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THE AMAZON MOLLY, POECILIA FORMOSA, AS A MODEL FOR STUDIES OF THE EFFECTS OF IONIZING RADIATION.

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Abstract

THE AMAZON MOLLY, POECILIA FORMOSA, AS A MODEL FOR STUDIES OF THE EFFECTS OF IONIZING RADIATION.

We have suggested that the viviparous teleost, *Poecilia formosa* the Amazon molly may have wide potential use for aquatic radiation studies. The Amazon molly is a naturally occurring gynogenetic species, in which the eggs are activated after mating with the males of closely related species, without the subsequent genetic contribution from the male. The offspring of a single original female constitute a clone, having identical genotypes. Clones of the genetically homogeneous Amazon molly may prove to be equally as valuable to aquatic radiobiologists as the inbred rodent lines have been to mammalian studies.

In many other respects the Amazon molly is a satisfactory laboratory animal. It is robust, easy to rear and has large broods of young when fully grown. Maintenance costs are low. Details are given of the conditions under which our colonies are presently kept.

DISCLAIMER

We have successfully used the Amazon molly in two lines of investigation: to study the molecular effects of cellular damage caused by physical and chemical agents, including irradiation. Secondly in studies of whole body irradiation. These experiments are described.

1. INTRODUCTION

There has been considerable experimental work directed towards assessing the effects of radionuclides upon aquatic ecosystems (reviewed by Templeton et al. [1], Chipman [2], Opel et al. [3]). Yet the data are insufficient to provide an overall evaluation of the findings. Much of this inadequacy is related to the use of different test animals, experimental protocols and statistical evaluations from laboratory to laboratory, so that comparisons are of doubtful validity. Indeed, there are a few instances in which totally opposed results have been obtained with the same species given the same irradiation exposure [4]. There is an acknowledged need for standardization of the biotic and abiotic experimental parameters, so that the data will be strictly comparable.

Studies of the effects of ionizing radiation upon mammals have greatly benefited from the availability of several genetically uniform strains of animals. Controlled inbred strains of mice were started as early as 1909 by C. C. Little and many of the cancer and irradiation studies since have been made with animals from these original strains [5]. The use of rodents, with limited genetic variability has given repeatable, consistent results, using only small numbers of animals in each assay.

A scant number of genetically controlled strains of fish exist (mostly members of the family Poeciliidae) which are the outcome of many generations of sibling matings. The most noted of these are the strains of Xiphophorid fishes (platyfish and swordtails) which have been widely used to study the genetic control of melanophore formation, including the effects of x-irradiation induced mutations upon color patterns [6]. Altogether there is a comprehensive literature describing the determination of color patterns in these fishes. But whether the Xiphophorids would be suitable more generally as animal models for the effects of irradiation upon other organ and tissue systems does not seem to have been looked at in any detail. There have also been several studies of irradiation effects made with inbred strains of *Lebistes reticulatus* [7]. More frequently, however, so called "selectively" bred species or inbred derived lines of aquatic animals have been used, in which there is rather little genetic homogeneity [8].

We suggest here that the teleost fish, *Poecilia formosa*, the Amazon molly, may fill the role of a standard test animal for aquatic radiation studies.

2. THE AMAZON MOLLY, *POECILIA FORMOSA* AS AN AQUATIC ANIMAL MODEL

The Amazon molly has an unusual mode of reproduction which makes it eminently suitable as a laboratory model for aquatic studies, quite the equal of the inbred rodent lines used by mammalian radiobiologists (Fig. 1). This molly is a small viviparous teleost, native to and abundant in the streams and rivers of southern Texas and Mexico. The species exists almost entirely as females; males are extremely rare, only three or four having been found since its first discovery by Hubbs and Hubbs in 1932 [9,10]. The Amazon molly reproduces gynogenetically, the eggs being activated after mating with a male of a closely related species. There is no genetic contribution to the offspring from the male; sperm provides only the stimulus for egg development. The young of a single original female constitute a clone, having identical genotypes. Because clones of this fish are genetically homogeneous, it has none of the attendant disadvantages often encountered in groups of inbred mice. Thus, even closely inbred rodent strains show some variability in response, and after some time separated colonies may differ as a result of gene drift, mutation or contamination of the population. Closely inbred rodent strains also tend to be less robust, less fecund and poor parents compared with randomly bred animals. Generally, they are shorter lived.

2.1. Husbandry

In many respects, the Amazon molly amply satisfies the requirements which are considered desirable in a laboratory animal. It is hardy, easy to rear and breed, and it withstands handling, injection and surgery well. The fish flourishes in a range of salinities, from fresh water to half strength sea water, and at temperatures from 16° to 26°. Clones have been kept successfully in outdoor pools for many years (C. P. Haskins, personal communication). The pools are artifically heated (with plastic covers to retain the heat) temperatures never falling below 16°; a good growth of water hyacinth provides cover for the young fish. The Amazon molly has no unusual food requirements and grows well on a high-protein porridge type food. Large numbers of fish can be housed in little space - currently we maintain fish at a density of 1 fish to a gallon of water. Maintainence costs are low, and we are able to simultaneously conduct several long term experiments. Details of the keeping conditions have been given [11].

For breeding purposes, groups of three or four Amazon mollies are kept together with a single host male fish. The broods produced are large, numbering up to 80 when the fish is fully grown. Isolated females, even those which can see the males do not usually become pregnant and physical contact seems to be essential for reproduction. We have had only two cases of spontaneous parthenogenesis in our clone. Occasionally, the Amazon molly will reproduce sexually. To monitor this happening, we have used for mating purposes the common black molly Poecilia spp. (readily available from most commercial aquaria), so that any hybrid young can easily be recognized by their color pattern (Fig. 1). During the first two years of culture 0.65% hybrids were born; seventy-five of these were produced when the brood number was 70 or greater, and principally during the months of December to May (Fig. 2). In the last two years, no hybrid offspring have occurred.

In early experiments, some fish died from bacterial (Mycobacterium fortuitum) or fungal infections. Isoniazid, at a concentration of 0.7 mg/ml proved an effective prophylactic agent for the bacterium restraining the spread of the disease, though not reversing it. The fungal infection was successfully treated with Maracyn at a concentration of 20 mg/gallon, applied daily for 4 days. In the last two years, the fish have been entirely free from infections and parasites and treatment has been unnecessary. Bacterial levels are held down by pumping the water first through a diatomaceous earth filter and then over a UV purifier at a circulation rate of 25 l/min [11].

We are currently working with a clone of mollies (Haskin's classification Clone 2) collected from a freshwater pool near Brownsville, Texas by C. P. Haskins in 1946 and kept under laboratory conditions since that time. The clone shows minimal differences in physiological response. Under our standard feeding regime fish of the same age show little variability in size (Fig. 3). Further, there is no behavioral hierarchy established within tanks, which might lead to differences in growth within a group, as is common in many species of fish. Poecilia formosa shows little individual variation in fecundity, fish of the same size producing almost equal numbers of young (Table I).

