DIETARY CHOLINE REQUIREMENTS FOR SPERM MOTILITY AND NORMAL MATING ACTIVITY IN DROSOPHILA MELANOGASTER[1]

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Reproduction of adult female insects is strongly influenced by the diet (Wigglesworth, 1960; House, 1961, 1962; Johansson, 1964). Nutrients required for normal female reproduction include amino acids, carbohydrate, lipid, sterol, certain minerals, and vitamins, although there is much interspecific variation. In contrast, few instances of dietary influence on male fertility have been found (Johansson, 1964). In a variety of insect species starvation results in males with smaller but functional reproductive organs than in males fed an optimal diet. Starvation also influences the sexual behavior of some species. Males of the flea *Ceratophyllus fasciatus* (Strickland, 1914) and the fruit fly *Dacus dorsalis* (Hagen, 1952) require a complete meal before they exhibit any copulatory activity. *Calliphora erythrocephala* females fed only a sugar and water diet will not accept courting males (Strangways-Dixon, 1961).

The carry-over of food stores from larval feeding poses a problem in studies of adult nutritional requirements. The small magnitude of the nutritional requirements of many adult insects, coupled with reduced feeding activity because of sizable food stores, makes depletion of carry-over food stores difficult and uncertain to obtain. Carnitine, one of the most effective substitutes for choline in the development of *Drosophila melanogaster* (Fraenkel *et al.*, 1955; Geer and Vovis, 1965), was used in the present study to obtain choline-free *D. melanogaster* adults. No larvae are able to pupate on a diet not supplemented with choline or a related compound. When carnitine is fed at a concentration equivalent to the optimal choline level in the larval diet, nearly as many larvae develop to eclosion as when choline is fed, but the larval growth period is 20% greater in duration (Geer and Vovis, 1965). Carnitine-raised adults are morphologically normal but they contain no detectable choline in their tissues. When carnitine-raised males and females are mated, however, they fail to reproduce (Geer, Vovis and Yund, 1967).

The current study presents evidence for a dietary choline requirement for the development of motile spermatozoa in *D. melanogaster* and quantitatively defines the requirement. Choline-deficiency is also shown to influence the normal sexual behavior of adult *D. melanogaster*.

Methods and Materials

Adults for test purposes were derived from the Canton-S, Riverside and Oregon-R strains by a three-way mating scheme. Canton-S females were crossed

[1] This investigation was supported by National Science Foundation Grant GB-4838.

to Riverside males and the hybrid female offspring were in turn mated to Oregon-R males. Eggs collected from females of the latter mating were sterilized by rinsing them thoroughly with sterile distilled water, washing with a 0.125% sodium hypochlorite (commercial Clorox) solution to eliminate egg-clumping, and exposing the eggs to a 0.4% peracetic acid-0.1% sodium alkylarylsulfonate solution for 10 minutes. Eggs were rinsed in 70% ethanol before transfer to cultures.

A defined medium consisting of amino acids, sucrose, yeast RNA, cholesterol, B vitamins and salts prepared as an agar gel as described by Geer and Vovis (1965) was employed for the main part. However, adults for mating activity observations and determination of male and female responses to dietary choline were raised on a diet containing 3.5% casein in place of the amino acid mixture; the modification of Sang's medium C (Sang, 1956) reported by Geer (1963). The choline requirement for reproduction was influenced in no way by this alteration of the dietary nitrogen source. Unless otherwise indicated, the diets were supplemented with either 5.7×10^{-4} *M* DL-carnitine HCl or 5.7×10^{-4}*M* choline chloride. Cultures were maintained at 23.8° C. in 6-dram shell vials containing 5 ml. of medium. These were sterilized by autoclaving for 20 minutes at 15 lb./in.^{2} pressure.

Males and females to be crossed to mates fed different test diets were separated within 12 hours after eclosion, using sterile laboratory instruments to avoid contamination of cultures. Cultures found to be contaminated during the course of the experiments were discarded. Males and females crossed to mates fed the same diet were moved to fresh cultures soon after eclosion. Egg production and the hatchability of eggs laid by females were determined by transferring the females with their mates to cultures for a 24-hour period, removing the adults, and then assessing the number of eggs laid and number to hatch within 24 hours after being laid. Observations of mating behavior were conducted by placing male and female pairs into 6-dram vials without anesthetizing and recording the time from introduction to copulation. Bastock and Manning's (1955) description of *D. melanogaster* courtship served as a guide for observations. The spermathecae and seminal receptacles of females that had copulated were dissected out in Drosophila Ringer's solution (Ephrussi and Beadle, 1936) and examined for sperm to determine whether the females had been successfully inseminated by their mates.

The development of motile sperm was ascertained by dissecting out the testes of adult males in Drosophila Ringer's solution and examining mature sperm for motility. The morphology of living spermatozoa was studied by phase microscopy or spermatozoa were stained with aceto-orcein or aniline blue-eosin (Gurr, 1965) before examination by light microscope methods.

In this paper the hatchability of eggs laid by females mated to test males is used as an assay for the development of motile sperm in the males. Results presented in the next section show that egg hatchability lags only slightly behind the appearance of motile sperm in the testes of males. Thus, the assay for motile sperm depends upon the frequency of hatchable eggs laid by females to increase as the quantity of motile spermatozoa increases in the testes of their mates.

