EFFECTS OF BENZYLADENINE AND ETHIONINE ON THE VESTIGIAL MUTANT OF DROSOPHILA MELANOGASTER

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EFFECTS OF BENZYLADENINE AND ETHIONINE ON THE VESTIGIAL MUTANT OF DROSOPHILA MELANOGASTER

An Abstract

Presented to

the Graduate Council of

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In Partial Fulfillment

of the Requirements for the Degree

Master of Science

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by

Ronald Wilson Hackney

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ABSTRACT

Various concentrations of benzyladenine and ethionine were added to the growth medium of wild and vestigial strains of Drosophila melanogaster to determine their effects on metamorphosis and wing length in these groups. The incorporation of tritiated leucine into larval protein of both strains was measured when development occurred at 25° and 30°.

Benzyladenine appears to speed up pupation and emergence in vestigial flies raised at 25° and 30°. The data indicate that ethionine delays metamorphosis in vestigial, wild, and heterozygous flies. The addition of methionine to the growth media of vestigial flies failed to reverse the inhibitory effect of ethionine on metamorphosis in the mutant strain cultured at 30°. A concentration of 0.01 M ethionine proved to be lethal to the vestigial strain, while 0.02 M ethionine was lethal to wild and heterozygous flies. When allowed to develop at 30°, wild-type wings were observed in the vestigial mutant.

The data show that 0.001 M ethionine severely inhibits wing length in vestigials cultured at 30°. The addition of supplementary methionine resulted in a significant reversal of the inhibitory effect of ethionine on wing length in the mutant strain. Ethionine may allow the expression of the vestigial trait at 30° by interfering with the enzymatic activity of transmethylases. A concentration of 0.01 M ethionine

resulted in severely shortened wings in wild and heterozygous imagoes cultured at 25°.

The data suggest that vestigial larvae cultured at 25° have a higher rate of protein synthesis than wild larvae developing at 25°. Wild larvae cultured at 30° exhibit a much greater rate of protein synthesis than vestigial larvae raised at the same temperature.

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August 1972

To the Graduate Council:

I am submitting herewith a Thesis written by Ronald Wilson Hackney entitled "Effects of Benzyladenine and Ethionine on the Vestigial Mutant of Drosophila melanogaster." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Biology.

Benj. L. Stamper
Major Professor

We have read this thesis and recommend its acceptance:

Charles S. Bolton
Second Committee Member

Floyd M. Ford
Third Committee Member

Accepted for the Council:

Wayne L. Stamper
Dean of the Graduate School

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Vestigial Raised at 30°	2,541	1,219	960	1.02	208
Vestigial Raised at 25°	717	1,123	440	2.04	64
Wild Raised at 30°	6,099	740	80	7.4	824
Wild Raised at 25°	469	588	320	1.47	77

Larvae were allowed to develop at 30° for 85 hours or 110 hours at 25° before feeding on L-leucine-4,5-^{3}H for 10 hours. Approximately 100 larvae were used in each experiment.

CHAPTER I

INTRODUCTION

The phenotype of the vestigial mutant of Drosophila is characterized by the absence of the posterior wing parts. Goldschmidt (1935) suggested that this was the result of degradation of specific parts of the wing during development. In a later study Waddington (1940) postulated that the missing parts simply failed to develop. Fristom (1968) utilized both light and electron microscopy to study wing discs of mature third instar larvae of both wild and vestigial Drosophila. She found degenerating cells present in the wing discs of vestigial larvae but absent in wild types. Her results concur with the mechanism proposed by Goldschmidt. Roberts (1918) reported that the temperature during development influences the expression of the vestigial phenotype. It was later reported that maximum wing length occurred in vestigial males at 30° and in vestigial females at 31° (Harnley, 1930). Akita (1955) observed that genotypically vestigial flies develop normal wild-type wings when cultured at 31°.

This study was undertaken to (1) observe the effects of benzyladenine and ethionine on metamorphosis in wild and vestigial Drosophila developing at 25° and 30°; (2) study the effects of benzyladenine and ethionine on wing length in wild and vestigial flies raised at 25° and 30°; (3) de-

termine the rate of protein synthesis in wild and vestigial flies cultured at 25° and 30° by assaying incorporation of ^{3}H leucine into protein of third instar larvae.

CHAPTER II

LITERATURE REVIEW

The mutant gene, vestigial, manifests a definite effect on growth and metamorphosis in Drosophila melanogaster. Alpatov (1929) found that vestigial larvae and pupae failed to attain the size of wild flies during the corresponding stages of development. He indicated that metamorphosis in the vestigial mutant occurred at a slower rate than in the wild strain. Pearl (1928) reported that vestigial imagoes under customary culture conditions averaged only one-third the life span of wild-type flies. Harnly (1929) found that the mutant gene for vestigial wings definitely retarded development in homozygous vestigial flies in comparison with their heterozygous wild-vestigial brothers and sisters during the larval period.

The vestigial condition can be recognized in very young pupa when the vestigial wings appear as small triangular lobes; whereas in wild pupae they are large flaps somewhat rectangular in shape (Chen, 1929). Fristom (1968) found that the vestigial condition was the result of degeneration of cells of the wing disc. This degeneration involved a shrinkage and condensation of the entire cell followed by phagocytosis by a neighboring cell.

Roberts (1918) reported that wing length in vestigial flies increased with a rise in temperature. Maximum wing

length occurs in vestigial males at 30° and in females at 31° (Harnly, 1930). This sexual dimorphism in the vestigial mutant was also reported by Stanley (1931). Bebak (1964) found that a temperature of 28° influenced the appearance of phenocopial changes in the strain $^{vg}/_{vg}$. At that temperature individuals with wings of large vestigial type appear in the first generation. Akita (1955) reported that normal wild-type wings occur in vestigial flies when they are cultured at 31°. Fristom (1968) found that vestigial flies growing at 30° show an increase in wing size and a decrease in the incidence of degenerating cells. Marchlewski (1961) regards the appearance of normal wings under the effect of high temperature as a phenocopy in the genetically vestigial winged Drosophila melanogaster.

Goldschmidt (1945) reported the existence of two types of mutant actions: one concerned with changing relative rates of integrated developmental processes; another concerned with interfering with definite steps of chemical synthesis. Marchlewski (1957, 1961) asserts that phenocopies have their background not only in the chromosomes or in direct processes connected with the activity of chromosomic genes, but also in cell cytoplasm. Bebak (1964) used Lindegren's (1961) theory of geneaction to account for the appearance of normal wings in the vestigial mutant. She states that temperature may induce partial deformation of DNA bases in the loci of polygenes.

Fristom and Knowles (1966) reported that the functional half-life of m-RNA in imaginal discs of Drosophila melano-

gaster was approximately 2½ hours. They proposed that no large differences in the pattern of protein synthesis in late larval and prepupal discs would be expected because existing m-RNA would buffer the system against changes. Hunt (1970) has proposed that de novo synthesis of thymine ribonucleotides in Drosophila requires the participation of active methionine for the methylation of deoxyuridylic acid to thymidylic acid.

Ethionine, an ethyl analogue of methionine, has been shown to be a metabolic inhibitor in both plant and animal systems. Schrank (1956) found that ethionine inhibits cell elongation in excised sections of Avena coleoptiles. Boll (1960) found that low concentrations of ethionine markedly increased growth in excised tomato roots while higher concentrations strongly inhibited cell elongation. He found that this inhibition was relieved by the addition of methionine. Cleland (1960) has suggested that ethionine inhibits cell elongation in Avena coleoptiles by interfering with protein synthesis.

S-adenosylmethionine is produced when methionine reacts with ATP in the presence of the enzyme, methionine adenosyltransferase (Schapiro and Schlenk, 1960). Shull et al (1966) suggest that free ethionine is rapidly converted to S-adenosylethionine in rat liver by this same enzyme. The S-adenosyl derivative of ethionine is apparently utilized to a much lesser degree than is the corresponding derivative of methionine in transalkylation and in other metabolic reactions (Farber, 1963). Shull (1962) reported that injection of ethionine into rats greatly decreased the level of ATP in the liver within a few

hours. These findings are in agreement with the adenine-trapping mechanism proposed by Schmidt et al (1956). According to this mechanism ATP or its derivatives are removed from the metabolic pathway by combining with ethionine. This removal occurs at a rate faster than the rate of synthesis of ATP from adenine precursors (Villa-Trevino et al, 1962). Villa-Trevino et al (1962) found that the administration of adenine or ATP counteracts ethionine induced inhibition of protein synthesis in rat liver. They concluded that the inhibition of protein synthesis by ethionine was secondary to a decrease in the concentration of adenosine triphosphate. Villa-Trevino et al (1962) suggested that ethionine affects the ATP level by reacting with the nucleotide to form s-adenosylethionine, and thus acting as a trapping agent. Stekol et al (1960) proposed that s-adenosylethionine blocks the synthesis of ATP by inhibiting mitochondrial oxidative phosphorylation. Shull et al (1966) found mitochondrial respiration and oxidative phosphorylation intact in isolated mitochondria of ethionine-treated rats. Norris (1964) found that ATP reversed ethionine induced inhibition of elongation in Avena coleoptiles.

Methylated purines and pyrimidines have been shown to occur in small amounts in RNA of a variety of organisms (Smith and Dunn, 1959). Dunn et al (1960) have shown that methylated purine and pyrimidine bases in nucleic acids are most numerous in the soluble RNA fraction. Natori (1963) has suggested that the methylated groups in RNA are derived from methionine by transmethylation. Evidence has been presented

for the ethylation of purines in liver RNA by ethionine (Farber and Magee, 1960). Swann et al (1971) have recently reported a small but definite ethylation of DNA in the liver of rats which have been injected with large doses of ethionine. Moore and Smith (1969) postulated that ethionine inhibited protein synthesis by blocking methylation of transfer RNA. Natori et al (1961) have proposed that ethionine induced inhibition of protein synthesis may be the result of ethionine becoming incorporated into protein. The protein would be abnormal since ethionine is substituted in place of some methionine. Natori (1963) has suggested that methyl groups function in altering the secondary and tertiary structure of the polynucleotide chain. He postulates that the administration of ethionine results in abnormal RNA which fails to function normally in protein biosynthesis.

Merrell (1970) reported that ethionine severely inhibits elongation of hypocotyls and roots in lettuce seedlings. He found that ethionine inhibited hypocotyl elongation which had been stimulated by giberellic acid. His results indicate that methionine reverses ethionine induced inhibition, but ATP does not. Harker (1971) found that ethionine severely inhibits incorporation of triated leucine into protein of Grand Rapids lettuce seedlings. Binion (1971) reported that ethionine drastically reduced the incorporation of methionine-methyl-^{14}c into both high molecular weight and transfer RNA of dwarf pea internodes. He also found that ethionine reduced cholorophyll synthesis in dwarf pea seedlings which had been treated with giberellic acid. Ethionine has been shown to inhibit inver-

tase synthesis in sugar beets (Stone, Whitty, and Cherry, 1970), and inhibit GA_3 stimulated α-amylase synthesis in the aleurone cells of barley (Chandra and Duynstee, 1971). Marzluf (1969) reported that ethionine produces a phenocopy of the eyeless mutant in Drosophila melanogaster, and converts the recessive eyeless factor into a semi-dominant gene. He found that the addition of supplementary methionine to the medium not only failed to reverse the effects of ethionine but increased the expression and penetrance of the eyeless phenocopy. Hunt (1970) observed that ethionine produced a reduction in eye size in the Pacific eyeless strain considerably in excess of equivalent concentrations of methionine. The action of ethionine was found to be independent of dietary RNA concentration.

Benzyladenine, an active phytokinin, delays senescence of both leaves and the entire shoot when applied to the primary leaves of intact bean plants. The retardation was characterized by higher levels of chlorophyll, protein, RNA and ribonuclease activity (Fletcher, 1969). Tuli, Dilley, and Whittwer (1964) have suggested that respiratory inhibition induced by benzyladenine is the result of inhibition of glycolytic kinases.

The application of benzyladenine to primary bean leaves of intact plants results in increased leaf area due to an extension of the duration of the period of leaf expansion (Jacoby and Dagan, 1969). Schaeffer and Sharpe (1969) reported that benzyladenine, applied to apical buds of tobacco plants, activates axillary bud growth and stimulates DNA

synthesis. Smolinski, Saniewski, and Peiniazek (1969) found that benzyladenine stimulated the growth of axillary buds of Pisum sativum, but retarded growth in roots and shoots. Lovell and Moore (1970) reported that excised mustard cotyledons treated with benzyladenine exhibited an increase in blade growth and a suppression of root initiation and development.

Fox (1965) found that a small amount of the benzyladenine added to kinin requiring tissues was incorporated into soluble RNA. He proposed that incorporation of benzyladenine into t-RNA confers amino acid transfer competency on a molecule much as methylation may do. This indicates that kinins function by serving as the biological equivalent of an RNA methylating enzyme (Fox, 1965). Fox (1965) alternately suggests that a small amount of benzyladenine may be incorporated into m-RNA, acting as a derepressing agent by preventing its normal repressing function. Carpenter and Cherry (1966) reported that low concentrations of benzyladenine enhanced nucleic acid synthesis in peanut cotyledons while higher concentration proved to be inhibitory.

Galston and Davies (1969) have proposed that phytokinins represent break down products of transfer RNA and not t-RNA precursors. Phytokinins, which stimulate bud formation in moss protonemata, are loosely attached to their target cells and are easily washed out (Brandes and Kende, 1968). They suggest that phytokinins control development by binding to specific sites on the target molecules.

