Chromosome Studies of *Xiphophorus helleri*, *Xiphophorus maculatus*, and Their Hybrid

by

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INTRODUCTION

Xiphophorus helleri, commonly known as the swordtail, and Xiphophorus maculatus, commonly called the platyfish, are different species of the family Poeciliidae. A cross between the platyfish bearing the gene for a dorsal fin spot, and the swordtail yields melanomatous offspring. Since these tumors are colored a dark brown to black and can be readily detected, and since these tumors always appear in the hybrid bearing this gene, they are excellent tools for research aimed at the physiological, histological, and cytological manifestations of cancerous growth. It has been shown then, that the two species do produce some fertile offspring; however, the number of hybrids which are produced is low, as well as the number of offspring of the hybrids. This failure of the melanomatous fish to reproduce does, of course, limit the number available for research and is detrimental to adequate testing, justifying the need to investigate this problem.

The basis for transmission of genetic material from parents to offspring and from cell to cell is by cell division; and therefore, must lie within the cell itself. More specifically, the part of the cell involved is the nucleus, a particular body in the cell. Within the nucleus are chromosomes composed of a

granular appearing substance, called chromatin, because it has a strong affinity for certain dyes. When the cell is ready to divide, the chromosomes become condensed into more definite, dark staining structures. (Herskowitz, 1962, p. 16)

Every species has a definite and characteristic karyogram, or chromosomal make up in regard to both number and structure. Chromosome number may be expressed as diploid (2n), the paired state found in somatic cells; or haploid (n), the unpaired condition of chromosomes found in the reproductive cells, or gametes. The condition in which more than the 2n number are present is known as polyploidy. The capacity of the cell to duplicate itself precisely at each cell division is responsible for the constancy of karyotype. (Hegner, 1959, p. 27)

Since chromosomes are visible as distinct structures only when the cell is dividing, it is during nuclear division, or mitosis, that their structure is observed. Mitosis includes a series of complex events. In the first stage, prophase, the duplicated chromosomes become visible as a mass of twisted and coiled structures; parallel fibers, called the spindle, appear across the nuclear space. During the next phase, metaphase, the chromosomes line up in the equatorial plane of the spindle. In the next stage, anaphase, the duplicated

chromosomes separate and go to opposite poles of the spindle. At telophase, the chromosomes are at the opposite poles and the events of prophase are reversed, leaving two interphase cells which are between mitotic divisions. (fig. 1) (Hegner, 1959, p.26) Because the chromosomes at metaphase are in somewhat the same plane at the equator of the spindle, it is this stage that is sought for observation as to chromosome structure and number.

In the somatic cell, chromosomes are present in homologous pairs; but in each gamete, as a result of meiotic division, there is only one of each chromosome pair. Meiotic division, or gamete formation, differs from mitotic division in that mitosis involves one chromosome duplication followed by separation, whereas meiosis involves one duplication followed by two separations. Thus meiosis results in gametes containing only one of each pair of chromosomes. (Herskowitz, 1962, p. 24)

For continuation of a species of sexually reproducing organisms, union of gametes must occur. When two gametes unite to form a zygote, the chromosomes are restored to their homologous paired state. If the chromosomes of the platyfish and swordtail are enough alike in structure and number, they should pair normally at meiosis of the hybrid and produce a viable gamete.

If, however, there is sufficient karyotype dissimilarity in the two species, chromosomes without homologues with which to pair line up alone on the equatorial plate. Consequently, at separation of chromosomes in the next meiotic division, deficient gametes would be produced which are often inviable, or which result in infertile offspring, as in the cross between the horse and ass which yields the mule. Such infertility may be due to variances in structure or in number as in polyploidy, due to a deficiency or overabundance of chromosomes from random separation in gamete formation. (Swanson, 1957, p. 178) Since the reproductive potential is dependent, therefore, upon the chromosomal mechanism and compatibility of complete haploid sets of chromosomes, an investigation was undertaken into the nature of the chromosomes of Xiphophorus helleri, Xiphophorus maculatus, and their hybrid.

Chromosomes are usually compared according to their shape in somatic metaphase because the shape characteristics are relatively constant and can, therefore, be used to identify chromosomes of the same and different species. This characteristic shape is determined by secondary constrictions, which in some cases form satellites, providing additional landmarks. (Swanson, p. 111f.) Although the diameter of any chromosome increases as the chromosome is contracted

in prophase, the size of the chromosomes within a species is constant. Conjointly with the use of characteristic size, metaphase length, satellites, and primary constrictions in identification and comparison, chromosome number is used as a criterion. (Swanson, p. 118)

Previous studies of chromosome morphology have used many approaches and a multitude of tissue types from both the plant and animal kingdoms. One such study made of the sex chromosomes of fishes, using the testes, first reported the presence of male heterogamy in fish. This study used the testes fixed in Champy's solution and stained with Heidenhain's iron-haematoxylin. (Nogusa, 1955, p. 12) Numerous studies employed embryonic tissue due to its rapid rate of mitosis and consequent plentiful appearance of characteristic metaphase chromosomes. The morphology of lake trout chromosomes was observed by using two-day old embryos fixed and stained in acetocarmine. (Wahl, 1960, p. 1065) Boothroyd (1959) carried out chromosome studies of salmon using young embryonic tissue fixed in acetic acid and stained with acetocarmine. However, all the authors who have worked with fish chromosomes refer to some difficulty in obtaining a reliable karyogram.