2.2. Genetics

The Amazon molly seemingly arose in nature from hybridization of *Poecilia latipinna* and *P. mexicana* [15]. The species is a permanent diploid, having 46 chromosomes (2n). Diploidy is achieved either by the re-entry of a polar body or doubling of the pre-meiotic chromosome without nuclear division [12,13]. In its normal habitat, *P. formosa* exists as several distinct clones. By means of tissue transplants Kallman [13] identified four separate clones from one geographically isolated population, and his successive surveys showed that the clones remained distinct and constant for at least 9 years. In the wild, new clones are thought to arise from separate matings by the gradual accumulation of mutations.

Significant numbers of unisexual triploid females have recently been found amongst natural populations of diploid Amazon mollies, collected from the headwaters of Mexican rivers [14]. These fish resemble the diploid *P. formosa* and its male hose, *P. mexicana*. Measurements of the amount of DNA have established that these fish are triploids, and that the triploids reproduce gynogenetically, maintaining the triploid genome in their daughters. Rausch and Balsano [14] suggest that stressful environmental conditions may lead to the production of these triploid forms. Breeding strains of the triploid have now been successfully maintained in the laboratory.

3. DEMONSTRATED USE OF THE AMAZON MOLLY IN EXPERIMENTAL STUDIES OF IRRADIATION

We have used the Amazon molly in two related lines of investigation. Firstly, to study the molecular effects of cellular damage cuased by physical and chemical agents, including x-irradiation. Secondly, in parallel studies of whole body irradiation exposure.

3.1. Molecular effects of irradiation of cells

Because there is immunological homogeniety within a clone, tissue and cell transplants between members of that clone survive as long as the recipient fish. Cells taken from specific tissues in donor fish can be treated in vitro, which allows precise physical or chemical measurements of the agent used, the cells then injected into isogenic recipients, and any resulting lesions scored in vivo some time later.

In a first series of experiments using this model, we have identified known specific damage to DNA caused by ultraviolet exposure [15]. Fish cells were exposed to UV radiation and subsequently injected into members of the clone. This caused the development of massive invasive thyroid hyperplasia in the recipients. The results were decisive; thyroid lesions were found in all fish given cells exposed to an average UV fluence of 20 J/m^2, whilst control fish, injected with untreated cells had none. If the UV-irradiated cells were exposed to photoreactivating light before injection, there was a 90% decrease in the number of fish lesions. Fish cells contain high levels of photoreactivating enzyme, and since enzymic photoreactivation only monomerized pyrimidine dimers in DNA and does not affect other UV induced lesions, these results indicated that the presence of pyrimidine dimers in DNA lead to the observed thyroid lesion. We confirmed by biochemical analysis that there is photoreactivation of dimers in DNA of UV-irradiated cells from P. formosa [16].

The results of this study were encouraging. We have therefore used this assay to study the nature of cellular damage caused by ionizing radiation. Again, fish cells were exposed in vitro to either a dose of 250 rad or to 500 rad of gamma rays (dose rate 45 rads/sec). Cell suspensions of the treated cells were then injected into the abdominal cavity of young recipients, and nine months later the animals were examined grossly and microscopically. Two conspicuous gross changes were found; there was development of massive thyroid hyperplasia and hypertrophy, secondly, there were large hemorrhages throughout the body of the treated fish (Fig. 4). From histological sections it was apparent that there had been a reduction in the amount of hematopoietic tissue in the head-kidney and spleen [17]. There were no consistent differences between the groups of fish which had been given 250 rad treated cells and those given cells exposed to 500 rads; in both cases the state and size of the thyroid growth and the extent of the hemorrhages were very similar. There was, however, a difference in the number of fish in each group which showed lesions (Table 2). All of the fish given 250 rad exposed cells were affected, but 28 individuals out of the group given 500 rad cells appeared entirely normal. A further 12 individuals in this group showed only thyroid lesions. We have suggested that the failure of some of the fish injected with 500 rad cells to respond, and the partial response of others, indicated that this dose may have been lethal to many of the injected cells.

We are unable, at present to give a satisfactory explanation for the differences we saw in the lesions induced by UV-irradiated and gamma-irradiated cells. An attractive, but speculative explanation for the difference in the two series is that the response reflects differences in the damage to the cell caused by uv and x-irradiation.

3.2. Whole body irradiation studies

The clinical and histological changes produced by ionizing irradiation of fish partially mimic those in mammals and man, despite considerable morphological differences in their organ systems. Our studies were initially made to compare the induced lesions with those resulting from the injection of x-irradiated cells into fish. They are presently being extended in order to obtain a timing of the onset of irradiation damage and the course of recovery. Groups of twenty, 3 month old, Amazon mollies were exposed to whole body irradiation doses of 1 kR to 7 kR from a Cs^{137} source, at a rate of 1 kR for 15 minutes. Similar groups of black mollies (which we use for mating purposes in our fish cultures) were also given doses of 1, 3, 5, and 7 kR. The mortality curves for both species are shown in Fig. 5. The Amazon molly are relatively resistant to x-irradiation, compared with other species. Thus, Schechmeister [18] found that goldfish, _Carassius auratus_, irradiated with 5 kR were all dead within 11 to 17 days, and within 8 to 27 days after 3 kR. Three inch long young channel catfish, _Ictalurus punctatus_, exposed to 2.5 kR had died by 13 days, and by 30 days after 2 kR [19]. Similarly, fingerling chinook salmon, _Oncorhynchus tshawytscha_, survive less than 30 days after exposure to 2.5 or 5 kR [20]. However, 90% of the group of Amazon mollies treated with 3 kR were still alive after 6 months when the experiment was terminated and all of the group of fish exposed to 1 kR are still living after 10 months.

There were notable histological changes in the hematopoietic system and the intestinal tract of the Amazon molly after treatment at all dosage levels. Marked depletion of hematopoietic tissue in head kidney and spleen was seen in the fish exposed to doses greater than 3 kR, the depletion of circulating blood cells was well seen in sections through the carotid sinus (Fig. 6a, b, c); below this dose the depletion was less marked. Reduced erythropoietic activity was confirmed by blood smears and by kidney imprints. At the lower radiation doses there was progressive recovery of the blood forming tissue, although the fish lived with reduced circulating levels of blood cells for some time before recovery was completed.

All groups of fish showed a marked depression of feeding activity for approximately 10 days after irradiation. Fish dying during this period had extensive degenerative changes in the alimentary tract (Fig. 7). Those fish which survived (at the lower doses) showed evidence of regeneration of the gut, which was eventually complete in groups irradiated with 3 and 1 kR (Woodhead and Scully, in preparation).