CHAPTER III

MATERIALS AND METHODS

Stocks and Experimental Cultures

Wild and vestigial stocks of *Drosophila melanogaster* were obtained from Carolina Biological Supply Company, Burlington, North Carolina. Additional cultures were prepared by allowing wild and vestigial flies to mate and deposit eggs on control medium. The control medium consisted of 10 ml of Formula 4-24 Instant Blue Drosophila medium, an equal volume of water, and a small pinch of Fleischmann's dry yeast. Flies were cultured in cylindrical plastic vials which were 10.16 cm tall, 3.275 cm in diameter, and were closed with polyurethane plugs. The Formula 4-24 Instant Blue medium was composed of: oat flour, soy flour, wheat flour, starches, dibasic calcium carbonate, citric acid, niacinamide, riboflavin, sodium chloride, sodium iron purophosphate, sucrose, thiamine mononitrate, brewers yeast, emulsifier, mold inhibitor, preservatives, and food coloring.

The stocks were maintained and propagated at a temperature of 25°. Experimental cultures were raised at 25° or 30°, but some groups were observed at both temperatures. A Model 808 Precision Scientific incubator with a temperature fluctuation of 24°-25° and a Model 805 Precision Scientific incubator with a variance of 28°-30° were used to obtain the two experimental temperatures. Both were set on a cycle of 16

hours of light and 8 hours of darkness. The only attempt to control the humidity was to keep an open vessel of water in each incubator.

The chemicals used in this study were acquired from the following sources: benzyladenine was purchased from Calbiochem, Los Angeles, California; ethionine from Sigma Chemical Company, St. Louis, Missouri; and methionine from Nutritional Biochemicals Corporation, Cleveland, Ohio. A stock solution of benzyladenine was prepared by dissolving the chemical in a small volume of 95% ethanol, adding distilled water, evaporating the ethyl alcohol by heating, and adjusting the volume to 1 liter.

The experimental media were prepared by pippeting 10 ml of a stock solution of benzyladenine, ethionine, or methionine into vials containing an equal volume of instant Drosophila medium. Controls consisted of equal volumes (10 ml) of instant Drosophila medium and distilled water. Larvae used in protein synthesis studies were raised on control medium. All experiments were run in duplicate.

Wild and vestigial virgins were collected within eight hours of eclosion and kept on control medium for at least two days. They were then transferred to experimental media and allowed to mate with males from one to several days old. This was necessary since females do not reach sexual maturity until approximately 48 hours after emergence, while males are capable of mating immediately after eclosion. In most experiments adults were not allowed to mate until they were placed on experimental media. This prevented fertilized females

from holding back eggs containing developing embryos. An exception to this procedure was employed when working with vestigial flies. Extremely small numbers of progeny made it necessary to place males and virgins on fresh control media for eight hours prior to the laying period. Stanley (1931) reported that females, in the presence of fresh fermenting food and in the absence of large quantities of eggs, were stimulated to lay almost continuously. The offspring obtained in this manner were still approximately the same age.

Six pairs of vestigial or wild imagoes were used in all experiments. When testing the effects of ethionine on heterozygotes, six vestigial females and six wild males were used.

Wild flies were anesthetized with ether and placed directly on experimental media. Vestigial adults were shaken out of vials containing control medium into vials of experimental food. In experiments with benzyladenine, ethionine, and methionine, laying periods were six hours in length and were conducted in continuous light at a temperature of approximately 24°. When protein synthesis at 25° and 30° was studied, the laying periods were extended to eight hours to obtain a larger number of larvae.

When benzyladenine, ethionine, or methionine were tested at both 25° and 30°, adults were allowed to mate and deposit eggs at 30°. They were then shaken into vials containing the same experimental media, and a second laying period proceeded. These vials were incubated at 25°. In this manner the same parents produced offspring to be raised at the two different experimental temperatures. Immediately following each laying

period, the imagoes were removed, and the vials were placed in an incubator set at the appropriate experimental temperature.

Wild and vestigial Drosophila were allowed to develop from egg to adult at 25°. To overcome the high mortality rate encountered at 30°, vials incubating at that temperature were transferred to 25° to complete development after pupae were observed. According to Harnly (1935) only the larvae stage demonstrated the temperature-effective period for wing length in vestigial flies at 30°.

The right wings of wild imagoes were measured using a Bausch and Lomb stereomicroscope equipped with a 0.7-3.0 power pod and 10X oculars, the right one containing a calibrated micrometer. Both right and left wings of vestigial flies were measured and the average length recorded. Measurements were made from the anterior edge of the pteropleura to the tip of the first posterior cell. A few flies had to be discarded due to excessive curling of the wings. Wing measurements were determined on slightly etherized flies. When extremely large numbers of flies emerged simultaneously, part of the population was etherized and preserved in 70% ethanol for one to three days before being measured. Some F_1 virgins and males were collected at eight hour intervals, measured, and kept on control medium for at least two days. These flies were placed on fresh experimental food, and a F_2 generation was observed using the procedure outlined previously. In some groups the F_1 adults died before a second cross could be made while in other instances no F_2 larvae developed. The

number of first generation imagoes used varied from group to group according to the number of progeny emerging.

Incorporation of Radioactive Leucine

Protein synthesis studies were conducted by measuring incorporation of ^{3}H-leucine into larval protein of Drosophila melanogaster. L-leucine-4,5,-^{3}H, specific activity 29.8 curies per millimole was obtained from International Chemical and Nuclear Corporation, Irvine, California.

The following procedure was used to determine protein synthesis by incorporation of L-leucine-4,5-^{3}H into protein of third instar Drosophila larvae. Approximately 100 wild and vestigial larvae which had been cultured at 30° for 85 hours or 25° for 110 hours were collected and washed twice with sterile distilled water. Sterile 9 cm filter paper was placed in glass petri dished which had been sterilized in an autoclave. The filter paper was moistened with 5 ml of incubation medium containing 3% sucrose and 10-^{4}M streptomycin and penicillin. The solution was sterilized by filtration through millipore H A 0.45 micron 25 mm filters into sterile flasks. Ten microcuries of L-leucine-4,5-^{3}H were added to the incubation solution giving a final concentration of 2 microcuries per ml. The larvae were added to the petri dishes and allowed to feed for 10 hours in continuous light at approximately 24°.

After incubation excess radioactive material was removed, and the larvae were washed three times with sterile distilled water. The larvae were homogenized in a chilled mortar containing 5 ml of 0.01 M tris buffer pH 7.5 and 0.2 mg/ml L-

leucine. The homogenate was collected in plastic centrifuge tubes. The mortar and pestle were washed with 5 ml of tris buffer and leucine, and this liquid was added to the tubes.

A 0.2 ml aliquot of the homogenate was added to a plastic tube containing 2 ml of 10% Trichloroacetic acid (TCA) and allowed to precipitate in a cold room at 0°. The precipitate was removed by centrifugation at 5,000 x g for 10 minutes in an International Clinical centrifuge. Fifty microliter aliquots of the supernatant were taken from each of the four tubes. The samples were placed on glass fiber filter paper and dried under a 250 watt General Electric infrared lamp for 15 minutes. The filters were placed in scintillation vials containing approximately 10 ml of scintillation fluid. The scintillation solution consisted of 4 grams of 2,5 diphenyloxazole and 50 milligrams of 1,4-bis-2-(5-phenyloxazolyl) benzene per liter of toulene. The vials were placed in a Nuclear Chicago Corporation, Unilux II liquid scintillation counter and counted for 10 minutes to determine the soluble amino acid precursor pool.

Incorporation of L-leucine-^{3}H into protein was assayed by a modification of the procedure of Mans and Novelli (1960). Two hundred microliter aliquots of the homogenate were added to filter papers cut in one inch squares, and the squares were dried under an infrared lamp.

The filter papers were placed in a beaker of cold (0°) 5% TCA and allowed to stand on ice for 15 minutes. The cold 5% TCA was decanted, and the filter papers were rinsed twice with cold 5% TCA containing L-leucine. The samples were in-

cubated at 90° for 10 minutes in a beaker containing 5% TCA. The TCA was decanted, cold 5% TCA added, and the beaker placed in a bucket of ice for 10 minutes. The TCA was decanted, and the papers were incubated in 95% ethanol for 3 minutes. After the ethanol was removed, the filter papers were extracted with ethanol, ether, and chloroform (2:2:1) at 55°-60° for 3 minutes. The liguid was decanted, and the papers were washed with acetone and dried under an infrared lamp for 15 minutes. The squares were placed in scintillation vials containing approximately 10 ml of scintillation fluid. L-leucine-^{3}H incorporation into protein was measured by counting the filters in a scintillation counter programmed for tritium counting.

An aliquot of 0.2 ml of the homogenate was dissolved in 1 ml of 3 N sodium hydroxide. This solution was diluted with 2 ml of distilled water and the protein of each sample was determined by the method of Lowry et al (1951).

CHAPTER IV

RESULTS

Effects of Benzyladenine on Metamorphosis in Wild and Vestigial Drosophila

Benzyladenine is a cytokinin which stimulates cell division and delays senescence in green plants (Tuli, Dilley, and Whittwer, 1964). Since the effect of benzyladenine in animal systems is uncertain, concentrations varying from 0.01 parts per million (ppm) to 20 ppm were added to the growth medium of wild and vestigial flies. Both strains were observed during metamorphosis at 25° or 30°.

The wild strain of Drosophila melanogaster was not significantly affected by benzyladenine, even at a concentration of 20 ppm. Wild flies developing on all concentrations of benzyladenine at 25° and 30° pupated and emerged at about the same time as control flies (Appendix I and II).

The data in Table I was collected from a population of vestigial flies raised on various concentrations of benzyladenine at 25°. The data indicate that benzyladenine promotes early pupation and emergence in this group. Vestigial larvae developing on all concentrations of benzyladenine at 25° pupated before control flies. The 0.1 ppm treatment was most effective, pupation occurring 42 hours before control flies (Table I). Vestigial larvae raised on 1 ppm and 10 ppm benzyladenine pupated 15 hours and 20 hours before the con-

TABLE I

EFFECTS OF BENZYLADENINE
ON METAMORPHOSIS IN F_1 VESTIGIAL
FLIES RAISED AT 25°

Concentration of BA	First Pupa Observed Time in Hours	First Adult Observed Time in Hours
Control	160	244.5
0.01 PPM	151	212.5
0.1 PPM	119	212.5
1 PPM	135	219
10 PPM	140	238

Flies were allowed to undergo complete development on the various concentrations of benzyladenine. Time was computed from the beginning of the laying period to the appearance of the first pupa or adult.

trol group. The least stimulation was observed in the 0.01 ppm treatment where larvae pupated 9 hours earlier than the control. Eclosion in vestigial flies raised at 25° was most affected by the 0.01 ppm and 0.1 ppm treatments of benzyladenine. Pupae raised on these concentrations at 25° emerged approximately 30 hours before control flies. One ppm benzyladenine resulted in eclosion 25.5 hours before the control group. Vestigial flies raised on 10 ppm benzyladenine emerged 6.5 hours before adults were observed in control vials.

Table II illustrates the effect of benzyladenine on metamorphosis in vestigial flies raised at 30°. Larvae raised on all concentrations of benzyladenine reached the pupa stage approximately 12.5 hours earlier than control flies. Vestigial pupae developing on 0.1, 10 ppm, and 20 ppm benzyladenine emerged 4 hours before the control group. Those pupae raised on 1 ppm benzyladenine emerged 8 hours before control flies. While none of the differences between experimental groups and the control were greater than 12.5 hours (Table II), the trend toward early pupation and emergence produced by benzyladenine in the vestigial strain is still apparent.

Effects of Ethionine on Metamorphosis in Wild, Vestigial, and Heterozygous Drosophila

Marzluf (1969) reported that a concentration of 0.01 M ethionine was lethal to the eyeless mutant and most wild strains of Drosophila melanogaster. Concentrations of ethionine varying from 0.0001 M to 0.02 M were added to the growth medium of wild and vestigial flies. The effects of ethionine on metamorphosis in both strains were observed when develop-

TABLE II

EFFECTS OF BENZYLADENINE
ON METAMORPHOSIS IN F_1 VESTIGIAL
FLIES RAISED AT 30°

Concentration of BA	First Pupa Observed Time in Hours	First Adult Observed Time in Hours
Control	130.5	214
0.1 PPM	118	210
1 PPM	118	206
10 PPM	118	210
20 PPM	118	210

Flies were allowed to develop on benzyladenine at 30° until most of the larvae within the vial had pupated. The flies were then placed in an incubator set at 25° to complete development. Time was computed from the beginning of the laying period to the appearance of the first pupa or adult.

ment occurred at 25° and 30°.

Wild and heterozygous larvae were observed to develop on media containing 0.01 M and 0.02 M ethionine when raised at 25° and 30°. Adults were observed in the 0.01 M ethionine treatments at both temperatures. Wild and heterozygous larvae developing on 0.02 M ethionine at 25° and 30° died before pupation.

Ethionine retarded pupation and eclosion in wild flies developing at 25° (Table III). Larvae grown on 0.001 M ethionine pupated 12 hours later than control flies. Wild larvae feeding on 0.0001 M ethionine pupated at about the same time as control flies. Eclosion was delayed 14 hours in the 0.0001 M and 0.001 M ethionine treatments at 25° (Table III). No reliable data were available on wild flies raised on 0.01 M ethionine at 25°.

The data contained in Table IV indicate that ethionine delays pupation and eclosion in wild flies raised at 30°. Pupation was delayed 50.5 hours in wild flies developing on 0.01 M ethionine at 30°. Wild larvae grown on 0.001 M ethionine pupated approximately 10.5 hours later than control larvae. The 0.0001 M ethionine treatment produced pupation at about the same time as the control. Wild pupae raised on 0.01 M ethionine at 30° emerged 46.5 hours after control flies. Eclosion was delayed 15 hours in the wild pupae developing on 0.001 M ethionine at 30°. A concentration of 0.0001 M ethionine produced wild imagoes 8 hours after eclosion was observed in the control group.

