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CONTENTS

THE EFFECT OF IRON ON HEMATOCRIT LEVELS IN TROPICAL FISH

by

Patricia Ellen Hopper

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APPROVAL SHEET

Approved by

Martin Roeder, Director

Laura G. Anderton

Charlotte Dawley

Hilda T. Harpster

Sarah Sands

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The Effect of Iron on Hematocrit Levels in Tropical Fish

INTRODUCTION

Although the amount of iron present in the body is small (in humans about 45 milligrams/kilogram of body weight), it is an essential constituent of hemoglobin and of the cytochrome respiratory enzyme system, and thus an element of fundamental importance. The absorption of iron is different from that of other elements; most minerals are absorbed freely and the excess excreted. Once iron is absorbed, however, most of it is retained within the body. Only minute amounts are excreted, and that present in the body is almost completely reutilized after metabolic breakdown of the iron containing compounds, e.g., hemoglobin. Thus if the excess iron is not excreted, there appears to be a mechanism for absorption control. (Granick, 1946).

Normally, iron is absorbed in inverse proportion to the body's reserves. Thus iron is absorbed only in trace amounts by a non-anemic animal, but is absorbed in abundance by an anemic animal depleted of its iron (Rabscheit-Robbins and Whipple, 1927; and Hahn, Bale, Lawrence, and Whipple, 1937).

The tissue directly responsible for acceptance or rejection of iron is the gastrointestinal mucosa, which, in general, preferentially

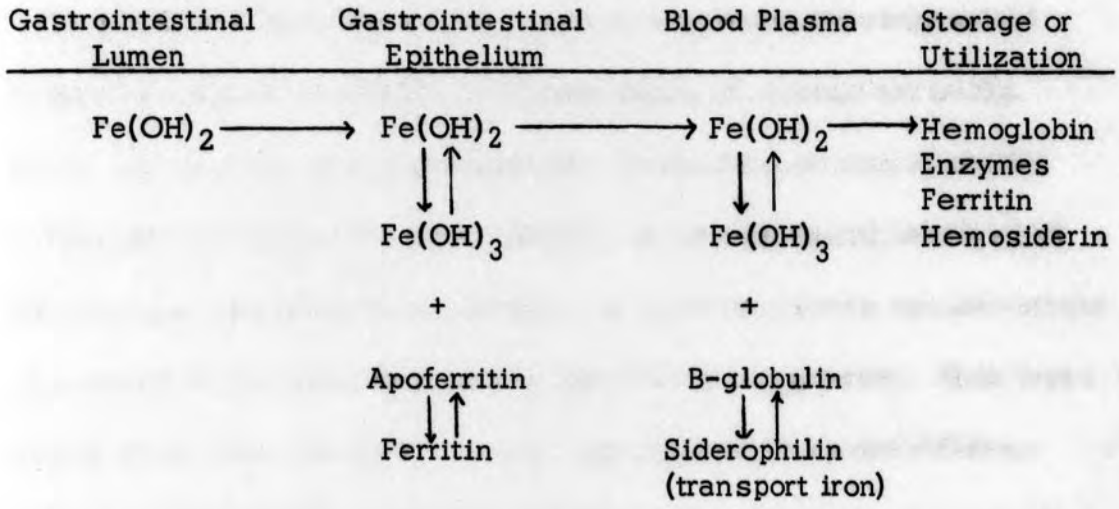
absorbs ferrous iron rather than ferric iron (Granick, 1946). It is thought that most of the absorption of iron in fish takes place through the mucous membranes lining the gills rather than in the gastrointestinal tract, but that a similar mechanism of absorption is involved. Whether or not the mucosal tissue will absorb iron is in turn regulated by the amount of ferritin (an iron storage compound) present in the epithelial cells, as shown by experiments with dogs and guinea pigs. (Hahn, Bale, and Ross, 1943; Granick, 1946).

The feeding of iron results in a rapid increase of ferritin in the mucosa, which parallels the rapid development of a mucosal block to the absorption of iron. Ferritin remains in the mucosa several days and slowly disappears, again paralleling the slow disappearance of the mucosal block to absorption. Thus, the term "physiological saturation" with iron may be applied to the gastrointestinal mucosa to explain its acceptance or refusal of the ingested salt. This mucosal block theory, however, is not always the complete control mechanism. When large amounts of iron are ingested in one group and small amounts of iron ingested in a second group, the former will absorb a greater quantity of iron if the body's stores and erythropoietic functions are approximately the same in both groups. The body's stored supply of iron is still the paramount regulation

factor. (Hahn, Bale, and Ross, 1943; Granick, 1946; and Miale, 1962).

Ferritin is an iron-protein complex containing iron in the form of aggregates of ferric hydroxide bound to the protein, apoferritin. Most of the ferrous iron on entering the mucosal cell is oxidized to ferric hydroxide which then combines with apoferritin to form ferritin. (Figure 1.). Small amounts of ferrous iron are located at the surface of the ferritin protein, apoferritin, but most of the iron in ferritin is in the ferric state and is internally situated. Thus only the ferrous iron at the surface is available for ready use. In the ferritin molecule there are free sulfhydryl groups associated with the ferrous iron to prevent the auto-oxidation of ferrous iron. Ferritin, however, gives up the iron bound to the sulfhydryl groups in the presence of a stronger ferrous acceptor, such as the iron binding plasma proteins. (Mazur, Baez, and Shorr, 1955.)

Figure 1. Mechanism of Absorption, Transport, and Storage of Iron. (Cantarow and Schepartz, 1962).



