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Growth of Platyfish (*Platypoecilus maculatus*) Free from Bacteria and Other Microörganisms.

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Since 1885 when Pasteur[1] made the statement that no life was possible in vertebrates without intestinal bacteria, there have been sporadic attempts to grow various animals in an environment free from microörganisms. In the early days, these experiments were conducted largely to prove or disprove this point. Schottelius[2] working with chicks, O. Metchnikoff[3] working with frog tadpoles, and Moro[4] working with toad tadpoles, were unable to obtain appreciable growth in their axenic* vertebrates and concluded that an intestinal flora was necessary for the development of these animals. On the other hand, Nuttall and Thierfelder,[5] Kuster (see Wollman), Cohendy,[6] and Wollman[7] were convinced that bacteria are not necessary in view of their experiments with young guinea pigs, a young goat, chicks, and frog tadpoles, respectively. Both Cohendy and Wollman found their axenic animals would live for weeks and that the growth paralleled that of controls. Wollman observed that failure to obtain growth in axenic animals might be due to the experimental procedures, as for instance the high temperatures at which food was sometimes autoclaved—a factor which is now known to be very important.

During the last decade, Reyniers and his associates at the University of Notre Dame[8] have had outstanding success in keeping axenic guinea pigs and chickens until they were several months of age. By employing special technics and equipment, together with carefully prepared and supplemented diets, they have been able to bring these animals to apparent physical maturity. As yet no offspring have been obtained in an environment free from microörganisms. Reproduction in an axenic vertebrate would indicate that the animals are physiologically normal, and multiplication through several generations would be definite proof that among higher animals life is possible without microörganisms.

Many invertebrate animals, both free-living and parasitic—particularly certain protozoa, nematodes, and insects—have been reared through successive generations in media free from microörganisms (See Trager's[9] review). The cultivation of these animals has required special diets and culture

1 Pasteur, L., *Compt. Rend. Acad. Sci.*, 1885, 100.

2 Schottelius, Max, *Arch. f. Hygiene*, 1899, **34**, 210; *Ibid.*, 1902, **42**, 48; *Ibid.*, 1903, **67**, 177.

3 Metchnikoff, O., *Ann. de l'Inst. Pasteur*, 1901, **15**, 631.

4 Moro, *Jahrbuch für Kinderheilkunde*, 1905, **2**, 467.

* The adjective *axenic* is introduced to denote a living organism that is free from all other demonstrable organisms. It is a more convenient term than expressions currently used, *e.g.*, "animals free from microörganisms," "animals free from contaminating organisms," and "animals free from contaminants," and in meaning it is more correct than the adjectives *sterile* and *germ-free*. *Axenic* is derived from two Greek words: *A*—meaning *without* or *free from*, and *Xenos*—denoting a *stranger* or *foreign life*. An axenic organism, as here defined, is a species free from any life apart from that produced by its own protoplasm.

The writers are greatly indebted to Professor A. C. Johnson, Department of Classics, Princeton University, for suggesting and defining the term *axenic*.

5 Nuttall, George H. F., und Thierfelder, H., *Z. f. Physiol. Chem.*, 1895, **21**, 109.

6 Cohendy, Michel, *Ann. de l'Inst. Pasteur*, 1912, **26**, 106.

7 Wollman, Eugène, *Ann. de l'Inst. Pasteur*, 1913, **27**, 154.

8 Reyniers, J. A., in *Micrurgical and Germ-Free Methods*, Charles C. Thomas, Springfield, Ill., (to appear in 1942).

9 Trager, William, *Physiol. Rev.*, 1941, **21**, 1.

conditions. It is reasonable to expect that methods will eventually be found which will permit an axenic vertebrate to grow and reproduce. When one or more species of axenic higher animals have been reared to physiological maturity, both invertebrate and vertebrate material will be available for the study of important biological problems.

Present-day investigators who desire to rear axenic animals have several ends in mind (Glaser).[10] They may be interested in the exact nutritional requirements of an animal without having to consider the role played in the digestion of food by organisms normally inhabiting the digestive tract. They may wish to study the reaction of an animal to a single species of pathogenic agent. Or they may be interested in the mechanism of acquired immunity developing in a host without interference from antibodies resulting from life in a contaminated state.

Warm-blooded, comparatively large laboratory animals were used by all the various workers, with the exception of the experiments with tadpoles; the nutritional and reproductive difficulties should be more readily overcome in some small cold-blooded vertebrate because of the size and ease of handling. A technic was therefore devised for removing axenic young from a small viviparous fish and certain nutritional work was done. A description of the operation and the results obtained are presented.

Material and Methods. The platyfish *(Platypoecilus maculatus)* is a small tropical fish, 4 to 5 cm in length, which is found in abundance in Mexican waters. In this country it is obtained readily from commercial fish hatcheries. The female platyfish bears large numbers of living young (each about 1 cm long), usually about 20 every 4 weeks. Seasonal variations in reproduction have been observed and it appears that the largest number of young are produced in the spring and early summer.