TABLE III

EFFECTS OF ETHIONINE ON
METAMORPHOSIS IN F_1 WILD FLIES
RAISED AT 25°

Concentration of Ethionine	First Pupa Observed Time in Hours	First Adult Observed Time in Hours
Control	134	222
0.0001 M	134	236
0.001 M	146	236

Flies were allowed to undergo complete development on the various concentrations of ethionine at 25°. Time was computed from the beginning of the laying period to the appearance of the first pupa or adult.

TABLE IV

EFFECTS OF ETHIONINE ON METAMORPHOSIS IN F_1 WILD FLIES RAISED AT 30°

Concentration of Ethionine	First Pupa Observed Time in Hours	First Adult Observed Time in Hours
Control	94	181.5
0.0001 M	94	189.5
0.001 M	104.5	196.5
0.01 M	144.5	228

Flies were allowed to develop on ethionine at 30° until most of the larvae within the vial had pupated. The flies were then placed in an incubator set at 25° to complete development. Time was computed from the beginning of the laying period to the appearance of the first pupa or adult.

A concentration of 0.01 M ethionine is apparently lethal to the vestigial mutant. No vestigial larvae were ever observed to develop on that concentration during the course of this study. Vestigial larvae cultured at 30° were allowed to feed on two concentrations of ethionine (Table V). The 0.001 M treatment delayed pupation 35.5 hours in vestigial larvae raised at 30°. Larvae developing on 0.0001 M ethionine pupated at the same time as the control flies. Emergence in both experimental groups was simultaneous with control pupae.

Methionine was added to the growth medium of vestigial flies in an effort to reverse the inhibitory effect on metamorphosis observed in that group at 30°. Pupation was delayed 36.5 hours in vestigial larvae raised on medium containing both 0.001 M methionine and 0.001 M ethionine (Table VI). A treatment of 0.001 M ethionine alone delayed pupation 26.5 hours. The same concentration of methionine at 30° resulted in pupation 8 hours after control larvae. Imagoes were observed in the 0.001 M ethionine group 20 hours after eclosion had occurred in control vials. Emergence was also delayed 20 hours in vestigial flies developing on medium containing both 0.001 M ethionine and 0.001 M methionine. The 0.001 M methionine treatment resulted in emergence 9.5 hours before control pupae.

The effects of ethionine on metamorphosis in heterozygous flies raised at 30° are recorded in Table VII. Pupation was delayed 17 hours in heterozygotes raised on 0.001 M ethionine. Pupation in the 0.0001 M treatment was simultaneous with control larvae. Eclosion was observed to occur in heterozygous

TABLE V

EFFECTS OF ETHIONINE ON METAMORPHOSIS IN F_1 VESTIGIAL FLIES RAISED AT 30°

Concentration of Ethionine	First Pupa Observed Time in Hours	First Adult Observed Time in Hours
Control	101.5	200
0.0001 M	101.5	200
0.001 M	137	200

Flies were allowed to develop on ethionine at 30° until most of the larvae in the vial had pupated. The flies were then placed in an incubator set at 25° to complete development. Time was computed from the beginning of the laying period to the appearance of the first pupa or adult.

TABLE VI

EFFECTS OF ETHIONINE AND METHIONINE ON METAMORPHOSIS IN F_1 VESTIGIAL FLIES RAISED AT 30°

Treatment	First Pupa Observed Time in Hours	First Adult Observed Time in Hours
Control	108	209.5
0.001 M Ethionine	134.5	229
0.001 M Methionine	116	200
0.001 M Methionine + Ethionine	144.5	229

Flies were allowed to develop on ethionine at 30° until most of the larvae in the vial had pupated. The flies were then placed in an incubator set at 25° to complete development. Time was computed from the beginning of the laying period to the appearance of the first pupa or adult.

TABLE VII

EFFECTS OF ETHIONINE ON METAMORPHOSIS IN F_1 HETEROZYGOUS FLIES RAISED AT 30°

Concentration of Ethionine	First Pupa Observed Time in Hours	First Adult Observed Time in Hours
Control	95	182.5
0.0001 M	95	191
0.001 M	112	204.5

Flies were allowed to develop on ethionine at 30° until most of the larvae in the vial had pupated. The flies were then placed in an incubator set at 25° to complete development. Time was computed from the beginning of the laying period to the appearance of the first pupa or adult.

pupae raised on 0.001 M ethionine 22 hours after emergence of the first control imagoes. A concentration of 0.0001 M ethionine delayed eclosion 8.5 hours in the heterozygotes developing at 30°. These same two concentrations of ethionine had little effect on pupation or emergence in heterozygotes cultured at 25° (Appendix III).

The Effects of Benzyladenine on Wing Length in Wild and Vestigial Drosophila

Benzyladenine at a concentration of 10 ppm resulted in an increase in wing length in vestigial males cultured at 25° (Figure 1). The increase was found to be significant at the 5% level of significance using Duncan's (1955) multiple range test. An F_2 generation of vestigial flies were allowed to develop on media containing benzyladenine at 25° (Appendix IV). No significant differences were observed between any of the experimental groups and the control.

Vestigial males raised on all concentrations of benzyladenine at 30° appear to have a smaller mean wing length than corresponding control males (Figure 2). Vestigial males raised on 0.1 ppm benzyladenine at 30° had wings which were significantly shorter than control males at the 5% level of significance. No significant differences were observed in vestigial females raised on benzyladenine at 30°. No F_2 generation was obtained at this temperature.

Wild males and females raised on 10 ppm benzyladenine at 25° exhibited a decrease in wing length when compared to corresponding control flies (Figure 3). The decreases were found to be significant at the 5% level. No significant dif-

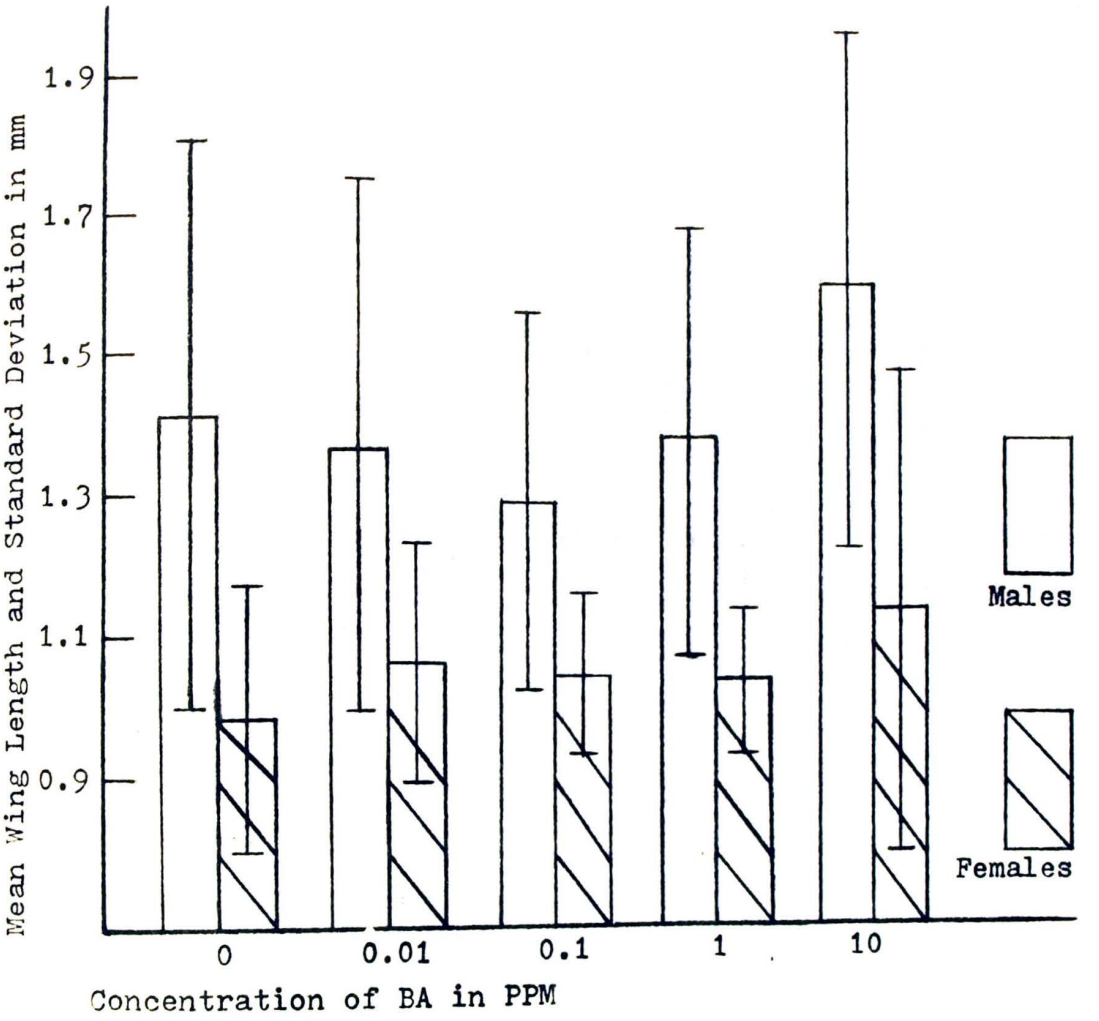


Figure 1. Effects of benzyladenine on wing length in F_1 vestigial flies cultured at 25°.

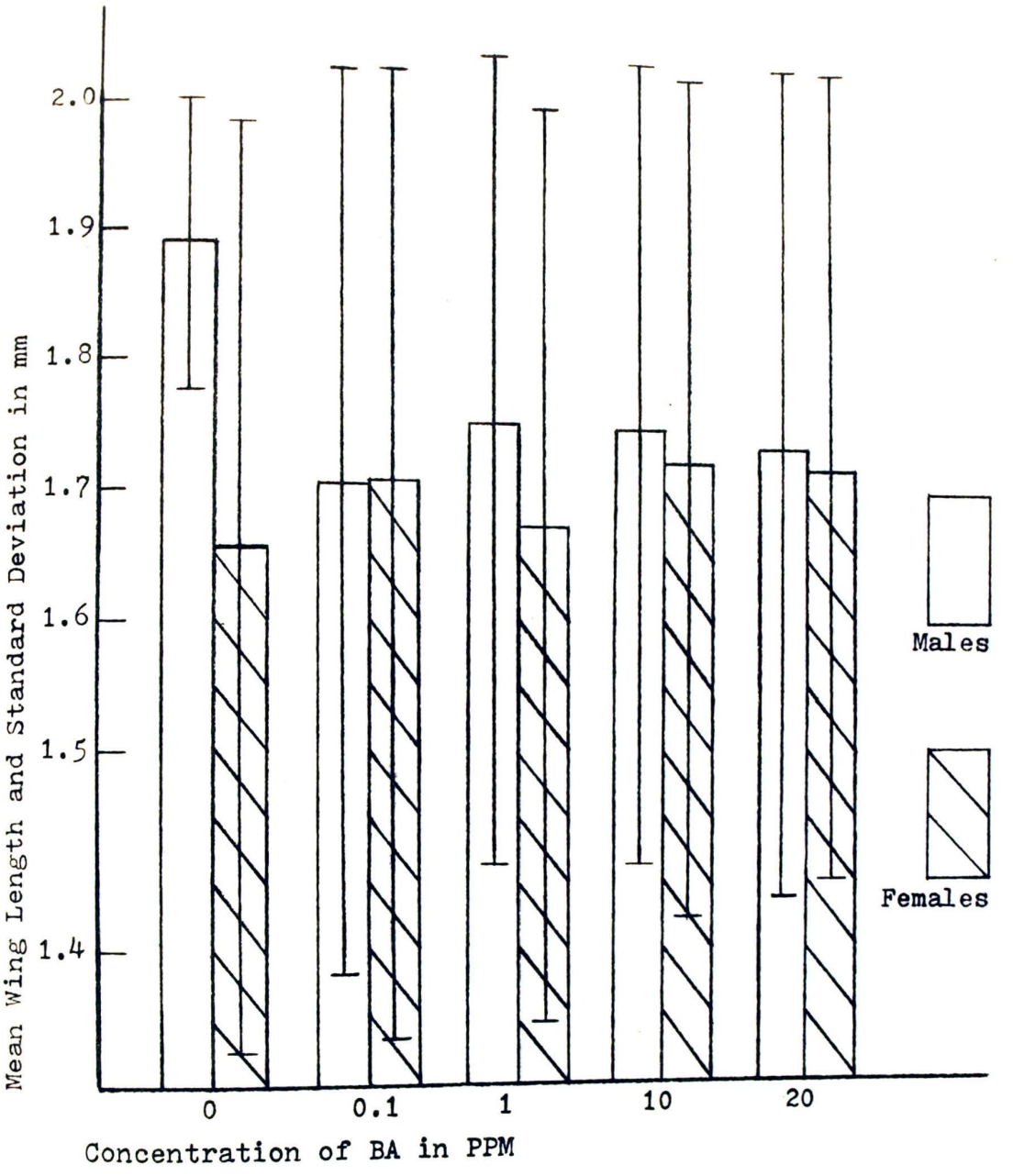


Figure 2. Effects of benzyladenine on wing length in F_1 vestigial flies cultured at 30^{o}.

