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Long-term Fish Toxicity Test Using the Zebrafish: Effect of Group Formation and Temporary Separation by Sex on Spawning

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The "Gesetz zum Schutz vor gefährlichen Stoffen" (act governing protection against dangerous substances in the FRG), in force since January 1st, 1982, and the 6th Amendment of Directive 67/548/EEC on the approximation of the laws, regulations and administrative provisions relating to the classification, packaging and labelling of dangerous substances (79/831/EEC) of September 18th, 1979, provide, in testing level 2, for a long-term study on fish which should also take into consideration the effect of substances on reproduction. This is laid down in the act; however, a test method has not yet been elaborated. It seems reasonable to study in a long-term test the effects of substances on growth and also on gamete formation in fish, as these two parameters not only represent important ecological processes but are also sensitive criteria for the toxicological evaluation of substances.

Whereas previous studies have focussed on growth in commercial fish species, reproduction was less commonly studied. However, it would be difficult to conduct reproductive studies with most commercial fish species, because most spawn only for a short period of the year and because body size of the adult renders them unsuitable for laboratory use. The fathead minnow, Pimephales promelas RAF., and the flagfish, Jordanella floridae Goode and Bean, were used in these studies predominantly in the USA and Canada (Mount 1973; Smith 1973). Both species are relatively small and can easily be kept in aquaria. In Europe, the zebrafish, Brachydanio rerio HAM.-BUCH., is under discussion as a test species. The fish spawns throughout the year. Earlier published experiments as to the reproduction of this species were reviewed by Laale (1977). Unlike the minnow and the flagfish, the zebrafish does not demonstrate aggressive behaviour in large groups.

The fish chosen as a test organism for the chemical

act should be sufficiently sensitive to substances to be representative of as many other fish species as possible. Different studies showed that for most substances tested the sensitivity of the zebrafish was of the same order of magnitude as that of the rainbow trout (Fogels and Sprague 1977; Dave et al. 1981; Newsome 1982; Pérès and Brichon 1982; Calamari et al. 1983).

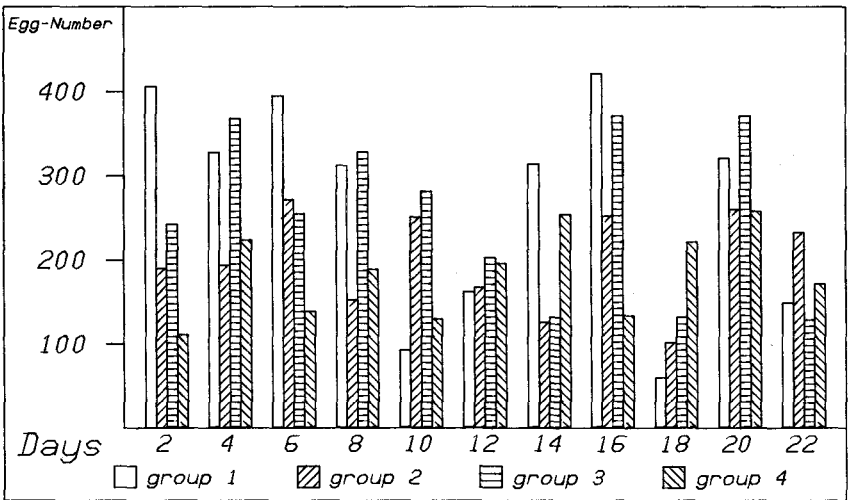
Previous spawning studies with zebrafish were conducted over a period of four months under controlled conditions (Bresch 1982). The quantities of eggs released in successive spawnings sometimes fluctuated considerably, although no trend was detected in the course of the experiments. Groups of male and female zebrafish were kept together continuously and eggs were counted at two-day intervals. The present paper reports results of additional spawning studies with zebrafish in which the number of animals per group and the sex ratio varied and males and females were separated between successive spawnings. It was further intended to discover how widely results vary when the experiments are performed under similar conditions in different laboratories.