The sources of chemicals were as follows: low vitamin casein—Gentosan Division, Fisons Pharmaceuticals Limited, Loughborough, Leicestershire, England; other nutrients and vitamins—Nutritional Biochemicals Corporation, Cleveland,

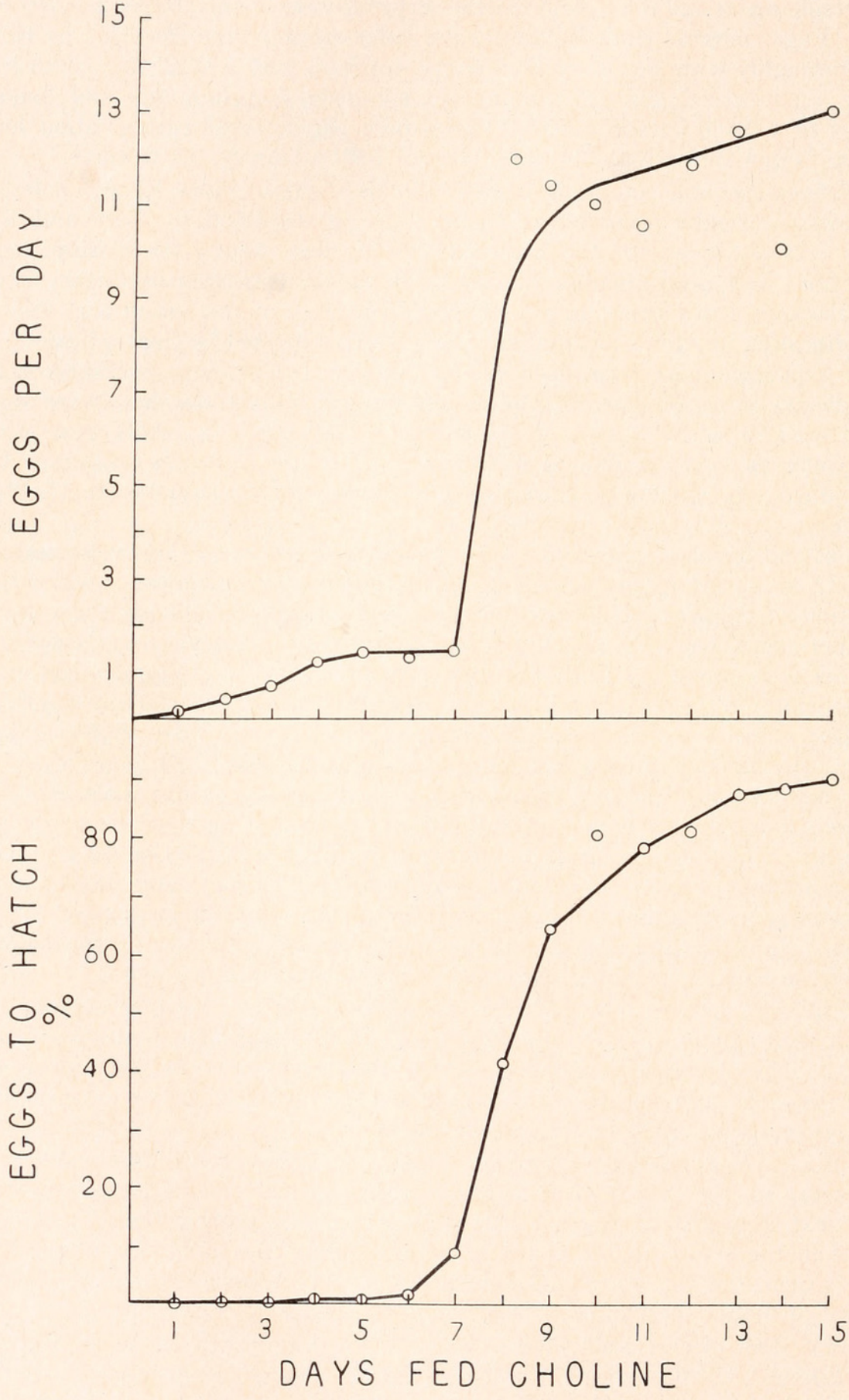
EGGS PER DAY
15
13
11
9
7
5
3
1
EGGS TO HATCH %
80
60
40
20
1
3
5
7
9
11
13
15
DAYS FED CHOLINE

FIGURE 1.

