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The killifish *Nothobranchius rachowi*, a new animal in genetic toxicology

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Summary

Nothobranchius rachowi, a tropical fish that belongs to the family of the Cyprinodontidae, is introduced as a new animal for genetic toxicological studies. The karyotype of *N. rachowi* consists of 16 large chromosomes. This species can be used for studies on chromosomal aberrations as well as for observations on sister-chromatid exchanges (SCEs). Exposure to 120 mg ethyl methanesulphonate per litre water induced 0.66 SCEs per chromosome, whereas the spontaneous frequency amounted to 0.10 SCEs per chromosome. A comparative study with *Umbra pygmaea* indicated that the sensitivity for this kind of mutagen is the same in both species. After exposure of *N. rachowi* to 50 mg cyclophosphamide per litre of water, 0.35 SCEs per chromosome were induced, showing that promutagens could be detected. It is postulated that *N. rachowi* can be used for screening both pure compounds and surface water for genotoxic potential. An advantage of *N. rachowi* over Umbra spp. is that the former species is more likely to breed under laboratory conditions.

In previous studies, Umbra spp. have proved to be appropriate models for genetic monitoring of toxic substances in water (Kligerman et al., 1975; Kligerman, 1979; Prein et al., 1978; Alink et al., 1980). For genetic toxicological research with Umbra spp. the specimens need to be captured from the wild, because breeding of these fishes under laboratory conditions is difficult. Moreover, the time required for an experiment is rather long and only small numbers of cells in metaphase show differential staining. Therefore, we looked for an alternative. According to the literature (Post, 1964), there are only few species of fish with karyotypes suitable for genetic toxicological studies. Apart from Umbra spp., one of the few species with a relatively small number of chromosomes of fair size is *Nothobranchius rachowi*

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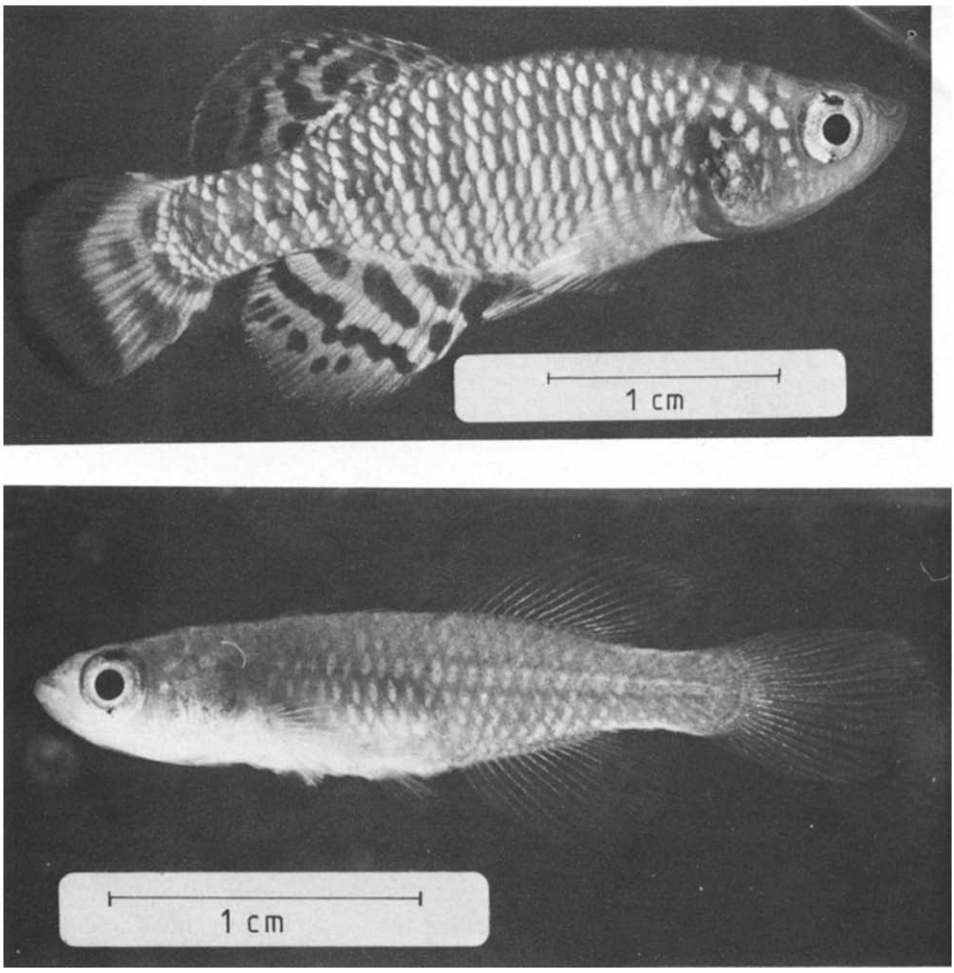

Fig. 1. *Nothobranchius rachowi*. Top, a male specimen; bottom, a female specimen.