The only previous study (Ralston, 1934) of the chromosomes of Xiphophorus helleri, Xiphophorus maculatus, and their hybrid, like the studies in other

fish, leaves much room for question. Ralston writes, "most of the hybrids are normal and fertile." (p. 434) This statement is contrary to what has actually been observed by others using this material. Ralston does not give his method but he observed gonial cells and admittedly remarks that "no equatorial plate stage was observed in which all chromatin elements were arranged regularly on the plate." (p. 426) Nevertheless, he reports that the haploid number is twenty-four chromosomes and that differences in morphology exist between Xiphophorus helleri and the other two under consideration, Xiphophorus maculatus and the hybrid. (p. 426) Ralston believes them to be morphologically compatible with regard to chromosomes.

The work of Fankhauser with amphibian chromosomes presents an interesting technique for the use of mitotically active embryonic tissue. This procedure involves Bouin's fixative, hydration in alcohol, and staining with Harris' acid haematoxylin followed by alkalinization and dehydration in alcohol with clearing in xylol. This procedure yields very visible chromosomes using only a distal third of the amphibian tail. (Rough, 1962, p. 130)

Various means are cited which may increase visibility of chromosomes. One such method is the use of colchicine, an alkaloid drug extracted from the root

of a lily plant. In many cases it has the effect of causing the chromosomes to shorten, due to a lengthened prophase, interfering with spindle formation, and arresting the division at metaphase. (Swanson, p. 382) Colchicine may, therefore, cause polyploidy. One such use of colchicine in plants produced beneficial results with uncoiled chromosomes spread throughout the cell for better observation. (O'Mara, 1939, p. 35) Krishan (1963) used colchicine and hypotonic salt solution on embryonic bird tissue and stained with haematoxylin after fixing in acid alcohol. Another study employed colchicine in hypotonic salt solution for studying insect larvae, producing a reported increase of readable figures. (French, 1963, p. 670) However, some investigators have found that pretreatment with colchicine before fixation resulted in clumping of the chromosomes and was worthless. (Meyer, 1945, p. 122)

Many procedures for fixing and staining chromosomes for viewing are known and recommended. Fixation is a process necessary to prevent post-mortem changes in the protein of the material and also to render all the cell constituents insoluble. Another objective is to protect tissue structures from distortion as well as to improve their staining potential. Various fixatives may be employed including alcohols, heavy metal compounds, acetic, picric, and other acids. (Humason, 1962, p. 106)

Staining is necessary for sufficient contrast for viewing with brightfield microscopes. One such stain important in mitotic study is the natural dye haematoxylin which stains chromosomes blue-black to black. (Lee, 1937, p. 150)

The techniques used in preparing chromosomes for observation are almost as numerous as the attempts at observation. Snow (1963) used acidic carmine in staining chromosomes which yielded intense staining of chromosomes with only lightly stained cytoplasm. Another method employed natural orcein in solution with lactic acid followed by staining for observation. (Dyer, 1963, p. 85)

Clearly then, the recipe must be devised or adapted to the material used.

MATERIALS AND METHODS

Initially, a large part of the problem was in obtaining information and adapting histological techniques to this particular material, the two species of fish. The first tissue chosen for use was the tail bud of the fish embryo because actively dividing cells are concentrated here and the chromosomes are visible in actively dividing cells. Because these fish are ovoviviparous, the embryo was difficult to obtain at an early age, before fin formation. Embryos were obtained from the stock of Dr. Martin Roeder, University of North Carolina at Greensboro. The pregnant fish was sacrificed and the embryos were removed and separated from each other. Tail buds were cut from the embryos before treatment in some instances, and after treatment of the entire embryo in other instances. The tail bud of the embryos was chemically treated to make the structures visible according to the technique of Fankhauser which is as follows:

1. Transfer tail bud with minimum fluid to the fixative for a minimum of three hours. Prepare fixative as follows:

Bouin's fluid	100 g.
Urea	1 g.
Glacial acetic acid	5 cc.

2. Transfer to 70% alcohol for removal of picric stain.
3. Transfer to watch glasses and hydrate through five minute changes in progressively dilute alcohols to distilled water.

4. Stain for 15 minutes in 1/3 Harris' acid haematoxylin. Harris' acid haematoxylin prepared as follows:

Grubler's haematoxylin	0.5	g.
Warm 95% alcohol	5.0	cc.
Potassium alum	10.0	g.
Warm distilled water	100.0	cc.
Red mercuric oxide	0.25	g.