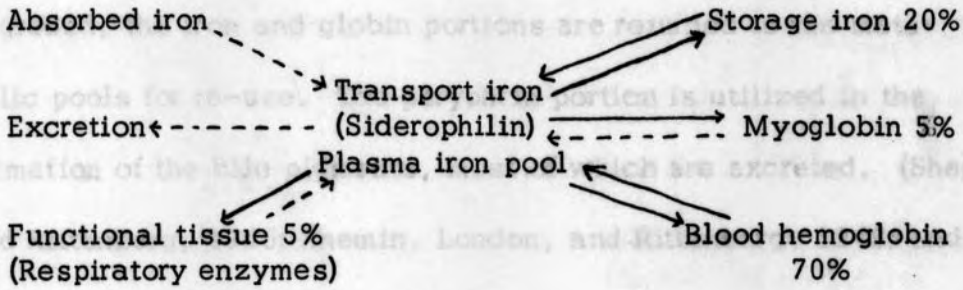
The reactions taking place in the mucosal cells in the formation of ferritin are also reversible depending on the body needs. Thus a slight decrease in circulating hemoglobin with a fall in oxygen content may favor the reduction of the bound ferric iron to ferrous iron which is then easily absorbed into the blood system (Figure 1). This conversion results also in the breakdown of ferritin to apoferritin. It is this decrease in ferritin concentration and increase in apoferritin concentration which permits the absorption of additional iron into the mucosal cells. The ferrous iron which is absorbed into the blood stream again undergoes oxidation to the ferric state, and it then combines with one of the plasma beta-globulins. This compound is siderophilin and is the transport form of iron in the body. (Granick, 1946 and Mazur, Baez

and Shorr, 1955).

After absorption into the tissues, iron is released from siderophilin, passes out of the capillaries and into the cells where it may be utilized or stored. The main areas of storage are in the liver, spleen, and intestinal mucosa. In the tissues iron is stored in the form of ferritin and hemosiderin. At physiological levels more ferritin than hemosiderin is present, but with increasing concentrations of injected iron, more hemosiderin than ferritin is present. Both types appear to be functionally the same, and probably they are different only in physical form. (Shoden, 1953).

The chief use of iron is in the synthesis of hemoglobin, myoglobin or muscle hemoglobin, and certain respiratory enzymes, such as the cytochromes, peroxidases, and catalase. The oxidative enzymes are probably formed in all cells, the myoglobin only in muscle cells, and hemoglobin in the developing red blood cells of the erythropoietic tissue. The circulating hemoglobin contains 70 per cent of the body iron; myoglobin, 5 per cent; storage iron, 20 per cent; functional tissue iron (respiratory enzymes), 5 per cent. Thus about 75 per cent of the body iron is in the form of hemoglobin. (Figure 2.)

Figure 2. Approximate Distribution of Iron in the Body. (Cantarow and Schepartz, 1962).



Due to the efficiency of iron return into the available plasma iron pool and the reutilization of this iron, only small quantities are lost under normal conditions. Thus, the exogenous requirement is correspondingly low. However, the increased requirement during periods of more rapid growth is reflected in increased absorption of iron at this time. (Darby, Hahn, Kaser, Steinkamp, Densen, and Cook, 1947).

The compound heme is formed by the addition of an atom of iron in the central position of the compound protoporphyrin IX. Heme may then be coupled with different proteins to form the conjugated hemoproteins, such as hemoglobin, myoglobin, cytochromes, catalase and peroxidase (Figure 3 and Figure 4). Synthesis of hemoglobin appears to proceed concurrently with the development of the erythrocytes. The early red blood cells contain free poryphrins rather than hemoglobin, but as the red blood cell matures, the

porphyrin content decreases, and that of hemoglobin increases. The average "life" of the erythrocyte is 120 days, and as the cells are degraded, the iron and globin portions are returned to the metabolic pools for re-use. The poryphrin portion is utilized in the formation of the bile pigments, most of which are excreted. (Shemin and Rittenberg, 1946; Shemin, London, and Rittenberg, 1948; and Walsh, Thomas, and Chow, 1949.)

Figure 3. Requirements for Hemoglobin Synthesis. (Miale, 1962.)

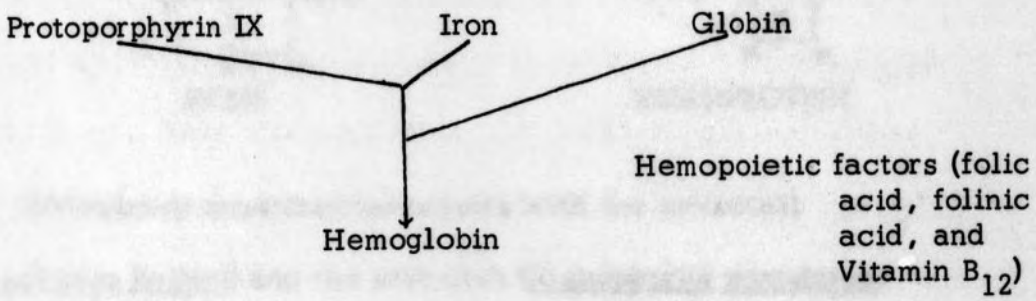
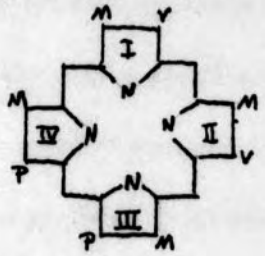
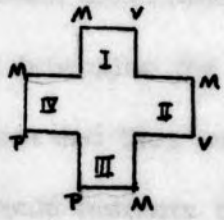


Figure 4. Structure of Protoporphyrin IX, Heme, and a Hemoprotein. (Cantarow and Schepartz, 1962.)