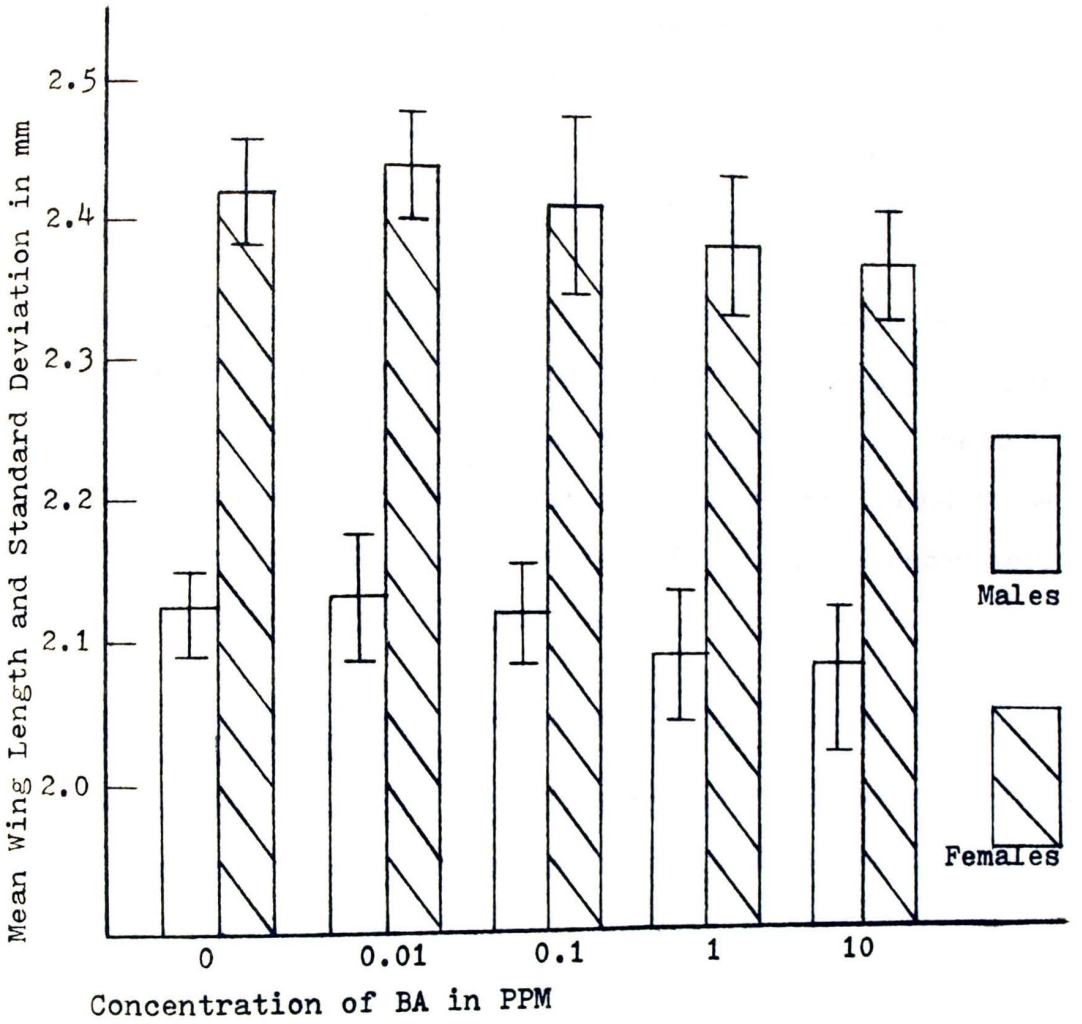


Figure 3. Effects of benzyladenine on wing length in F_1 wild flies cultured at 25^{o}.

ferences in wing length were observed in F_2 wild flies raised on benzyladenine at 25° (Appendix V).

When wild flies were allowed to develop on various concentrations of benzyladenine at 30° (Figure 4), their mean wing lengths were greater than those of corresponding controls. Wild females raised on 10 ppm benzyladenine at 30° had a mean wing length which was significantly larger than the mean wing length of control females at the 5% level of significance.

A second generation of wild flies were raised on the same concentrations of benzyladenine at 30° (Figure 5). Wing length was reduced in F_2 wild males cultured on 0.01 ppm and 1 ppm benzyladenine at 30°. Both reductions were found to be significant at the 5% level. No significant differences were observed in the F_2 wild females.

The Effects of Ethionine on Wing Length in Vestigial, Wild and Heterozygous Drosophila

Ethionine, an inhibitor of RNA and protein synthesis, has been found to produce a phenocopy of the eyeless mutant in Drosophila melanogaster (Marzluf, 1969). Various concentrations of ethionine were added to the growth medium of wild, vestigial, and heterozygous flies to determine its effect on wing length.

Figure 6 illustrates the effects of ethionine on a F_1 generation of vestigial flies cultured at 30°. The 0.001 M ethionine treatment resulted in a severe reduction in wing length in vestigial males and females raised at 30°. These reductions were found to be significant at the 5% level of

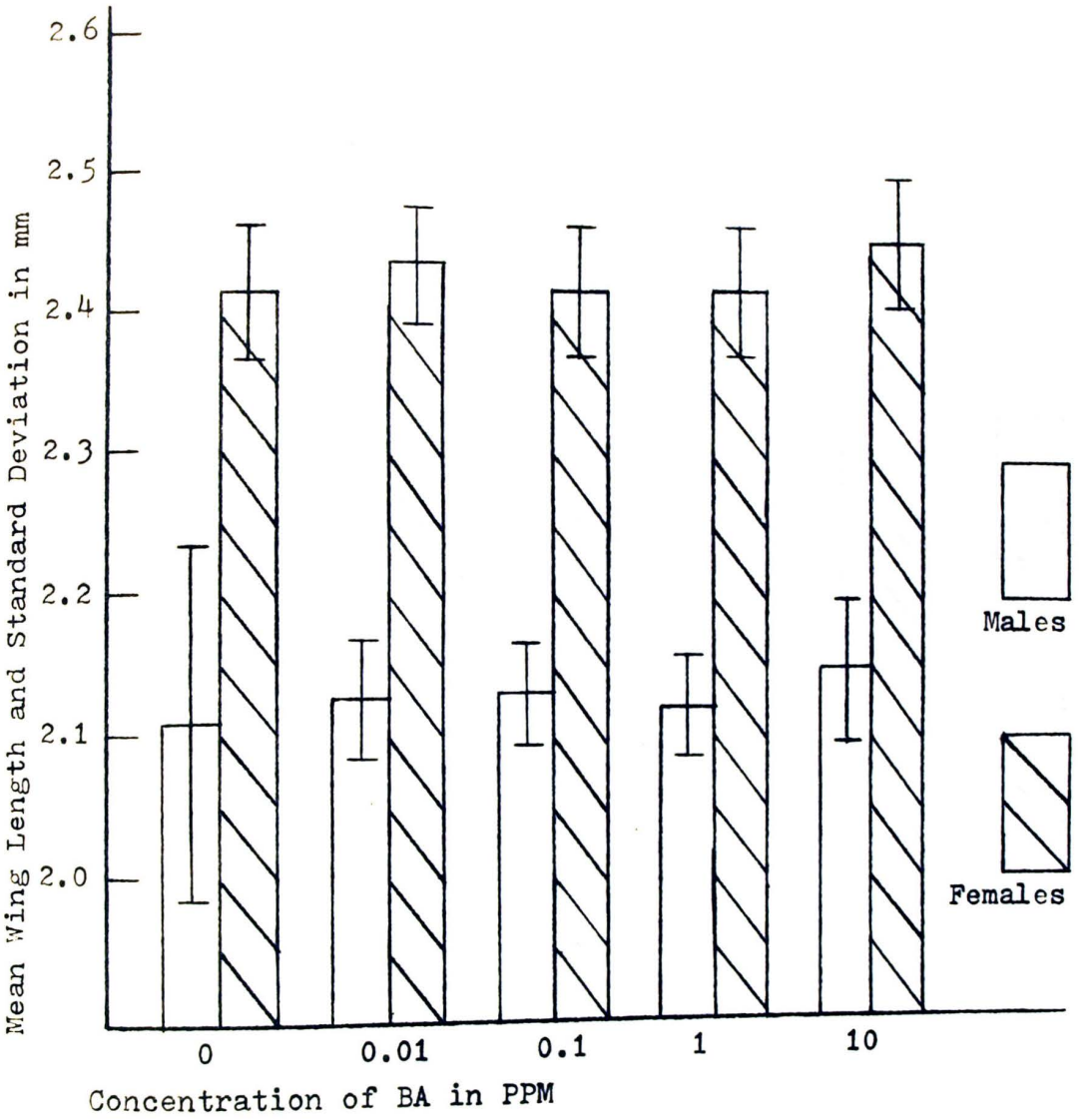


Figure 4. Effects of benzyladenine on wing length in F_1 wild flies cultured at 30^{o}.

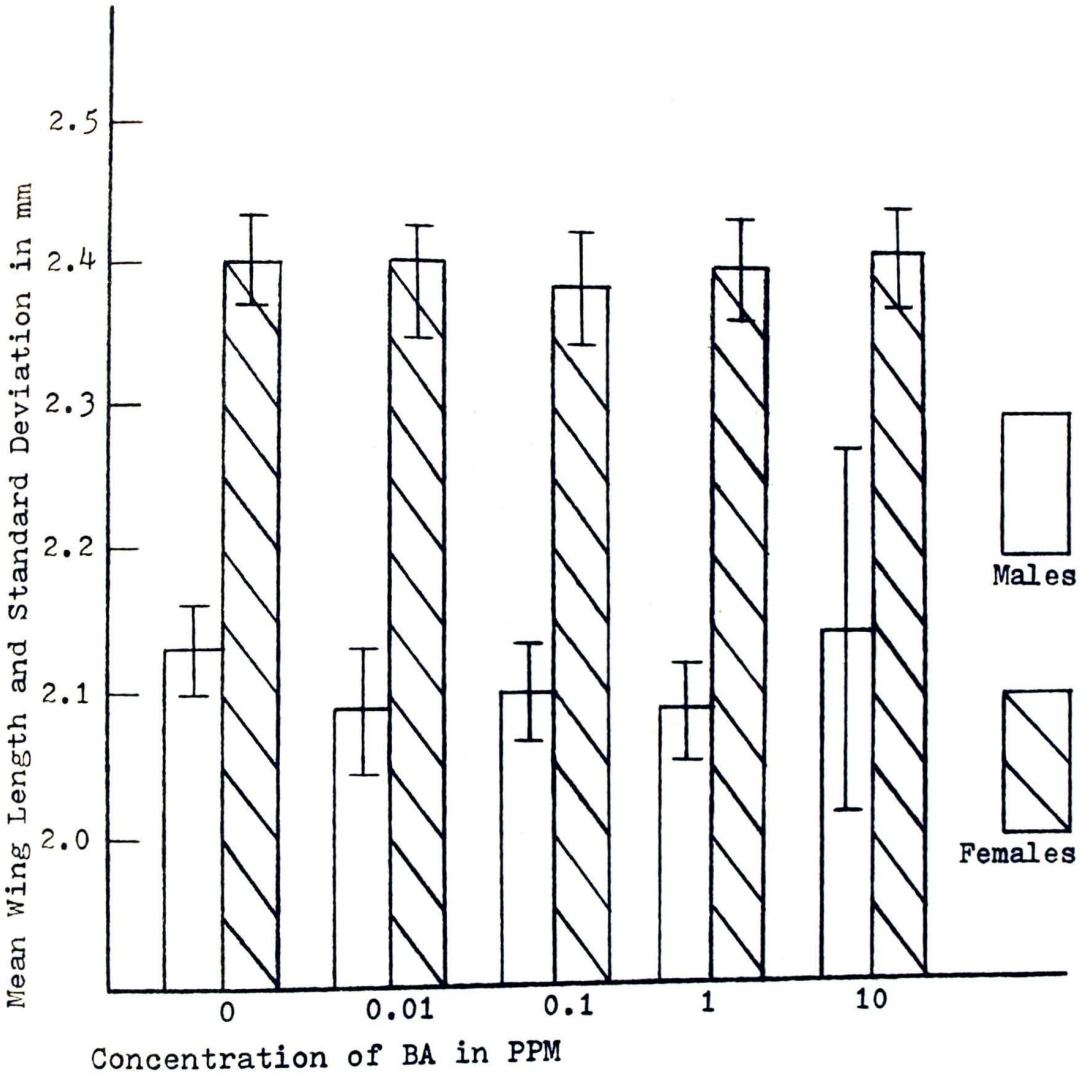


Figure 5. Effects of benzyladenine on wing length in F_2 wild flies cultured at 30^{o}.