(Fig. 1). Therefore, this species was chosen for the present study.

As we reported previously (10th Annual Meeting of the European Mutagen Society, 14–19 September 1980, Athens), preliminary studies indicated that ethyl methanesulphonate (EMS) induced SCEs in this species (Van der Hoeven et al., 1981). In this paper the results obtained with EMS are reported in more detail. Furthermore, the sensitivity of *N. rachowi* for the induction of SCEs by the promutagen cyclophosphamide was determined. A number of *Umbra pygmaea* specimens were subjected to EMS for comparison.

Materials and methods

Nothobranchius rachowi

Nothobranchius rachowi is a tropical fish that belongs to the family of the Cyprinodontidae of the order of Microcyprini or tooth-carp (Ahl, 1926). *N. rachowi* belongs to a group of Cyprinodontid fish known as "annual" fish. These animals are found in Africa and South America in temporary bodies of water that dry seasonally. The population survives the dry season as embryos encased in the dry substrate. Under laboratory conditions the eggs may be kept in almost dry peat for 9 months at room temperature. The species reaches its adult stage 6 weeks after hatching. Males reach a length of 3.5–4.5 cm, and females, which are slightly smaller, reach a length of 3.0–3.5 cm. The genus Nothobranchius occurs along the coastal lowlands of East Africa. The strain used in the present study was obtained from a tropical-fish -breeding farm in The Netherlands. It originated from a sample of fish taken from the Kruger National Park in South Africa about 30 years ago.

Sister-chromatid exchange test with N. rachowi

At the start of each experiment, 2 male fish were placed in 1 l of tap water. To the water was added 150 mg 5-bromo-2'-deoxyuridine (BrdU; Sigma Chemicals) and the compound under investigation. Ethyl methanesulphonate (EMS; Merck-Schuchardt) was dosed directly into the water, whereas cyclophosphamide (Asta-Werke AG, Germany) was dissolved in a small amount of water before application.

The fish were fed throughout the experiment with living chironomid midge larvae. Experiments were carried out at a water temperature of 25–27°C. Colchicine (50 mg/l; Fluka) was added to the water 44–64 h after the start of the experiment. The fish were decapitated 6 h after addition of the colchicine, and the gills were taken out (Fig. 2). The gills were placed in a 0.4% hypotonic solution of KCl for 30 min. The tissues were then fixed in a methanol–acetic acid mixture (3 : 1). Cell preparations were made by the solid-tissue technique as described by Kligerman and Bloom (1977). The cells were dried for at least 18 h and then stained according to a

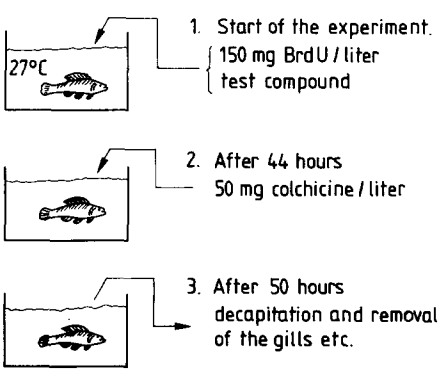

Fig. 2. Experimental protocol for the sister-chromatid exchange technique with *Nothobranchius rachowi.*

modified FPG technique (Perry and Wolff, 1974). Preparations were first treated with Hoechst fluorochrome 33258 (5 μg/ml) in phosphate buffer (pH 7.0) for 10 min in the dark, rinsed in distilled water and then exposed to UV radiation (HBO-Hg, 100 W) for 4 h in a phosphate-citrate buffer (pH 7.0). Subsequently, preparations were heated in 2 × SSC at 60°C for 25 min and stained in 5% Giemsa in Sörensen's buffer (pH 6.8) for 10 min. The preparations were dried overnight, and SCEs were scored.

