichloromethane, hexane and acetonitrile. Following concentration, the elutes were chromatographed between standards using gas-liquid chromatography (Shimadzu GC-8A) and an electron capture detector. Identification was confirmed with columns of different polarity and GC-MS. The method has a detection limit

of $1.0 \mu\text{g kg}^{-1}$ for alpha and beta endosulfan and $2.5 \mu\text{g kg}^{-1}$ for endosulfan sulphate, giving mean recoveries of 86.2% for alpha endosulfan, 83.2% for beta endosulfan and 88.8% for endosulfan sulphate (Nowak & Ahmad 1989). The residues of total endosulfan were calculated as the sum of the residues of alpha endosulfan, beta endosulfan and endosulfan sulphate. The residues under the limit of detection were considered as zero for further calculations and statistical analyses.

Light microscopy (LM) and electron microscopy (EM). After fixation in glutaraldehyde, the samples were rinsed 3 times in 0.1 M cacodylate buffer and postfixated in 1% osmium tetroxide for 1 h. Tissue samples were dehydrated in a series of acetone. They were placed in a mixture of 100% acetone:Spurr's resin (1:1) and allowed to infiltrate overnight. Infiltration was completed by transfer to fresh Spurr's resin for 12 h. The following day, the tissue blocks were embedded in Spurr's resin (Spurr 1969).

The tissue blocks were sectioned using an ultramicrotome (OmU2). Semi-thin sections for LM (0.5 to $1.0 \mu\text{m}$) were cut with a glass knife, attached to glass slides and stained with 0.5% toluidine blue in 1% borax. The slides were examined under a light microscope. Thin sections for EM (50 to 90 nm) were cut with a diamond knife and collected on copper grids coated with Parlodion film (Parlodion, a purified pyroxylin, Ted Pella). They were stained with uranyl acetate and Reynolds' lead citrate (Reynolds 1963). The sections were examined in a Phillips 400 transmission electron microscope, TEM (accelerating voltage = 100 kV).

Qualitative observations. The structure of the liver was examined using light and electron microscopy. All observations were made on a morphological basis (Ghadially 1982).

Quantitative observations. Quantitative observations focused on the ultrastructural changes previously reported in catfish exposed to endosulfan (Nowak 1996). The percentages of cells with structural alterations (changes in RER, presence of concentric membranous bodies, presence of pyknotic nuclei) were determined at the EM level (6000× magnification) in all experimental fish. Replication was based on previous results (Nowak 1996): From each fish we randomly selected 2 replicate tissue blocks; 1 section was chosen from each block by random selection; 100 hepatocytes were randomly chosen from each section and the number of hepatocytes displaying ultrastructural changes was counted. The sections were coded, and were examined without prior knowledge of the treatment or the residue level in the tissue.

Ageing. The age of each fish was determined by examination of sections of the dorsal spine using the method described for processing and examining pectoral spines in ictalurids (Blouin & Hall 1990).

After fish dissection, the spine was removed by pressing it flat against the body, grasping its base with pliers, and twisting it clockwise while pushing towards the body. After removal, the spine was placed in a paper envelope and allowed to dry. The spines were sectioned with a low-speed saw equipped with a single lowconcentration diamond wafering blade. Sections were cut as thinly as possible (0.12 to 0.14 mm) and mounted on microscope slides using Spurr's mounting media. They were examined under the LM at 100× magnification and interpreted according to Davis (1977), who concluded that annual increments are deposited in the spines of *Tandanus tandanus* between May and June.

Experimental design and statistical analyses. Statistical analysis used analysis of variance (ANOVA). The results were considered to be statistically significant at $p < 0.05$. When statistically significant differences were found, the means were compared using the Student-Newman-Keuls (SNK) test. Before ANOVA was performed, the homogeneity of variances was tested using Cochran's test (Winer 1971). If the variances were heterogeneous, the data were transformed and resubmitted to the test again. Calculations of power were made where the results were not statistically significant, whereby we determined if the null hypothesis of no effect could be confidently accepted. Calculations of power were done using the observed values for means, error variance and degrees of freedom, and were based on Cohen's effect size index f and an alpha value of 0.05. The power of the tests was determined using tables for power of the F -test in ANOVA (Cohen 1988).