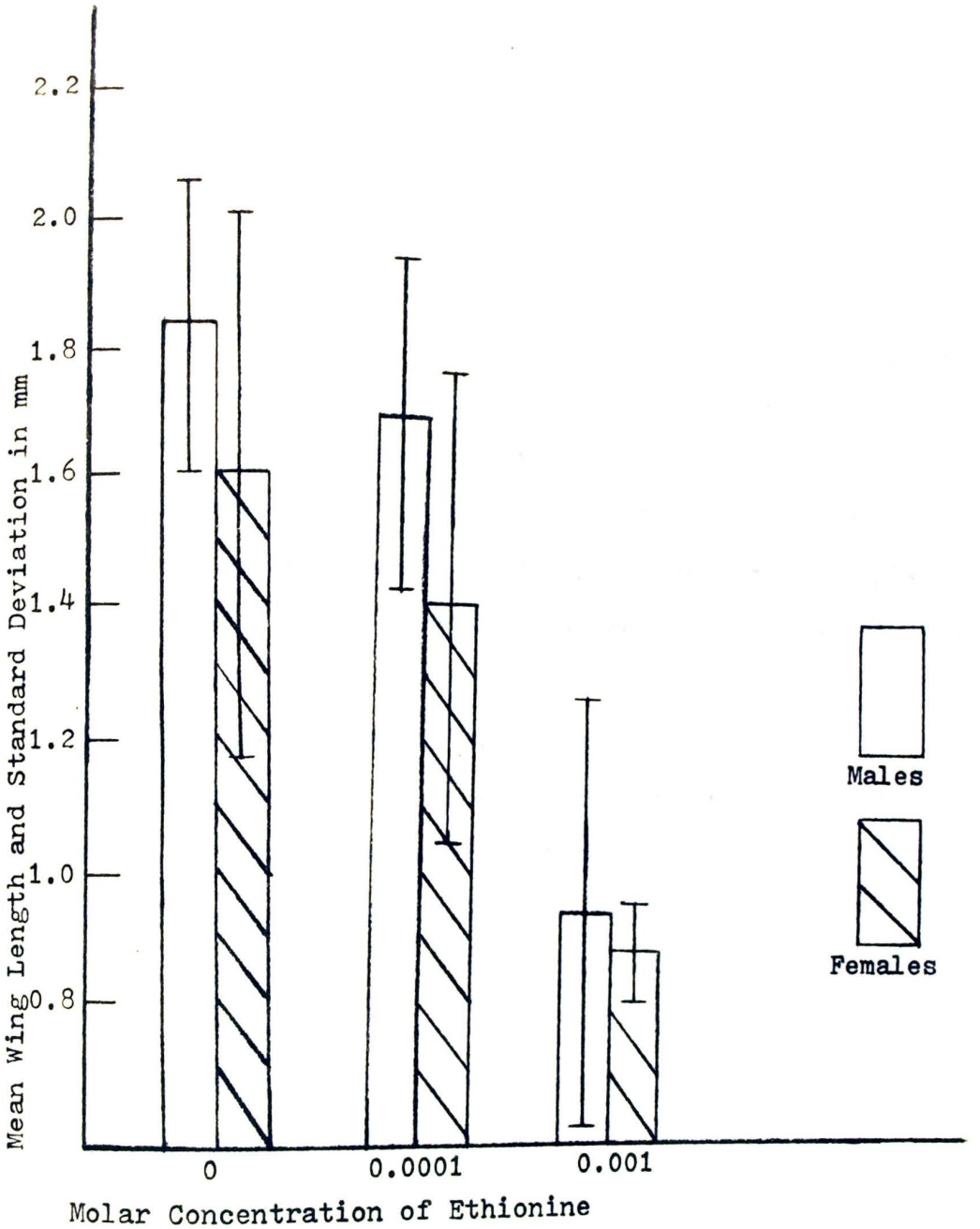


Figure 6. Effects of ethionine on wing length in F_1 vestigial flies cultured at 30^{o}.

significance. The wings of many vestigials in the control group and the 0.0001 M ethionine treatment at 30° were indistinguishable from those of the wild strain.

Another population of vestigial flies was allowed to develop on the same concentrations of ethionine at 30° (Figure 7). The 0.001 M concentration of ethionine again resulted in greatly reduced wing length in vestigial males and females raised at 30°. Mean wing lengths for both males and females were significantly smaller than control means at the 5% level of significance. Vestigial females raised on 0.0001 M ethionine at 30° had wings significantly shorter than those of control females. The F_2 progeny of vestigial flies raised on ethionine at 30° was too small for statistical comparison.

Methionine was added to the growth medium of vestigial flies developing at 30° in an attempt to reverse the inhibitory effect of ethionine on wing length in that group. Male and female imagoes raised on 0.001 M ethionine exhibited a decrease in wing length when compared with the control group (Figure 8). The decreases proved to be significant at the 5% level. Vestigial males and females raised on medium containing both 0.001 M ethionine and 0.001 M methionine had wings that were longer than flies raised on 0.001 M ethionine but shorter than those of control imagoes. Both differences were found to be significant at the 5% level.

The data in Figure 9 indicate that ethionine results in a decreased wing length in wild flies cultured at 25°. Wild males raised on 0.001 M ethionine and wild females raised on

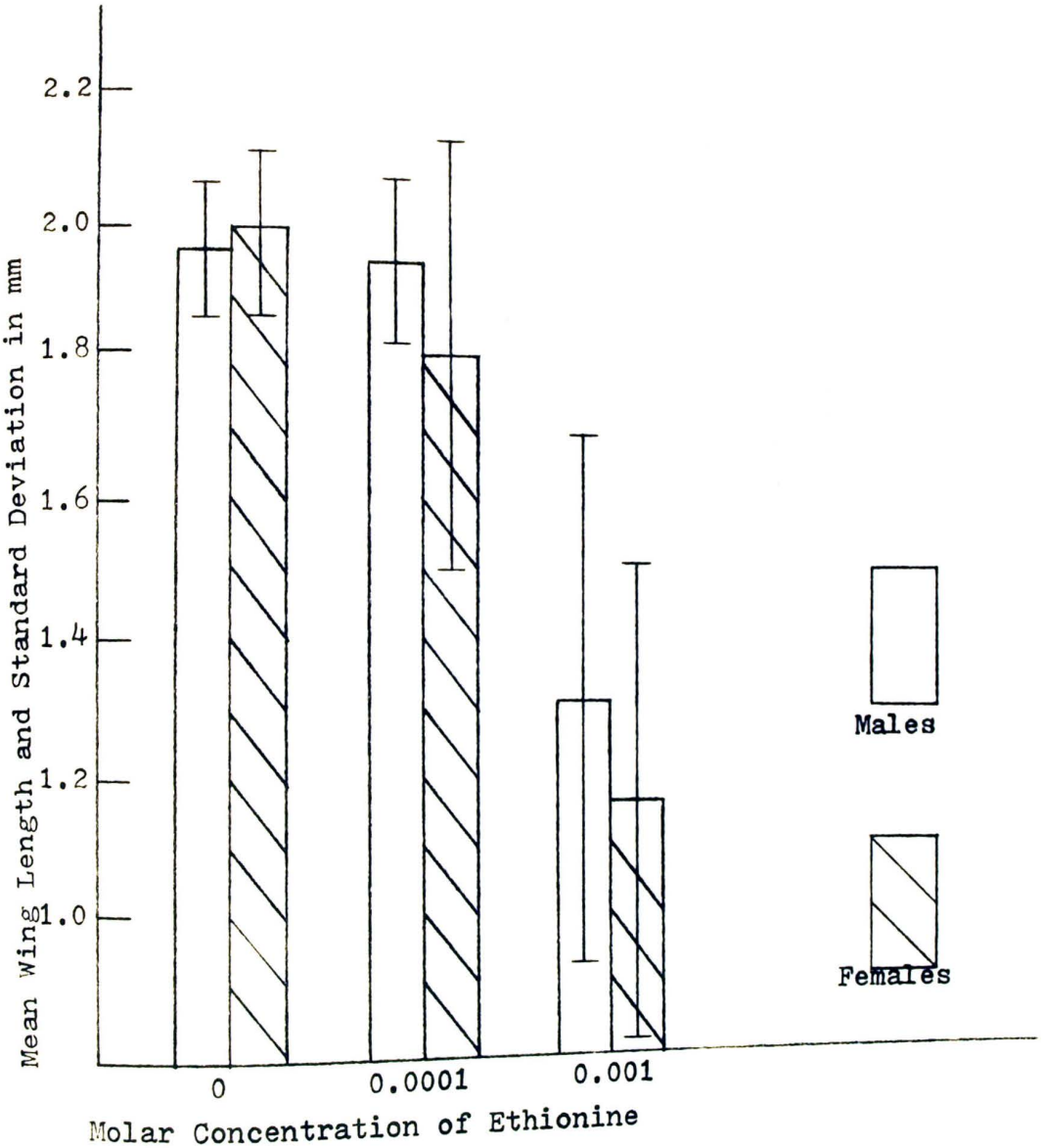


Figure 7. Effects of ethionine on wing length in F_1 vestigial flies cultured at 30^{o}.

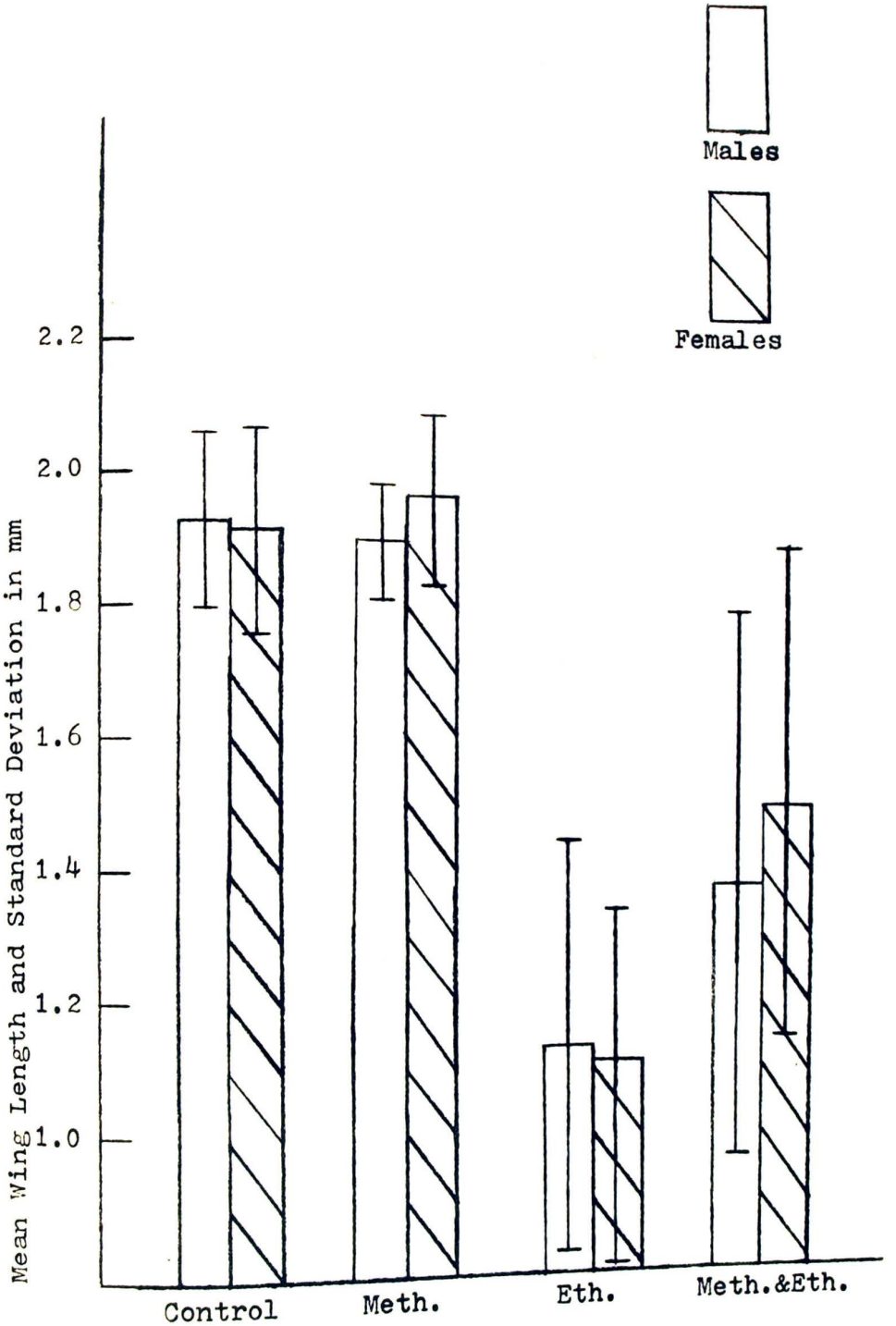


Figure 8. Effects of ethionine and methionine on wing length in F_1 vestigial flies cultured at 30°.