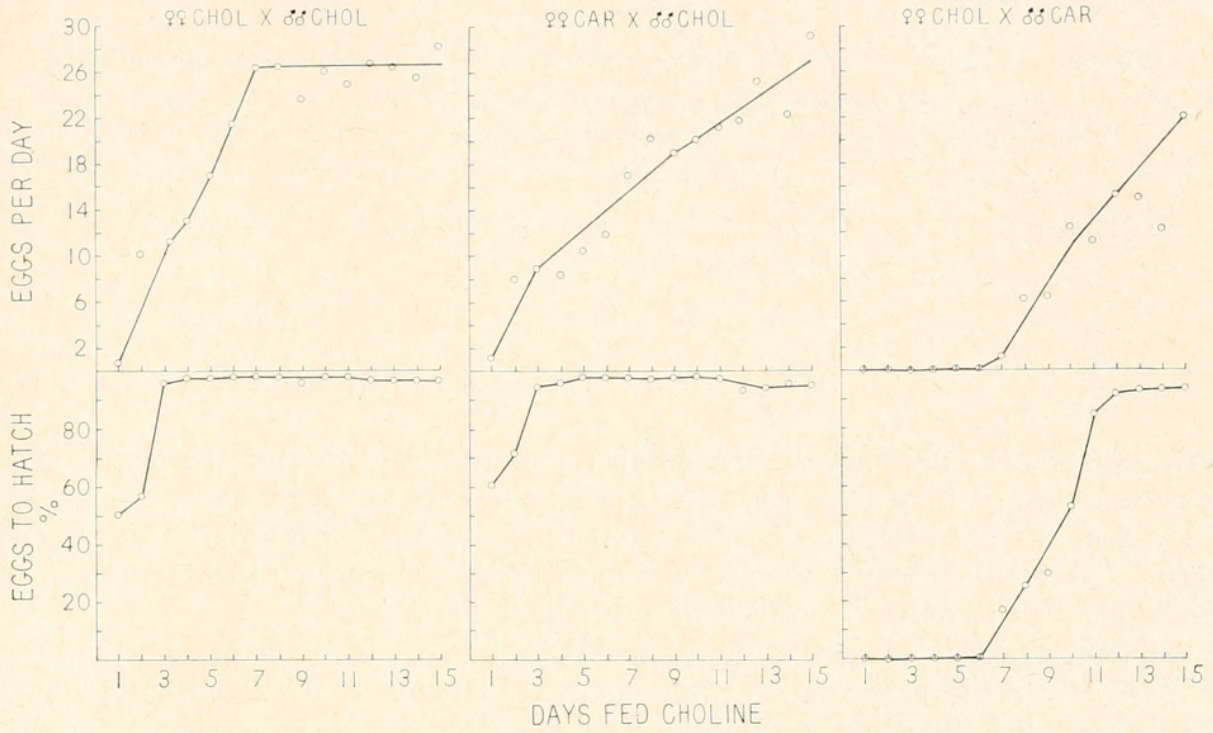

FIGURE 2. The reproductive capacities of choline-raised females mated to choline-raised males (left), carnitine-raised females mated to choline-raised males (center), and carnitine-raised females mated to carnitine-raised males (right) when both mates were fed a diet containing 5.7×10^{-4} *M* choline. The upper half of the graph indicates the eggs laid per female per day and the lower half shows the percentage of eggs to hatch for each day of the test period. Circles in the left graph represent mean values for 40 females and 50 males in 5 cultures, circles in the center graph are for 120 females mated to 120 males in 12 cultures, and circles in the right graph are for 96 females mated to 120 males in 12 cultures.

Ohio; inorganic salts—J. T. Baker Chemical Company, Phillipsburg, New Jersey; 40% peracetic acid—Inorganic Chemicals Division, FMC Corporation, New York, New York; sodium alkylarylsulfonate—Fisher Scientific Company, Chicago, Illinois; Orcein—Eastman Organic Chemicals, Rochester, New York; aniline blue—National Aniline and Chemical Company, Inc., New York, New York; Eosin B—Matheson Coleman Bell Division, Matheson Company, Inc., East Rutherford, New Jersey.

RESULTS

Response to dietary choline

The initial investigation was to find if the sterility of carnitine-raised adults could be corrected by feeding choline. Adults raised on a carnitine-supplemented diet were fed a diet containing 5.7×10^{-4} *M* choline for 15 days. The number of eggs laid per female and the percentage of these eggs to hatch was determined for each day during the test period (Fig. 1). Almost none of the eggs hatched

FIGURE 1. The reproductive capacity of carnitine-raised females mated to carnitine-raised males when both mates were fed a diet containing 5.7×10^{-4} *M* choline. The upper half of the graph indicates the eggs laid per female per day and the lower half shows the percentage of eggs to hatch for each day of the test period. Circles represent mean values for 80 females mated to 100 males in 10 cultures.

TABLE I

Increase of sperm motility in carnitine-raised males fed a choline-containing diet

Days fed choline	Number of males	Males with motile sperm
0	12	0
4	12	0
5	12	2
6	12	3
7	12	8
8	12	12

until the seventh day, then the hatchability increased steadily for the next 9 days. Females raised on a carnitine diet also laid very few eggs. After choline was added to the diet, the egg productivity of females remained low until adults had been fed choline 7 days; then egg productivity jumped from slightly more than one egg per female per day to more than 10 eggs per female per day.

The responses of carnitine-raised males and females to dietary choline were compared by crossing carnitine-raised males and females to choline-raised mates and determining the egg hatchabilities and egg production of the females of these two matings when maintained on a choline diet (Fig. 2). Choline-raised males and females were mated as a control. Carnitine-raised females when mated to choline-raised males laid a relatively large quantity of eggs after 2 days on a choline diet and 71.5% of the eggs hatched. Egg hatchability for carnitine-raised females increased rapidly, reaching an optimal level at the end of day 5 of the choline feeding period. These results were similar to those of the choline control matings. Egg production continued to climb throughout the test period but did not reach the productivity level of the choline control matings until after 15 days of feeding.

Carnitine-raised males, on the other hand, did not reproduce until after 7 days of choline feeding. Eggs laid by their choline-raised mates began to hatch after day 7 and egg hatchability increased through day 15, reaching a level only slightly less than that of the choline control matings. Egg production increase paralleled the climb in egg hatchability but did not attain the levels reached by choline- or carnitine-raised females when mated to choline-raised males.

TABLE II

The reproduction of D. melanogaster grown on a carnitine-supplemented diet and fed either a choline- or carnitine-supplemented diet for 8 days as adults

Mating*	Total eggs	Eggs to hatch (%)	Egg productivity (eggs/female/day)
♀ ♀ ♂ ♂			
Carnitine × Carnitine	31	0	1.3
Carnitine × Choline	170	11.1	7.7
Choline × Carnitine	73	0	4.3
Choline × Choline	309	74.4	14.7

* Adults were mated for 4 days while being fed a carnitine-supplemented diet before being tested.

TABLE III

The reproduction of D. melanogaster grown on either a choline- or carnitine-supplemented diet and maintained on a carnitine-supplemented diet for 7 days as adults

Mating	Total eggs	Eggs to hatch (%)	Egg productivity (eggs/female/day)
♀♀ ♂♂			
Carnitine × Carnitine	14	0	0.4
Carnitine × Choline	295	39.6	9.8
Choline × Carnitine	41	0	1.7
Choline × Choline	201	84.5	7.7

The choline response experiments show that the sterility of carnitine-raised adults is primarily due to the inability of males to reproduce, a condition that can be corrected by feeding choline. Upon examination, the testes of carnitine-raised males were found to be devoid of motile sperm although spermatozoa in all stages of development were present. When choline was fed, some males possessed motile sperm after 5 days of feeding but all males did not have motile sperm until after 8 days of feeding (Table I). The development of motile sperm preceded only slightly the increase in hatchability of eggs laid by their mates.

Choline fed during the larval period

Matings were made between carnitine-raised adults maintained on a carnitine diet and those fed a choline diet (Table II). Carnitine-raised and -fed adult females when mated to carnitine-raised and choline-fed males laid relatively large numbers of eggs, and a significant percentage of the eggs hatched, 11.1%. Carnitine-raised and fed males were sterile since females mated to these males laid very few eggs and none of the eggs hatched.