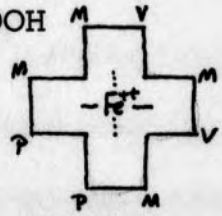


PROTOPORYPHRIN IX

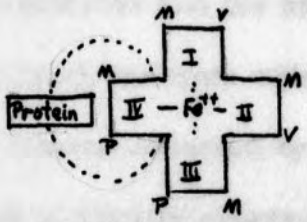


PROTOPORYPHRIN IX
(Simplified Structure)

M $-CH_3$
V $-CH=CH_2$
P $-CH_2CH_2COOH$



HEME



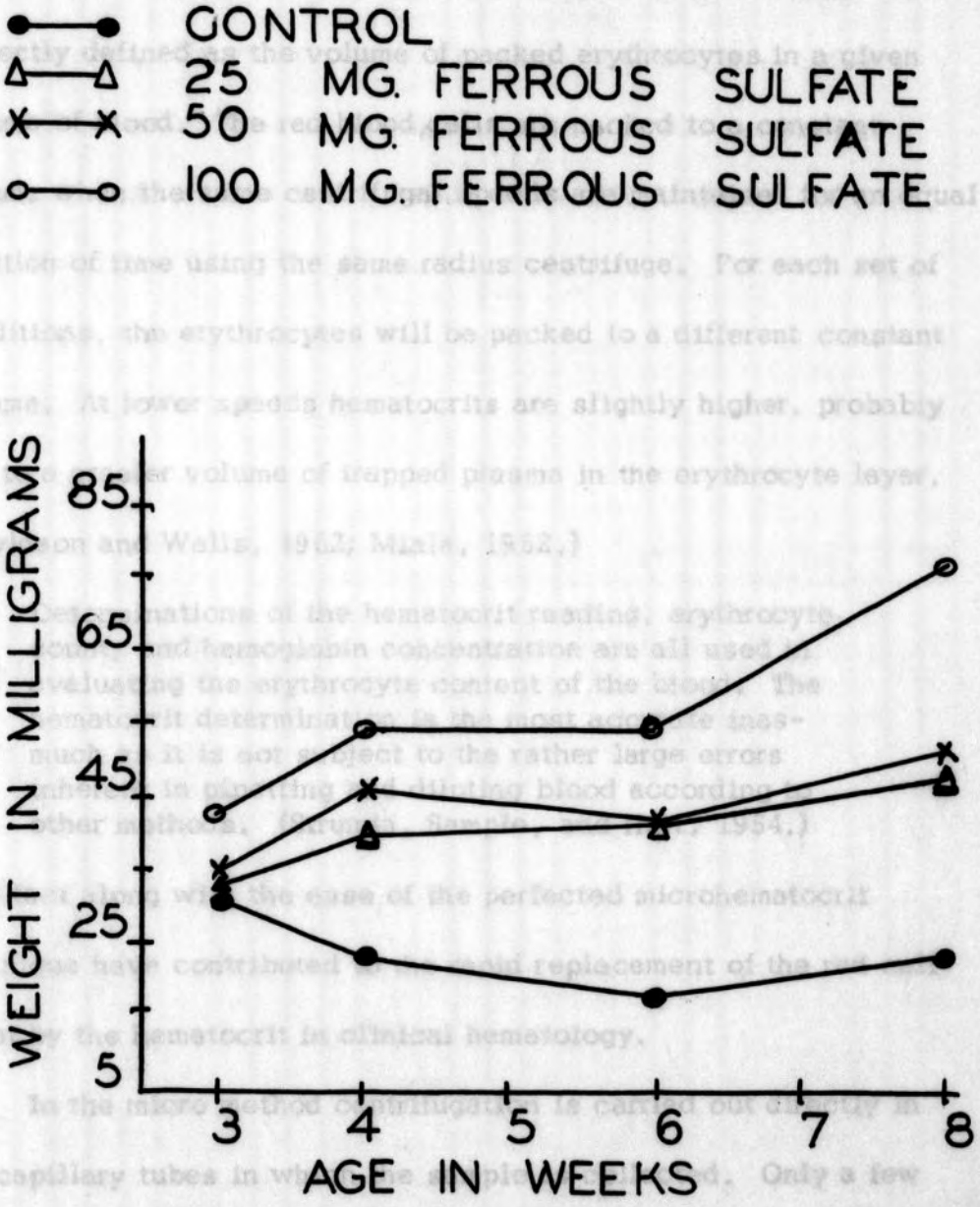
HEMOPROTEIN

Preliminary experimental results with the swordtail (Xiphophorus helleri) and the platyfish (Xiphophorus maculatus) indicated a doubling of the growth rate of inbred strains in the presence of small amounts of ferrous sulfate. The growth increment was roughly proportional to the amount of iron salt supplement (Figure 5). It was also found that at the end of seventeen weeks the swordtail fry receiving the added ferrous sulfate showed signs of sexual differentiation. This change was most prominent in the groups receiving the highest concentration of ferrous sulfate, but

differentiation was also evident in the groups receiving less iron. No sexual change had appeared in the controls (Roeder and Roeder, 1963). Subsequently, an investigation was planned to determine the effect of this iron on the number of erythrocytes present and thus on the amount of hemoglobin formed. These values would indicate the relative absorption of the supplementary iron. The microhematocrit was chosen as the experimental measurement to obtain these results, since it is related to both the number of erythrocytes and the amount of hemoglobin formed, although it does not directly measure either.

The hematocrit may be defined as the volume occupied by erythrocytes in a given volume of blood, and is usually expressed as a per cent volume of erythrocytes per 100 milliliters of blood. The hematocrit obtained by using a sample of venous blood is called venous hematocrit and represents the per cent of erythrocytes in the peripheral blood. Although this value is the one most commonly used, it may not indicate accurately the proportion of red blood cells to plasma in the entire circulation, a value known as the body hematocrit. (Davidson and Wells, 1962; Miale, 1962.) In order to accelerate the sedimentation rate of the cells, the blood sample, with an anticoagulant added, is centrifuged. After centrifugation the erythrocytes are packed in the bottom of the tubes with a thin

FIGURE 5


CONTROL
25 MG. FERROUS SULFATE
50 MG. FERROUS SULFATE
100 MG. FERROUS SULFATE
WEIGHT IN MILLIGRAMS
85
65
45
25
5
3
4
5
6
7
8
AGE IN WEEKS

layer of leukocytes and platelets, the "buffy coat," on top. Above the cell layers is the clear plasma. Thus hematocrit is more correctly defined as the volume of packed erythrocytes in a given volume of blood. The red blood cells are packed to a constant volume when the same centrifugal speeds are maintained for an equal duration of time using the same radius centrifuge. For each set of conditions, the erythrocytes will be packed to a different constant volume. At lower speeds hematocrits are slightly higher, probably due to a greater volume of trapped plasma in the erythrocyte layer. (Davidson and Wells, 1962; Miale, 1962.)