Effects of 3 factors: treatment (control and treated), time after treatment (1 and 28 d) and individual fish were tested. Treatment and time were considered fixed and orthogonal, while fish was nested in each treatment-time combination. For each fish, 2 replicate blocks were examined. The relationship between endosulfan residues and the percentage of pyknotic nuclei as well as between relative residues of endosulfan isomers and the percentage of pyknotic nuclei was determined for catfish containing endosulfan residues by calculating the correlation coefficient. The age of the control and treated fish was compared using a Student's t -test.

RESULTS

Endosulfan residues

None of the control fish contained endosulfan, whereas all exposed fish had detectable residues in their liver and bile (Table 1). Time after exposure did

not have any effect on the total endosulfan residue. There was high individual variation, particularly for the total endosulfan residues in the bile (Table 1). In the liver, beta endosulfan was not detected; in the bile there was a trend of increased percentage of beta endosulfan over time, but there was much individual variation.

Qualitative structural changes

Control livers showed the normal cellular structure (Fig. 1a) previously described *Tandanus tandanus* (Nowak 1996). In most treated fish, many hepatocytes had lost the compartmentation in areas of high metabolic activity and storage (results not shown) that was apparent in the control fish. This was (at least partly) a consequence of the proliferation of the endoplasmic

reticulum (ER), which often comprised dilated rough endoplasmic reticulum (RER) and filled the whole cytoplasm (Fig. 1b). Fragmentation and vacuolation of ER was common, particularly in fish sacrificed 28 d after treatment, when vesiculated ER filled most of the cytoplasm in some hepatocytes (Fig. 1c, Table 2). Morphological changes were much more prominent 28 d after treatment than 1 d after treatment (Table 2). These changes included increased ultrastructural heterogeneity (Fig. 1d), progressive vacuolation and fractionation of RER (Fig. 1c), accumulation of lysosomes and residual bodies (Fig. 2), intranuclear inclusions and pseudoinclusions (Fig. 1c), membrane whorls, and myelinated bodies (Fig. 2a). Fibrous, highly electron-dense material of granular nature filled the residual bodies, which were present only in treated fish (Fig. 2); 28 d after treatment; they were present in up to 69% (mean 27.8%) of all examined hepatocytes of treated fish (Table 1). They contained lipopigment granules morphologically identified as lipofuscin (Fig. 2).

Table 1. *Tandanus tandanus*. Total residues of endosulfan (sum of alpha endosulfan, beta endosulfan and endosulfan sulphate) and quantitative effects of endosulfan on catfish hepatocytes after 1 and 28 d exposure to 1.0 $\mu\text{g l}^{-1}$ endosulfan. Data are means (SE). ER: endoplasmic reticulum

| Effect | 1 d | | 28 d | |
|---|-----------------|--------------------|----------------|--------------------|
| | Control | Treated | Control | Treated |
| Endosulfan residue in liver ($\mu\text{g kg}^{-1}$) | 0 | 227.47 (21.97) | 0 | 282.83 (65.38) |
| Endosulfan residue in bile ($\mu\text{g kg}^{-1}$) | 0 | 313.97 (119.13) | 0 | 334.53 (127.26) |
| Pyknotic nuclei (%) | 1.10 (0.90) | 3.80 (0.40) | 2.10 (1.50) | 9.60 (2.30) |
| Altered ER (%) | 12.93 (4.07) | 14.30 (4.59) | 7.50 (1.06) | 35.00 (7.64) |
| Lipofuscin (%) | 0 | 0 | 0 | 27.80 (11.45) |