Choline obtained by feeding during the larval period was sufficient to insure the fertility of adult males (Table III). Results similar to those described for the previous matings were obtained when adults raised on either choline- or carnitine-supplemented diets were mated. Fewer of the eggs laid by carnitine-raised females

TABLE IV

The effect on male fecundity of adding choline to the diets of larvae fed a carnitine-supplemented diet

Larva age when choline added* (days)	Larvae to become adults (%)	Growth period† (days)	Eggs to hatch‡ (%)
3	81.3	12.0 ± 1.1	96.6
6	80.0	12.0 ± 1.1	95.5
8	76.6	12.4 ± 1.0	88.8
10	69.6	13.3 ± 1.0	95.6

* Eight mg. of choline chloride were added to each culture in two drops of 70% ethanol.

† Figures represent mean ± S.D. The growth period was the time from inoculation of the cultures with eggs until the larvae pupated

‡ The adults were fed a carnitine-supplemented diet 6 days before being tested.

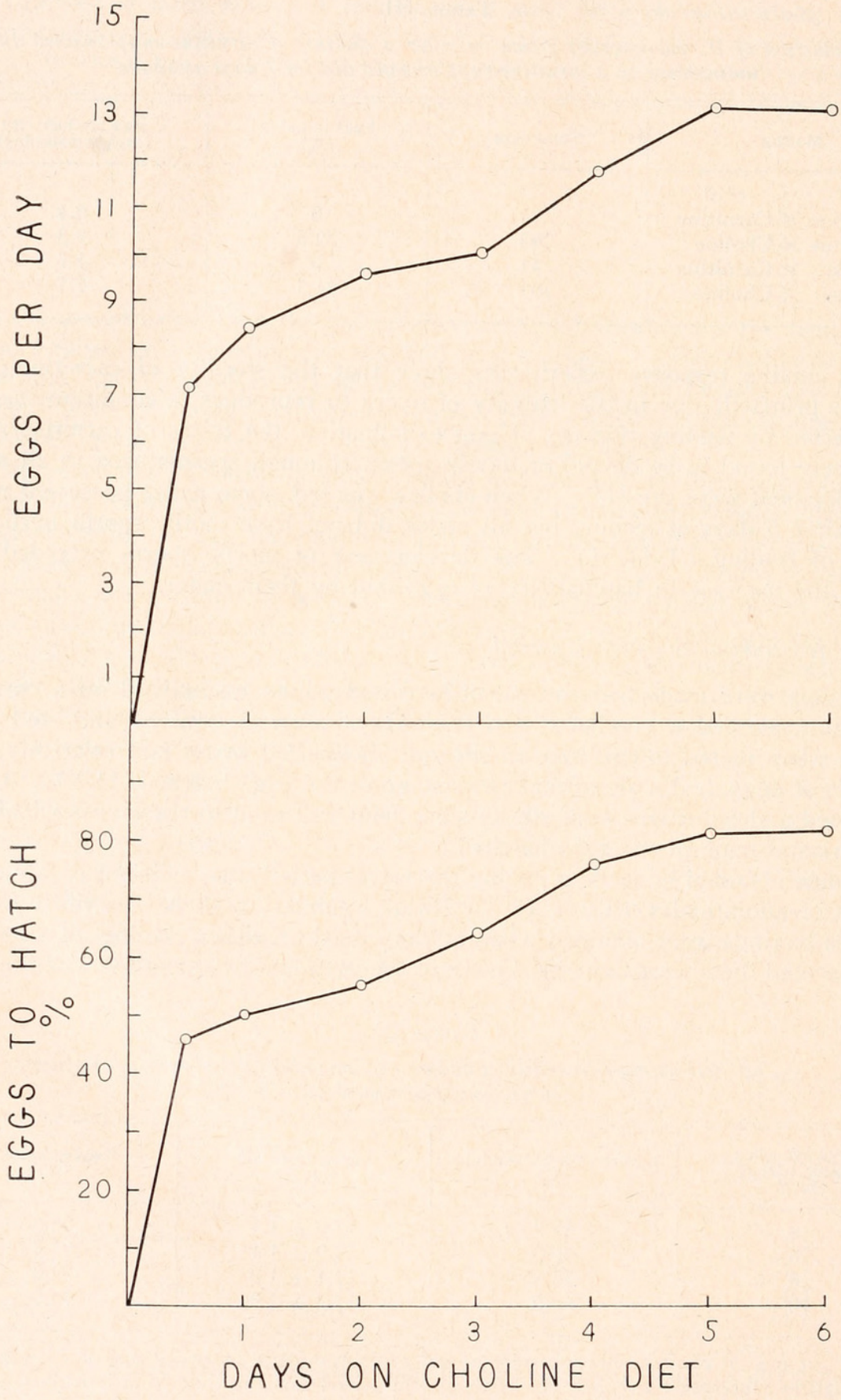
EGGS PER DAY
15
13
11
9
7
5
3
1
EGGS TO HATCH %
80
60
40
20
1
2
3
4
5
6
DAYS ON CHOLINE DIET

FIGURE 3.

TABLE V

The mating activity of males and females raised and maintained as adults for 7 days on a diet supplemented with either choline or carnitine

Female diet	Male diet	Number of pairs observed	Pairs to copulate within 2½ hours	Average time to copulation (seconds)	Number of females inseminated
Choline	Choline	20	14	2133 (437–3327)*	14
Choline	Carnitine	20	5	3058 (1219–4991)	0
Carnitine	Choline	20	3	6164 (2293–8565)	3
Carnitine	Carnitine	20	1	6741	0

* The range in time to copulation of mating pairs is indicated in parentheses.

mated to choline-raised males hatched than did when choline-raised females were mated to choline-raised males. Carnitine-raised males were sterile regardless of the larval diet of their mate.

Choline fed late in the larval period appears sufficient to insure adult male fertility (Table IV). Although there was a significant extension of the larval growth period and a decrease in the number of larvae to become adults, the percentage of hatchable eggs mated to the eclosing males remained at a high level. It is possible that a more curtailed larval choline feeding period might result in a measureable degree of sterility; nevertheless, enough choline is consumed during a 3-day larval meal for sperm motility.

