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A Simple Way of Storing Numerical and Metrical Parameters of Fish Progeny for Toxicological Studies by Photograms

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Recently a test was reported to determine the influence of xenobiotics on the reproduction of fish whereby the zebrafish, Brachydanio rerio, was chosen as a model. The number of eggs released at certain intervals was the most important parameter to be determined. The eggs were counted while they were being taken out of the spawning vessel by means of a Pasteur pipette (Bresch 1982). This procedure is time-consuming. A technique by which the eggs are allowed to settle on a glass or plastic support provided with a known number of small holes capable of trapping one egg respectively, probably would be a quicker one (Ozoh 1979). Since it is desirable in toxicological studies not only to count the eggs but also to conduct metrical measurements, we tried to develop a method by which the counting of eggs and of later developmental stages is simplified and which also allows to store the habitus of these stages for evaluation at a later date. The method must not influence the eggs in any negative way as their development is to be pursued later. In principle a photographic procedure could fulfil these conditions. Photographing the eggs is unproblematical and rapidly done; counting and measuring could be postponed to a later date. A photographic method would be completely harmless to the eggs. The simplest way seems to be to photograph the spawning vessel directly, preferably by a miniature camera for economic reasons, and to evaluate the experiment in a paper picture. However, reproduction of the spawning vessels on a 35 mm film and the necessary enlargement of the negative to a size which could be easily evaluated involved a loss of dissolution. But there was still another problem: the vessels indeed contain not only eggs but also disturbing rests of feed and particles of faeces. These disturbing factors should be eliminated before the picture is taken to allow an accurate counting of the eggs on the paper picture. Since the eggs settle at a different speed in water than the particles to be removed, the latter can be eliminated to a sufficient

degree by repeated washing. To obtain a smaller object to be photographed, the washed eggs were placed into a Petri dish of conveniable size. The picture obtained was well suited for a determination of the total egg number, but less so to supply further additional information. Due to the lack of contrast it was difficult to distinguish between living transparent and dead opaque eggs. To determine metrical parameters such as, for instance, the egg diameter or the length of the fish larva, it was necessary to photograph a rule as well together with each object. For this procedure which was found to be time-consuming, also complete dark-room equipment was required. We finally found a simpler way to obtain better reproductions which shall be described in the following.

MATERIALS AND METHODS.

The zebrafish were ordered from the West-Aquarium Bad Lauterberg/West Germany. Eggs were obtained as described (Bresch 1982). The Petri dishes used had a diameter of 20 cm and were made of glass. They were free of visible streaks. Before photographing, the outside of the bottom of the dish was carefully cleaned and dried. As photopaper Agfa-Brovira-Speed BH 310 PE was used. Eggs and larvae were measured under a Wild-M3 microscope. Larvae were sedated with MS 222.

RESULTS AND DISCUSSION.

The eggs on the bottom of the Petri dish or the more advanced stages swimming closely above the ground of the vessel were photostated directly as silhouettes on the photopaper; for this procedure no camera is required. This method which yields very sharp scale pictures containing also structural details of the object in dependence of its transparency, is extremely simple. The photopaper is placed under the Petri dish and exposed to light coming from directly above. Flashlight mounted over the Petri dish at a distance of about 1 m has proven well. If flashlight is used, minor vibrations transferred from the environment to the dish do not influence the sharpness of the reproduction, also swimming stages of fish are conserved as well. The method is quick, particularly if plastic photopaper is used which can be developed, fixed, rinsed and dried within a few minutes. No expensive equipment and no enlarger are needed.

Figure 1 shows a photogram of eggs. It allows to distinguish clearly between living and dead material. The simplest way of counting the eggs is by using a suitable pen for marking each registered egg. Counting by applying a marker connected to an electronic impulse

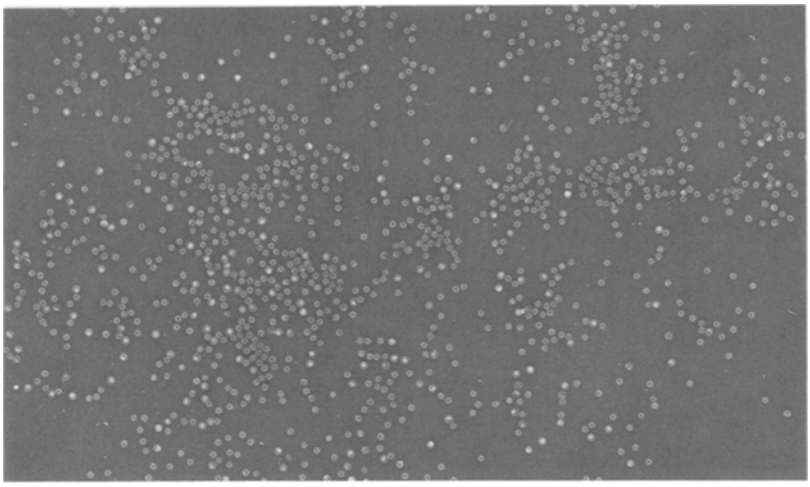

Fig. 1. Photogram of eggs from the zebrafish, Brachydanio rerio. Dead eggs are opaque. Transparent eggs are distinguished by a "black hole" in the centre of the egg, caused by refraction of the light.

recorder is even quicker. Such markers are used by microbiologists to count bacterial colonies in Petri dishes. Rapid counting is facilitated if one makes sure that the eggs lie not too closely together, i.e. that the Petri dish does not contain too many eggs. In the size of the dish we used, 500 eggs should not be exceeded. Counting such a number takes about 5 min.