> Determinations of the hematocrit reading, erythrocyte count, and hemoglobin concentration are all used in evaluating the erythrocyte content of the blood. The hematocrit determination is the most accurate inasmuch as it is not subject to the rather large errors inherent in pipetting and diluting blood according to other methods. (Strumia, Sample, and Hart, 1954.)

This fact along with the ease of the perfected microhematocrit technique have contributed to the rapid replacement of the red cell count by the hematocrit in clinical hematology.

In the micro method centrifugation is carried out directly in the capillary tubes in which the sample is collected. Only a few drops of blood, 20 to 40 microliters, are required. In the ultra microhematocrit only 5 to 10 microliters of blood are used, or less

than one drop. The hematocrit is read as the length of the cell column to the length of the whole blood column x 100. Using the high speed capillary technique, the results are precise, and the packing of erythrocytes is so complete that any plasma trapped in the erythrocyte partion is negligible. (Stramia, Sample, and Hart, 1954; Natelson, 1951; and Snieszko, 1960.)

EXPERIMENTAL PROCEDURE

The fish used in the experiment were the green swordtail and the spotted swordtail, two inbred strains of Xiphophorus helleri, and the hybrid offspring of the swordfish and the platyfish, Xiphophorus maculatus.

Aquaria holding thirteen liters of water were used to maintain the fish. Each aquarium contained small granite gravel which covered one-third to one-half of the tank floor, a block of calcium carbonate, sprigs of Elodea which were implanted in the gravel, and ten to twenty snails. These aquaria would remain balanced for about a year if the fish were not overfed. However, if fouling did occur, as indicated by a cloudy appearance, the fish were placed in a fresh aquarium.

Soon after birth the brood of fry was separated into groups of equal number, depending on the number of fish and the variations in procedure. No more than ten fry were placed in one aquarium, and generally only two to three adult fish were retained in a single tank. At the beginning of the experiment, each group of fish was placed in a separate aquarium, and the ferrous sulfate supplement, if any, was added daily. The iron salt was introduced in the form of a solution containing one milligram ferrous sulfate per milliliter

of distilled water. To different test groups was added 25, 50, or 100 milligrams daily. The controls were maintained with no additions of salt nearby the experimental colony.

The young fry were fed an average of 90 milligrams of dry food daily per tank and 300 milligrams of a liquid supplement containing 50 milligrams of dry material. Older fish were fed 150 milligrams of dry food each day and were given weekly supplements of live Enchytraeus, a worm. The amount of iron present in the tap water, less than 0.2 milligrams in a new aquarium, and the available iron in the food, less than 0.07 milligrams daily, is considered negligible in comparison to the amounts used as growth supplements. The determination of exogenous iron content was made using the alpha-alpha' dipyridyl method. Using this procedure good correlations are obtained between the iron which reacts with the reagent and that which is available for utilization by the animal. (Sherman, Elvehjem, and Hart, 1934.)

After a growth period of seven to nine months, the fish are large enough for hematocrit determinations to be made. The fish which were removed from the aquaria for the blood studies were kept in a small container filled with water from the same aquaria. The blood sample was taken immediately after the fish was removed from the container to minimize the effects of asphyxia. It has been

shown that the blood of fish may undergo marked and rapid change when the fish have been exposed to partial asphyxia. Most blood constituents appear to have a higher concentration in fishes during asphyxiation because of a release of water from the blood which may result in a 50 per cent decrease in blood volume. It is thought that increased tissue acidity during asphyxiation brings about transfer of water from blood to tissue. (Hall, Gray, and Lepkovsky, 1926.)

The technique used for collecting the blood sample is a modification of the orbital bleeding technique described by Riley (1960) with certain adaptations for use with the fish. To prevent drying out and to minimize movement, the fish was completely wrapped in a moist Kimwipe except for the head region, and placed under a dissecting microscope. (In order to clearly see the eye structure and the capillary bed, it was necessary to use magnification.) A folded Kimwipe placed under the head region enabled the posterior of the optic cup to be reached more easily.

Using a semi-micro glass probe and a slightly curved semi-micro glass needle, the eye was gently pushed just out of the eye socket in a dorsal anterior position. The tip of the probe and the needle were fire polished to avoid unnecessary damage to the eye.

Holding the eyeball in this position, and using the glass needle, a portion of the conjunctiva membrane was ruptured. This operation revealed the posterior portion of the optic socket containing the rectus and oblique muscles; the optic nerve; the cranial nerves, V, III, IV, VI, and VII; and the optic capillaries.

Due to the rapid clotting time of the fish blood, it was necessary to introduce approximately 0.5 microliters of heparin (heparin sodium, 1000 U.S.P. units/cc.) into the capillary area of the optic cup. This procedure was not found to affect the constancy of the hematocrit levels as all the fish were treated in the same manner.

The pipettes used to collect blood were heparinized capillary tubes with an outside diameter of 1.0 millimeter and a length of 32 millimeters.[1] The tips of the capillary tubes were drawn out in a microburner so as to have an outside diameter of approximately 0.6 millimeter.