The platyfish was found readily adaptable to certain types of laboratory experiments by Gordon,[11] in his genetical and tumor research, and Baker,[12] in his work on tuberculosis of platyfish. The small size of these fish, their adaptability, and the ease with which they could be kept in a laboratory environment led to the conclusion that they would be ideal subjects for work under sterile conditions. A method of operation was devised by which young could be removed from the mother without contamination.

The scales of the fish are covered by a continuous layer of epithelium that retains all bacteria on a relatively smooth surface (Fig. 2). By application of a germicidal agent to this surface, it was found that an area for operation could easily be made sterile. From studies of sagittal sections, it was known that the embryos are located posterior and dorsal to the intestinal tract (Fig. 3). Hence, by extracting the young through the ruptured walls of the ovary, contamination from the intestines could be avoided.

Pregnant females, determined by obvious distension of the abdomen, were immersed in alcohol for 3 min and then placed in tincture of iodine for 7 min. The iodine was removed by rinsing with alcohol and finally ether, which by evaporation gave a dry, sterile surface. The fish were fastened with a stapler to a board of balsa wood and the scales over the operating area were stripped off with forceps. An incision slightly posterior and dorsal to the anus (Fig. 1) was made in the abdominal wall and a sterile pipette (tip 3 mm in diameter) was introduced into the body cavity. Gentle suction with a rubber bulb caused rupture of the ovary, and drew a number of embryos into the pipette, from which they were transferred temporarily to a Petri dish containing sterile water. This procedure was repeated several times for each female, and usually 10 to 15 young were obtained. All of the young were not removed because it was feared that continued pressure might rupture the intestinal tract and cause contamination. The young

[10] Glaser, R. W., in *Micrurgical and Germ-Free Methods*, Charles C. Thomas, Springfield, Ill., (to appear in 1942).

[11] Gordon, Myron, *Am. J. Cancer*, 1937, **30**, 362.

[12] Baker, James A., *J. Inf. Dis.*, 1942, **70**, 248.