4. POTENTIAL USEFULNESS OF THE AMAZON MOLLY

The discovery of the gynogenetic Amazon molly by Hubbs and Hubs in 1932 generated much excitement amongst zoologists, providing the first record of this type of reproduction in any vertebrate. The Amazon molly stood alone as the only naturally occurring unisexual vertebrate until 1959. The majority of investigations made with the species have centered around the usualy reproductive mechanisms of this and other closely related <u>Poeciliidae</u>, and on the origin and development of unisexuality. These studies culminated in the laboratory synthesis of a unisexual Poecilopsid fish by Schultz in 1972 [21].

In other respects, the Amazon molly has been largely neglected. It has been used in a limited number of transplantation experiments. Recently Hart and Setlow [22] selected the Amazon molly for their analysis of molecular damage to DNA by uv irradiation. The molly was chosen for this purpose since it grows in clones, and tissue can be transplanted successfully. During the work, it became apparent that the fish has wide potential research use. In the context of aquatic radiobiological studies, the molly may prove to be equally as valuable as the inbred rodent lines have been to mammalian studies. Further, the Amazon molly is an important, naturally occurring component of the freshwater and brackish ecosystem of the southern United States and Mexico and is not a "laboratory" species only. As such, it should readily lend itself to controlled pond culture experiments, which can start to approach the complexities of the natural environment.

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Table 1. The number of oocytes in different stages of development in the gonads of 3 untreated groups of 3 month old Amazon mollies. The counts were made from sagittal histological sections.

	Minor growth phase oocytes	Maturing oocytes
Group 1	31 to 34	76 to 86
Group 2	29 to 34	70 to 83
Group 3	31 to 35	72 to 86

Table 2. Comparison of the response of fish injected with 250 rad and 500 rad cells. (From Woodhead, Setlow and Hart, in press)

Dose to injected cells (rads)	Percentage with indicated lesions	
	hemorrhage	thyroid
0	0	0
250	100	100
500	60*	72

*All 60 had thyroid lesions.

FIGURE CAPTIONS

FIG. 1. The gynogenetic Amazon molly, *Poecilia formosa*, and its male host, the black molly, together with a hybrid offspring (from Hart, Livesey and Setlow 1976).

FIG. 2. The numbers of isogenic and hybrid young born in two years of culture (from Hart Livesey and Setlow 1976).

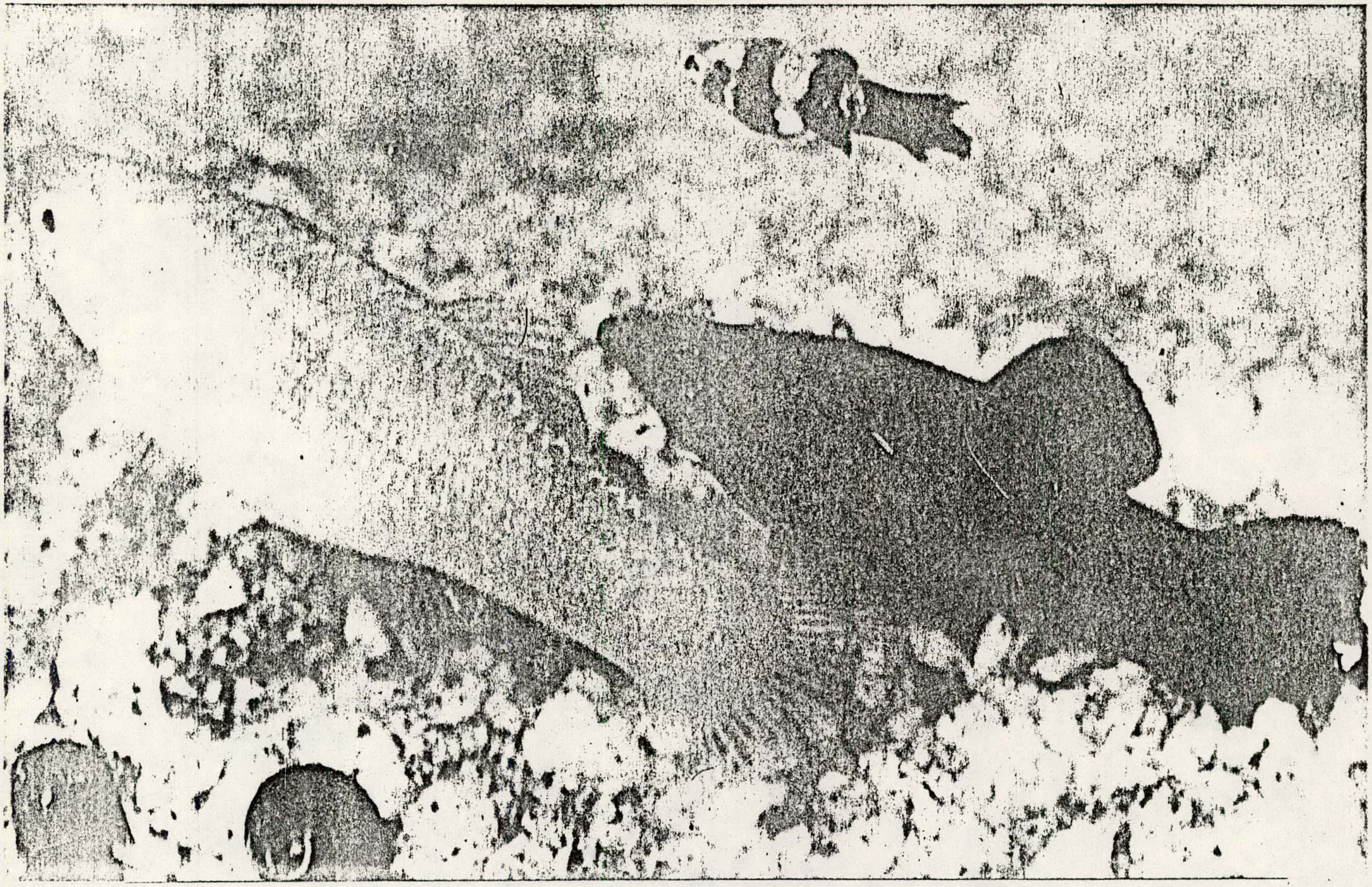
FIG. 3. The length distribution of groups of 50 untreated *P. formosa*. For comparison the length distribution is shown of an inbred untreated line of *Lebistes reticulatus* (Woodhead, unpublished data).

FIG. 4. Dissection of an irradiated fish (a) and a control fish (b) showing massive thyroid hypertrophy and conspicuous hemorrhages in the treated animal. (from Woodhead, Setlow and Hart, in press).

FIG. 5. The mortality curves after various doses of x-irradiation for the black molly (above) and the Amazon molly (below).

FIG. 6. Sections through tissues of an unirradiated Amazon molly (above) and an Amazon molly sampled 60 days after 4 R (below). (a) kidney (b) spleen (c) carotid sinus. These sections show marked depletion of the hematopoietic tissue and circulating blood cells.

FIG. 7. Sections through the intestine of an unirradiated Amazon molly (above) and an Amazon molly sampled 60 days after 4R (below). Degeneration and atrophy of the gut is apparent in the irradiated fish.