Mating behavior

Observation of the mating activity of carnitine- and choline-raised adults showed that mating was inhibited by choline deprivation (Table V). Carnitine-raised and -fed males copulated more readily with choline-raised and -fed females than carnitine-raised and -fed females, but could not successfully inseminate females. Carnitine-raised and -fed females, though they did not copulate readily, were successfully inseminated by choline-raised and -fed males when copulation occurred. Carnitine-raised females appear less receptive to male courtship advances than do their choline-raised counterparts. Carnitine-raised and -fed males court vigorously but seldom get beyond the licking stage of courtship with carnitine-raised and -fed females (the stage preceding mounting and copulation). When they do, the males have difficulty mounting the females. That male activity is also inhibited by choline deprivation is shown by the rapid and successful mating of choline-raised and -fed males when mated to choline-raised and -fed females as compared to carnitine-raised and -fed males when mated to choline-raised and -fed females.

FIGURE 3. The reproductive capacities of carnitine-raised adults maintained on diets supplemented with 5.7×10^{-4} *M* choline for periods ranging from ½ to 6 days. All test groups were aged to 8 days on carnitine-supplemented medium before being tested. The upper half of the graph indicates the eggs laid per female per day and the lower half shows the percentages of eggs to hatch for each test group. Circles represent mean values for 80–120 males and females in 6 cultures for each test group.

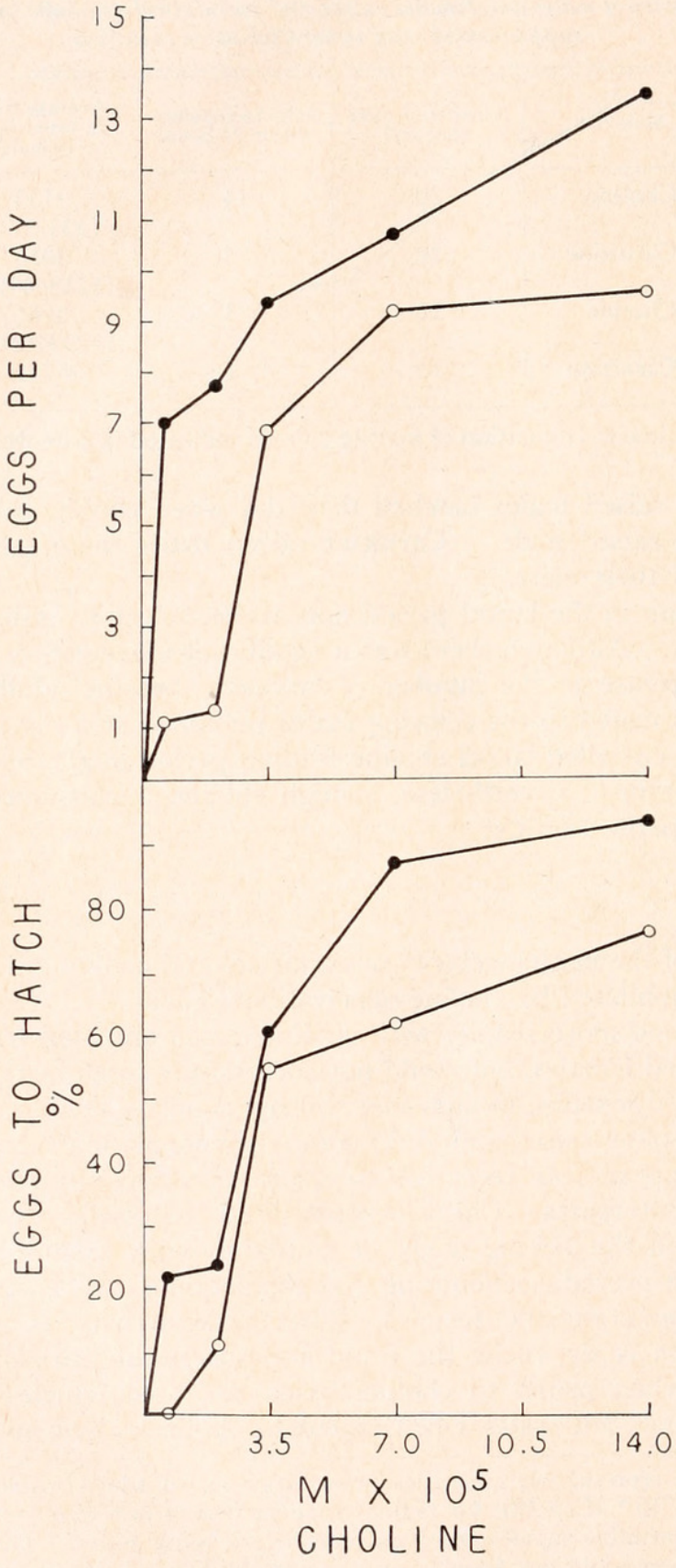
EGGS PER DAY
15
13
11
9
7
5
3
1
EGGS TO HATCH %
80
60
40
20
3.5
7.0
10.5
14.0
M X 10⁵
CHOLINE

FIGURE 4.

Choline feeding period

Because a lag period of several days occurred before carnitine-raised males became fertile, experiments were performed to find if a long choline adult feeding period is necessary for the development of motile sperm or whether a short choline feeding period plus a period on a choline-free diet are sufficient. Carnitine-raised adults were fed a diet containing 5.7×10^{-4} *M* choline for periods of time ranging from 0 to 6 days and maintained on a carnitine-supplemented diet until 8 days old. As little as $\frac{1}{2}$ day on a choline diet increased egg hatchability significantly and subsequent feeding up to 5 days on a choline diet further increased egg hatchability (Fig. 3). Egg hatchability for the females fed a $\frac{1}{2}$-day choline meal was 46.2%, increasing to 81% for females fed choline for 5 days. Egg production followed a similar pattern. Egg production for females fed choline for $\frac{1}{2}$ day was 7.1 eggs per female per day, whereas females given a 5-day meal laid an average of 12.9 eggs per female during the test day. Thus, if choline is fed at a concentration of 5.7×10^{-4} *M*, 5 days of feeding as an adult are needed to accumulate sufficient choline for optimal male fertility.