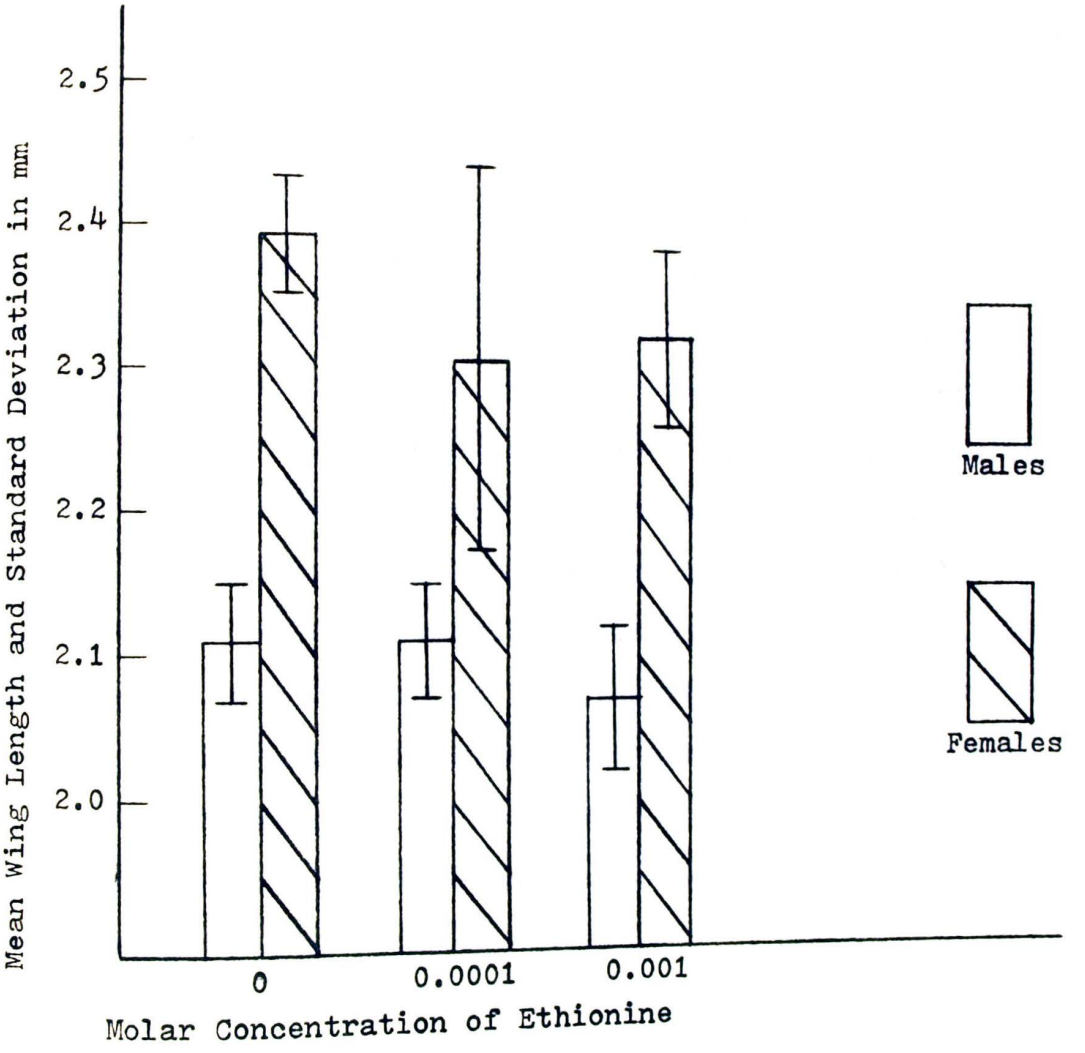


Figure 9. Effects of ethionine on wing length in F_1 wild flies cultured at 25^{o}.

both concentrations of ethionine had mean wing length significantly smaller than corresponding control imagoes. Two wild females with severely shortened wings emerged in the 0.01 M ethionine treatment at 25°.

A second generation of wild progeny was measured after development had occurred on 0.0001 M ethionine at 25° (Figure 10). This concentration resulted in a decrease in wing length in males and an increase in wild females. Both the increase and decrease were found to be significant at the 5% level.

Ethionine also appears to decrease wing length in F_1 wild flies raised at 30° (Figure 11). Males and females raised on all concentrations of ethionine had a mean wing length significantly smaller than corresponding means of control flies. The shortest wings were observed in the 0.01 M ethionine treatment. Since this concentration is near lethal, the numbers of progeny were much smaller than in other groups. An antagonistic effect was observed in F_2 wild males raised on ethionine at 30° (Figure 12). Concentrations of 0.001 M and 0.0001 M ethionine resulted in an increase in wing length in males. Both increases were determined to be significant at the 5% level using Duncan's (1955) multiple range test.

Populations of heterozygous flies were raised on various concentrations of ethionine at 25° and 30° to determine its effect on wing length. Figure 13 indicates that 0.0001 M and 0.001 M concentrations of ethionine had little effect on wing lengths in heterozygotes raised at 25°. The 0.01 M ethionine treatment decreased wing length in heterozygous

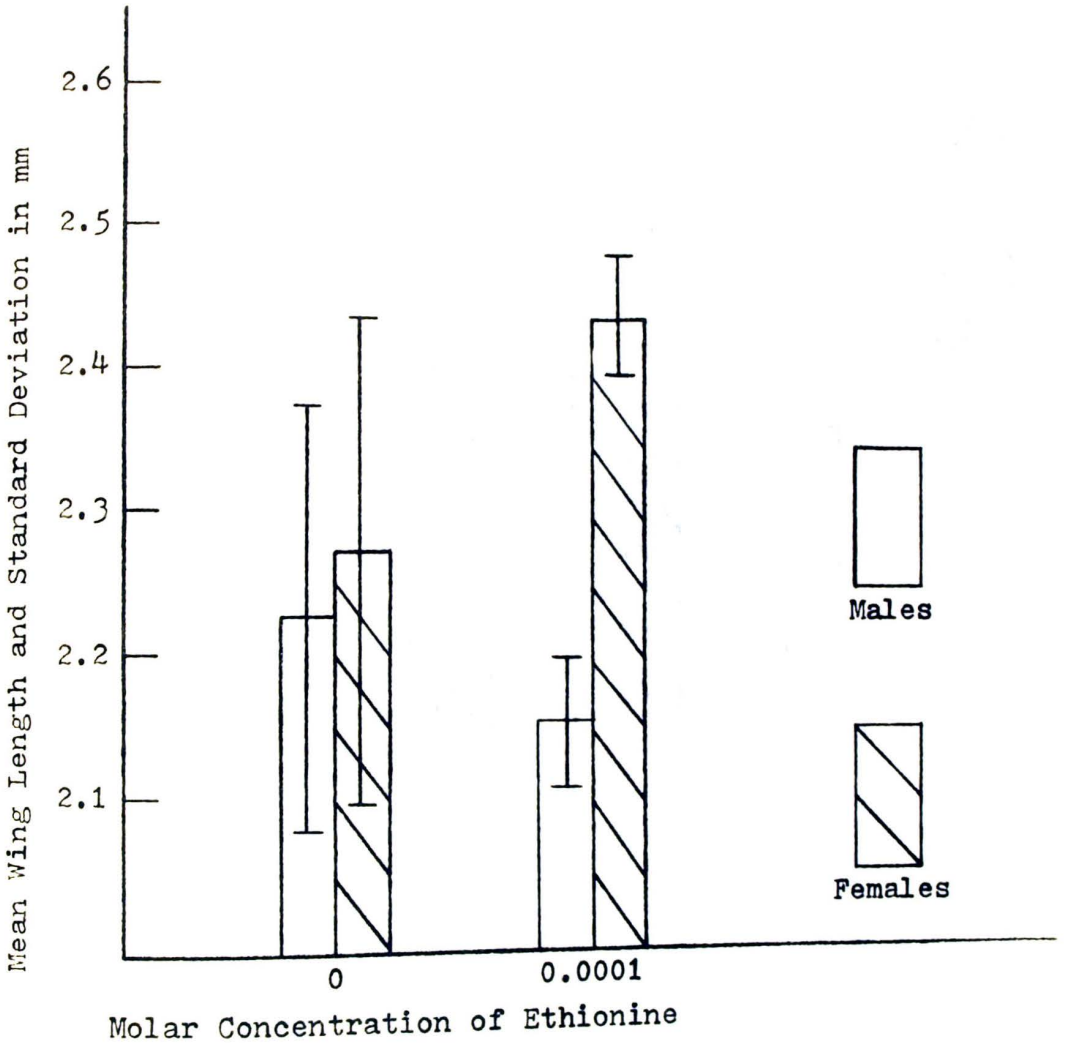


Figure 10. Effects of ethionine on wing length in F_1 wild flies cultured at 25^{o}.

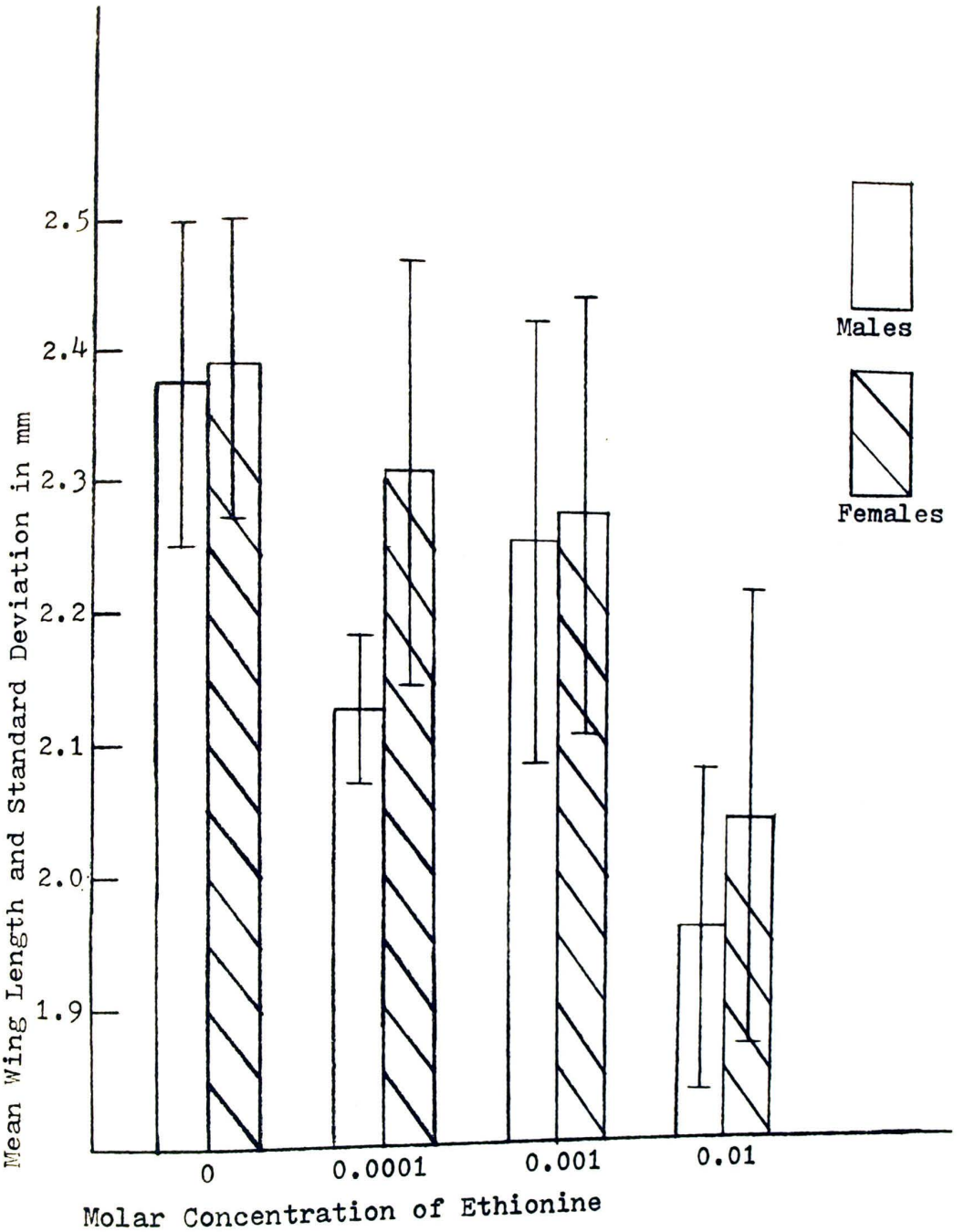


Figure 11. Effects of ethionine on wing length in F_1 wild flies cultured at 30°.

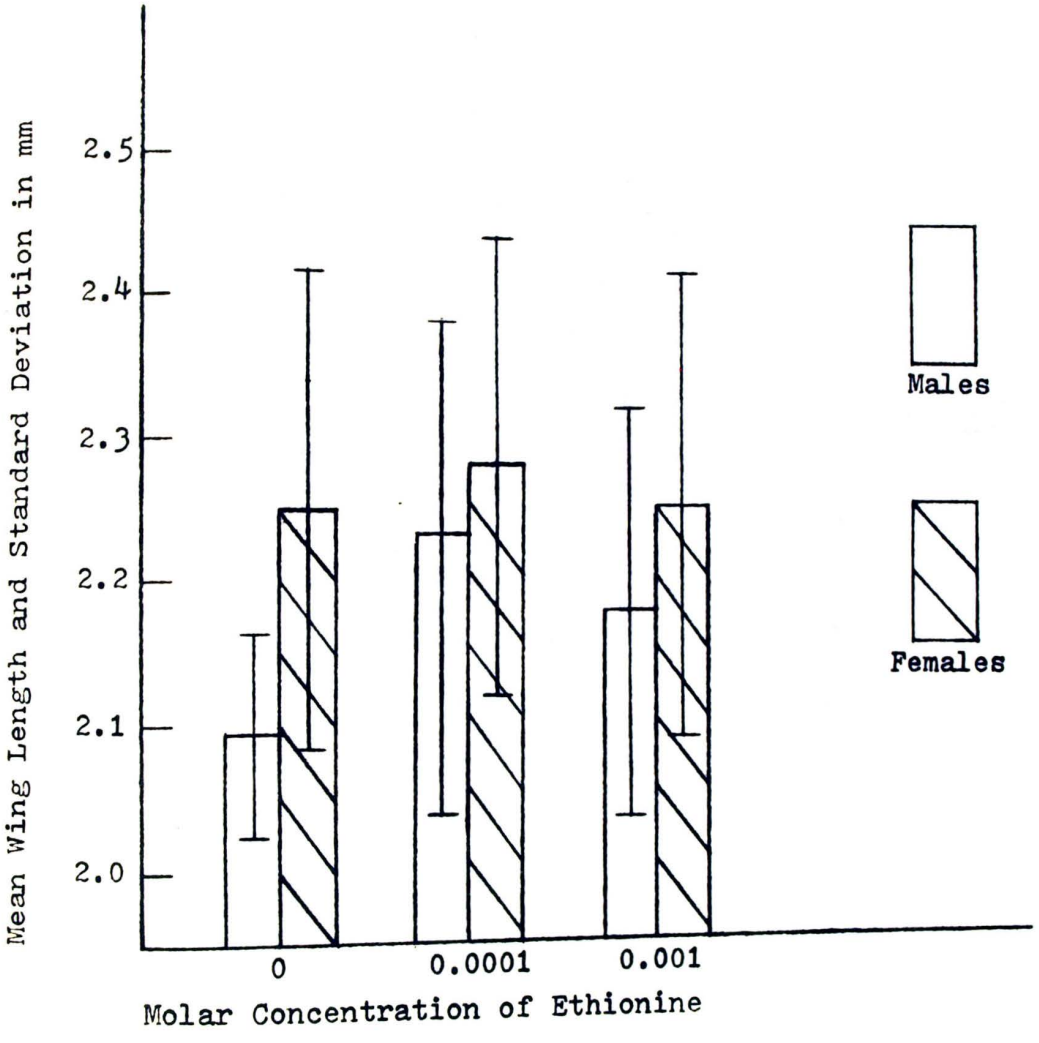


Figure 12. Effects of ethionine on wing length in F_2 wild flies cultured at 30^{o}.