Just before using add 2.5 cc. of glacial acetic acid to 50 cc. of Harris' and dilute for use.

5. Transfer through two changes of water containing sodium bicarbonate to alkalize the stain and cause a blue color.
6. Dehydrate with five minute changes in progressively more concentrated alcohols to 95% where two changes are made, followed by four changes in absolute alcohol. Transfer to carbol-xylol for five minutes, then into two changes of xylol.
7. Mount and squash.

(Rough, p. 130)

This exact procedure directly from Fankhauser was employed and subsequently, various changes were made. The dehydration time in xylol and carbol-xylol was shortened due to the brittleness it produced in the tissue. The stain was taken into the tissue more effectively if the material remained in the 70% alcohol for a minimum of twenty-four hours.

The chromosomes of mitotic figures seemed to be clustered together, as shown in the results. Consequently, colchicine was used in an attempt to obtain mitotic figures in which the chromosomes would be more evenly distributed. This procedure used embryos taken from the pregnant fish and kept alive as long as

possible in the colchicine solution. More exactly, the procedure used was as follows:

1. Extract embryos and remove from capsular membranes.
2. Place in 0.025% w/v colchicine solution for one hour.
3. Fix in Bouin's and continue with procedure from Fankhauser as previously given.
(Moorhead, 1960, p. 613)

Pretreatment in hypotonic salt solution also reportedly expands cells and causes chromosomes to spread out in the cell. The salt treatment was carried out be pretreatment in colchicine followed by dissection of the tail bud in hypotonic sodium citrate solution. Fixing and staining was according to Fankhauser.

In addition to embryonic tail bud tissue, blood from mature fish was used in an attempt to view chromosomes. Blood was obtained from the heart of a sacrificed fish and placed on a slide. Coagulation time is so short in these fish that difficulty was encountered in preparing smears unless the drops were obtained quickly from the heart. Small amounts of heparin, an anticlotting agent, were also used. The procedure followed was the standard Wright's stain technique, as follows:

1. Touch a drop of blood to the end of the slide.
2. Push across with another slide to smear.
3. Dry rapidly in air.
4. Cover with Wright's stain for 1-2 minutes.

5. Add buffer of distilled water for 2-4 minutes.
6. Rinse in distilled water and blot with filter paper.

(Humason, p. 220)

RESULTS AND DISCUSSION

Work with the tailbud technique using haematoxylin yielded some mitotic figures. However, it proved impossible to work out a complete and reliable karyogram. Failure could be attributed to the nature of the material. The haematoxylin was often taken up by tissues other than the chromosomes resulting in squashes too heavily stained for chromosome identification. Inadequate clearing was believed to be partly responsible for the opaqueness and therefore, various adjustments were made in time and solutions, without success. However, even if dehydration and clearing were improved, the tissue was not soft enough for squashing. No photomicrographs were taken of the slides made by the Fankhauser technique because the mitotic figures were unclear and not in a definite visible stage such as metaphase. Those chromosomes observed were rod shaped.

The effect of the colchicine pretreatment was the same as that reported by Meyer. Clumping resulted and the chromosomes obtained for viewing were too bunched to count. Failure could be due to the nature of the material in taking up the drug or the concentrations used. The embryos at times did not live long after being placed in the solution, possibly accounting

for some of the failures. The tissue may not be susceptible to the drug's effects as was that used by Meyer.