The drawn out tip of the pipette was placed in the lower posterior corner of the eye where the blood vessels could be clearly seen. The rough tip of the tube was slid gently but firmly

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[1]Heparinized capillary tubes. Clay-Adams, Inc., New York.

over the vessels. The fragile venous capillaries ruptured upon contact with the tip of the pipette, and the orbital cavity, serving as a reservior, was allowed to quickly fill with blood. The pipette was filled by slightly withdrawing the tube to free the tip and holding it in a horizontal position just touching the drop of blood. As the orbital cup filled, the blood was drawn into the tube by capillary action. To prevent blood from clotting in the flamed pipette tip, the point was washed with heparin solution. The capillary tube was filled one-half to two-thirds full, an estimated volume of 10 to 20 microliters. The blood clotted in the eye cup rapidly as the anticoagulant was removed; therefore, bleeding had stopped by the time the pipette was removed.

The capillary tube was sealed by pushing the blunt end through a small disc of light-colored modeling clay about 2 millimeters in thickness.[1] The sealed blood capillaries were centrifuged for three minutes,[2] and the hematocrit was then determined in a microhematocrit reader.[3]

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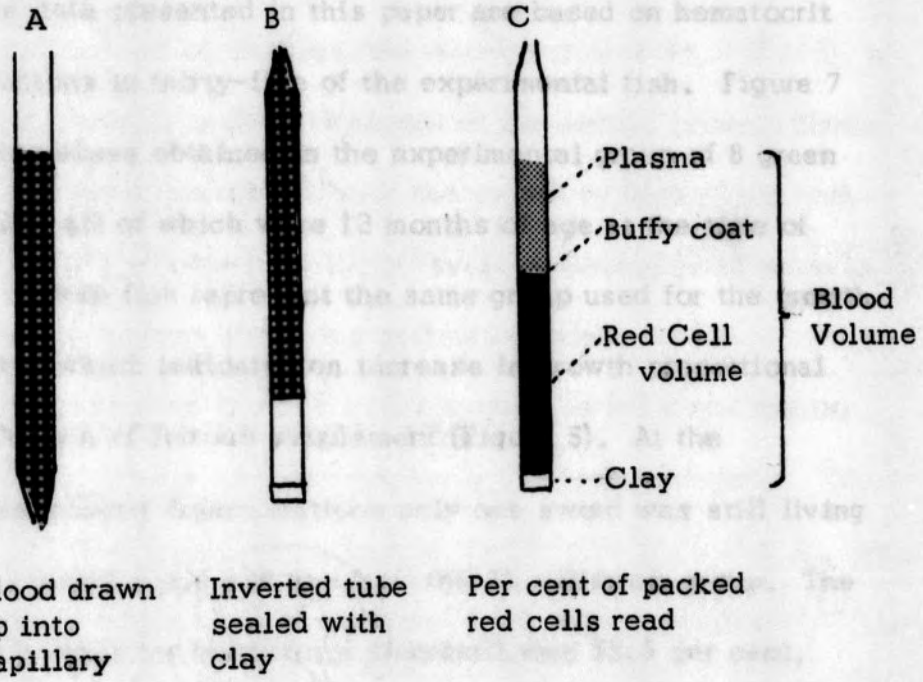
[1]Seal Ease. Clay-Adams, Inc., New York.
[2]Adams Microhematocrit Centrifuge. Clay Adams, New York.
[3]Adams Microhematocrit Reader. Clay Adams, New York.

Figure 6. Sequence in Determining Hematocrit Values.



RESULTS

The data presented in this paper are based on hematocrit determinations in thirty-five of the experimental fish. Figure 7 shows the values obtained in the experimental group of 8 green swordtails, all of which were 13 months of age at the time of testing. These fish represent the same group used for the growth rate study, which indicated an increase in growth proportional to the addition of ferrous supplement (Figure 5). At the time of hematocrit determinations only one sword was still living from the control group and one from the 25 milligram group. The hematocrit value for the control swordtail was 35.5 per cent. This value was accepted as accurate for normal swordtails since other hematocrit determinations with non-experimental swordtails had indicated an average per cent of 35.1 ($\pm$3.5[1]). The per cent of red blood cells for the fish receiving 25 milligrams was 35 per cent, a value accepted as being within the normal range, and indicating no effect on the erythrocyte content from the addition of iron. The average for the two swords receiving 50 milligrams of ferrous sulfate was found to be 37.2 ($\pm$2.1), a 5 per cent increase

[1]The figure $\pm$3.5 indicates the average mean deviation. Other variances given also represent the average mean deviation unless otherwise indicated.

in the per cent of packed red blood cells over the fish receiving no iron. The 4 swordtails receiving the 100 milligram supplements were found to have an average hematocrit value of 38.5 ($\pm$1.0), a 9 per cent increase in the hematocrit of the control group. These values indicate a correlation with the growth in these fish; both the growth rate and the hematocrit levels increased in relation to the increased amount of ferrous sulfate addition.

Eight fish were present in the spotted sword experimental group, having an age of eight months at the time of testing. The average for three control swords was found to be 35.5 ($\pm$6.3), the same average as that for the controls in the green swordtail experimental group (Figure 8). An average hematocrit of 38.2 ($\pm$0.9) was obtained for the three fish in the 25 milligram test group, an increase of 8 per cent over the control group. The two swordtails in the 100 milligram group were found to have an average hematocrit of 40.2 ($\pm$0.75) per cent. This indicated an increase of 12 per cent over the control group.

Thirteen fish, age 10 months were present in the hybrid fish colony (Figure 9). The average hematocrit for the five control fish was determined to be 31.7 ($\pm$0.4). It is of interest that the average for the hybrid control fish is less than the hematocrit for the swordtail controls. The iron test group in the hybrid colony

received 50 milligrams of ferrous sulfate daily. This group of 8 fish was found to have an average packed red cell volume of 40.2 ($\pm$1.4) per cent. This value showed an increase of 27 per cent over the controls. This increase is almost three times that found in the two swordtail groups, and may be correlated with the faster growth rate of the hybrid fish over the swordtails.