Concentration of dietary choline

Concentration of choline in the diet, as well as the length of the choline feeding period, was found to be important in the degree of fertility attained by carnitine-raised adults. To assess the effect of dietary choline-concentration, carnitine-raised adults were fed diets containing from 0.7 to 14×10^{-5} *M* choline and then were fed the diets for 4 more days and retested. Distinct differences existed between the hatchabilities of these eggs laid by females fed the test diets for 8 days (Fig. 4). None of the 45 eggs hatched that were laid by females fed a diet with 0.7×10^{-5} *M* choline but 10.3% and 55.1% of the eggs hatched laid by females fed 2.1 and 3.5×10^{-5} *M* choline, respectively. Egg productivity was slightly more than 1 egg per day for females fed a diet containing either 0.7 or 2.1×10^{-5} *M* choline, but was 6.9 eggs per day for females fed 3.5×10^{-5} *M* choline. Both egg productivities and egg hatchabilities for adults fed 7 and 14×10^{-5} *M* choline were also markedly improved by the higher choline levels.

Egg productivity and egg hatchability were higher for adults fed choline for 12 days instead of 8 days regardless of the concentration of choline. Egg hatchabilities had risen to 22.3% and 23.4%, and egg productivity had risen to 7.0 and 7.7 eggs per female for the two lower choline levels tested. There was a continuous gradient from 9.3 to 13.5 eggs per female per day and 61.5% to 92.0% hatchability for the higher choline levels fed. Clearly, choline can be accumulated in the tissues of the adults, the rate of accumulation depending upon the dietary choline concentration and the amount accumulated depending upon the duration of the feeding period. These results show that the development of motile sperm is dependent upon the accumulation of a minimal amount of choline but degrees of

FIGURE 4. The reproductive capacities of carnitine-raised adults maintained on diets supplemented with 0.7 to 14×10^{-5} *M* choline for 8 days (open circles) and 12 days (filled in circles). The upper half of the graph indicates the eggs laid per female per day and the lower half shows the percentages of eggs to hatch for each test group. Circles represent mean values for 72 to 110 males and females in 6 cultures for each test group.

fertility between absolute sterility and optimal fertility result when limiting amounts of choline are fed.

Choline substitutes

Betaine, 2-dimethylaminoethanol and homocholine were fed to larvae with carnitine to see if these combinations would stimulate sperm motility (Table VI). Larval growth was influenced but not adult fertility. Betaine was without effect whereas 2-dimethylaminoethanol and homocholine shortened the larval growth period. 2-Dimethylaminoethanol also reduced the number of larvae to become adults. Adults raised on diets supplemented with carnitine and either 2-dimethylaminoethanol or homocholine may have produced a slightly greater quantity of eggs than adults fed only carnitine. These eggs, however, failed to hatch, indicating the absence of motile sperm.

Adults raised on a diet supplemented with either homocholine or β-methylcholine alone were also found to be sterile. Homocholine-raised adults produced more eggs than carnitine-raised adults, whereas β-methylcholine adults produced no more eggs than carnitine-raised adults. Thus, homocholine appears to stimulate Drosophila females to oviposit when they have not been inseminated with motile sperm.

The abilities of choline-related compounds to promote male fertility were also tested by feeding carnitine-raised adult diets supplemented with one of the related compounds. The fertility of these adults was tested after 8 and 12 days of feeding. Table VII summarizes the present findings and reviews the activities of the test compounds in promoting larval growth and development. None of the nine choline-related compounds was effective in promoting male fertility. In contrast, all but betaine have some activity in promoting adult growth and development. Thus, the requirement for sperm motility is quite specific for the intact choline molecule, much more specific than the requirement for development.

TABLE VI

The effects on adult reproduction of feeding larvae choline-related compounds

Larva supplement*	Larvae to become adults (%)	Growth period† (days)	Eggs to hatch‡ (%)	Egg productivity (eggs/female/day)
Carnitine	73.5	14.1 ± 1.5	0	0.2
Carnitine + betaine	72.5	14.5 ± 1.8	0	0.2
Carnitine + 2-dimethylaminoethanol	61.1	13.1 ± 1.1	0	0.7
Carnitine + homocholine	70.3	12.1 ± 1.1	0.9	1.3
Homocholine	71.0	12.2 ± 1.1	0	1.9
β-methylcholine	72.8	13.1 ± 1.6	0	0.2

* Carnitine was fed in equimolar amounts with other supplements. The total supplement concentration was 5.7×10^{-4} *M* in all diets.

† Figures represent mean ± S.D.

‡ The adults were fed a carnitine-supplemented diet 8 days before being tested.

TABLE VII

Comparison of the activities of choline-related compounds in the development and fertility of Drosophila melanogaster

Compound	Development†	Male fertility
Choline	++*	++
Monoethylcholine	++	−
Diethylcholine	+	−
2-Dimethylaminoethanol	++	−
2-Methylaminoethanol	+	−
Carnitine	++	−
β-methylcholine	++	−
Homocholine	++	−
Sulfocholine	+	−
Betaine	−	−

† Taken from Geer and Vovis (1965).

* ++ indicates at least 60% as effective as choline, + less than 60% as effective as choline, and − ineffective. All compounds were tested at 5.7×10^{-4} *M*.

Sperm morphology

Spermatozoa of carnitine-raised males have been examined with the light microscope. There is no indication of morphological abnormalities but, as in Kiefer's study (1966) of the spermatozoa of X/O males, examination with the electron microscope may reveal structural deficiencies. The numbers of mature sperm in terms of sperm bundles were reduced in carnitine-raised males.

DISCUSSION

To interpret the results of the present study, the position of carnitine-raised Drosophila in terms of choline metabolism must be clarified. During the growth period of the first generation cultured on a carnitine-supplemented diet, larvae are able to use carnitine as a choline substitute effectively. If provided with adequate quantities of carnitine, nearly as many larvae pupate and become adults as when choline is fed, although the growth period is slightly extended (Geer and Vovis, 1965). When carnitine supplementation is delayed, larvae that have been maintained on choline-free diets become increasingly less able to utilize carnitine as well as choline (Geer, Vovis and Yund, 1967). Carnitine is less than 10% as effective as choline in promoting development during the pupal period and, as indicated in the present study, the adult male is incapable of using carnitine as a choline substitute for sperm motility. Bridges, Ricketts and Cox (1965) have also noted that adult *Musca domestica* cannot readily incorporate carnitine into their tissues.