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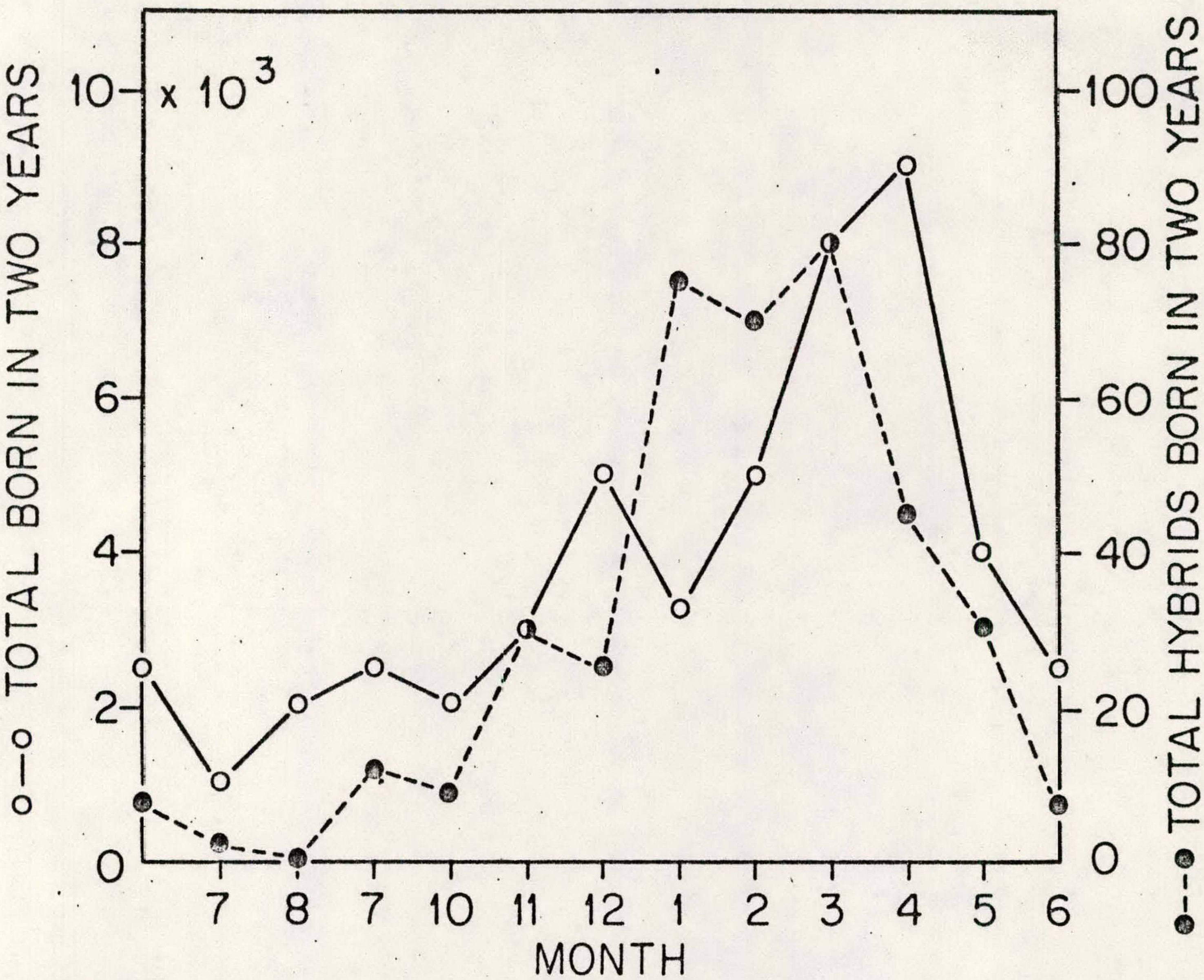

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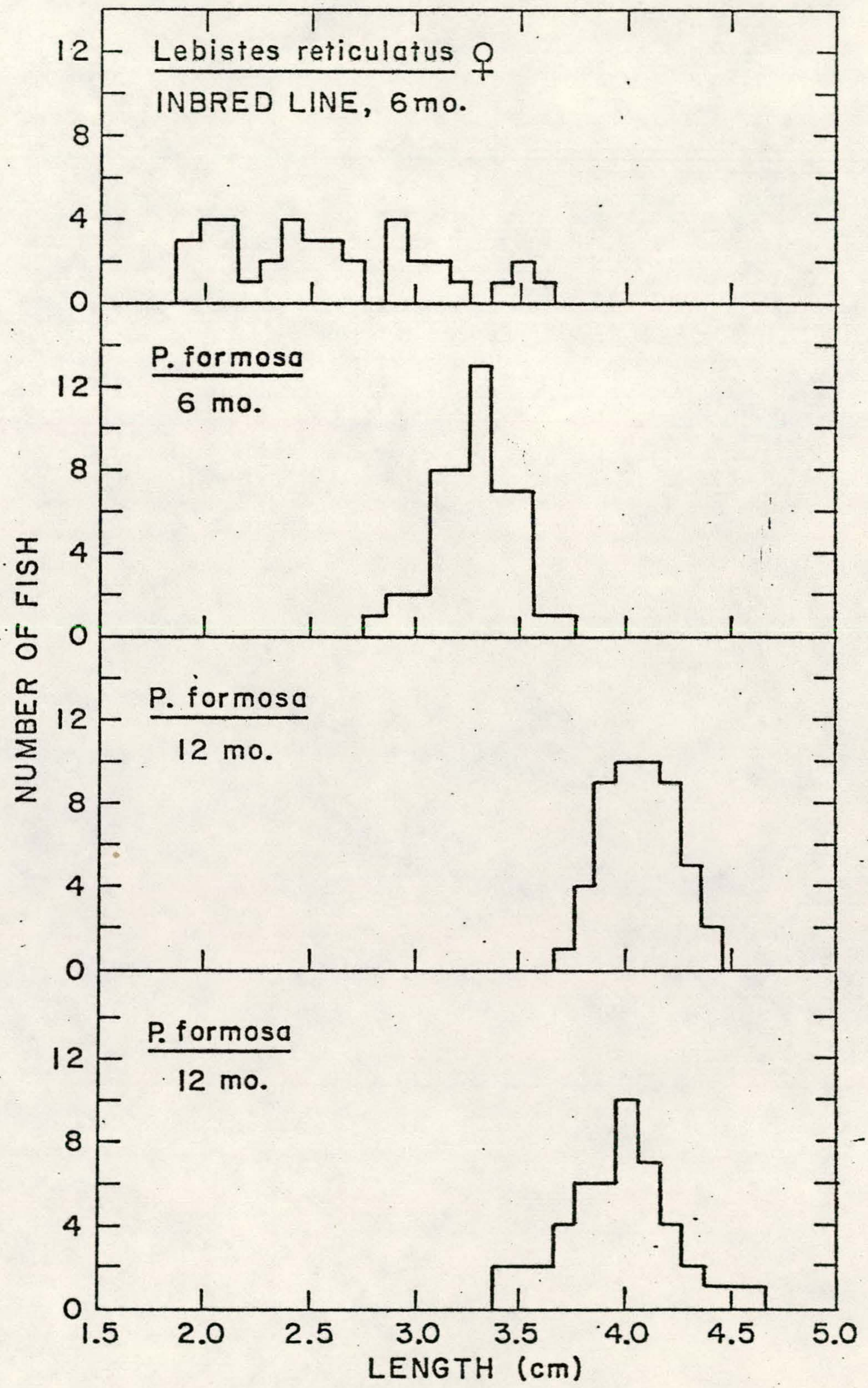