Table 2. *Tandanus tandanus*. Morphological signs of cellular senescence present in hepatocytes. -: absent; +: present; ++: common; +++: very common

| Sign | 1 d | | 28 d | |
|---|---------|---------|---------|---------|
| | Control | Treated | Control | Treated |
| Increased ultrastructural heterogeneity | - | + | - | +++ |
| Progressive vacuolation and fractionation of RER | - | + | - | +++ |
| Drastic accumulation of lysosomes and residual bodies | - | - | - | +++ |
| Presence of lipofuscin | - | - | - | +++ |
| Intranuclear inclusions and pseudoinclusions | - | - | - | + |
| Membrane whorls | - | - | - | + |
| Myelinated bodies | - | - | - | + |

Quantitative ultrastructural changes

Both treatment and time after treatment had a statistically significant effect on the percentage of pyknotic nuclei. The mean percentage of cells with changed endoplasmic reticulum ranged from 12.93% on Day 1 to 7.50% on Day 28 for control fish, while for exposed fish it increased from 14.30% on Day 1 to 35.0% on Day 28. After 28 d ER changes were observed in 39.5% of hepatocytes in treated females and 46.3% in treated males. The mean percentage of cells with pyknotic nuclei increased from 1.1 to 2.1% in control fish and from 3.8 to 9.6% in exposed fish. The mean percentage of pyknotic nuclei was greater in the liver of treated fish (ANOVA $p = 0.005$). This difference significantly had increased by Day 28 (ANOVA $p = 0.032$). 'Fish' was also a significant factor suggesting high individual variability (ANOVA $p = 0.0005$). The percentage of cells with pyknotic nuclei was positively correlated with relative residues of endosulfan isomers in the bile ($r = 0.9411$, $p < 0.01$), but not with total endosulfan in the bile ($r = 0.2053$), indicating that this effect is isomer-specific.