Table 1. Comparison between direct counting of eggs (B.rerio) from the spawning vessel and counting from the photogram. +Numbers from direct counting: 100%. a transparent eggs, b dead eggs.

Petri Dish	Egg Number Direct		Egg Number Photogram		Deviation+ (%)	
	a	b	a	b	a	b
1	492	46	494	45	+0.4	-2.0
2	374	56	374	56	0.0	0.0
3	333	77	334	77	+0.3	0.0

Table 1 shows that the counting errors are small and negligible. If the spawning vessel contains more than

500 eggs, these should be distributed to several Petri dishes. It should be mentioned that we also tried to produce a picture of the Petri dish by xerography, but the results were not satisfactory. The individual eggs could indeed be recognized, but a differentiation between living and dead individuals was not possible, however.

By using the photogram method, not only the number of eggs can be recorded, but also their metrical parameters determined since the exact dimensions of the eggs are reproduced by exactly vertical exposure of the photopaper to parallel light. As Table 2 shows, the real diameters are practically identical with those in the photogram.

Table 2. Diameter of transparent eggs and of yolk, about 5 hrs. after fertilization (B.rerio). As egg and yolk do not represent perfect spheres, the largest diameter was measured. P1,P2: Measurements done by two persons.

Number of Eggs		49	
Diameter of the Egg	Direct	1.13 (S:0.04)	
	Photogram (mm)	1.14 (S:0.04)	P1
		1.14 (S:0.04)	P2
Diameter of the Yolk Sphere	Direct	0.74 (S:0.05)	
	Photogram (mm)	0.73 (S:0.02)	P1
		0.72 (S:0.03)	P2

The metrical parameters of eggs can be measured directly in the object; measurements from the paper picture are more comfortable, however. Mobile developmental stages cannot be counted and measured without previous narcotization of the individuals. Here the photogram is especially helpful. By using flashlight, the stages capable of swimming may be photographed without narcotics. Whereas chemicals to sedate the fish cannot be applied as often as desired without lasting damage to the animals, exposure to flashlight is harmless, and the procedure may be repeated as often as necessary. However, the silhouettes produced correspond, as the geometrical optics show, to the actual lengths only if

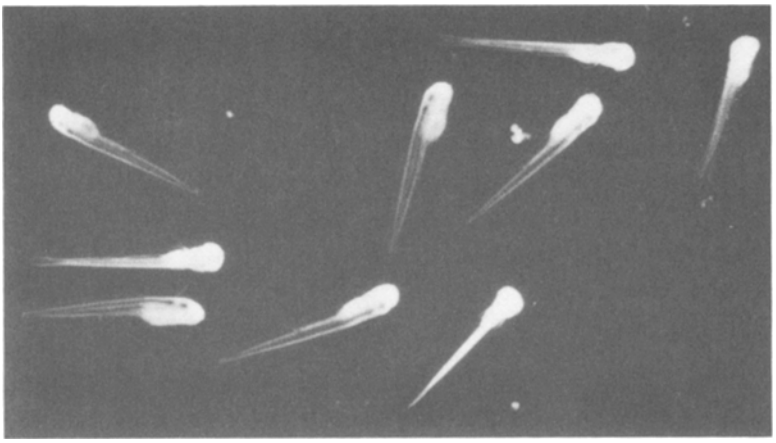

Fig. 2. Larvae of the zebrafish, fixed in a photogram. This picture demonstrates an enlargement (reproduction) out of a photogram and was taken through the microscope. True length of the larvae is about 4 mm.

the fish are swimming horizontally. One can force them to do so by maintaining a low water level in the Petri dish. In 7 days old zebrafish of about 4 mm length, for instance, a water depth of about 2 mm was found sufficient to keep the fish in horizontal position. Figure 2 shows a silhouette of 7 days old zebrafish. As the detailed enlargement out of a photogram demonstrates, the fish already capable of swimming appear as pure silhouettes, whereas the younger individuals, still lying on their sides appear also by transmitted light. In the latter, individual structures such as somites and fins are well recognizable.

The recording of larvae through the picture is of great advantage as far as the measurement of lengths is concerned. Whereas the total lengths of the larvae may be well determined in the photogram, a direct measurement of the total fish length by the microscope is more difficult, as the fins of young stages have practically the same refraction index as water. In order to demonstrate that the larvae stored in the picture appear true to scale, only the distances from head to end of trunk are indicated in Table 3. These distances are clearly visible also by evaluating the larvae directly under the microscope.

These examples show that numerical and metrical parameters of fish eggs and of advanced stages may be rapidly and very precisely stored in a photogram. This is a universal method which may be applied also to

Table 3. Mean length of 7 days old larvae of the zebrafish, measured directly and out of the photogram. Tail fin not included in the measurements. (Total length of the larvae as measured in the photograms 4.37 (S:0.22) mm.)
+Length directly determined: 100%.

Number of Larvae	Length Direct (mm)	Length Photogram (mm)	Deviation+ (%)
85	4.10 (S:0.18)	4.06 (S:0.20)	-0.8

other biological objects. So far the pictures were measured in our laboratory by hand, but evaluation could be accelerated by using an electronic picture analyzer. Reproduction tests in fish combined with an early-life stage test could so be accomplished at little personnel expense.

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