Journal of Fish Diseases 1979, **2**, 35–42

Vertebral dysplasia in young fish exposed to the herbicide trifluralin

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Abstract. Sheepshead minnows, *Cyprinodon variegatus* Lacépède, exposed to 5·5 to 31 μg/l of the herbicide trifluralin, throughout their first 28 days of life, developed a heretofore undescribed vertebral dysplasia. This dysplasia consisted of semi-symmetrical hypertrophy of vertebrae (three to 20 times normal), characterized by foci of osteoblast and fibroblasts actively laying down bone and bone precursors. Effects of the abnormal vertebral development were dorsal vertebral growth into the neural canal, ventral compression of renal ducts, and longitudinal fusion of vertebrae. Fish, exposed for 51 days to 16·6 μg/l trifluralin and thereafter depurated for 41 days, showed no increase in vertebral dysplasia during depuration; however, residual spinal column damage was evident. Serum calcium concentrations were elevated in adult fish exposed for 4 days to 16·6 μg/l trifluralin. Fluorosis or mimicry of hypervitaminosis A are considered possible mechanisms for the osseous effect, but are not considered to be the only possible causes. The highly predictable nature of this disorder in experimental exposures strengthens the probability that young fish may serve as experimental models for determining effects of chemicals on early vertebrate ontogeny, particularly in regard to skeletal development.

Introduction

Trifluralin (2, 6 dinitro-N, N-dipropyl-4-(trifluoromethyl) Benzamine) is a fluorine-containing, pre-emergent herbicide widely used in the United States (Wiswesser 1976). Continuous laboratory exposure of early life stages of the sheepshead minnow *Cyprinodon variegatus* Lacépède to relatively low concentrations of trifluralin results in marked vertebral dysplasia. Vertebral anomalies such as kyphosis, lordosis or scoliosis of unknown etiologies have been reported in natural populations of fresh water and marine fish (Bengtsson 1975a; Moore & Hixson 1977). Other investigators have reported vertebral abnormalities, especially scoliosis, occurring in several species of fish as a result of parasitic infections (Hoffman 1962), dietary deficiencies (Halver 1972; Horak 1975), heavy metal exposure (Bengtsson 1975b; Holcombe, Benoit, Leonard & McKim 1976), organochlorine (Meyer 1966; McCann & Jasper 1972; Couch, Winstead & Goodman 1977), organophosphate (Mount & Stephan 1967; Weis & Weis 1976) and carbamate poisoning (Carter 1971).

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0140-7775/79/0100-0035 $02.00

The present report describes heretofore undescribed vertebral tissue response of sheepshead minnows during early life and adult exposure to trifluralin. To our knowledge, no pathological response identical to the subsequently described effects has been reported for any animal experimentally exposed to chemicals.

Materials and methods

Two trifluralin exposure tests of sheepshead minnow life stages (zygote, embryo, larval, juvenile and young adults) were completed in flowing seawater. The methods of continuous experimental exposure of the fish from zygote to adult have been described by Hansen, Goodman & Wilson (1977). The first test involved exposure of different groups of fish (zygote to 28 days) to 1·2, 2·7, 5·5, 20 and 31 μg/l of trifluralin (average measured concentrations); lethality data and histological specimens were obtained. In the second test, early life stages (zygote up to 51 days) and adults were exposed to 16·6 μg/l trifluralin (average measured) to obtain further histological specimens. Chemical analyses of trifluralin was conducted by gas chromatography of exposure water and fish tissue.

Pooled serum samples were taken from adult sheepshead minnows exposed for 4 days to 16·6 μg/l trifluralin for determination of serum calcium concentrations. Similar measurements were made on control and feral fish serum calcium concentrations. Also, a comparable group of fish exposed to 1·6 μg/l of the insecticide Kepone (sublethal concentration) was bled for comparable serum calcium measurements. Kepone-exposed fish were available and examined for serum calcium concentrations because Kepone has a severe scoliotic effect upon *Cyprinodon variegatus* (Couch *et al.* 1977) and thus could serve as a chemical control for comparison with serum calcium concentrations, of feral, control and trifluralin exposed fish. Blood samples were pooled within each of the above groups (10–20 fish per sample) because

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Figure 1. (a) Longitudinal, horizontal section from fish with marked hypertrophy of vertebral walls and zygapophyseal regions; fish were exposed to 31 μg/l trifluralin from zygote to 28 days post-fertilization; note obvious cellular notochord (N) and focus of osteoblasts and fibroblasts (O) (×160).