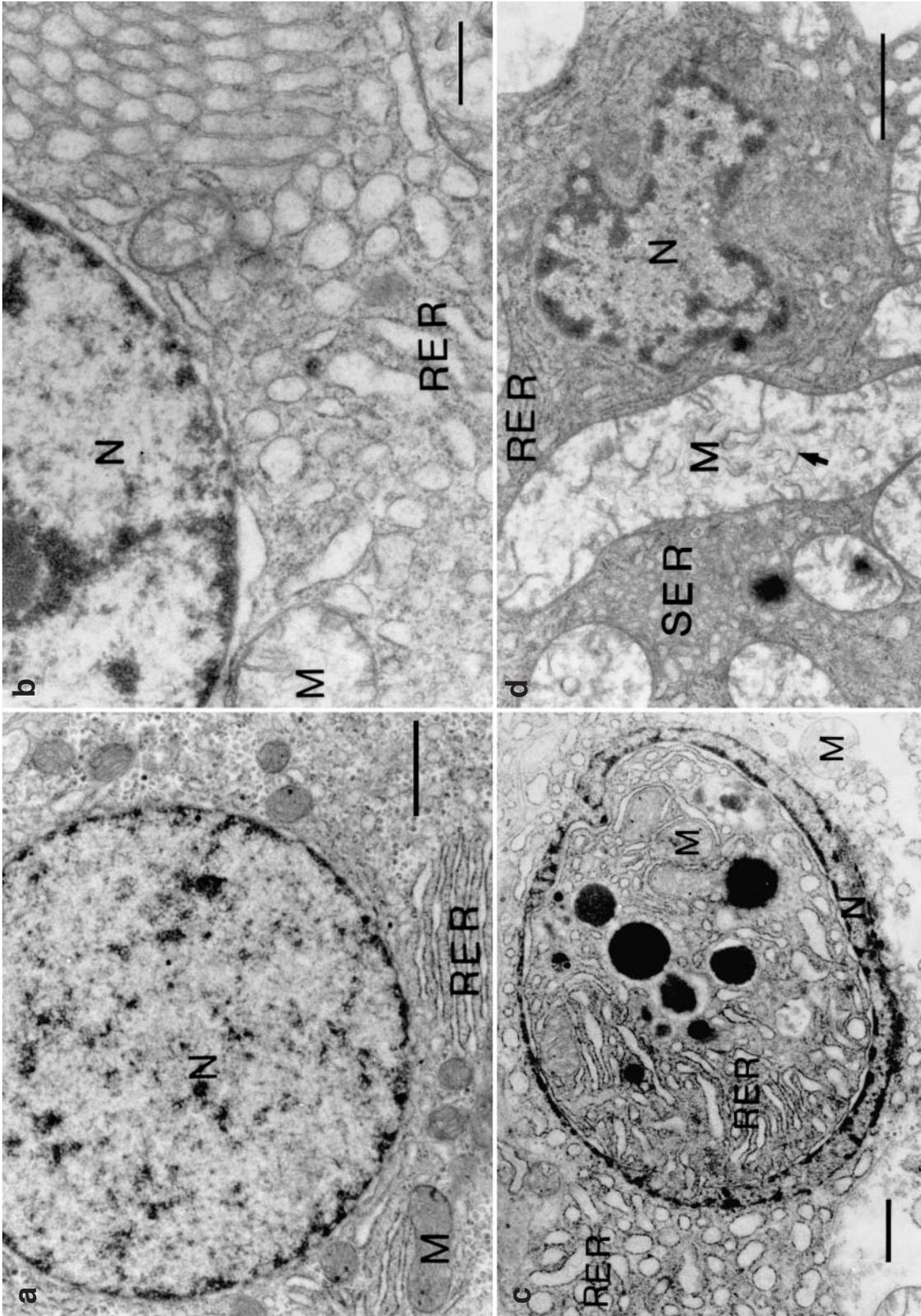


Fig. 1. *Tandanus tandanus*. (a) Hepatocyte from control catfish showing a few profiles of rough endoplasmic reticulum (RER) and mitochondria (M) concentrated around the centrally located nucleus (N); note extensive glycogen deposit evenly distributed in cytoplasm. (b) Hepatocyte from catfish containing endosulfan showing a nucleus (N) surrounded by dilated rough endoplasmic reticulum (RER); note marked swelling of mitochondria (M). (c) Hepatocyte nucleus (N) showing large double-membrane-bound pseudoinclusion containing dilated and vesiculated rough endoplasmic reticulum (RER), and mitochondria (M) in catfish containing residues of endosulfan. (d) Shrunken hepatocyte containing enlarged, dilated mitochondria (M), pyknotic nucleus (N), abundant rough endoplasmic reticulum (RER) and smooth endoplasmic reticulum (SER) in catfish containing residues of endosulfan; note dilation of cristae (arrow) within enlarged mitochondria. Scale bar in (a), (c), (d) = 1.0 µm; scale bar in (b) = 0.5 µm