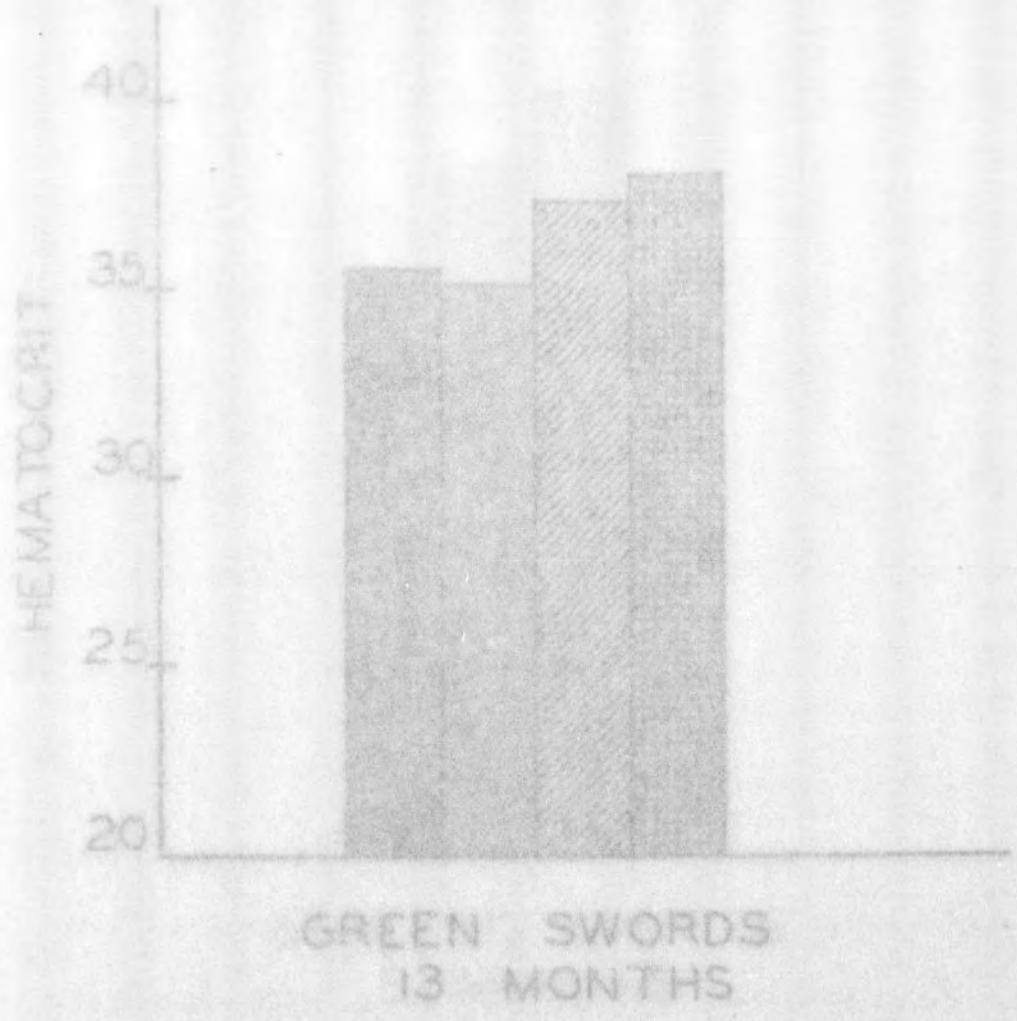


FIGURE 7

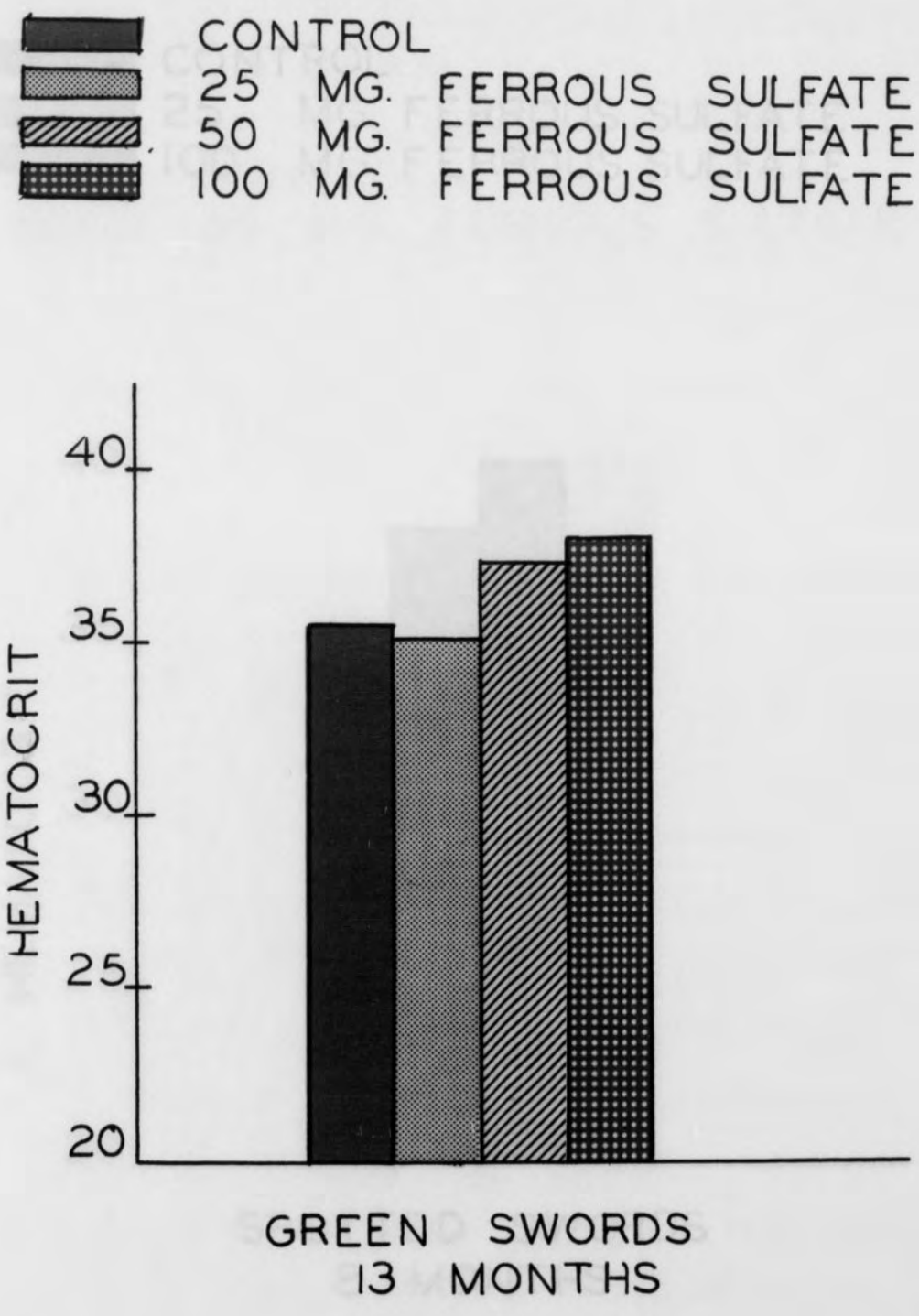

CONTROL
25 MG. FERROUS SULFATE
50 MG. FERROUS SULFATE