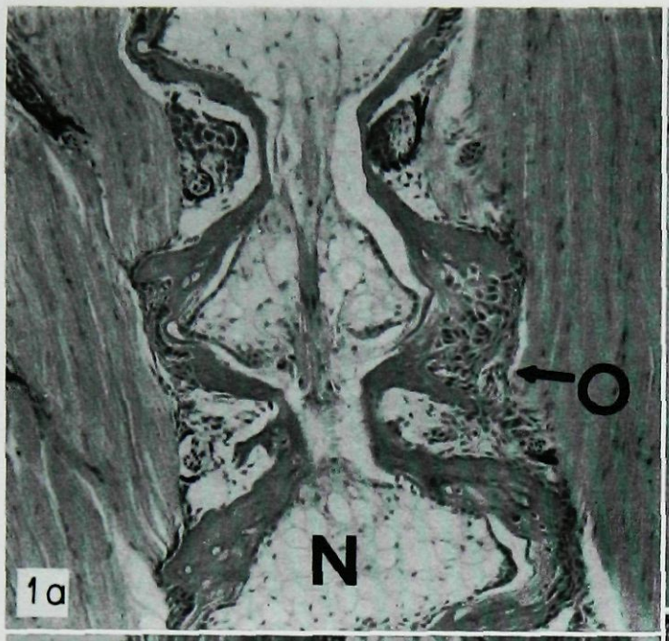
(b) Advanced vertebral hypertrophy in fish exposed to 16·6 μg/l trifluralin for 51 days beyond zygote stage. Note large foci of osteoblasts (O) and some osteocytes (Oc) (×160).

Figure 2. Longitudinal, horizontal section of vertebral column from normal control fish. Compare with Fig. 1(a, b) at approximately same magnification. Note the thin walls of centra of vertebrae (W) and the vestigial notochord (N) (×160).

Figure 3. Sagittal section from fish exposed for 28 days to 31 μg/l trifluralin. Normal form of vertebra is not recognizable. Note bony fusion of zygapophyseal regions (F), prominent notochord (N), thickened walls of vertebrae, and intrusion against spinal cord of the zygapophyses of the vertebrae (Z) (×160).

Figure 4. Sagittal section from fish exposed to 16·6 μg/l trifluralin. Note compression of duct that drains kidney (arrows) by outgrowth of osteoblastic focus (×160).