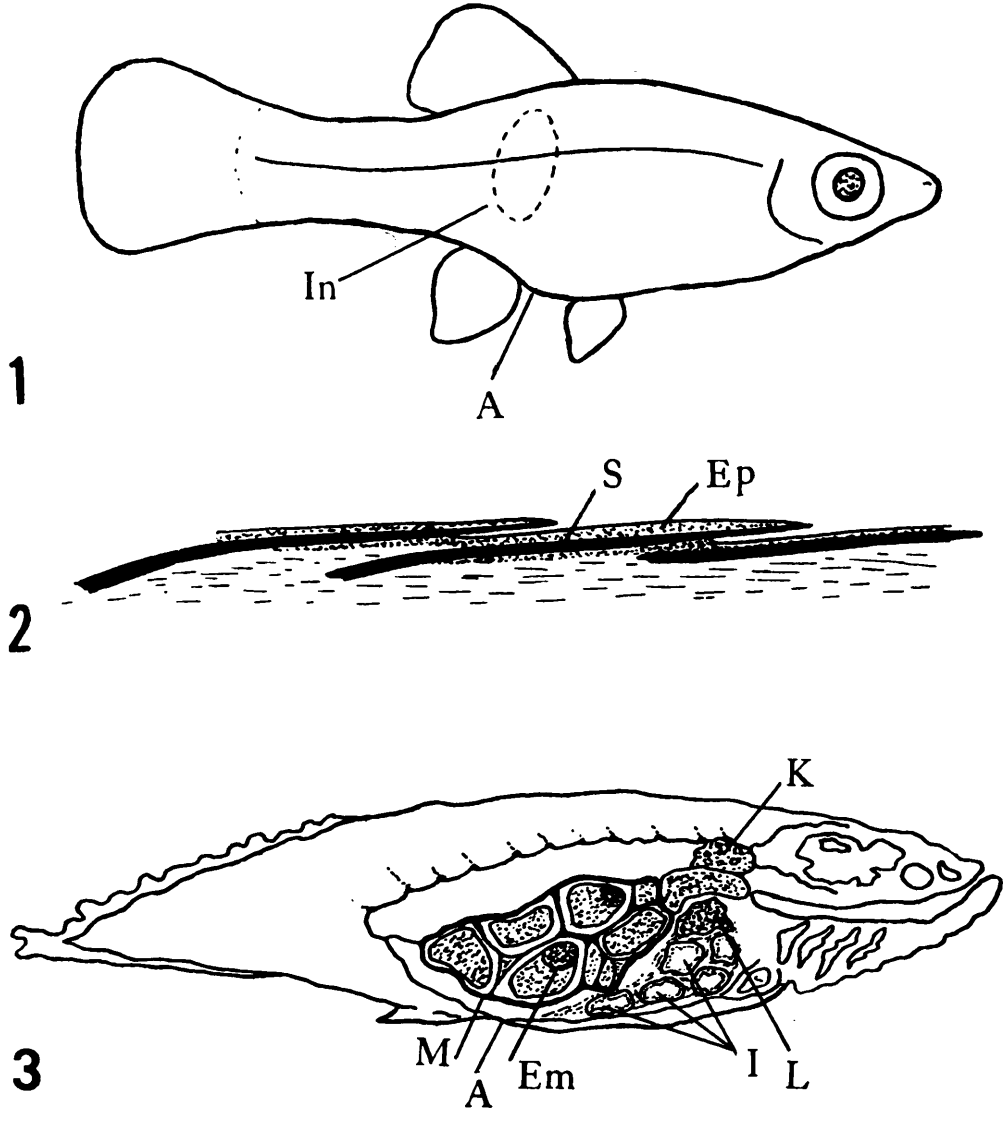

FIG. 1.

The drawings show: (1) Location of incision in body wall for removal of embryos; (2) Relation of epithelium to scales on body surface; (3) Location of embryos in pregnant fish with respect to other visceral organs as seen in sagittal section. A—anus; EM—embryo; EP—epithelium; I—loops of intestine; In—location of incision; K—kidney; L—liver; M—ovarian wall; S—scale.

fish were distributed singly or in pairs into cotton-plugged bottles containing ½ to 2 liters of autoclaved tap or pond water. Small amounts of sterile food were given at semi-monthly or monthly intervals and the fish were observed to eat in a normal manner.

Nutrition. It can be seen from the table that fish fed agar particles lived for 2 to 3 weeks without apparent growth and died when the contents of the yolk sac were depleted. The decline was gradual with progressive emaciation and weakness signaling death. Those fed an autoclaved, commercially prepared fish food, known to be adequate under usual aquarium conditions, lived for a period longer than 2 months and showed some increase in size. Growth was rapid during the first month but it gradually ceased. Thereafter the fish appeared to be in a static condition for 2-3 weeks and then

declined as if unfed. Additional growth and longer survival were obtained in fish given nematodes *(Neoaplectana glaseri)* that had been reared free from microörganisms.[13] A diet of autoclaved fish food plus nematodes was not markedly superior to that of nematodes alone. Nematodes supplemented with heat-killed algæ or autoclaved housefly larvæ proved to be the best diets in that life was maintained longer than 4 months, but they were not completely satisfactory. Pond water showed no advantage over tap water, and a change of water did not promote better growth. Probably certain nutritional requirements were not supplied.

TABLE I.
Effect of Various Foods on Size and Survival of Axenic Platyfish.

Food	Effect on fish: Size, cm	Effect on fish: Survival, days
Agar particles	1	14- 21
Commercial fish food	2	60- 90
Commercial fish food + nematodes	2-2.5	75-120
Nematodes	2-2.5	75-109
Nematodes + heat-killed algæ	2-2.5	93-127
Nematodes + autoclaved housefly larvæ	2-2.5	113-127

[13] Glaser, R. W., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 512.

Bacteriological Examination. Whole fish, as well as samples of the water in which they had lived, were tested for contaminating microörganisms under aerobic and anaerobic conditions by culture in infusion broth and sealed piece-meat media incubated at both room temperature and 37°C. Practically all of the fish had remained free of microorganisms. Molds occasionally developed in some containers, and, more rarely, bacterial contamination occurred. Usually it was possible to determine contamination merely by inspection of the aquariums, which showed molds growing or a marked turbidity when bacteria were present.

Summary. An operative procedure on pregnant platyfish, a small viviparous Mexican fish, was devised that regularly yielded young free from contamination. These axenic fish were maintained under conditions free from microörganisms for as long as 4 months and during this period grew appreciably. Under usual aquarium conditions these fish would have reproduced at this age. Several diets were tested for their effect on maintenance and growth and some of them were definitely superior to others—an indication that nutritional factors may be the principal cause of the lack of continued growth and reproduction.

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Mechanism of Vitamin B_1 Destruction by a Factor in Raw Smelt.

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The destruction of vitamin B_1 by certain species of fish when incorporated into a diet in the raw form has aroused considerable interest. The work of Green *et al.*[1-4] on carp has treated the nutritional aspects of this problem in some detail. Recent work by Woolley[5] on the causative agent of this destruction in fresh carp indicated either an enzymatic degradation or a tying up of the thiamine molecule in such a manner that it is not available to the living organism. Spitzer *et al.*[6] likewise suggest that the destruction may be due to an enzyme.

We have investigated the vitamin B_1 de-

[1] Green, R. G., Carlson, W. E., and Evans, C. A., *J. Nutrition*, 1941, **21**, 243.

[2] *Ibid.*, 1942, **23**, 165.

[3] Green, R. G., and Evans, C. A., *Science*, 1940, **92**, 154.

[4] Green, R. G., Evans, C. A., Carlson, W. E., and Swale, F. S., *J. Am. Vet. Med. Assn.*, 1942, **100**, 394.

[5] Woolley, D. W., *J. Biol. Chem.*, 1941, **141**, 997.