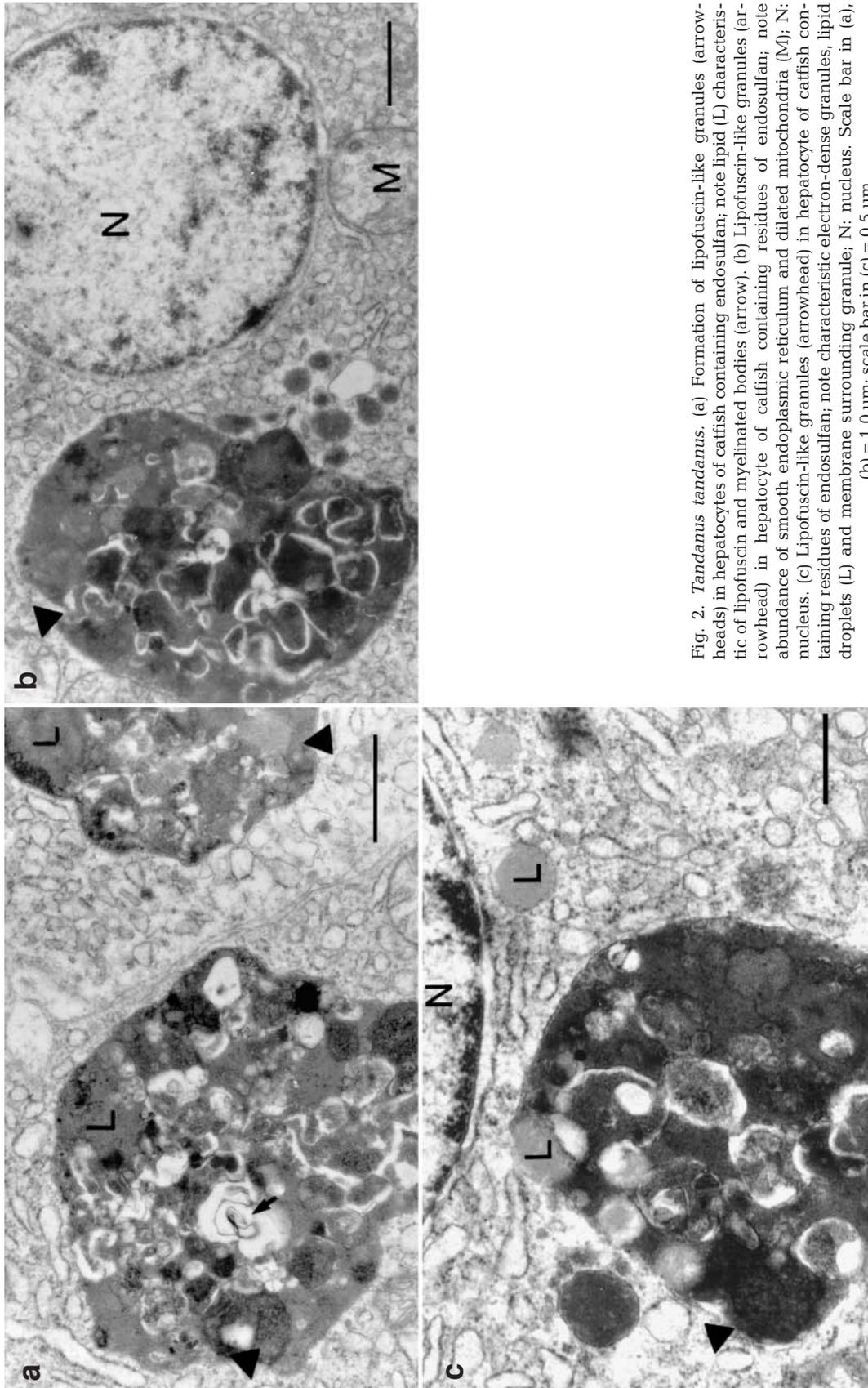


Fig. 2. *Tandanus tandanus*. (a) Formation of lipofuscin-like granules (arrowheads) in hepatocytes of catfish containing endosulfan; note lipid (L) characteristic of lipofuscin and myelinated bodies (arrow). (b) Lipofuscin-like granules (arrowhead) in hepatocyte of catfish containing residues of endosulfan; note abundance of smooth endoplasmic reticulum and dilated mitochondria (M); N: nucleus. (c) Lipofuscin-like granules (arrowhead) in hepatocyte of catfish containing residues of endosulfan; note characteristic electron-dense granules, lipid droplets (L) and membrane surrounding granule; N: nucleus. Scale bar in (a), (b) = 1.0 μm ; scale bar in (c) = 0.5 μm

The mean percentage of cells with altered ER was greater in fish containing endosulfan than in controls, particularly after 28 d. However, this trend was not statistically significant (ANOVA: treatment, $p = 0.07$; time, $p = 0.29$; fish (treatment–time), $p = 0.08$; treatment \times time, $p = 0.16$), and was at least partly due to high individual variability. The power of the test was poor, and as a consequence the probability of accepting a false null-hypothesis of no effect was higher than desirable (β for treatment = 0.50, β for time = 0.75). The size effect was very large (0.45 for treatment, 0.70 for time), indicating a high departure from the null hypothesis.