Figure 5. Silver stained section of dysplastic vertebra from fish exposed to 31 μg/l trifluralin from zygote to 28 days of age. Note the reticulin fibers (R) surrounding the scleroblastic cells in hypertrophied bone (×400).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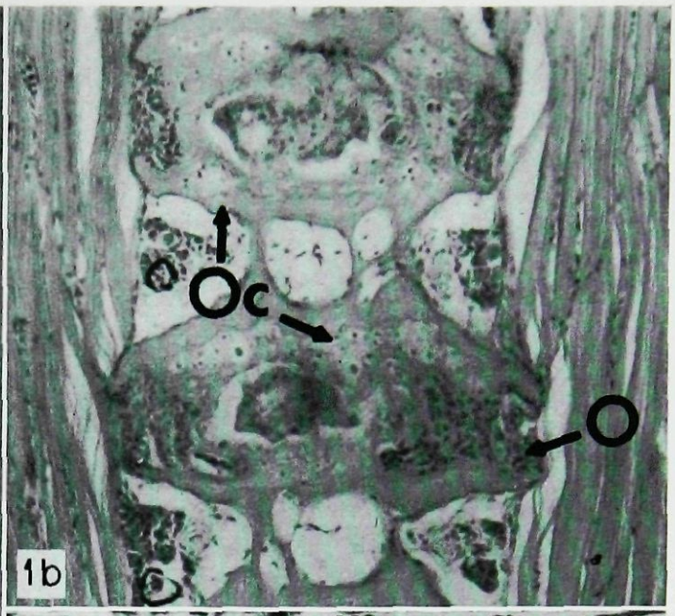
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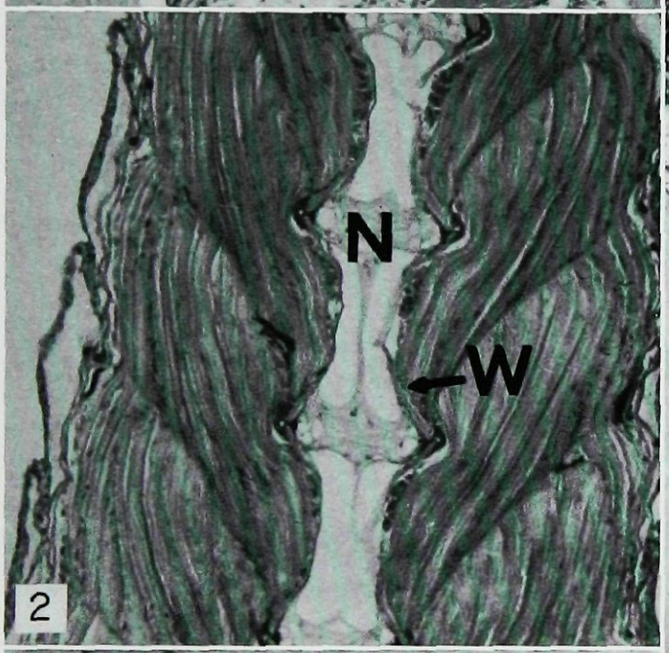