MATERIALS AND METHODS

All studies were performed on fish from the Wesu Aquarium, D-3422 Bad Lauterberg, FRG. Only animals from the same batch were used for each experiment. They were between 6 mo and 1 yr old. The fish were fed exclusively with 20 mg Tetramin® per fish per day. Studies were conducted largely as described (Bresch 1982), but the water in the 30L tanks was exchanged continuously at 100 mL/min. In two laboratories, the spawning vessels were covered with stainless-steel net (BASF and Hoechst) instead of plastic net. Water temperature was maintained throughout at 24°C. Water hardness was constant during the experiments but differed among the laboratories (200-380 mg/L as $CaCO_3$, pH 7.5-8.1). The light period was 12 h. Throughout the first series of studies, the sexes were kept together and spawning vessels were present. Eggs were counted every 2 days, or, taking account of weekends, every 3 days. In subsequent series, the sexes were kept separated and only brought together for spawning.

RESULTS AND DISCUSSION

The results of the studies are shown in Figures 1 - 7. The egg counts are drawn versus the time. The results do not differentiate between intact and dead eggs as

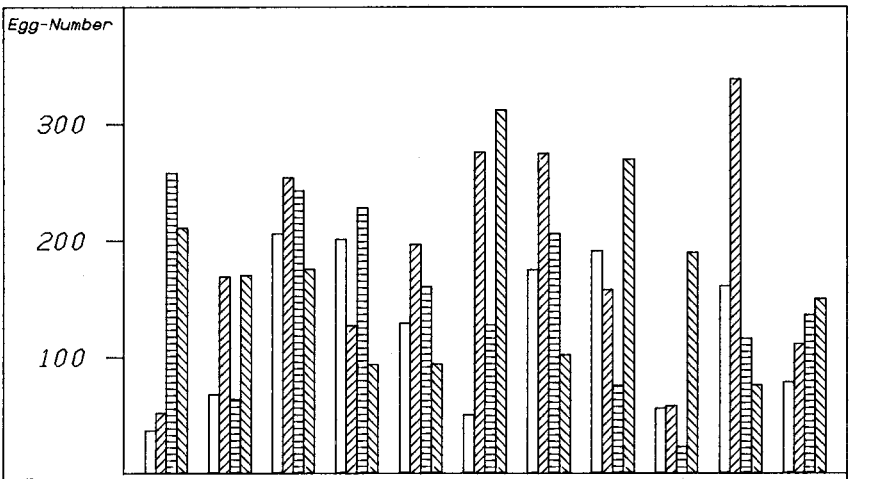


	group 1	group 2	group 3	group 4
E.P.	2959	2200	2813	2028
E.Q.	187	140	179	129

Figure 1. Spawning of zebrafish (Egg counts plotted versus time)
Five females and 5 males per group.
Sexes kept together continuously.
Federal Research Centre for Nutrition (BFE)

separate studies are presently being conducted on this problem. Generally, however, it can be stated that the proportion of dead eggs within 24 hours after spawning was 20-30 %. The data were not evaluated mathematically, because the basic principles for a statistical evaluation have not yet been clarified. Some data are listed in the figures. The overall egg production (E.P.) means the sum of all eggs counted in a group. In order to be able to compare data from different experiments, an "egg quotient" (E.Q.) was calculated expressing the number of eggs produced by one female in one week.

The first experiment was intended to demonstrate the extent to which the egg production of fish from the same batch and kept under similar conditions differs among different laboratories, i.e. how unchangeable conditions in the laboratories, such as the location of the aquaria or water quality, influence spawning. As in the previous study (Bresch 1982), three groups consisting of 10 females and 17 males each were set in parallel. The studies proceeded in all laboratories without any problems for the first two weeks. Subsequently, however, several fish in two laboratories exhibited deteriorated condition, and, for as yet unknown reasons, egg production was reduced. Egg counts