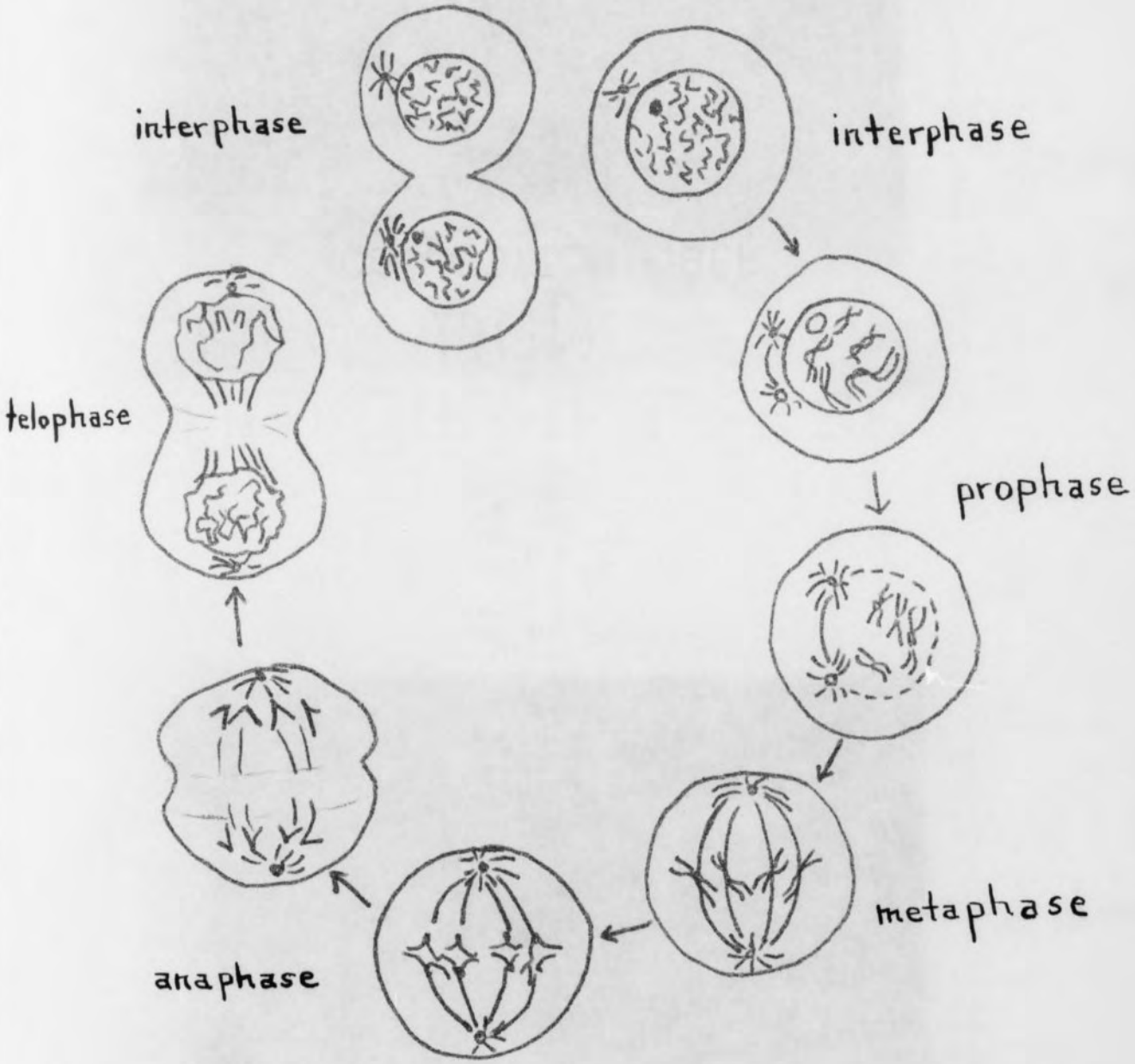
The most promising results were those obtained by the blood smear technique. The smears contained lymphocytes, monocytes, eosinophilic granulocytes, thrombocytes, and erythrocytes. The erythrocytes averaged 8.1 microns in diameter with the elliptical nuclei averaging 4.1 microns. The nuclei stained a violet and the cytoplasm pink to red. Catton (1954) reports that the formation of blood cells in at least some teleosts is amitotic. However, structures were visible in the nuclei of the erythrocytes. Stained a dark purple to black, the appearance of these structures interpreted to be chromosomes, varied in the erythrocytes. (figs. 2 and 3) Chromosomes were observed during what appeared to be spireme of early prophase (fig. 4), prophase (figs. 5 and 6), anaphase (fig. 7), telophase (fig. 8), and a questionable metaphase. The fact that the metaphase stage is usually very short may account for the difficulty in obtaining this stage. In some cells the chromatin did not appear as condensed into chromosomes. It appears that some blood cells must be formed mitotically. Limiting factors such as available magnification, as well as time, prevented any further pursuit of the answer to the

problem.

Several reasons may be postulated to account for the fertility barrier. A difference in chromosomes between the two species may cause their hybrid to be fertile due to reasons already mentioned. More specifically, the difficulty may lie in the sex chromosomes. Sex chromosome abnormalities resulting in infertility are known in man and could conceivably occur in any sexually reproducing organism. The fertility barrier may be entirely of a mechanical nature, due to genetically dictated traits in structure of the fish, which prevent the egg and sperm from coming together for fertilization. There is definitely a fertility barrier between the Xiphophorus helleri-Xiphophorus maculatus. The reason for this remains unsolved but should be explainable by obtaining a reliable chromosomal picture and accurate methods of studying the fish grossly with regard to structure related to breeding habits.

CONCLUSIONS

This work was based on the possibility that the difficulty in producing offspring of the Xiphophorus helleri-Xiphophorus maculatus cross is on a chromosomal basis. Since the data obtained were too meagre to provide a reliable karyogram, no conclusive statement may be given as to the exact basis for the fertility barrier. It may also be said that reports of difficulty in using this type of tissue are confirmed.



(fig. 1) Diagramatic sketch of mitosis in a generalized animal cell.