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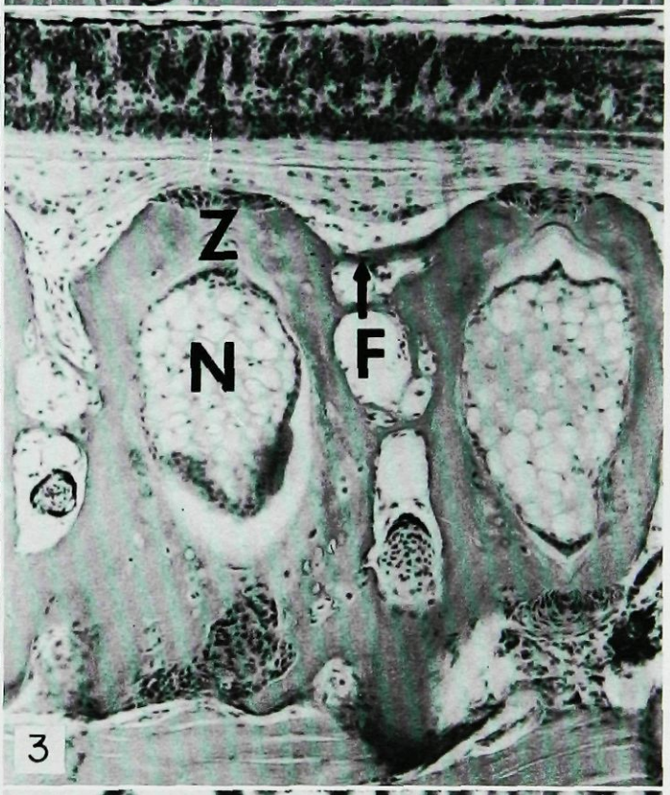
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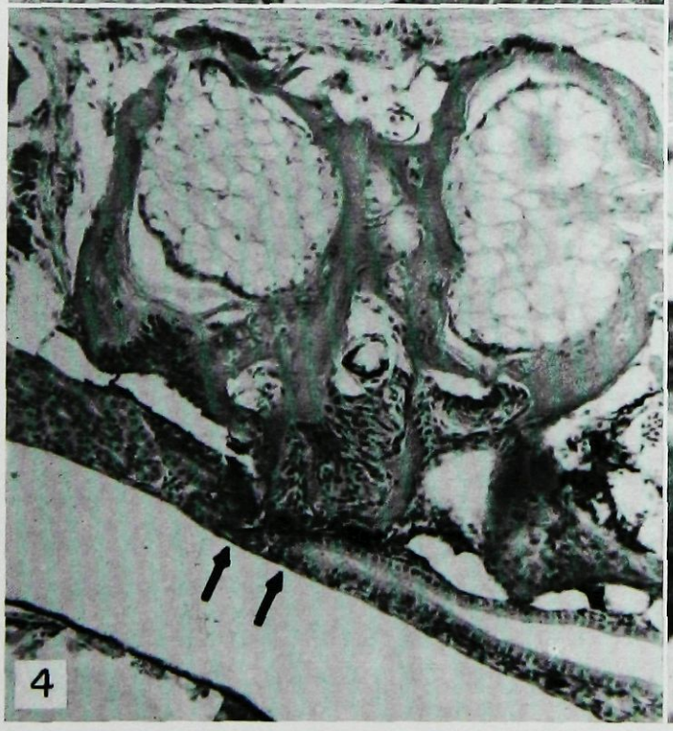
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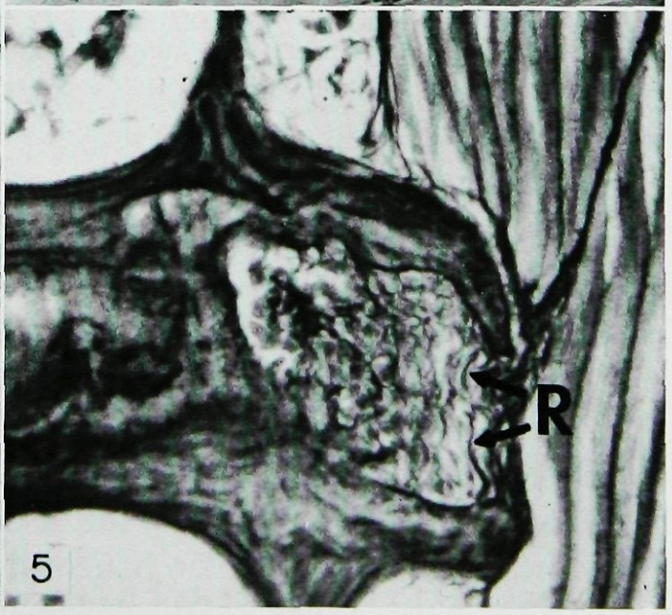
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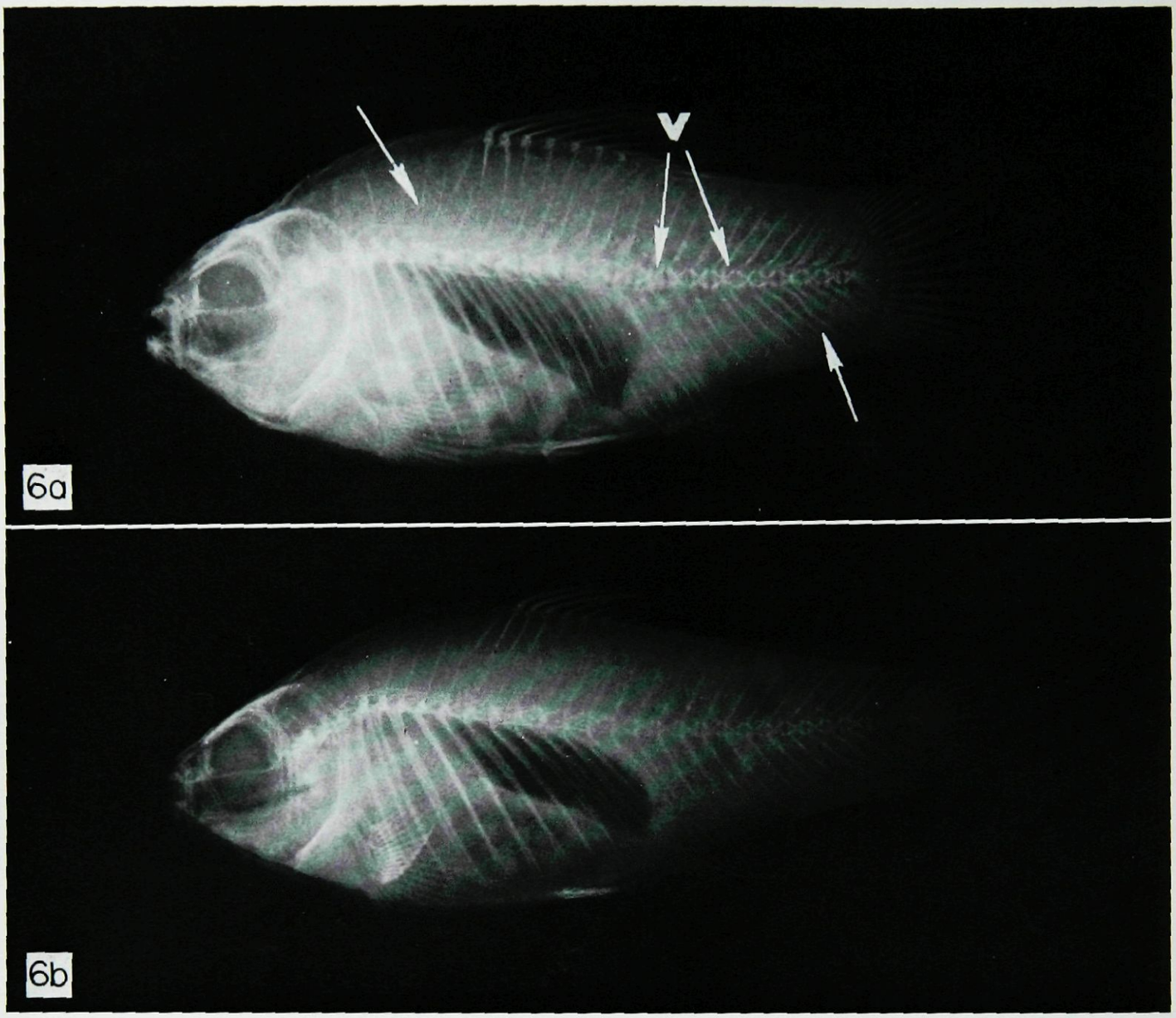