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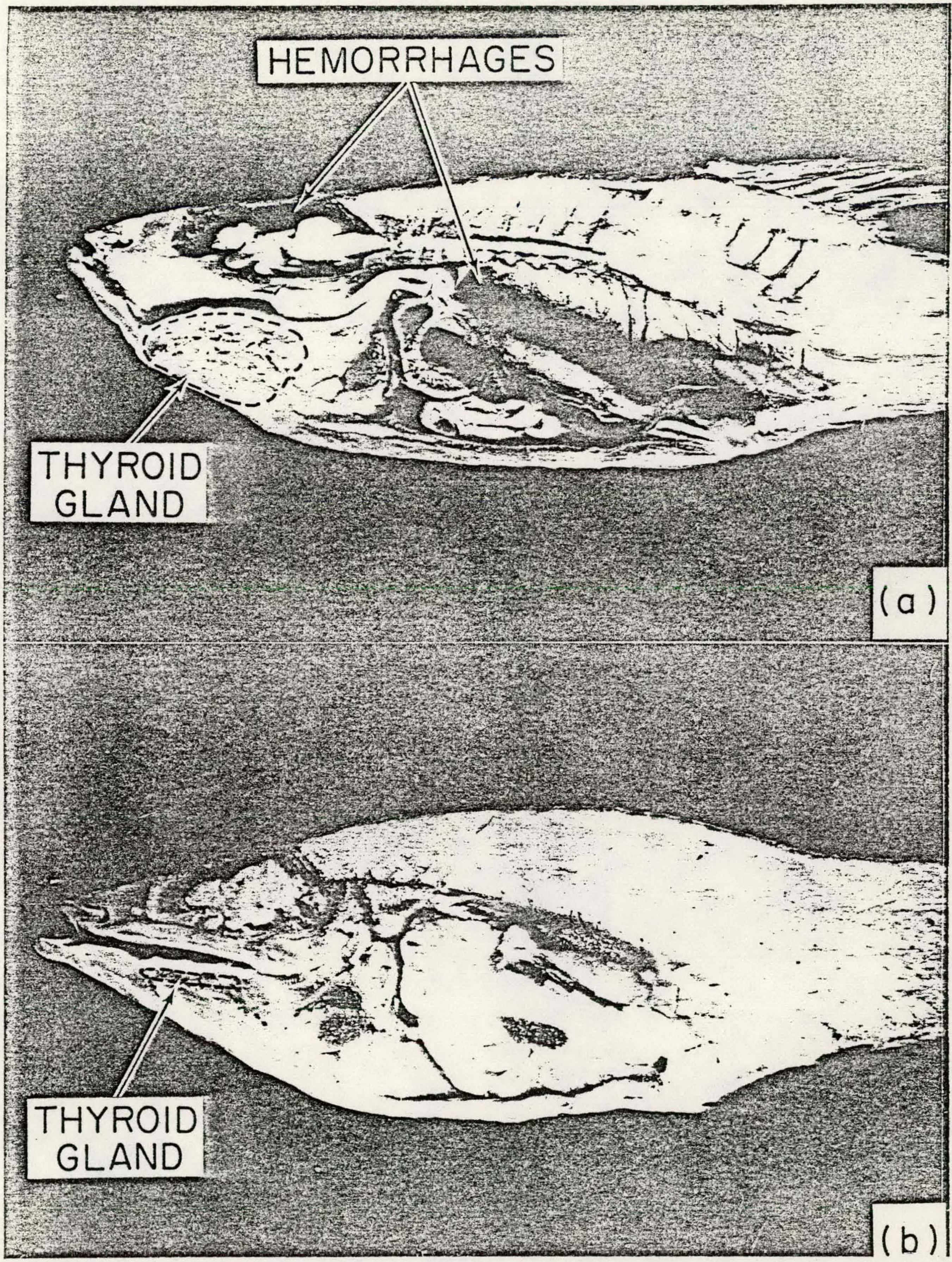

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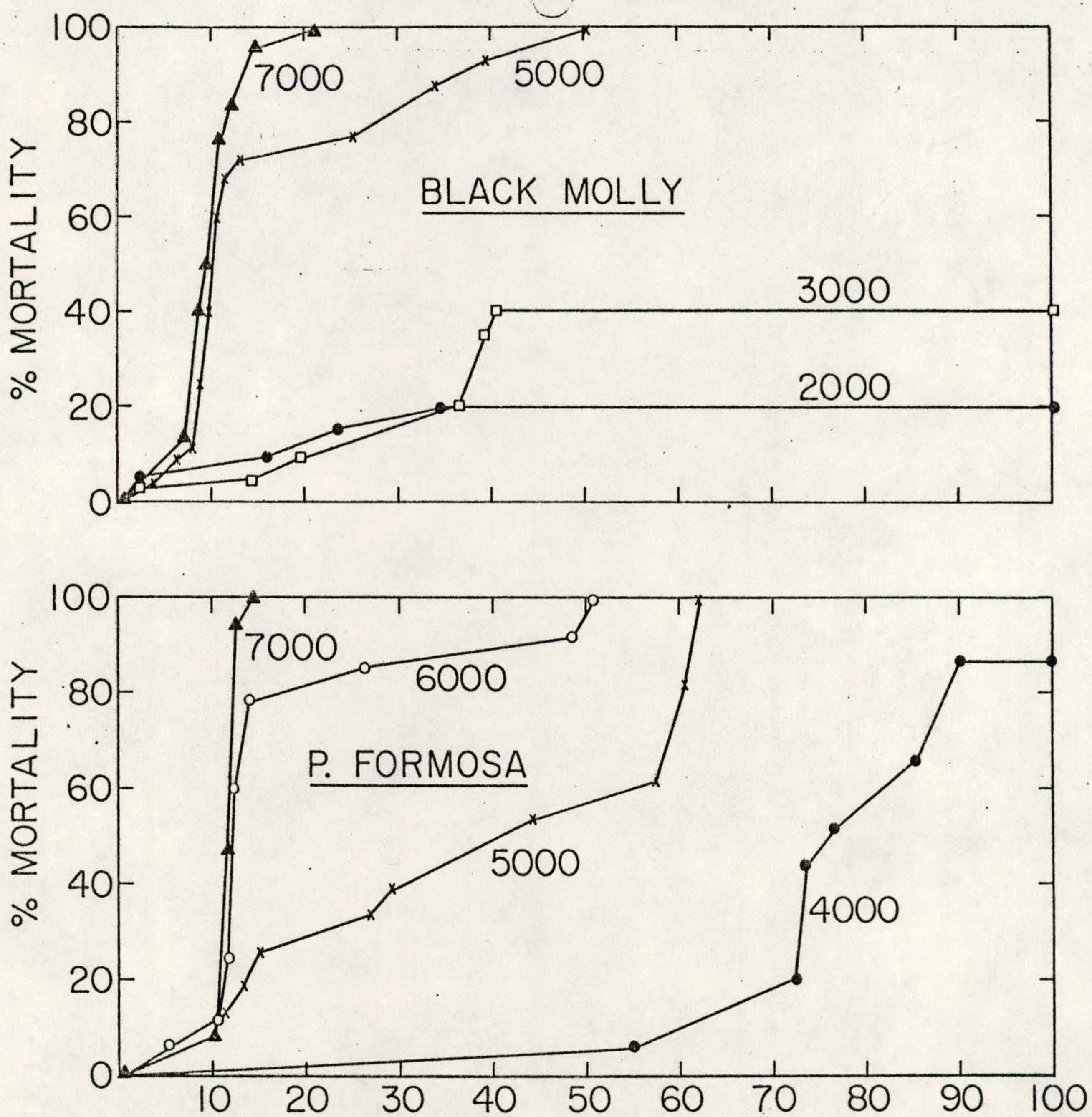