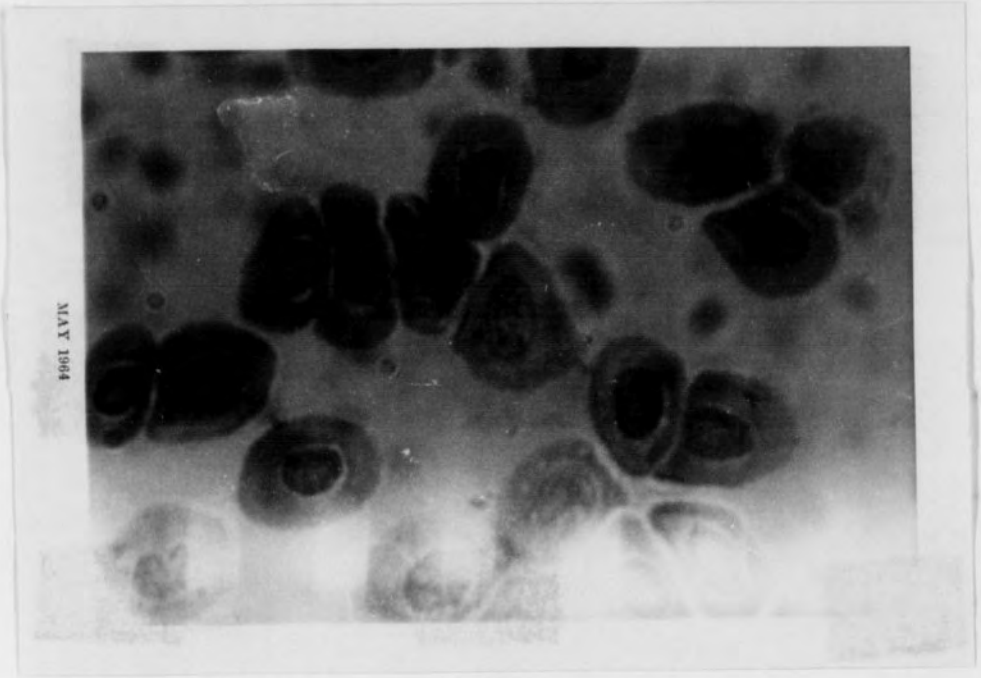


fig. 2

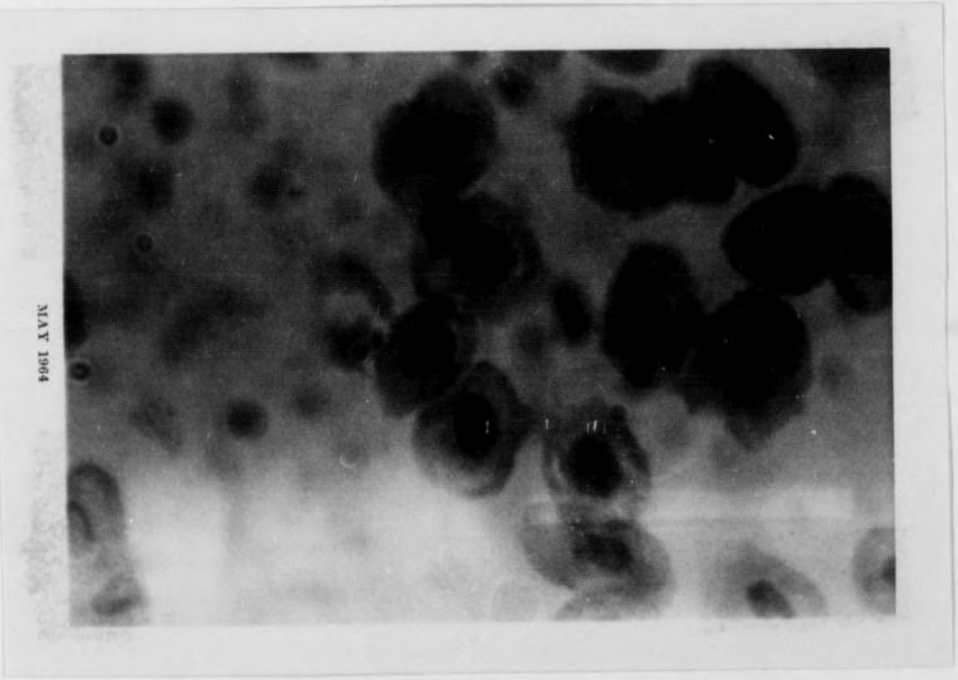


fig. 3

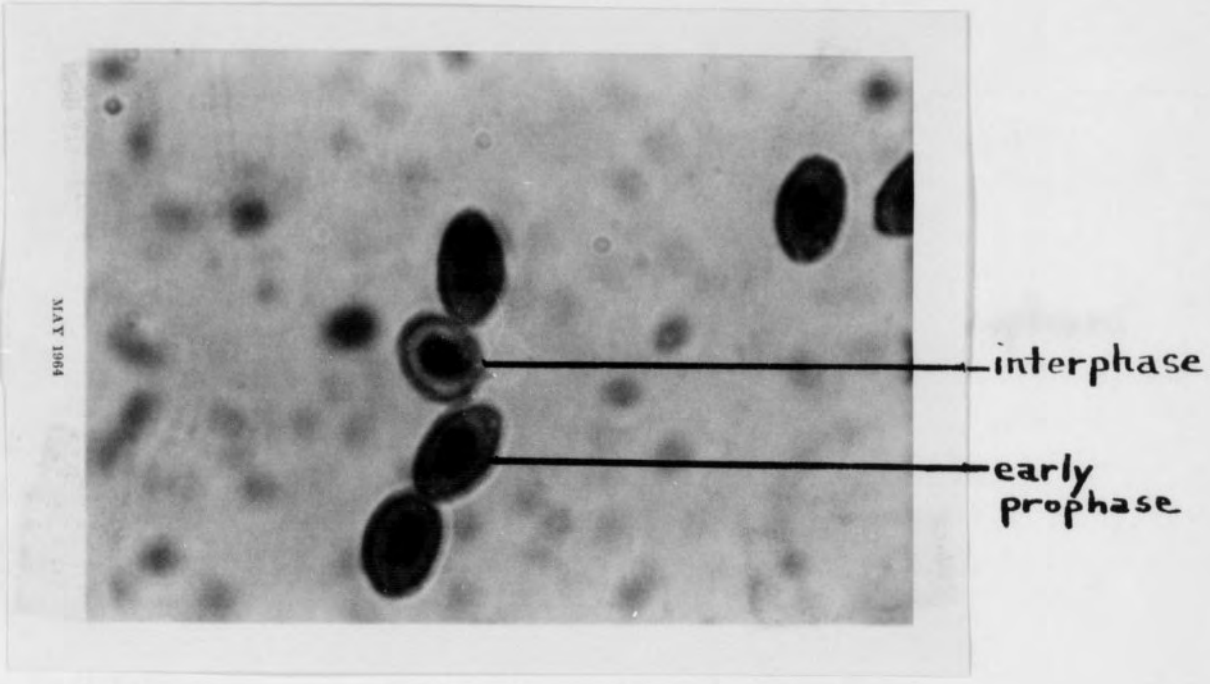


fig. 4

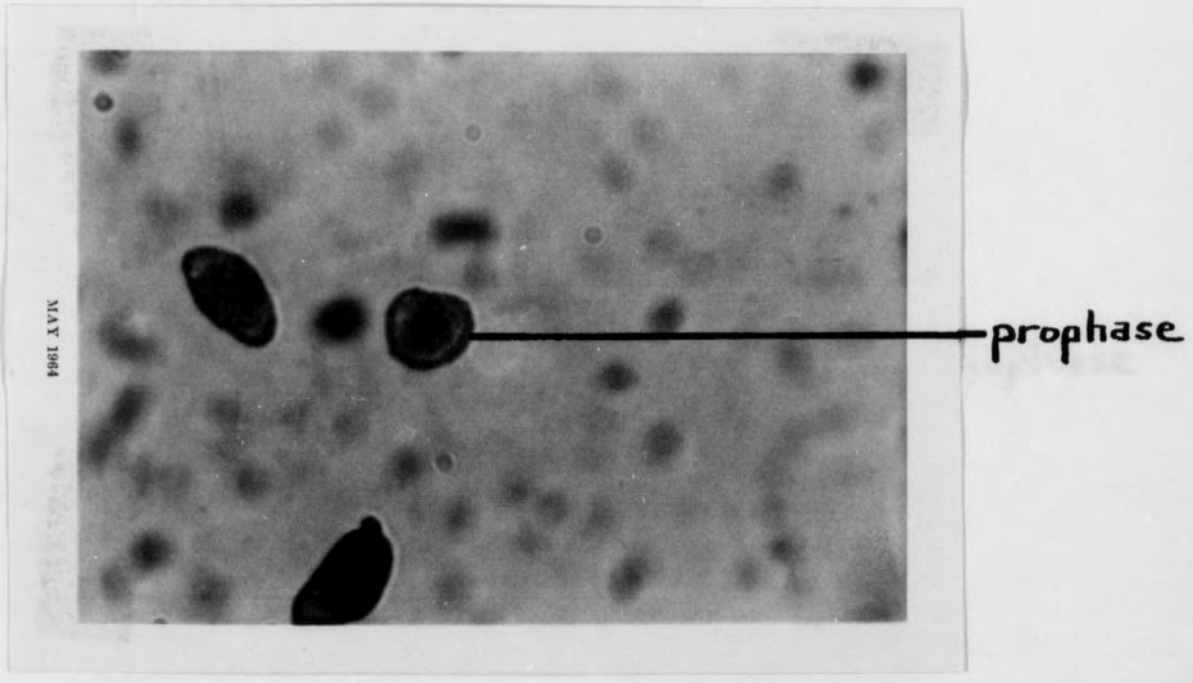


fig. 5

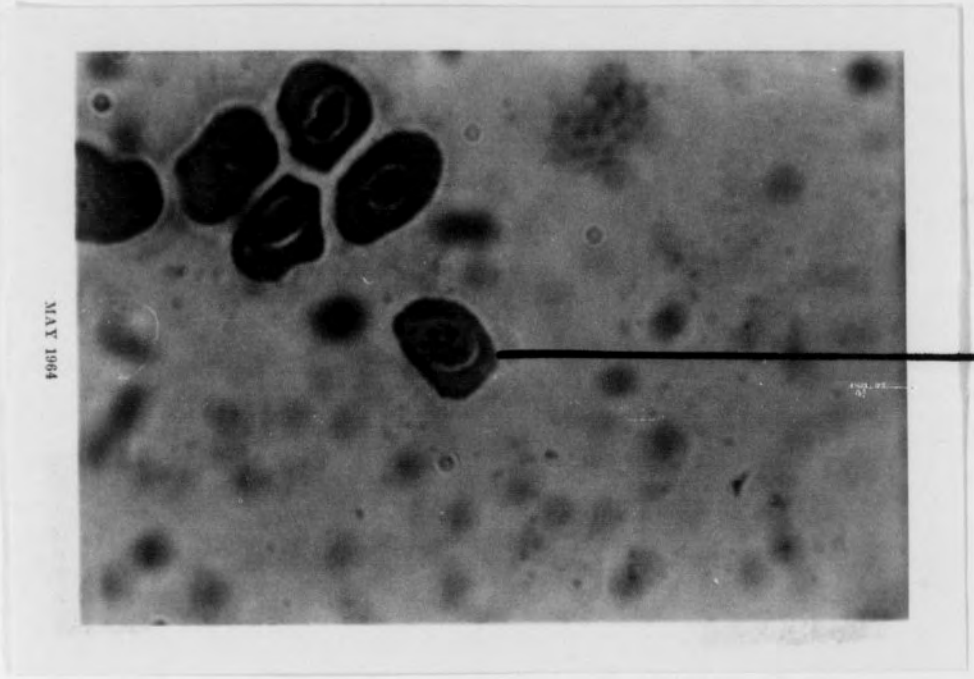


fig. 6

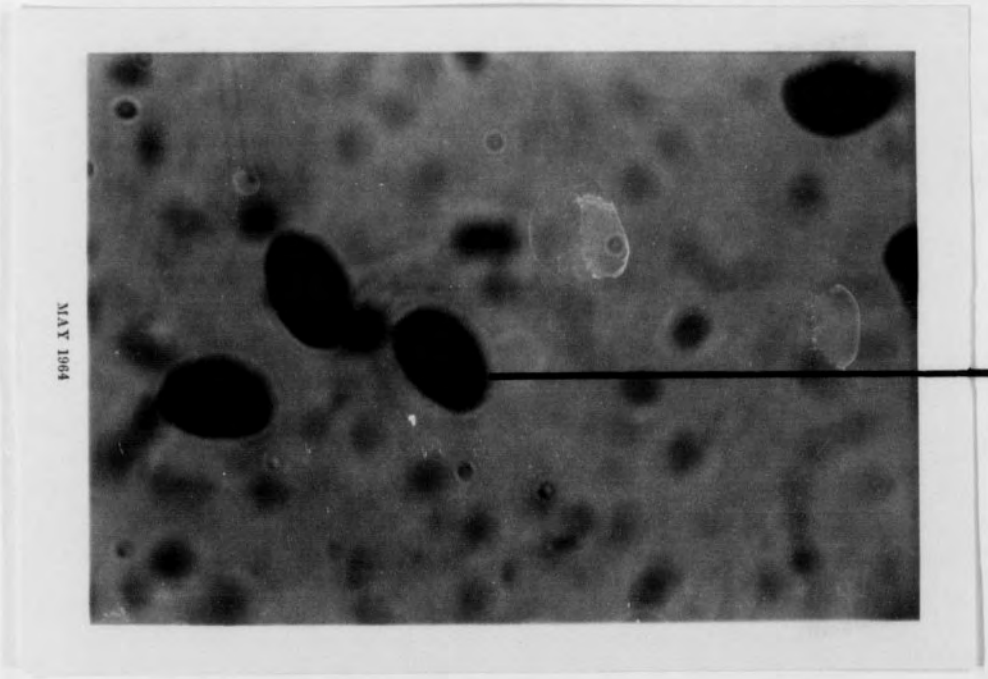


fig 7

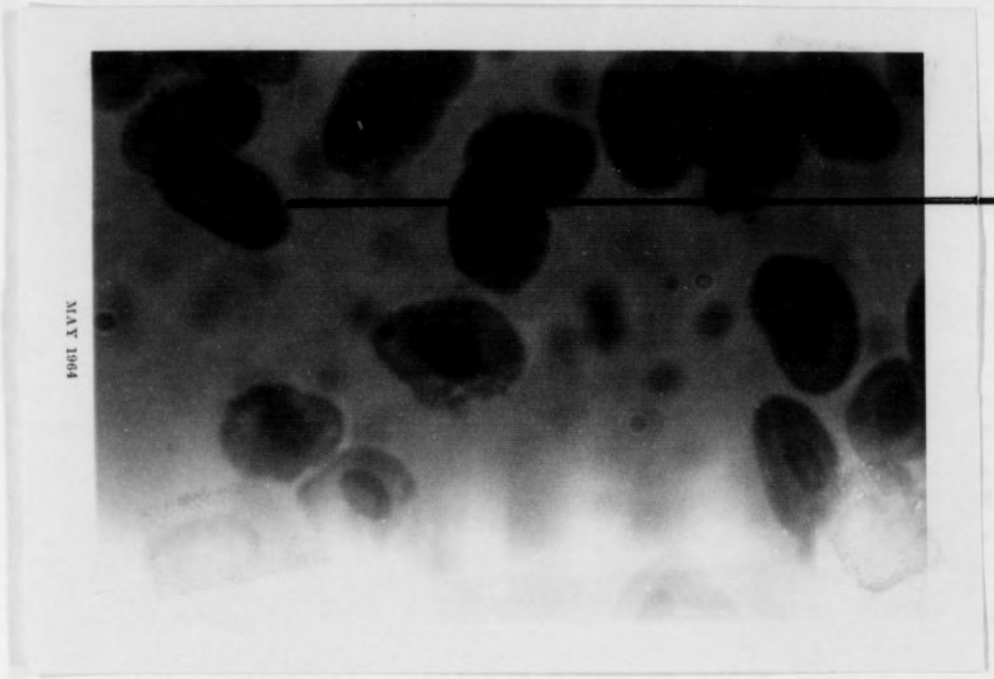


fig. 8

BIBLIOGRAPHY

Baker, J. R. 1960. Cytological Technique. New York: John Wiley and Sons.

Boothroyd, E. R. 1959. Chromosome Studies on three Canadian populations of Atlantic Salmon. Canad. J. Genet. and Cytol., 1, 161.

Catton, W. T. 1954. Blood cell formation in certain teleost fishes. Blood, 6 (1), 39.

Cole, Arthur. A molecular model for biological contractility: Implications in chromosome structure and function. 1963. Biological Abstracts, 42, 728.