Figure 6. Radiographs of control, normal sheepshead minnow (b) and minnow (a) exposed to 16·6 μg/l trifluralin for 51 days and then depurated for 41 days. Compare the regular period of neural and haemal spines in the control to the irregular and displaced pattern in the exposed fish (arrows). Exposed fish has enlarged, dysplastic (malformed) vertebrae (V) with irregular articulations between vertebrae (×2·5).

the fish were small (35–60 mm standard length). Fish were bled from the caudal peduncle. Serum calcium concentrations were determined by an EDTA titration method (Tietz 1976).

Histological specimens (usually less than 1 cm long) were fixed in Davidson's fixative, paraffin embedded, sectioned at 7 μm in longitudinal, horizontal and sagittal planes, and stained with Harris' haematoxylin and eosin or Lillie's silver oxide method for reticulin and collagen. Fish, exposed for 51 days to 16·6 μg/l trifluralin and allowed to depurate for 41 days, were radiographed for spinal column study. Stock trifluralin was also analysed to detect the presence of contaminants (such as nitrosamines; see acknowledgment, p. 41).

Results

Histological study of longitudinal horizontal sections of 28-day-old fish, exposed to 5·5, 20 and 31 μg/l trifluralin throughout their early development (from zygote) revealed an extreme dysplasia of vertebrae: the dominant feature was hyperostosis (Fig. 1a), primarily involving acellular bone. Control fish of the same stage of development and feral fish of approximately the same size had normal, thin vertebral walls (from 5 to 6 μm thick) (Fig. 2). Trifluralin-exposed fish had vertebrae with hypertrophied walls (from 18 to 75 μm thick); in some instances, bizarre hypertrophy resulted in wall thicknesses of over 100 μm (Fig. 3). All exposed fish examined (10 fish for each of the above concentrations) had hypertrophied vertebrae; all controls (10 fish) were normal. The second test exposure to 16·6 μg/l trifluralin produced the same results in 28-day-old fish. All exposed fish, but no control fish had the vertebral dysplasia. Furthermore, fish exposed in the second test for 51 days (from zygote to adults) had a more pronounced dysplasia of their vertebrae than fish exposed for only 28 days. A few fish that survived 51 days exposure to 16·6 μg/l trifluralin were then depurated for 41 days and showed no increase in vertebral dysplasia after cessation of exposure. However, residual damage in the form of malformed and displaced vertebrae was revealed by radiographs (Fig. 6). Other bony elements in the fish showed no obvious effects from exposure to trifluralin. Cranial bone and cartilage appeared normal.