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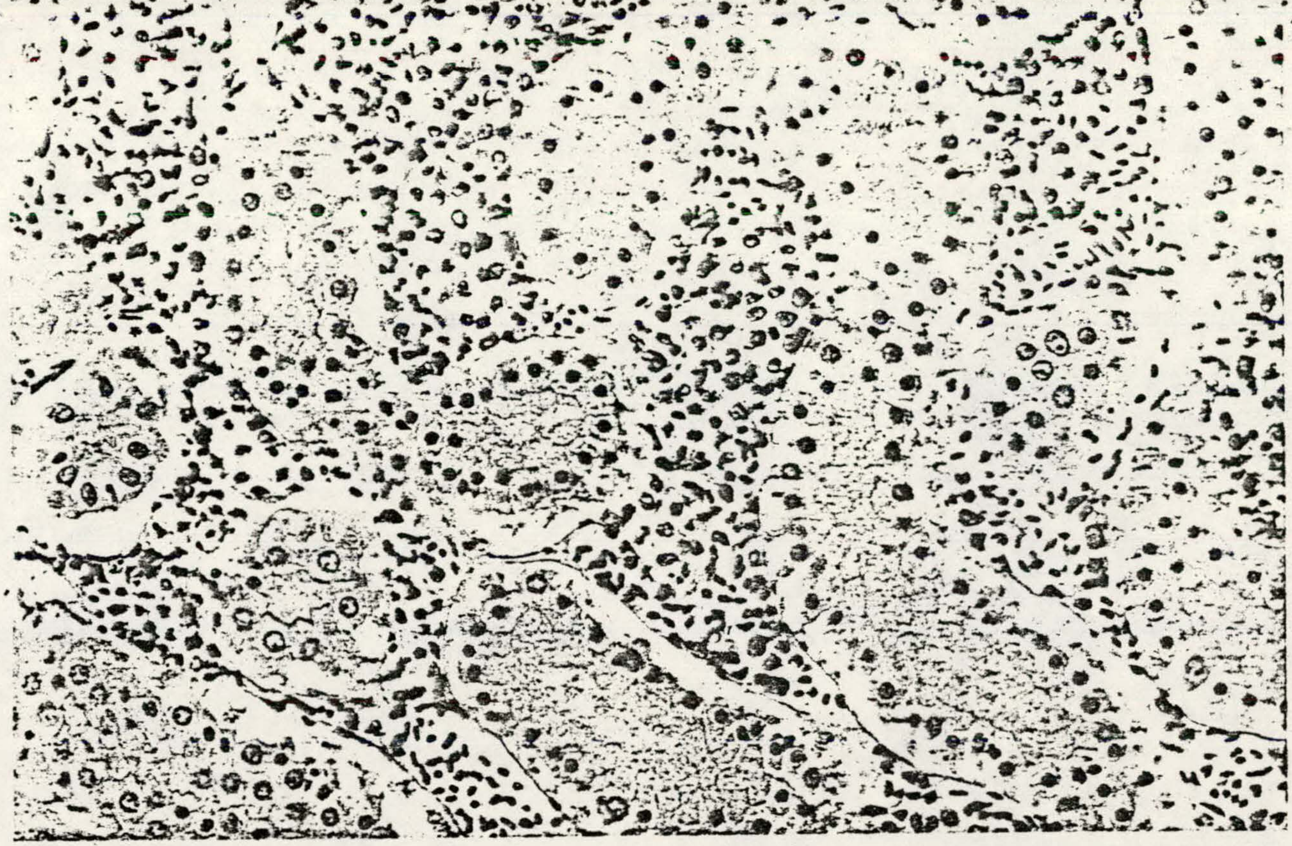