Sister-chromatid exchange test with U. pygmaea

For the induction of SCEs in *U. pygmaea* in vivo, the sister-chromatid differentiation technique as described by Kligerman and Bloom (1976) was used. Each fish was placed in 1 l of tap water (20°C), injected once intraperitoneally (i.p.) with 0.5 mg BrdU (dissolved in Hanks' balanced salt solution, HBSS) per gram fish. After injection of BrdU, EMS was added to the water. After 112 h the fish were injected i.p. with 0.25 mg colchicine (dissolved in HBSS) per gram fish, and decapitated 8 h later. The gills were taken out and treated in the same way as described for *N. rachowi.*

Analysis of data

From each fish a cell suspension of the gill tissue was prepared. 3 droplets of this suspension were put on a microscope slide (20 cm^2), according to the method of Kligerman and Bloom (1977). 3 microscope slides were prepared per fish. For *N. rachowi* at least 10 metaphases containing 12–16 chromosomes were scored, and for *U. pygmaea* these numbers were 7 and 18–22, respectively. The SCE frequency was calculated by dividing the total number of SCEs by the total number of chromosomes scored per fish. Dose–response curves were plotted for each experiment using linear regression. Differences between the dose–response curves obtained for *N. rachowi* and *U. pygmaea* were statistically analysed by analysis of variance.

Results

As Fig. 3 shows, the karyotype of *N. rachowi* consists of 16 large chromosomes. This confirms the earlier observation by Post (1964). At the water temperatures used, maximal numbers of cells showing differential staining were found after between 50 and 96 h of exposure to BrdU. At a water temperature of 25°C an exposure time of 70 h was used; at 27°C this was 50 h. A rather large variation was found in the percentage of cells showing differential staining for different individuals. At an exposure time of 70 h, percentages varied from 15 to 50% per fish. At an exposure time of 96 h, metaphases were found that had divided more than twice. At least 100 cells in metaphase (first division) were scored for each fish. The experimental protocol adopted on the basis of these findings is described in Fig. 2.

Table 1 presents the results obtained after exposure of *N. rachowi* to EMS. The concentrations of EMS were 24, 60 and 120 μg/ml. The spontaneous SCE frequency was 0.1 SCE per chromosome. A marked increase in the SCE frequency was found after exposure to EMS.