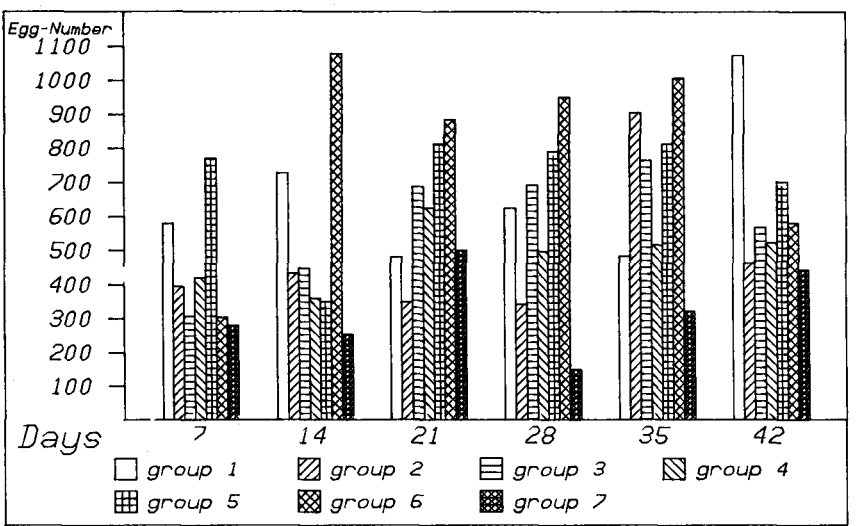
	group 1	group 2	group 3	group 4
E.P.	1352	2015	1636	1843
E.Q.	86	128	104	118

Figure 2. Spawning of zebrafish
Five females and 10 males per group.
Sexes kept together continuously. BFE

in the first two weeks fluctuated by less than 20 % in all laboratories, thus indicating that the fish reacted similarly in all laboratories. Local conditions do not therefore severely influence egg production, provided that all important parameters are constant. This was further confirmed by studies described below.

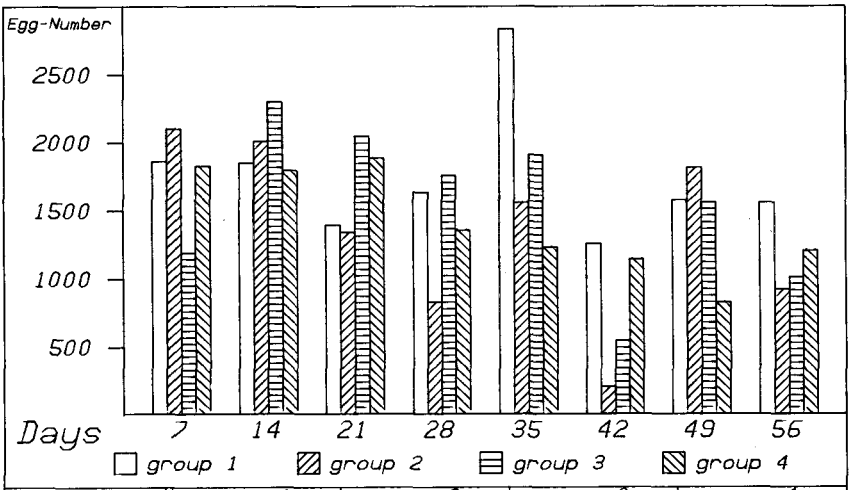
The next study examined the influence of the number of males on spawning. In most reports on zebrafish spawning, the groups contained more males than females. In one of the present studies, a 1:1 ratio (5 females, 5 males) was chosen; in another, the number of male fish was double the number of female fish. The results are shown in Figures 1 and 2. When the number of males was doubled, less eggs were counted. Based on the values indicated in the figures under E.Q., the coefficient of variation is less than 20 % in both studies.

Counting eggs every two days would be very tedious for many laboratories. As previous studies have shown that females do not spawn in the absence of males, it should be determined to what extent egg production is affected if the sexes are kept separated for a while. A six-day separation would be convenient, as eggs would only have to be counted once a week. In the first study, the group consisted of 5 females and 5 males. The sexes