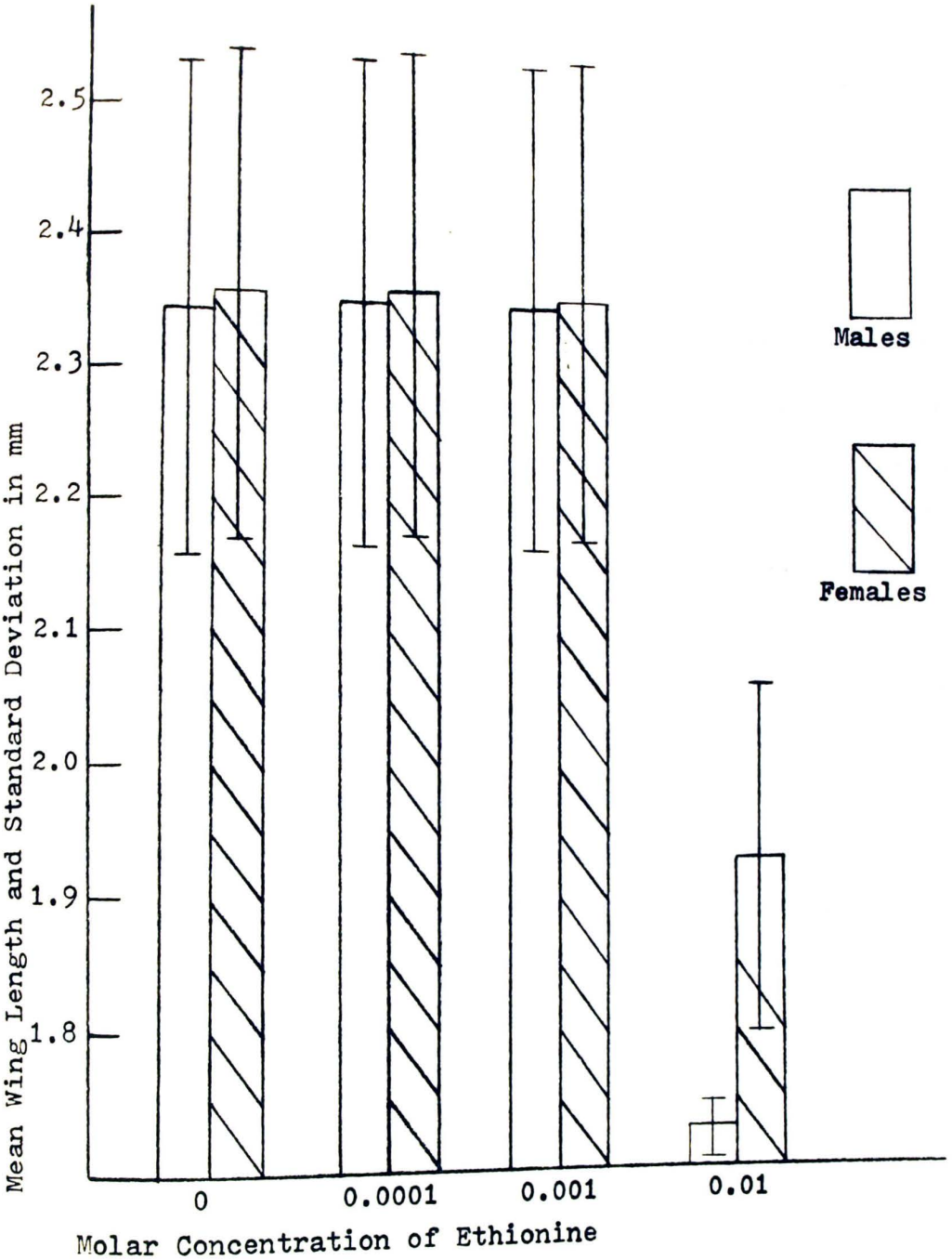


Figure 13. Effects of ethionine on wing length in F_1 heterozygotes cultured at 25^{o}.

males and females cultured at 25°. These decreases were found to be significant at the 5% level of significance. Heterozygous males raised on 0.0001 M and 0.001 M ethionine at 30° had longer wings than those of corresponding control males (Figure 14). Both increases were found to be significant at the 5% level of significance.

Incorporation of Triated Leucine into Larval Protein of Wild and Vestigial Drosophila

Cultures of wild and vestigial Drosophila were raised on control medium at 25° and 30°. Approximately 100 third instar larvae from each group were allowed to feed on medium containing L-leucine-4,5-^{3}H for 10 hours prior to the extraction of proteins. The data contained in Table VIII indicate that vestigial larvae cultured at 25° had a higher rate of protein synthesis than wild larvae raised at 25°. Wild larvae cultured at 30° incorporated over six times as much ^{3}H-leucine into protein as vestigial larvae raised at the same temperature. No valid comparisons of protein synthesis with a strain can be made due to the great variation in the soluble leucine pools between 25° and 30°.

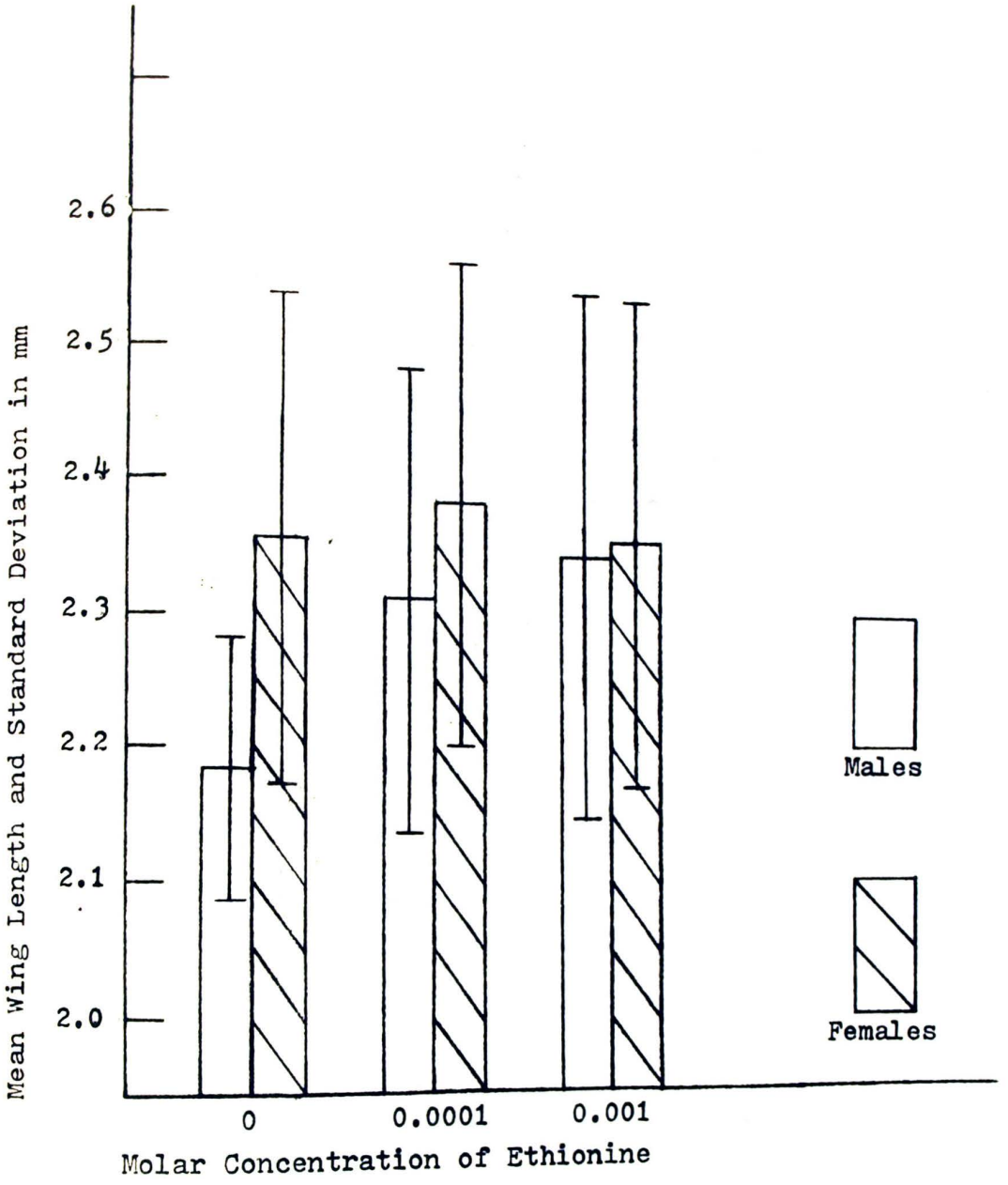


Figure 14. Effects of ethionine on wing length in F_1 heterozygotes cultured at 30°.

TABLE VIII

INCORPORATION OF TRITIATED LEUCINE
INTO LARVAL PROTEIN OF WILD
AND VESTIGIAL DROSOPHILA

Strain	Soluble Leucine cpm	L-leucine-4,5-^{3}H Incorporated Total Protein cpm	Total Protein mgm	cpml ug Protein	Percent Soluble Leucine in Protein
Vestigial Raised at 30°	2,541	1,219	960	1.02	208
Vestigial Raised at 25°	717	1,123	440	2.04	64
Wild Raised at 30°	6,099	740	80	7.4	824
Wild Raised at 25°	469	588	320	1.47	77

Larvae were allowed to develop at 30° for 85 hours or 110 hours at 25° before feeding on L-leucine-4,5-^{3}H for 10 hours. Approximately 100 larvae were used in each experiment.

CHAPTER V

DISCUSSION OF RESULTS

The data in Tables I and II indicate that benzyladenine speeds up metamorphosis in vestigial flies developing at 25° and 30°. Benzyladenine appears to have little effect on pupation and emergence in wild flies developing at either temperature (Appendices I and II). Cytokinins are known to stimulate cell division in plant tissue (Tuli et al, 1964). Benzyladenine may speed up metamorphosis in the vestigial mutant by increasing cell division in larvae developing at 25° and 30°.

The 10 ppm benzyladenine treatment resulted in a significant increase in wing length in vestigial males cultured at 25° (Figure 1). All concentrations of benzyladenine decreased wing lengths in vestigial males cultured at 30° (Figure 2). A concentration of 10 ppm benzyladenine produced a significant reduction in wing length in wild males and females cultured at 25° (Figure 3) but increased wing length in wild females raised at 30° (Figure 4).

Fox (1965) proposes that benzyladenine is incorporated into t-RNA conferring amino acid transfer competency on a molecule much as methylation may do. Brandes and Kende (1968) suggest that cytokinins function by binding to specific sites on target molecules. Fox (1965) has also suggested that benzyladenine may be incorporated into m-RNA, acting as a dere-

pressing agent by preventing its normal repressing function.

Fristom (1968) has shown that the vestigial condition is the result of condensation of the wing disc followed by phagocytosis by neighboring cells. She states that less degeneration occurs in the wing discs of vestigials raised at 30°. At this temperature the expression of the vestigial genotype is greatly reduced with many mutants exhibiting normal wild type wings. Kuraishi (1968) has proposed that cytokinins delay the degradation of protein in leaf discs rather than stimulate the synthesis of bulk protein. Benzyladenine may increase wing length in the vestigial mutant developing at 25° by retarding the decomposition of proteins in the embryonic wing discs at that temperature. This is apparently the first report of the effects of benzyladenine on the vestigial mutant of Drosophila.

The data contained in Tables III and V indicate that ethionine exerts a definite inhibitory effect on metamorphosis in wild flies raised at 25° and vestigial flies cultured at 30°. Ethionine was also found to inhibit pupation in wild flies raised at 30° (Table IV). The data in Table VII show that a concentration of 0.001 M ethionine inhibits metamorphosis in heterozygous Drosophila developing at 30°.

Akita (1955) has reported the appearance of normal wild-type wings in vestigials cultured at 31°. The number of vestigial flies with wild-type wings were observed and recorded for a population of vestigials raised at 30° (Figure 6). Approximately 79% of the control males and 21% of control females exhibited wild-type wings. In the 0.0001 M ethionine

treatment only 35% of the males and 5% of the females had normal wings. There were no normal winged vestigials observed in the 0.001 M ethionine treatment. These results are in agreement with Harnly (1930) who reported that maximum wing length occurs in vestigial males at 30° and in vestigial females at 31°. The results of this study indicate that a concentration of 0.001 M ethionine permits the expression of the mutant trait in vestigial flies cultured at 30°. A concentration of 0.01 M ethionine proved to be lethal to the vestigial mutant. Ethionine at a concentration of 0.01 M severely inhibited wing length in wild and heterozygous imagoes (Figures 11 and 13). A concentration of 0.02 M ethionine was lethal in both the heterozygotes and the wild strain. These experiments suggest that the occurrence of a normal winged phenocopy in vestigials cultured at 30° involves altered biochemical pathways.