Fish age

There was no significant difference in chronological age between the treated and control fish (t -test, $p = 0.13$), although the treated fish tended to be slightly younger (mean age 2.8 yr, SE = 0.26) than controls (mean age 3.6 yr, SE = 0.37).

DISCUSSION

The percentage of pyknotic nuclei was significantly affected by both treatment and time. An increase in the percentage of pyknotic nuclei was also observed in the liver of the catfish *Tandanus tandanus* 24 h after exposure to endosulfan in an earlier study (Nowak 1996). However, the percentage of pyknotic nuclei in catfish 24 h after pulse exposure to endosulfan was 15% in the earlier study, whereas in the current study it was 4%. In the present study, in all fish, the percentage of pyknotic nuclei was related to the relative residue of beta endosulfan but not to the total endosulfan residue, regardless of the time after exposure; this is in agreement with the previous results (Nowak 1996).

The presence of endosulfan residues in this catfish was associated with changes in the RER. This is in agreement with previous observations (Nowak 1996) that the percentage of hepatocytes exhibiting changed ER depends on the residues of total endosulfan in the liver of the fish. However, the degree of change in the endoplasmic reticulum 1 d after application of endosulfan in the present experiment differed from that recorded in the previous experiment (Nowak 1996). This difference is consistent with the discrepancy in the results for the pyknotic nuclei, and may be due to qualitative and quantitative differences in the residues in the livers of the fish used in the experiments (Nowak 1996). Whereas in the 1996 experiment the mean residue was $57 \mu\text{g kg}^{-1}$ total endosulfan, in the present

experiment the mean was $220 \mu\text{g kg}^{-1}$ total endosulfan. These values are within the range found in the same species in cotton-growing areas in New South Wales (Nowak & Julli 1991). The liver residues consisted of 34.1% alpha, 23.2% beta and 42.7% sulphate in the 1996 experiment, whereas in the present experiment the residues in the liver consisted only of the alpha isomer. Catfish collected in the field contained a greater proportion of endosulfan sulphate (around 50%); however, the livers of most of these fish did not contain beta endosulfan. Sex and different collection areas of the fish may be other factors responsible for the difference in the magnitude of the response between the 2 experiments. While in the present experiment the sex ratio was 1:1 (3 of the treated fish were males, 3 were females), the previous work was based mostly on females (Nowak 1996). Braunbeck et al. (1989) found that proliferation of RER was a sex-specific change, characteristic only for females, in the zebrafish *Brachydanio rerio* treated with 4-nitrophenol. This may be due to the function of RER in the hepatocytes in the production of vitellogenin during the female reproductive cycle (Peute et al. 1978). While sex determination was not possible in the catfish until dissection at the termination of the experiment, sex must be taken into consideration when interpreting the toxicity tests.

The ER changes observed in the present experiment are consistent with changes induced by endosulfan in other fish species. Vesiculation and dilation of RER and proliferation of SER were present in hepatocytes of male rainbow trout *Oncorhynchus mykiss* after exposure to 0.01, 0.05 or $0.1 \mu\text{g l}^{-1}$ endosulfan for 28 d (Arnold et al. 1996). Proliferation of both RER and SER in liver cells were apparent after exposure of male rainbow trout *O. mykiss* to $0.05 \mu\text{g l}^{-1}$ endosulfan and 1 to $10 \mu\text{g l}^{-1}$ organophosphate disulfoton for 18 or 34 d (Arnold et al. 1995). This suggests that these changes are not species-specific. The other studies investigated changes only in males (Arnold et al. 1995, 1996). Our results were very similar for males and females (after 28 d ER changes were observed in 39.5% of hepatocytes in treated females and 46.3% in treated males).