Carnitine-raised *D. melanogaster* adults contain no measureable phospholipid-bound choline in their tissues (Geer, Vovis and Yund, 1967); yet, a phospholipid is present that is very similar chromatographically to the lecithin of the choline-raised adults (Geer and Dates, unpublished). The lecithin-like compound is probably similar to the β-methylcholine-containing phospholipid isolated from carnitine-raised *Phormia regina* (Bieber *et al.*, 1961; Bieber, Cheldelin and Newburgh, 1963).

Carnitine may act by replacing choline in certain metabolic activities, thus releasing choline for those activities for which carnitine is an ineffective substitute. Experiments being conducted in this laboratory indicate that significant amounts of choline are derived by larvae from both the egg and sperm. Delay in supplying dietary carnitine may result in the depletion of sperm- and egg-transmitted choline, thus the reduced effectiveness of carnitine as a choline substitute for the offspring of choline-fed adults when supplementation is delayed. An alternate explanation is that the physiological activities for which choline is required are less specific during early development than during the pupal period or adult stage.

Choline-related compounds are known to supplement the activity of choline in development when fed with choline in the diet. Choline is several times more effective when fed in suboptimal quantities if 2-dimethylaminoethanol is also included in the diet (Geer, Vovis and Yund, 1967). 2-Dimethylaminoethanol when fed alone sponsors the development of only a small percentage of larvae to the adult stage. Carnitine, however, does not complement the activity of 2-dimethylaminoethanol, indicating further the existence of choline-specific developmental activities. Carnitine-raised adults are morphologically normal but possess no detectable choline in their tissues for physiological activities that specifically require choline. One can speculate that the ability to utilize choline-related compounds such as carnitine for certain metabolic activities is of adaptive value to dipterous insects since these insects lack the ability to synthesize choline. Carnitine is widely distributed in nature and might be available under some circumstances when choline is the limiting dietary factor.

The choline requirement of carnitine-raised adult males for sperm motility is very specific. None of the choline substitutes exhibiting activity in development are active in sponsoring sperm motility. This is consistent with the interpretation of both specific and less specific activities for choline in insect metabolism.

Although the primary reason for the sterility of carnitine-raised adults is the immotility of spermatozoa, a secondary reason is the ineptness of both carnitine-raised females and males in courtship. Carnitine-raised males are more successful in courtship with a choline-raised mate than are carnitine-raised females when crossed to a choline-raised mate. Also, carnitine-raised males copulate more readily with choline-raised females than with carnitine-raised females. Several observations of courtship activity indicate that carnitine-raised males court females vigorously but seldom get beyond the licking and probing stage. When they do, they have difficulty in mounting the female. Carnitine-raised females are less active than their choline-raised counterparts and are less receptive to the advances of the male. However, the differences in mating activities of carnitine- and choline-raised adults are quantitative since carnitine-raised males and females have been observed to copulate but carnitine-raised males never successfully inseminate the female, due to the absence of motile sperm.

Carnitine-raised females do not require choline for oogenesis since they lay large numbers of viable eggs after mating with choline-related males. This agrees with the observation by Sang and King (1961) that choline-raised females do not have a choline requirement for normal oogenesis.

Three variables must be determined to adequately define the choline requirement for sperm motility. There is a distinct lag period after choline is fed before adult

males become fertile. Some variation exists in the time that individual males become fertile but all males possess motile sperm by the eighth day on a choline-supplemented diet. No attempt was made to quantify the number of motile sperm after choline feeding periods of different lengths but differences were evident. It seems likely that choline is utilized in the formation of motile sperm rather than activation of the mature immotile sperm since the lag period is great enough for this process.

Although there is a lag period of five to eight days before motile sperm are formed if choline is fed at a dietary level of 5.7×10^{-4} *M*, choline may be accumulated in the body if present in a lower concentration in the diet so that sperm motility may ensue though delayed. If choline is supplied at a dietary concentration of 1.4×10^{-4} *M*, for example, 12 days of feeding is necessary for optimal male fertility.

The length of the feeding period for optimal male fertility for a choline level of 5.7×10^{-4} *M* was 5 days if a sufficient lag period was allowed before testing. In fact, choline-feeding periods as short as $\frac{1}{2}$ day were effective in promoting male fertility with a $7\frac{1}{2}$-day time lapse before testing. Thus, to define the choline requirement for the development of motile sperm in *D. melanogaster* males, the choline concentration in the diet, the length of the feeding period, and the time period between the initial choline meal and fertility test must be stated. A time lapse sufficient for uptake of a minimal amount of choline into the adult body, incorporation of choline into the reproductive tract, and successful insemination of females with motile sperm are necessary. Thus, the choline requirement for optimal fertility of *D. melanogaster* males may be stated as a feeding period of 5 days on a diet supplemented at a level of 5.7×10^{-4} *M* with a total time lapse of 12 days between the initial feeding and examination of eggs laid by females mated to the test males. Eight days are required for the appearance of motile sperm in the testes of all males after choline is fed, whereas the choline response experiments indicate that reproduction is not at an optimal level until 12 days after the initial choline meal. The number of motile sperm must reach an optimal level and mating must occur between day 8 and day 12.

The existence of a dietary choline requirement for spermatozoan motility in Drosophila is significant in light of observations on sperm metabolism in other animals. The primary lipids of spermatozoa are known to be choline-containing phospholipids, the principal phospholipid of invertebrate spermatozoa being lecithin (Mohri, 1957; Hartree and Mann, 1959; Barnes and Dawson, 1966) with choline plasmalogen and lecithin both being prominent in vertebrate spermatozoa (Lovern *et al.*, 1957; Hartree and Mann, 1959; Gray, 1960; Scott, Dawson and Rowlands, 1963; Hartree, 1964; Bratanov, Dikov and Angelova, 1965; Minassian and Terner, 1965).