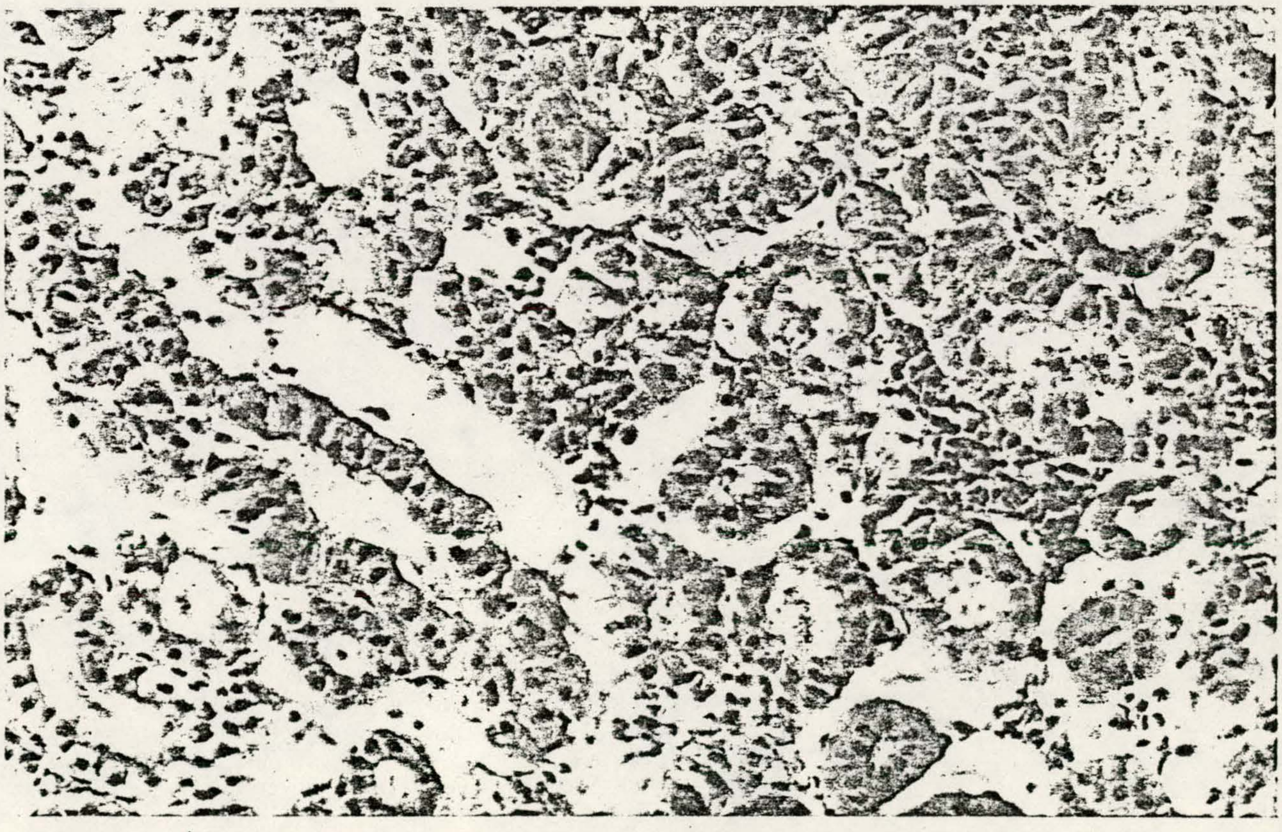
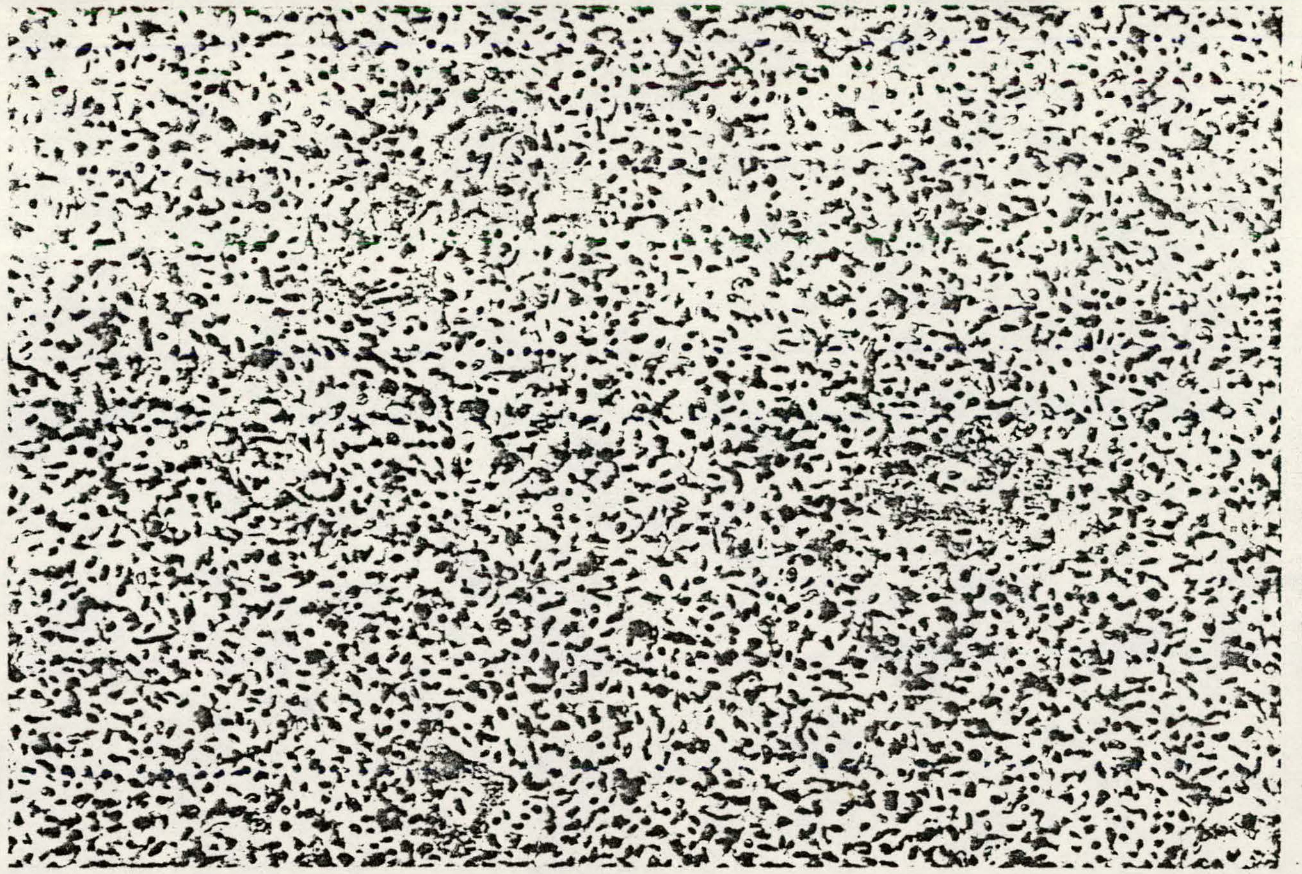


Figure 6. Sections through tissues of an unirradiated Amazon molly
(6a) (above) and a molly sampled 60 days after 4R (below).
(a) kidney, (b) spleen (c) carotid sinus x400.
These sections show marked depletion of the hematopoietic
tissue and circulating blood cells.