The salient cytological and histological findings in the exposed fish are best illustrated in Figs 1(a), 1(b), 3, 4, 5, 7 and 8. Foci of scleroblasts (probably osteoblasts and fibroblasts) were abundant in the zygapophyseal region of the amphicelous vertebrae (Figs 1, 3, 7, 8). Considerable reticulin (Fig. 5) was present surrounding the osteoblasts, demonstrating the genesis of new bone or bone precursors. The abundance of osteoblasts and fibroblasts reflected an abnormal persistence or proliferation of these cells (Figs 7, 8). Osteoblasts or osteocytes were absent in the vertebrae (Fig. 2) of control or feral fish of the same stage of growth. A striking histological feature of the hypertrophied vertebrae was the marked presence of cellular notochord in the central canal (Figs 1, 3, 4, 7, 8). In contrast, notochord in comparable control fish regressed and usually appeared vestigial (Fig. 2). Abnormal processes of vertebrae in their zygapophyseal and central regions further characterized advanced hyperostosis in the fish exposed for 28 and 51 days (Figs 3, 4, 7).

Pathological effects of the dysplasia included: (1) dorsal outgrowth of vertebrae into the neural canal (Fig. 3), thus compressing the spinal cord, (2) ventral outgrowth of vertebrae, thus compressing the mesonephric ducts draining the kidneys (Fig. 4) and (3) fusion of vertebrae (Fig. 3), resulting in apparent loss of somatic flexibility.

A significant increase in serum calcium concentration occurred only in the pooled serum sample from adult fish exposed for 4 days to trifluralin. Serum calcium concentrations in this sample (27·4 mg/dl) were almost twice that found in control (15·7 mg/dl), feral (15 mg/dl) and kepone-exposed fish (14·8 mg/dl) samples.