100 MG. FERROUS SULFATE
HEMATOCRIT
40
35
30
25
20
GREEN SWORDS
13 MONTHS

FIGURE 8

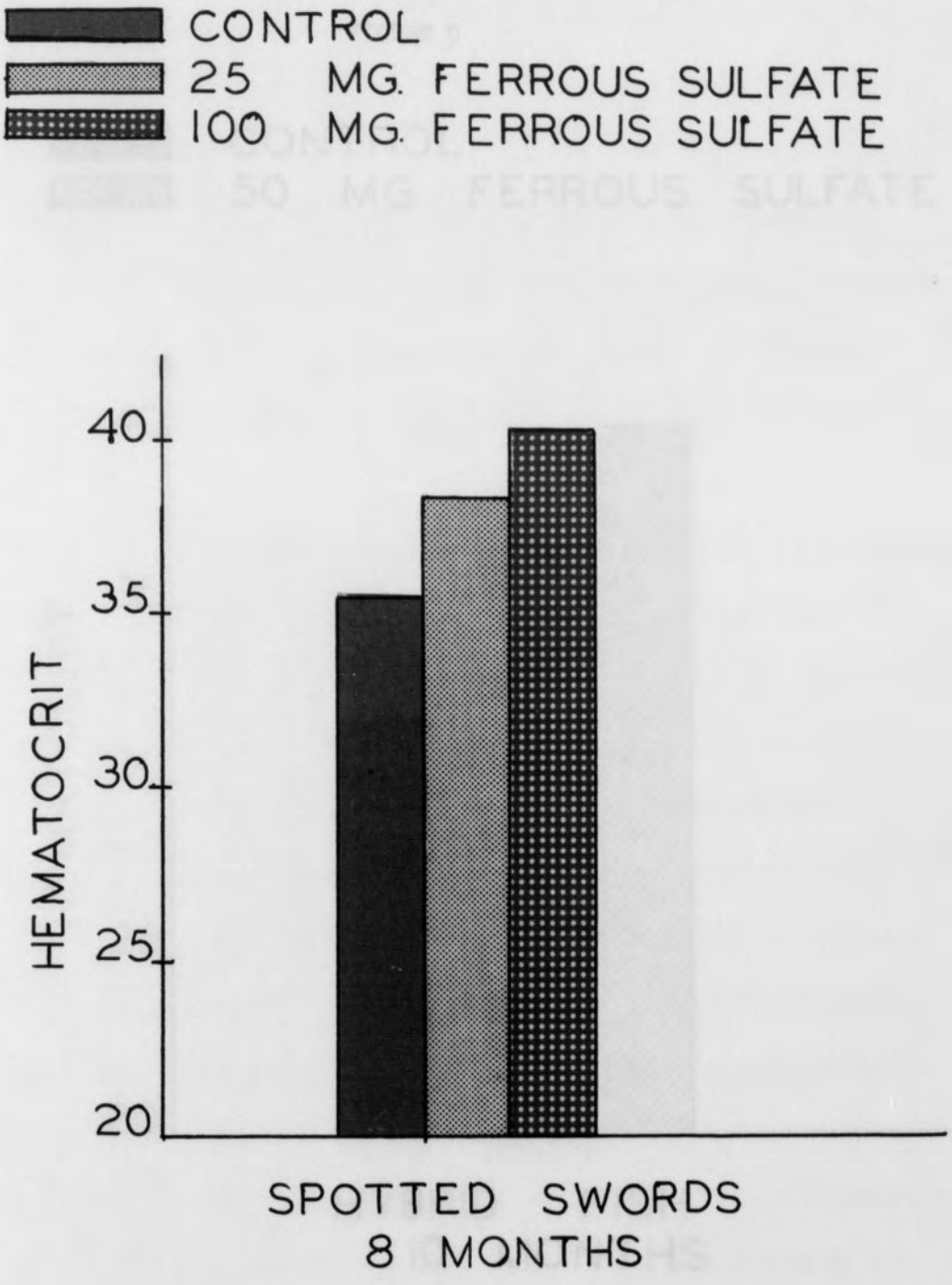
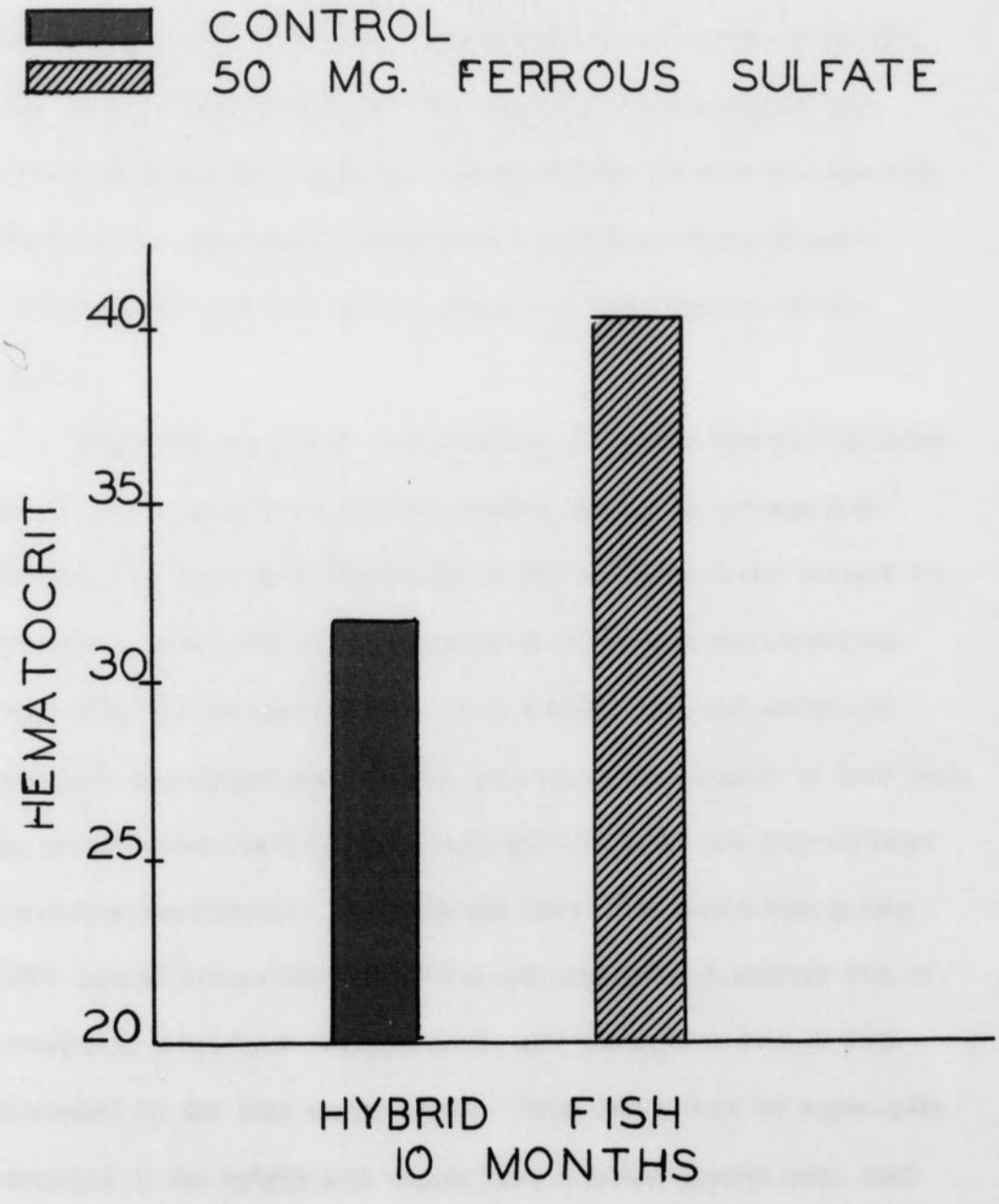