Dyer, A. F. 1963. The use of lacto-propionic orcein in rapid squash methods for chromosome preparations. Stain Technology, 38 (2), 85-90.

Fankhauser, G. 1963. personal communication. Department of Biology. Princeton University, Princeton, New Jersey.

French, W. L., R. H. Baker, and J. B. Kitzmiller. 1963. Preparation of Mosquito Chromosomes. Biological Abstracts, 42, 670.

Glucksman, M. A. and M. Gordon. 1953. Hematology of normal and melanomatous fishes: a preliminary report. Zoologica: New York Zoological Society, 38, 151-153.

Gray, P. 1958. Handbook of Basic Microtechnique. New York: McGraw Hill.

Hegner, Robert W. and Karl A. Stiles. 1959. College Zoology. New York: Macmillan Company.

Herskowitz, Irwin H. 1962. Genetics. Boston: Little, Brown and Company.

Hill, Helen D. and R. C. Buckner. 1963. Fertility of Lolium-Festuca hybrids as related to chromosome number and meiosis. Biological Abstracts, 42, 934.

Humason, Gretchen L. 1962. Animal Tissue Techniques. San Francisco: W. H. Freeman and Company.

Krishan, Awtar. 1962. Avian microchromosomes: As shown by prefixation treatment with colchicine, squashing, and haematoxylin staining. Stain Technology, 37 (6), 335-337.

Lee, B. 1937. The Microtomist's Vade-Mecum. Philadelphia: Blakiston's Son.

Meyer, J. R. 1943. Colchicine-Feulgen leaf smears. Stain Technology, 18, 53-56.

Meyer, J. R. 1945. Prefixing with paradichlorobenzene to facilitate chromosome study. Stain Technology. 20, 121-125.

Miner, Roy W., ed. 1948. The Biology of Melanomas. vol. 4. Special publications of the New York Academy of Sciences.

Nagusa, Sh. 1955. Chromosome studies in Pisces: the chromosomes of Mogrunda obscura (Gobiidae), with evidence of male hetergamety. Cytologia, 20, 11-18.

O'Mara, J. G. 1939. Observations on the immediate effects of colchicine. J. Heredity, 30, 35-37.

Ralston, E. M. 1934. A study of the chromosomes of Xiphophorus, Platypoecilus and of Xiphophorus-Platypoecilus hybrids during spermatogenesis. J. Morphology, 56, 423-443.

Rough, R. 1962. Experimental Embryology. Minneapolis: Burgess.

Snow, Richard. 1963. Alcoholic hydrochloric acid-carmine as a stain for chromosomes in squash preparations. Stain Technology, 38 (1), 9-13.

Swanson, Carl P. 1957. Cytology and Cytogenetics. Englewood Cliffs, New Jersey: Prentice Hall, Inc.

Wahl, R. W. 1960. Chromosome morphology in lake trout Salvelenius namaycush. Copeia, 16-19.

Wolf, K. and M. C. Quimby. 1962. Established eurythermic line of fish cells in vitro. Science, 135, 1065-1066.