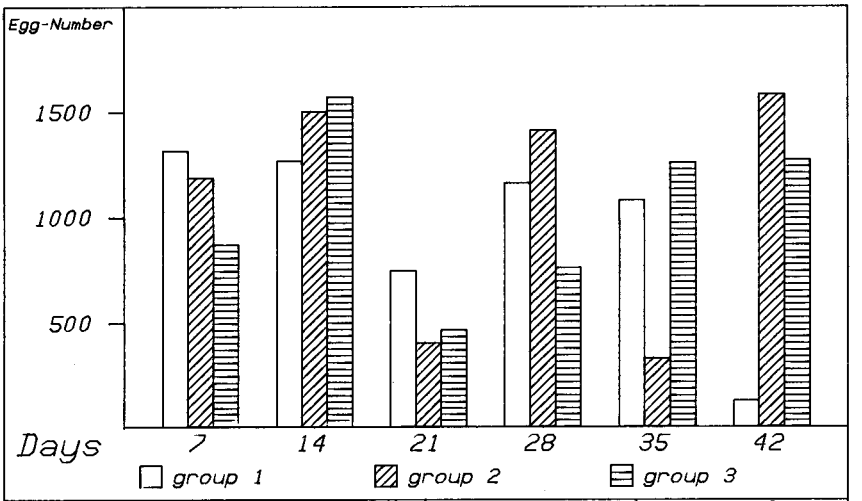
	group 1	group 2	group 3	group 4	group 5	group 6	group 7
E.P.	3966	2891	3466	2939	4238	4803	1950
E.Q.	132	97	116	98	141	160	65

Figure 3. Spawning of zebrafish. Five females and 5 males per group. Sexes kept separated for 6 days. In the afternoon of day 6, spawning vessels were placed in the aquaria and sexes reunited. Vessels were removed in the afternoon of the following day and sexes separated again. BFE



	group 1	group 2	group 3	group 4
E.P.	13972	10794	12331	11278
E.Q.	175	135	154	141

Figure 4. Spawning of zebrafish
Ten females and 10 males per group. The experiment was conducted as indicated in Figure 3. BFE

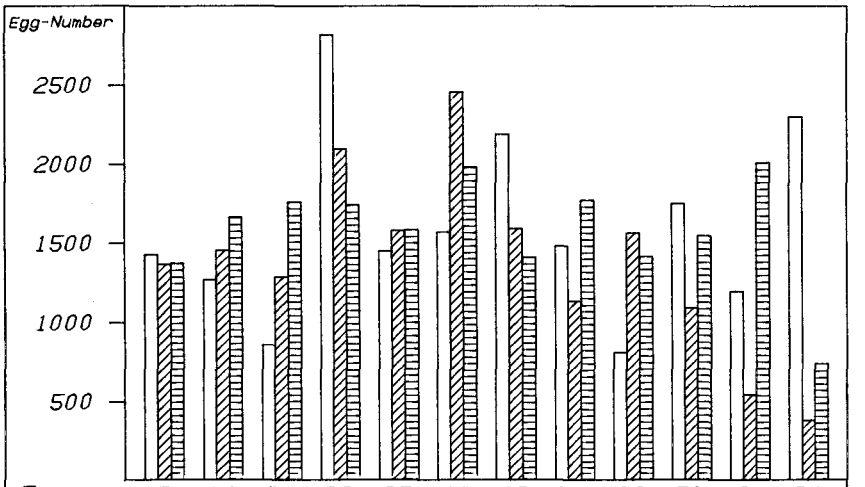


	group 1	group 2	group 3
E.P.	5706	6431	6210
E.Q.	95	107	104

Figure 5. Spawning of zebrafish. Ten females and 10 males per group. The sexes were separated for 6 days. Immediately following reunion of sexes the spawning vessels were installed and left in the aquaria for 24 hours. The sexes were then kept separated for another 6 days. HOECHST

were left together for 24 hours for spawning. The results are shown in Figure 3. The data indicate that the fish produced the same quantity of eggs per week and per female as when kept together all the time. The variation among the groups was greater than in the last study, probably due to chance. To reduce such variation, the number of females and males was doubled in the following study, on the assumption that individual characteristics are less pronounced if the group is bigger. The results in Figure 4 correlated well with those from earlier studies and differences between the groups were less marked. It may therefore be advisable to work with larger groups. In addition to the aforementioned series, other groups also consisting of 10 females and 10 males were assembled in another laboratory. The results are presented in Figure 5. Comparison of the results obtained by both laboratories reveal a difference of 30 % in overall egg counts. Taking account of the complexity of this reproductive study, the results may be considered to conform well. The variation may be due to differing conditions among the laboratories and to the fact that fish came from different batches.