In this experiment, lipofuscin was present in up to 69% of hepatocytes of catfish 28 d after exposure to endosulfan. Lipofuscin accumulation was also observed after exposure of juvenile grey mullet *Liza ramada* to 0.17 mg l^{-1} atrazine (Biagianni-Risbourg & Bastide 1995) and in necrotic hepatocytes of cutthroat trout *Salmo clarki* after repeated aqueous pulse exposure to 0.01 mg l^{-1} endrin (Eller 1971). Intraperitoneal injection of benzo[a]pyrene resulted in an increase in lipofuscin in hepatocytes of immature oval sole *Solea ovata* (Au et al. 1999). Formation of lipofuscin granules, in close association with lipid droplets, was observed in the flounder *Platichthys flesus* contami-

nated with HCBs and PCBs (Kohler 1989, 1990). The size and absolute volume of lipofuscin granules per hepatocyte were greater and the granules were in higher numbers in the livers of immature oval sole *S. ovata* collected from contaminated sites than in the individuals sampled at reference sites (Au & Wu 2001). Lipopigments represent peroxidised forms of lipid, and their accumulation may affect the functional capacity of the liver. Fish are particularly susceptible to lipopigment formation because of their high concentration of unsaturated fatty acids (Agius & Agbede 1984).

An increase in lipofuscin accumulation is one of the signs of ageing (Hammer & Braun 1988), and is considered to be one of the most consistent events in all senescing nonmitotic cells. Thus, measurement of lipofuscin concentration has been suggested to be a reliable ageing technique for fish (Hill & Radtke 1988) and crustaceans (Sheehy 1990, Nicol et al. 1991, Sheehy et al. 1994). However, organisms age in 2 ways: relative to time (chronological age) and relative to their rate of living (physiological age) (Sohal 1984). While the effects of some factors other than age on lipofuscin accumulation have been evaluated (e.g. temperature: Hammer 1988; solvent, ration, photoperiod: Hill & Womersley 1993; sample preservation: Nicol 1987), the potential influence of pollution on physiological ageing has not been investigated. Care should be taken when using lipofuscin content as the only indicator of fish age (Hill & Womersley 1993). As this study has shown, other factors such as toxicants may cause an increase in the accumulation of lipofuscin. Thus, fish exposed to xenobiotics can contain more lipofuscin than fish of the same chronological age from uncontaminated sites.

Endosulfan residues in the fish tissues had not significantly decreased by the time the experiment was terminated (28 d post-exposure), long after it had ceased to be detectable in the tank water. At the same time, the extent of ultrastructural changes in treated catfish remained the same, confirming that these are related to the residues of endosulfan in the tissues (in particular to the relative residues of endosulfan isomers in the bile) and not to the concentration of endosulfan in the surrounding water. This relationship between residues and ultrastructural pathology was not affected by time after exposure. The results of this study suggest that the effects of pulse exposure to sublethal level of endosulfan applied as Thiodan® can continue for at least 28 d after exposure. Longer periods of time are therefore necessary to investigate the regeneration of fish tissues after exposure to Thiodan®. Furthermore, while endosulfan is not as persistent as other organochlorines, it can accumulate in fish organs, and the residues can affect the fish for long periods after exposure. During the field sampling, endosulfan was detected in all catfish from cotton-growing areas,

including fish sampled months after endosulfan had been used (Nowak & Julli 1991). It can therefore be assumed that the organ structure of wild fish populations is affected. This could have implications for the physiology of the fish, including reproduction and/or growth.

Acknowledgements. This project was supported by a Sydney University Postgraduate Research Grant and funding from the New South Wales State Pollution Control Commission. The experiment and chemical analysis were performed at the Centre for Environmental Toxicology, State Pollution Control Commission. The ultrastructure investigation was done at the Electron Microscope Unit, University of Sydney. We are grateful to Dr. T. Braunbeck, J. Bryan, Dr. J. C. Chapman, M. Julli, Dr. R. D. Otaiza, and 2 anonymous reviewers for their advice and critical comments at various stages of this project.

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Editorial responsibility: Thomas Braunbeck, Heidelberg, Germany

*Submitted: May 12, 2002; Accepted: March 21, 2003
Proofs received from author(s): August 21, 2003*