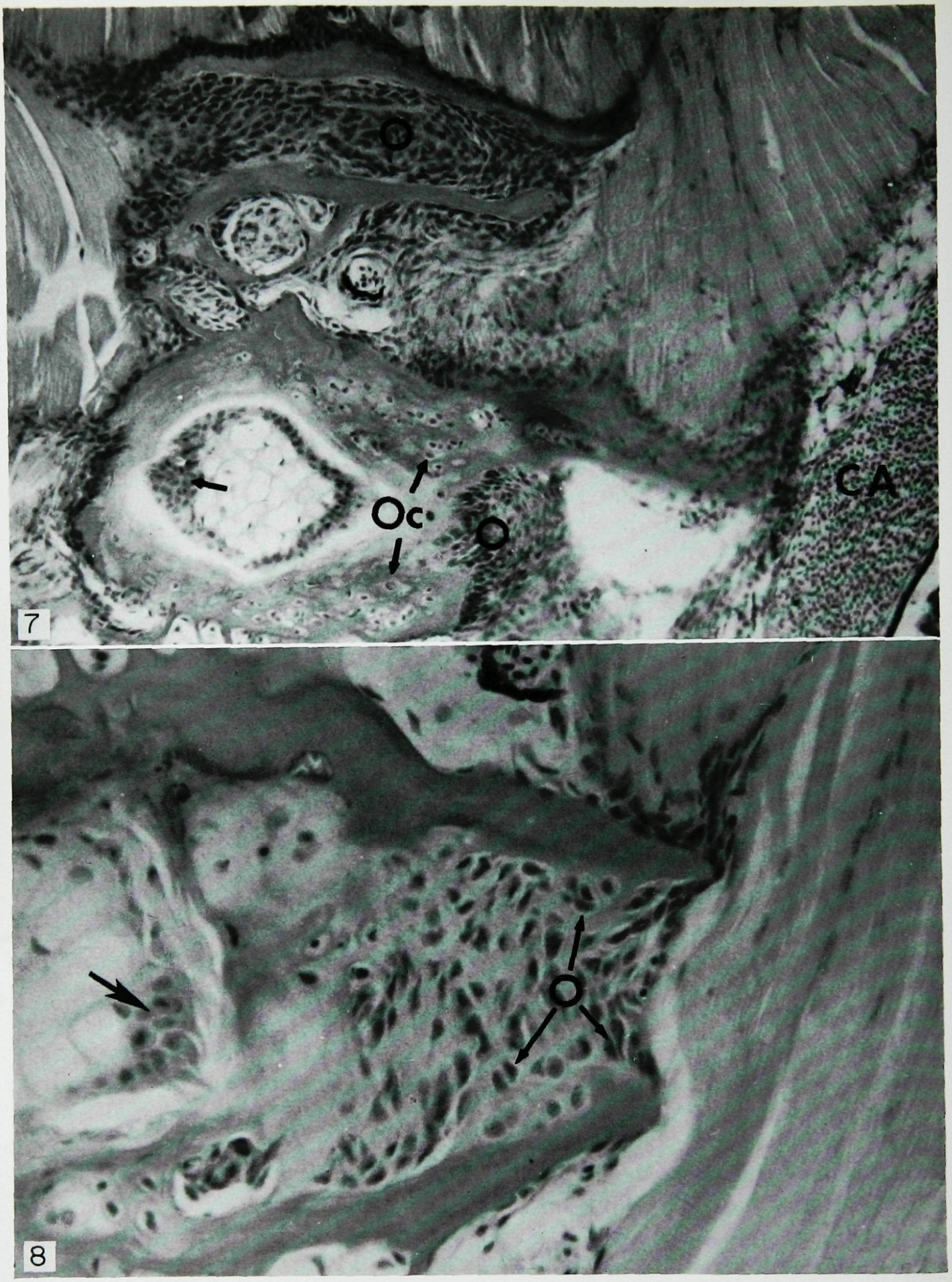

Figure 7. Hypertrophied vertebra of trifluralin-exposed-fish showing osteocytes (Oc) and intense accumulation of fibroblasts and osteoblasts (O), caudal artery (CA); note nucleated notochord cells in peripheral position (arrow) (×400).

Figure 8. Higher magnification of osteoblasts (O) in vertebral zygapophysis of trifluralin-exposed fish; note relationship between nest of osteoblasts and notochord (arrow) (×1200).

Discussion

Suketa, Mikani & Hayashi (1977) reported a significant increase in calcium content (14·3 × normal) of the kidneys in rats given a single large dose of fluoride. We suggest a possible relationship between the relatively high concentration of fluorine in exposed fish that concentrated up to 123 mg/kg trifluralin (whole body) in 10 days (16·6 μg/l trifluralin exposure) and the rather rapid rise in serum calcium concentration. Fluorosis in man and other animals leads to osseous changes and abnormalities correlated with elevated calcium concentrations in tissues (Suketa *et al.* 1977; Roholm 1937). A difficulty arises here if we propose fluorosis as a mechanism in that fluorosis is usually related to exposure to the ionic fluorides (fluoride salts) rather than to non-ionic fluorine. However, in view of the osseous changes found in the growing, trifluralin-exposed fish, serum calcium elevation in exposed adult fish, and the relatively high concentration of trifluralin in exposed fish, we suggest the possible risk of osseous dysplasia in other vertebrates exposed to unusually high concentrations of trifluralin, particularly in early ontogeny.

It is possible, but not probable, that contaminants (such as nitrosamines) in the trifluralin actually induced the condition. Other cellular or molecular mechanisms may also explain the vertebral dysplasia. The Corpuscles of Stannius and the ultimobranchial glands are known to control calcium concentrations in teleost fishes as the parathyroid glands do in other vertebrates (Hoar & Randall 1969). Possibly trifluralin has a direct effect on the cells of the Corpuscles of Stannius or ultimobranchial glands in sheepshead minnows, thus influencing their hormonal control on calcium utilization and compartmentalization in the fish. The above would hardly explain the continued high activity of fibroblasts and osteoblasts. Another possibility is that trifluralin could have a direct or indirect stimulatory effect on the osteogenic cells by mimicking chronic hypervitaminosis A (Clark 1977) or by inducing direct stimulation of osteogenic tissues, thus causing the hypertrophy of vertebrae. Such effects on fish in nature would reduce their individual survival potential, particularly in regard to escape from predators and in competition for prey. Reproductive behaviour (courtship, etc.) would probably be inhibited in dysplastic individuals.

The consistent vertebral dysplasia (hyperostosis) produced in the sheepshead minnow by exposure of early life stages to trifluralin suggests an animal model to study the effects of compounds on skeletal systems of vertebrates, particularly in their early development.

Acknowledgments

We wish to thank Dr W. Bontoyan USDA, Beltsville, MD, for analysis of our stock trifluralin for the presence of nitrosamines. His findings showed 6 μg/g dipropylnitrosamine in our original trifluralin powder. When this powder was diluted to 50 μg/l or less in seawater, the resulting concentration of dipropylnitrosamine was

0·30 ng/l or less, an extremely low concentration. We, therefore, do not consider the nitrosamine contaminant to be related to the vertebral dysplasia.

Contribution No. 346, Environmental Research Laboratory, Gulf Breeze, Florida, USA.

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