While the aforementioned studies were being performed, other studies were being conducted simultaneously in a

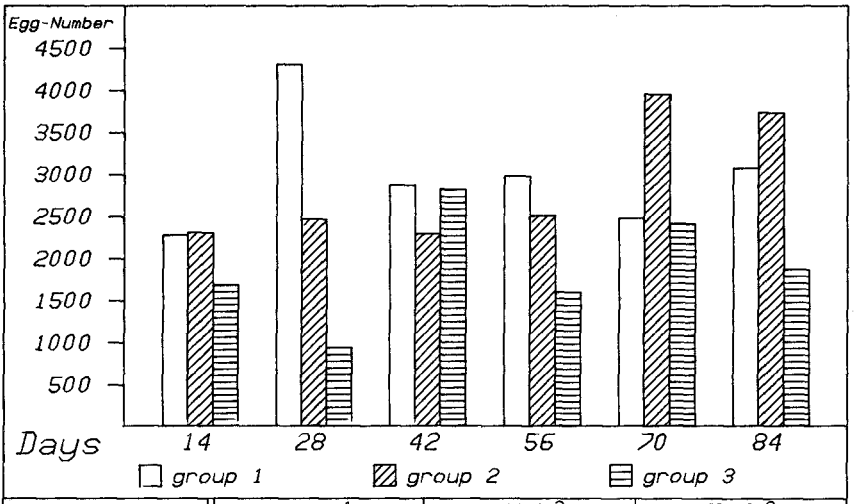


	group 1	group 2	group 3
E.P.	19119	16567	19001
E.Q.	159	138	158

Figure 6. Spawning of zebrafish
Ten females and 20 males per group. The experiment was conducted as indicated in Figure 5. BASF

third laboratory under slightly changed conditions. There, in the first series, 10 females and 20 males per group were assembled and, as in the other laboratories, the sexes were kept separated for 6 days. From the overall egg counts of the groups shown in Figure 6 a coefficient of variation of less than 10 % was calculated. The figures conform to those of experiments already reported with less fish per group. In a second parallel series, the sexes were separated for 12 days and subsequently kept together for 48 hours. Figure 7 demonstrates that about the same amount of eggs per week and per female was registered as in the first series. A separation for a period exceeding 12 days resulted in reduced egg production.

From the studies the following conclusions can be drawn. The amount of eggs released increases in proportion to the number of animals, at least in groups containing 5 to 10 females, the range in which these studies have been conducted. In a group with less than 5 females, individual characteristics of the fish may cause pronounced fluctuations in the quantities of eggs. On the other hand, the upper limit of animals per group is determined not only by the resultant pollution, but also because too many fish in each vessel would cause general disturbance among the



	group 1	group 2	group 3
E.P.	17980	17262	11335
E.Q.	150	144	95

Figure 7. Spawning of zebrafish. Ten females and 20 males per group. The sexes were separated for 12 days. Immediately following reunion of sexes the spawning vessels were installed and left in the aquaria for 48 hours. The sexes were then kept separated for another 12 days. BASF

animals over the spawning substrate.

The differences in the overall egg counts between the experiments conducted with a male : female ratio of 1:1 or 2:1 are not regarded as being pronounced. The extent to which the fertilization rate depends on sex ratio must be investigated in further studies.

If the sexes are kept separated for one week or at most two weeks, egg production rates are not significantly different from those obtained when the sexes are kept together continuously. Separation would mean less work each week, but a longer period would be needed to observe trends, as only four sets of data would be obtained per group in one month; on the other hand, far more data could be recorded if the sexes were kept together and the eggs counted every two days or even daily.

A fundamental principle of toxicity testing is that studies deliver results which are reproducible not only in one laboratory, but also in other laboratories, if conducted under similar conditions. It has been demonstrated here that zebrafish spawning tests conducted under controlled conditions can give similar results in different laboratories.

Egg counts decreased slightly in the course of most studies and may have been due to the diet. This problem will be investigated in further studies.

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