CONTROL
25 MG. FERROUS SULFATE
100 MG. FERROUS SULFATE
HEMATOCRIT
40
35
30
25
20
SPOTTED SWORDS
8 MONTHS

FIGURE 9


CONTROL
50 MG. FERROUS SULFATE
HEMATOCRIT
40
35
30
25
20
HYBRID FISH
10 MONTHS

CONCLUSION AND DISCUSSION

The increased erythrocyte volumes found in the iron treated fish indicated an increase in hemoglobin content in these groups, showing that the ferrous iron was actually being absorbed and utilized by the fish. This fact indicated that the iron was directly affecting the growth rate rather than the change being brought about indirectly by altering the flora and fauna content of the aquaria.

Since the amount of iron absorbed is predominantly regulated by the body requirement and the amount of ferritin storage iron present, the increased absorption in the iron test group seemed to indicate a basic deficit of storage iron in the fish not receiving supplementary ferrous sulfate. It is thought that the amount of available absorption iron usually present in the aquaria is less than the amount necessary to physiologically saturate the iron-storage regulation mechanism. It is thought then, that since fish grown under normal laboratory conditions may not receive enough iron to completely meet their requirements, and the growth rate is thus increased by the iron supplements. This factor may be especially pertinent in the hybrid fish which have a faster growth rate, and

thus a greater requirement for iron during development, than do the swordtails.

SUMMARY

Inbred strains of Xiphophorin fish were given daily supplements of ferrous sulfate. Initial results indicating an increase of growth rate in the group receiving the ferrous sulfate led to hematocrit studies in the fish. An increase in the packed cell volume of erythrocytes was found for the fish receiving the ferrous sulfate, indicating an increased absorption and use of iron. This increment was roughly proportional to the amount of salt added and to the growth rate in these fish. Since absorption of iron is related to the body's need, it is thought that fish raised under normal laboratory conditions may be partially anemic, and that small amounts of iron may be used as dietary supplements to increase the growth rate. This appears to be especially true in the hybrid fish which have a faster growth rate and greater requirement for iron.

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