Schmidt *et al* (1956) have suggested that ethionine combines with ATP or its derivatives removing them from the metabolic pathway. Stekol *et al* (1960) proposed that s-adenosylethionine blocked the synthesis of ATP by inhibiting mitochondrial oxidative phosphorylation.

Natori (1961) states that ethionine may inhibit protein synthesis by becoming incorporated into protein forming abnormal enzyme molecules. Moore and Smith (1969) postulate that ethionine inhibits protein synthesis by blocking methylation of transfer RNA.

The data in Table VIII indicate that vestigial flies cultured at 30° have a reduced rate of protein synthesis as

compared to wild flies raised at the same temperature. It appears that the occurrence of normal winged vestigials at 30° is associated with a reduced rate of protein synthesis. Consequently, ethionine may allow the expression of the vestigial trait at 30° by modifying a macromolecule such as t-RNA rather than directly inhibiting protein synthesis. Undermethylated t-RNA has been shown to be a poor acceptor of amino acids (Orthwert and Novelli, 1969). Stone, Whitty, and Cherry (1971) have shown that ethionine interferes with the charging of leucine-t-RNA in sugar beets. They suggest that leucine charging of t-RNA may be correlated with methylation of t-RNA in vivo. Hunt (1970) states that de novo synthesis of thymine ribonucleotides in the eyeless strain of Drosophila melanogaster requires the participation of active methionine for methylation of deoxyuridylic acid to thymidylic acid. The data in Figure 8 show that methionine significantly reverses the inhibitory effect of ethionine on wing length in vestigials cultured at 30°. Ethionine may permit the expression of the vestigial trait at 30° by interfering with the enzymatic activity of transmethylases.

Supplementary methionine failed to reverse the inhibitory effect of ethionine on metamorphosis in vestigial flies cultured at 30° (Table VI). The data suggest that factors inhibiting wing length in vestigials at 30° may not significantly affect metamorphosis in that group.

The experiments carried out in this study indicate that the phenomenon of phenocopy in the vestigial at 30° may be altered by ethionine which is a metabolic inhibitor of trans-

methylation.

The results obtained from this study suggest that benzyladenine significantly increases wing length in the vestigial mutant cultured at 25°. This effect of a plant hormone upon wing growth in the vestigial mutant should stimulate investigation into the effects of benzyladenine on other patterns of insect development.

CHAPTER VI

SUMMARY

This study was conducted to (1) observe the effects of benzyladenine and ethionine on metamorphosis in wild and vestigial strains of Drosophila melanogaster developing at 25° and 30°; (2) study the effects of benzyladenine and ethionine on wing length in wild and vestigial flies cultured at 25° and 30°; (3) determine the rates of protein synthesis in wild and vestigial larvae developing at 25° and 30° by assaying incorporation of ^{3}H-leucine into protein of third instar larvae.

The data indicate that benzyladenine stimulates early pupation and emergence in the vestigial stain raised at 25° and 30°. Ethionine at a concentration of 0.01 M proved to be lethal to the vestigial strain. Vestigials developing on 0.001 M ethionine were characterized by a delayed metamorphosis. When supplementary methionine was added to the growth medium of vestigial flies cultured at 30°, it failed to reverse the inhibitory effect of ethionine on metamorphosis in that group. A treatment of 0.02 M ethionine was lethal to wild and heterozygous Drosophila. Pupation and eclosion were delayed in wild flies raised on 0.01 M ethionine at 30°.

At a temperature of 30° wing length in the vestigial mutant is greatly increased. Many vestigials in the control and 0.0001 M ethionine treatment exhibited normal wild-type

wings. Wing length was severely reduced in vestigial flies raised on 0.001 M ethionine at 30°. The addition of methionine to the growth media of the vestigial mutant produced a significant reversal of the inhibitory effect of ethionine on wing length. Ethionine may allow the expression of the vestigial genotype at 30° by blocking the enzymatic activity of transmethylases or protein synthesis.

The data indicate that vestigial larvae cultured at 25° exhibit a higher rate of protein synthesis than wild larvae raised at 25°. Wild larvae cultured at 30° had a rate of protein synthesis six times greater than vestigial larvae developing at that temperature.

APPENDIX I

EFFECTS OF BENZYLADENINE ON METAMORPHOSIS IN F_1 WILD FLIES RAISED AT 25°

Concentration of BA	First Pupa Observed Time in Hours	First Adult Observed Time in Hours
Control	124	208
0.1 PPM	115	194
1 PPM	112	194
10 PPM	112	194
20 PPM	120	208

Flies were allowed to undergo complete development on media containing various concentrations of benzyladenine at 25°. Time was computed from the beginning of the laying period to the appearance of the first pupa or adult.

APPENDIX II

EFFECTS OF BENZYLADENINE ON METAMORPHOSIS IN F_1 WILD FLIES RAISED AT 30°

Concentration of BA	First Pupa Observed Time in Hours	First Adult Observed Time in Hours
Control	104	196
0.01 PPM	104	196
0.1 PPM	107	204
1 PPM	107	196
10 PPM	107	196

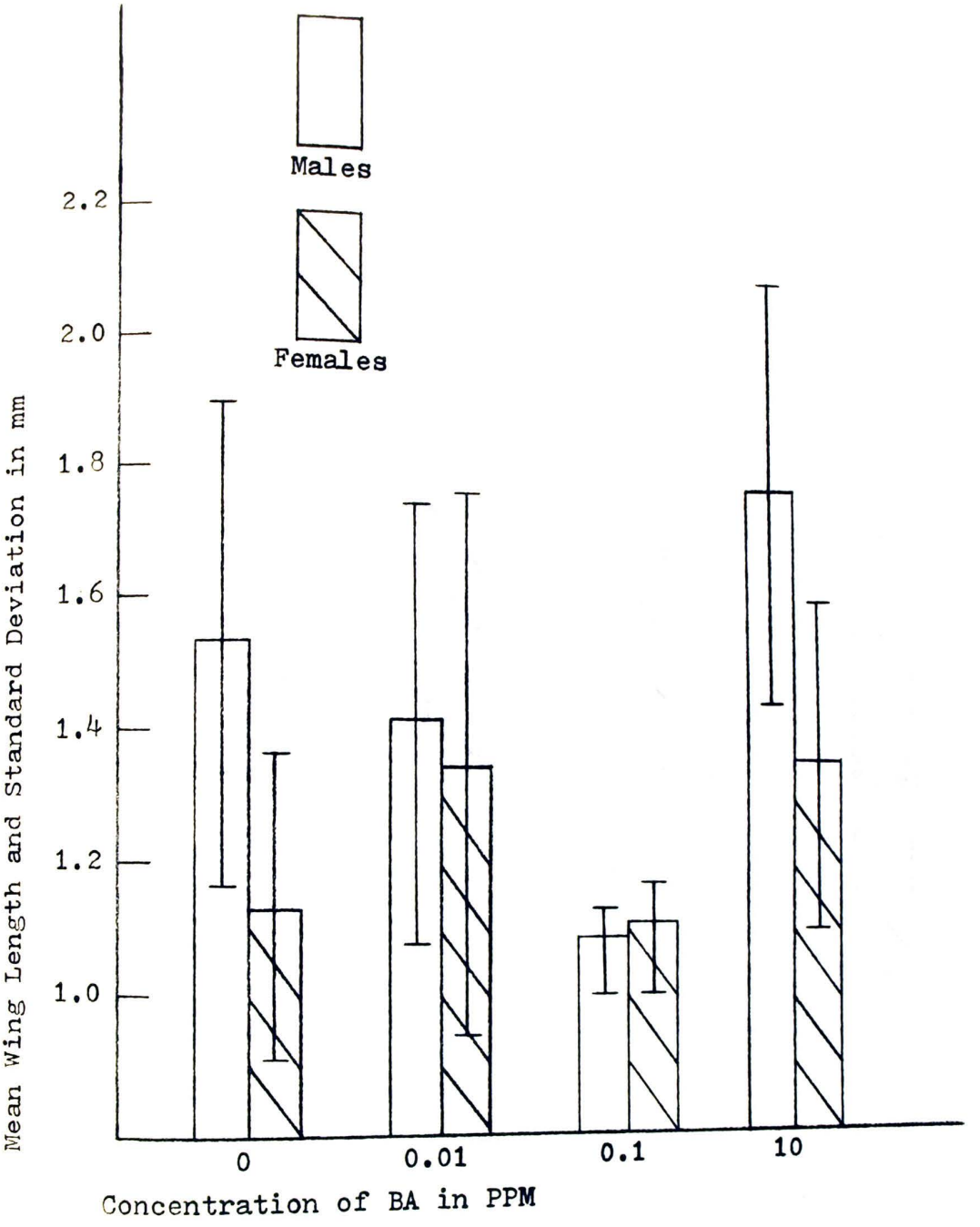
Flies were allowed to develop on media containing various concentrations of benzyladenine at 30° until the first pupa was observed. The flies were then placed in an incubator set at 25° to complete development. Time was computed from the beginning of the laying period to the appearance of the first pupa or adult.

APPENDIX III

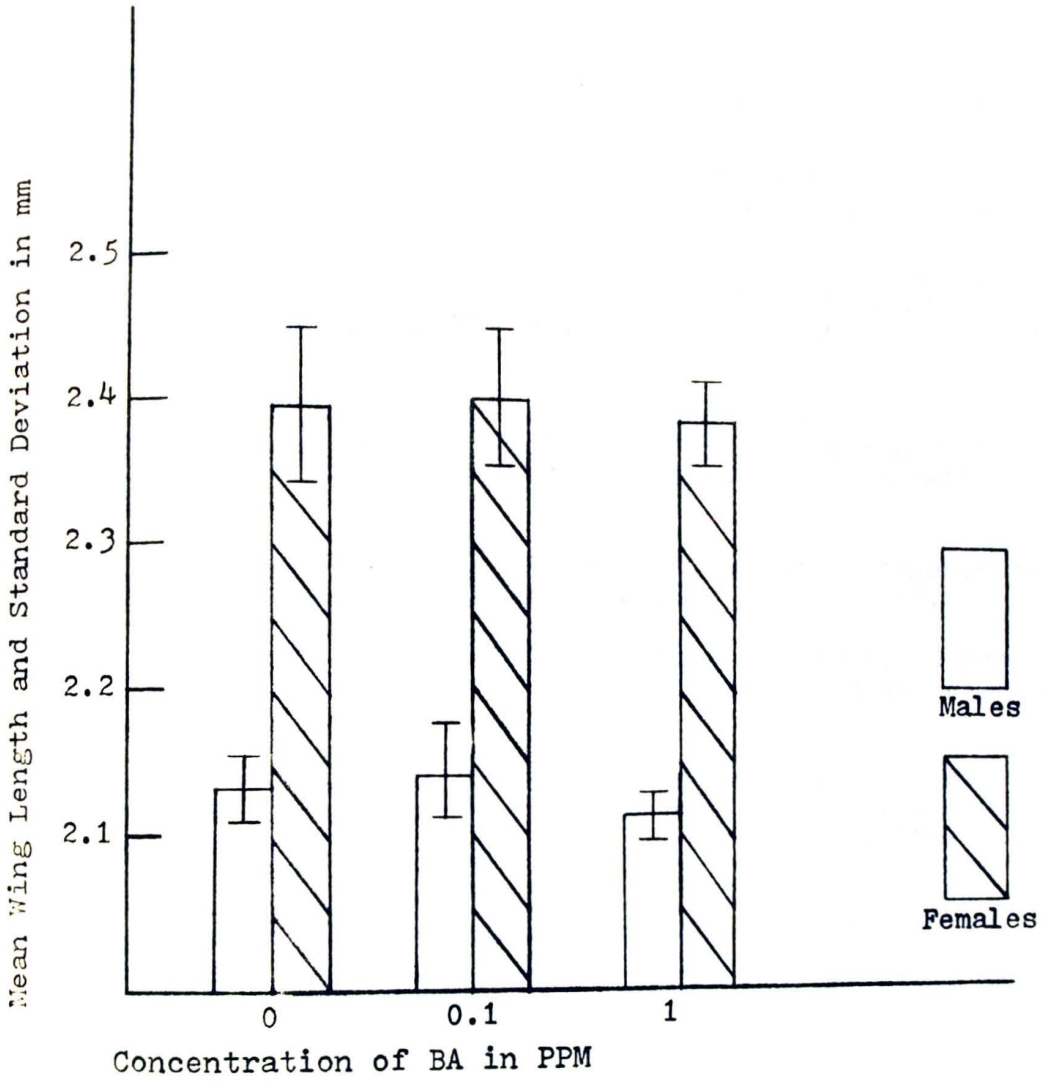
EFFECTS OF ETHIONINE ON METAMORPHOSIS IN F_1 HETEROZYGOUS FLIES RAISED AT 25°

Concentration of Ethionine	First Pupa Observed Time in Hours	First Adult Observed Time in Hours
Control	114	202.5
0.0001 M	106	198.5
0.001 M	123	202

Flies were allowed to undergo complete development on various concentrations of ethionine at 25°. Time was computed from the beginning of the laying period to the appearance of the first pupa or adult.



APPENDIX IV. Effects of benzyladenine on wing length in F_2 vestigial flies cultured at 25°.



APPENDIX V. Effects of benzyladenine on wing length in F_2 wild flies cultured at 25°.

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