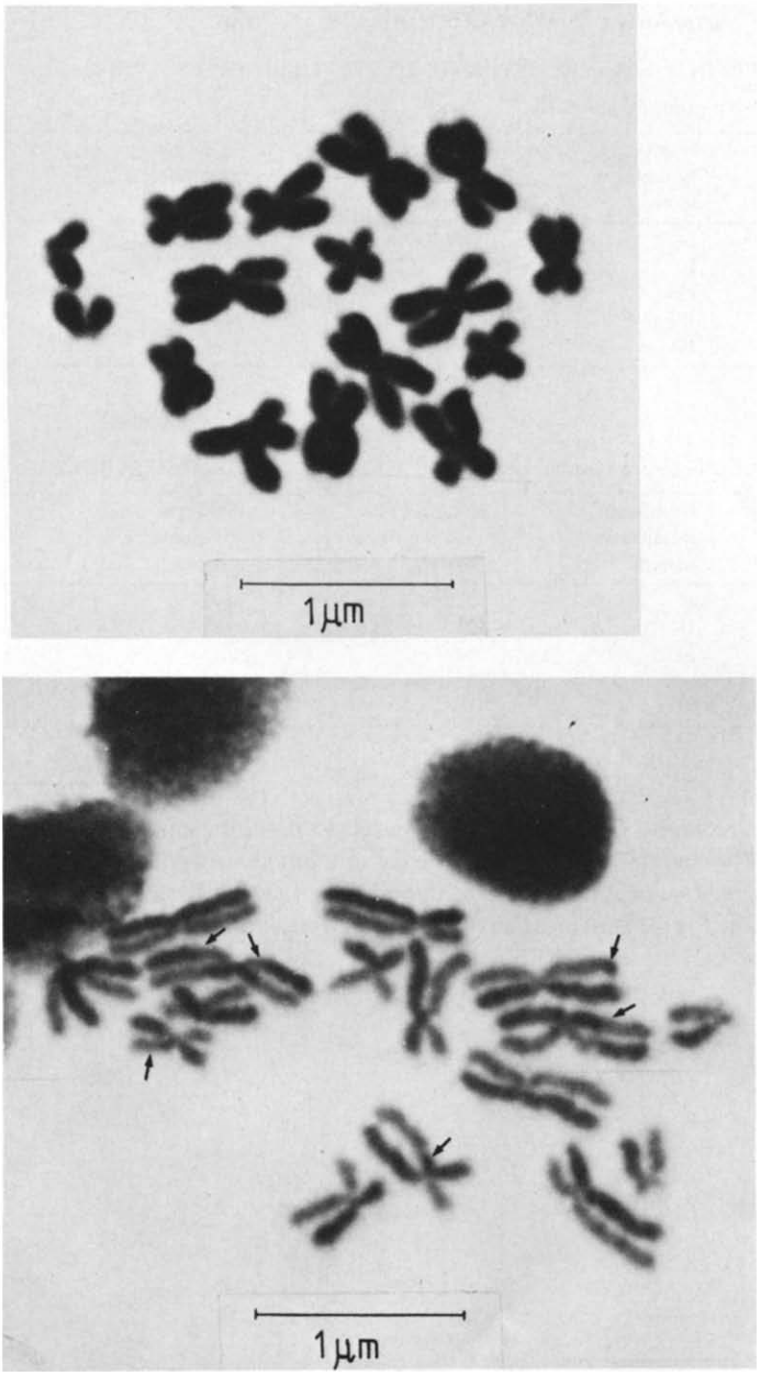

Fig. 3. Metaphase spreads from gill tissue of *Nothobranchius rachowi*. Top, a normal picture; bottom, a metaphase showing differential staining.

TABLE 1

INDUCTION OF SCEs IN *N. rachowi* BY EXPOSURE TO ETHYL METHANESULPHONATE

Water temperature 25°C; exposure time 70 h.

Concentration (μg/ml)	Number of metaphases scored	Number of chromosomes scored	SCEs per chromosome [a]
0	24	360	0.10
24	10	156	0.30
60	12	183	0.26
120	18	277	0.61
120	17	265	0.68

[a] Average per fish.

TABLE 2

INDUCTION OF SCEs IN *U. pygmaea* BY EXPOSURE TO ETHYL METHANESULPHONATE

Concentration (μg/ml)	Number of metaphases scored	Number of chromosomes scored	SCEs per chromosome [a]
0	10	220	0.05
24	18	361	0.37
24	23	449	0.35
120	9	177	0.57
120	7	148	0.68

[a] Average per fish.

In a comparative study, *U. pygmaea* was exposed to the same concentrations of EMS (Table 2). The spontaneous SCE frequency amounted to 0.05. The increase after EMS exposure was comparable to that found in *N. rachowi*. The dose–response curves are shown in Fig. 4. Statistical analysis did not reveal a significant difference between the 2 curves.

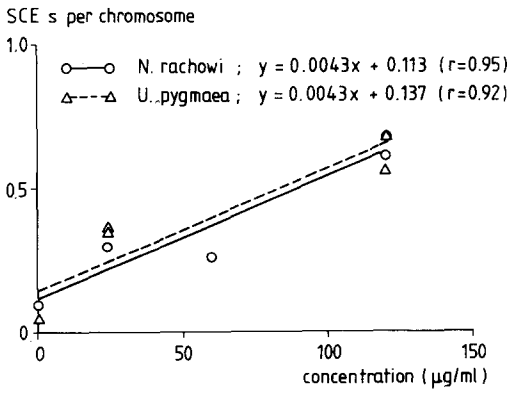

Fig. 4. Dose–response curves for the induction of SCEs in *N. rachowi* and *U. pygmaea* by ethyl methanesulphonate.

TABLE 3

INDUCTION OF SCEs IN *N. rachowi* BY EXPOSURE TO CYCLOPHOSPHAMIDE

Water temperature 27°C; exposure time 50 h.

Concentration (μg/ml)	Number of metaphases scored	Number of chromosomes scored	SCEs per chromosome [a]
0	23	363	0.04
0	29	286	0.04
0	15	226	0.05
2.5	23	344	0.10
2.5	15	221	0.11
5.0	16	250	0.10
10.0	17	245	0.12
25.0	10	156	0.12
25.0	18	280	0.15
25.0	16	235	0.27
25.0	20	303	0.30
50.0	17	258	0.35

[a] Average per fish.