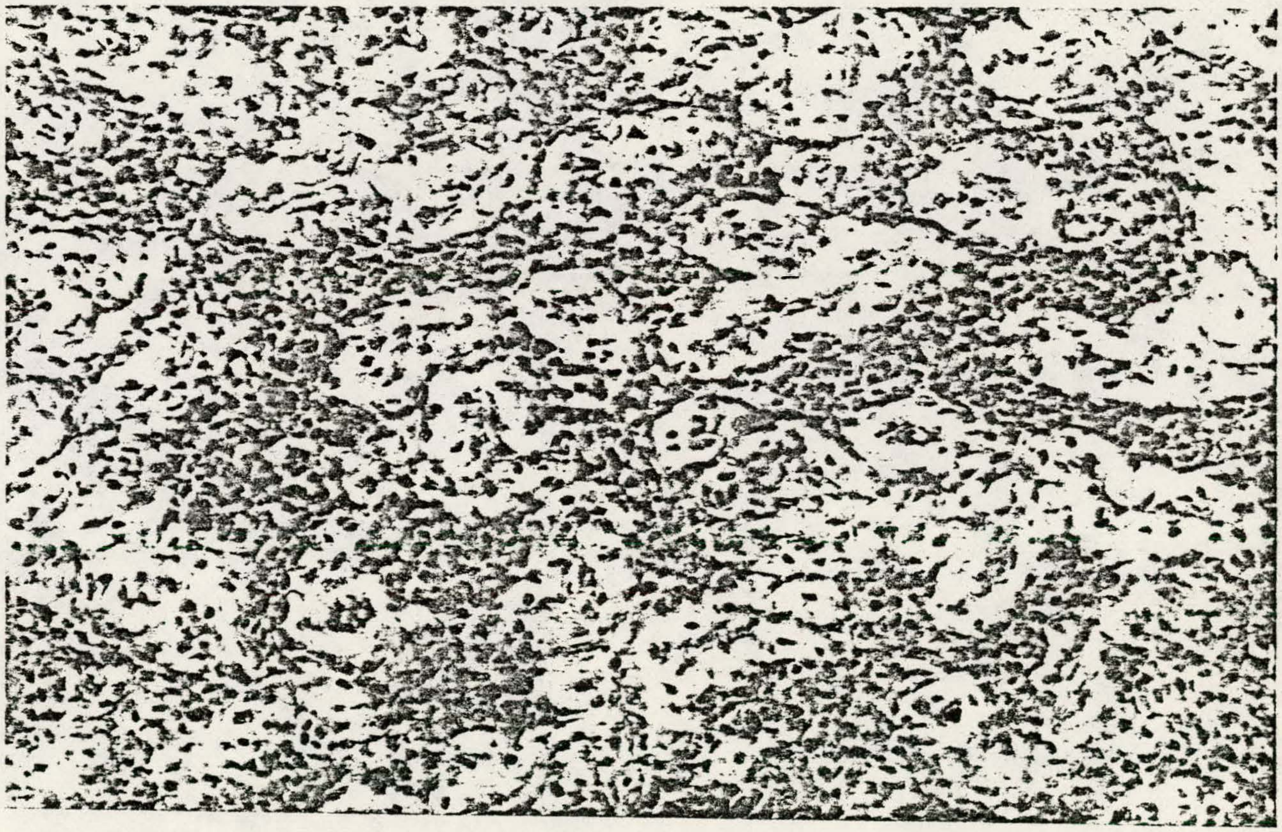


Figure 6. Sections through tissues of an unirradiated Amazon molly
(6b) (above) and a molly sampled 60 days after 4R (below).
(a) kidney, (b) spleen (c) carotid sinus x400.
These sections show marked depletion of the hematopoietic tissue and circulating blood cells.

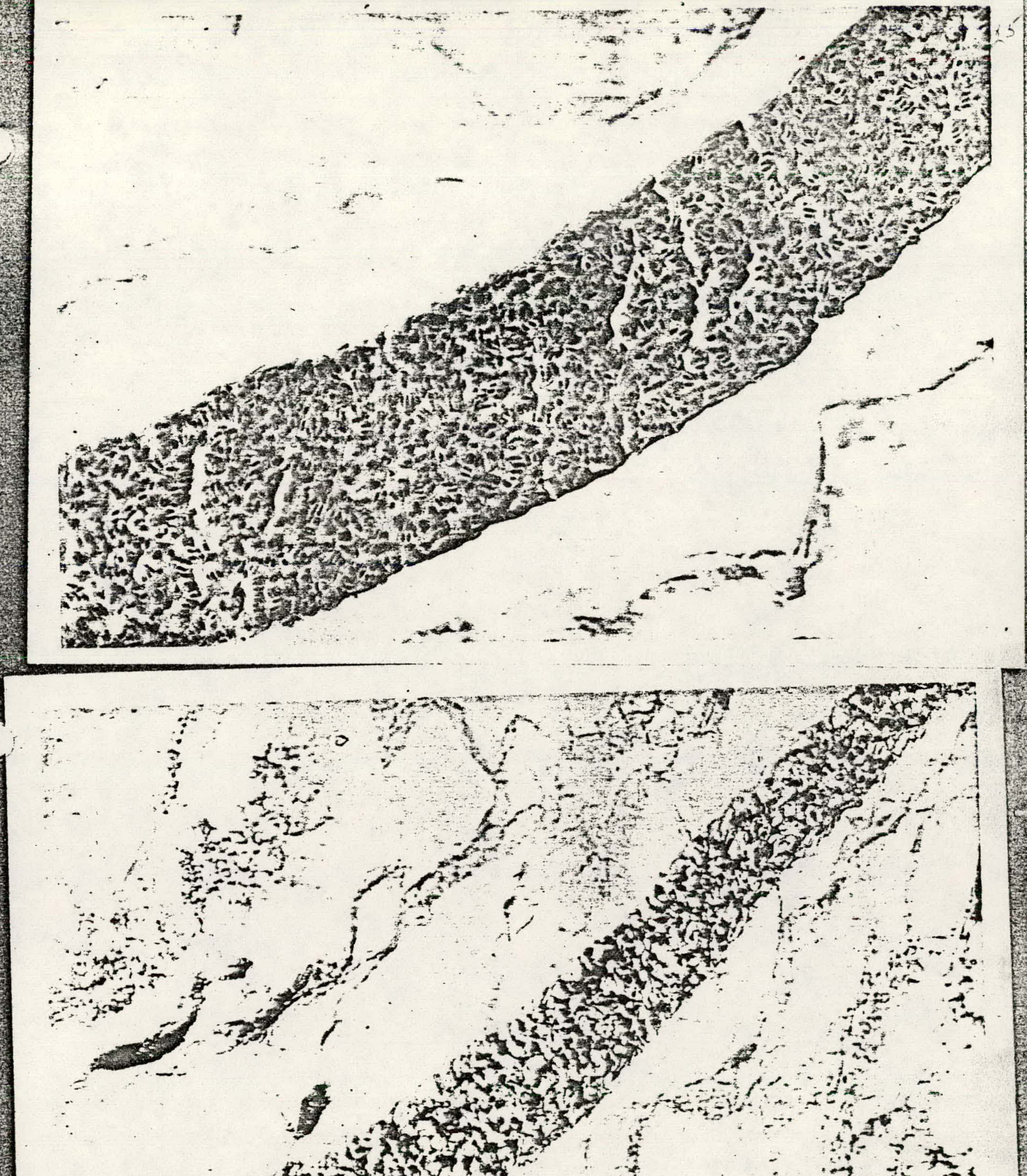


Figure 6. (6c) Sections through tissues of an unirradiated Amazon molly (above) and a molly sampled 60 days after 4R (below). (a) kidney, (b) spleen (c) carotid sinus x400. These sections show marked depletion of the hematopoietic tissue and circulating blood cells.

Figure 7. Sections through the intestine of an unirradiated Amazon molly (above) and a molly sampled 60 days after 4R (below). x 400 Degeneration and atrophy of the gut is apparent in the irradiated fish.