Table 3 shows the results obtained after exposure of *N. rachowi* to cyclophosphamide. This compound was applied at 2.5, 5.0, 10.0, 25.0 and 50.0 μg/ml. The spontaneous SCE frequency amounted to 0.04 SCE per chromosome, and a dose-related increase of the SCE frequency was found (Fig. 5). Although the number of data points is small, the sample regression of SCE on concentration was calculated to give a preliminary idea about the dose–response relationship. On a molar basis, cyclophosphamide was an almost 3 times more effective inducer of SCEs in *N. rachowi* than was EMS.

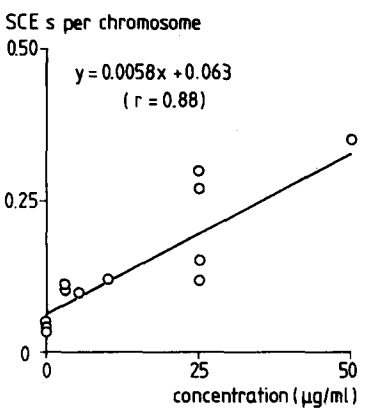

Fig. 5. Dose–response curve for the induction of SCEs in *N. rachowi* by cyclophosphamide.

Discussion

Until now only mudminnows (Umbra spp.) have been used successfully for the assessment in vivo of genotoxic properties of surface water. In this paper we report that *Nothobranchius rachowi* can be used as an alternative to Umbra species.

The use of *N. rachowi* offers a number of advantages over Umbra: a larger number of metaphases per specimen can be scored; the test can be carried out more quickly; the species can be bred comparatively easily under laboratory conditions (almost during the whole year); and the number of chromosomes is only 16 as compared with 22 for Umbra.

One advantage of Umbra over Nothobranchius is that the former can be kept at water temperatures that prevail in the Dutch environment. The 2 species show a similar sensitivity towards the direct-acting mutagen, EMS. Compounds that require metabolic activation, such as cyclophosphamide, can be detected with *N. rachowi*. This has also been reported for *Umbra limi* (Kligerman, 1979). Because the SCE test with *N. rachowi* is rather simple and can be carried out quickly, it is suggested that this species is a useful tool for screening compounds for genotoxic properties.

Acknowledgements

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References

Ahl, E. (1926) Neue oder selten importierte Fische, Bl. Aquarium- u. Terrariumk., 37, 346.

Alink, G.M., E.M.H. Frederix-Wolters, M.A. van der Gaag, J.F.J. van der Kerkhoff and C.L.M. Poels (1980) Induction of sister chromatid exchanges in fish exposed to Rhine water, Mutation Res., 78, 369–374.

Kligerman, A.D. (1979) Induction of sister chromatid exchanges in the central mudminnow following in vivo exposure to mutagenic agents, Mutation Res., 64, 205–217.

Kligerman, A.D., and S.E. Bloom (1976) Sister chromatid exchanges in adult mudminnows (*Umbra limi*) after in vivo exposure to 5-bromodeoxyuridine, Chromosoma, 56, 101–109.

Kligerman, A.D., and S.E. Bloom (1977) Rapid chromosome preparations from solid tissues of fishes, J. Fish. Res. Bd. Canada, 34, 266–269.

Kligerman, A.D., S.E. Bloom and W.M. Howell (1975) *Umbra limi*, A model for the study of chromosome aberrations in fishes, Mutation Res., 31, 225–233.

Perry, P., and S. Wolff (1974) New Giemsa method for the differential staining of sister chromatids, Nature (London), 251, 156–158.

Post, A. (1964) Vergleichende Untersuchungen der Chromosomenzahlen bei Süsswasser-Teleosteern, Z. Zool. Systematik u. Evolutionsforsch., 3, 47–93.

Prein, A.E., G.M. Thie, G.M. Alink, C.L.M. Poels and J.H. Koeman (1978) Cytogenetic changes in fish exposed to water of the river Rhine, Sci. Tot. Environ., 9, 287–291.

Van der Hoeven, J.C.M., I.M. Bruggeman, G.M. Alink and J.H. Koeman (1981) *Nothobranchius rachowi*, a new experimental animal for cytogenetic studies, Mutation Res., 85, 240–241.