Phospholipid serves as an endogenous energy source for spermatozoan motility. Lardy and Phillips (1941a, 1941b) first suggested this after observing that bull spermatozoa washed free of seminal plasma maintain motility under aerobic conditions. Under anaerobic conditions in the presence of the carbohydrate of seminal plasma, energy for the motility of many vertebrate spermatozoa is supplied by fructolysis preferentially (Mann, 1946, 1954; Gonse, 1962). Under aerobic sugar-free conditions some mammalian spermatozoa metabolize choline plasmalogen selectively in lieu of lecithin, the fatty acids of choline plasmalogen being utilized as a

substrate in spermatozoan respiration (Carlson and Wadstrom, 1958; Hartree and Mann, 1959, 1961; Hartree, 1964). Phospholipid may also serve as the source of energy for mammalian spermatozoa during the maturation period in the epididymis (Scott, Voglmayr and Setchell, 1967). In contrast to the capacity of mammalian spermatozoa to acquire energy by aerobic or anaerobic means, invertebrate spermatozoa, which are shed into an aquatic environment without the benefit of an accessory fluid, are dependent upon the oxidation of intracellular phospholipid as an energy source (Rothschild and Cleland, 1952; Mohri, 1957, 1964; Gonse, 1962).

Whether Drosophila spermatozoa resemble either vertebrate or marine invertebrate spermatozoa in the utilization of phospholipid as an energy source is unknown. Certain observations suggest the importance of phospholipid in the metabolism of Drosophila spermatozoa and also indicate resemblances to the vertebrate pattern. Faludi, Csukás-Szatlóczky and Széplaky (1960) found that dietary P^{32} is incorporated into the lipid of Drosophila during the larval and pupal developmental periods and that adult males raised on a P^{32}-containing diet contribute an amount of P^{32}-containing lipid to their offspring sufficiently large to influence the larval lipid composition. Other reports indicate that the primary source of phospholipid in the male ejaculate is the seminal fluid and not the spermatozoa and that the fluid is critical to male fertility. King (1954) observed that dietary phosphorus accumulated during a 24-hour adult feeding period, a period too short for the incorporation of phosphorus into sperm DNA, is stored in the accessory glands and ejaculatory bulb and is released from these glands with ejaculated sperm during copulation. Oftedal (1959) confirmed that the most important pathway of phosphorus transfer from male to female during the mating of *D. melanogaster* is the seminal fluid, and Lefevre and Jonsson (1962) found that inability to transfer sperm due to excessive mating results not from lack of mature sperm, but from the lack of accessory gland secretion.

The synthetic capacities of vertebrate spermatozoa are well documented. Human, fish and bull spermatozoa can synthesize phospholipid readily (Terner and Korsh, 1962; Minassian and Terner, 1966) using glucose or glycerol as substrates for the glycerol moiety and acetate as substrate for fatty acids. Scott, Dawson and Rowlands (1963) noted an increase in choline plasmalogen content of rat spermatozoa as they passed through the epididymis and Terner (1965) found that human spermatozoa can use glucosamine of the cervical secretion as substrate for energy metabolism and synthesis of lipids. Utilization of maternal derivatives by Drosophila spermatozoa has been suggested but, as yet, not adequately demonstrated. Anderson (1945), following studies of the *lozenge* mutant of *D. melanogaster* which lacks or has defective spermathecae, suggested that substances essential to the survival of sperm in the seminal receptacles of the female are derived from the spermathecae, a questionable hypothesis since Bender and Green (1962) have shown that the low reproductive capacities of *lozenge* females may largely be due to ovarian pathologies. Herskowitz (1963) demonstrated a maternal effect on restitutional events leading to the healing of breaks induced in mature sperm chromosomes. The rate of paternal mutations increased 50% and the rate of partial loss of the paternal sex chromosomes increased 300 to 600% in eggs oviposited by females undernourished before mating and irradiation, but did not increase greatly

when females were well fed. Although the maternal effect shown by Herskowitz may not be representative of normal sperm metabolism, the utilization of nutrients derived from the female by spermatozoa seems almost a certainty due to the relatively long period of sperm storage in Drosophila females. Although vertebrate and Drosophila spermatozoa may share the capacity to use exogenous nutrients derived from tissues of the female reproductive tract, the permeability of spermatozoa of marine invertebrates such as *Spisula* and *Arbacia* to exogenous substrates is known to be limited (Gonse, 1962; Hartree, 1964).

The requirement for choline for Drosophila spermatozoa motility is dictated by two factors: (1) the inability of Drosophila to synthesize choline from available substrate substances (Geer, Vovis and Yund, 1967); and (2) the requirement for choline as a constituent of phospholipid. Utilization of choline-containing phospholipid as an energy-yielding substrate has not been demonstrated but is strongly suggested by the present study. Another need for choline for normal sperm function is suggested by the postulate that acetylcholine esterase activity is critical to coordination and propagation of the flagellar wave of spermatozoa (Tibbs, 1962; Nelson, 1964). The present experimental results are also consistent with Nonidez's observation (1920) that sperm motility is critical for the successful insemination of female Drosophila.

The current study represents the first case of sperm immotility in Drosophila associated with a dietary requirement. *D. melanogaster* males that lack the Y chromosome are sterile and the production of functional sperm is dependent on seven fertility factors located in the Y chromosome (Brosseau, 1960). By employing electron microscopy methods, Kiefer (1966) found abnormal development of Nebenkern derivatives and incomplete axial fiber complexes in the spermatozoa of X/O males. In another case of male sterility in *D. melanogaster,* Shoup (1967) found that a translocation of parts of the X chromosome to chromosome 2 blocks differentiation of the sperm head as well as preventing the formation of arginine-rich histone.

Gene activity in the Y chromosome critical for the development of functional sperm in Drosophila has been observed. Chromosomal differentiations of the lampbrush type in the Y chromosomes of *D. hydei* and *D. neohydei* in growing spermatocytes are necessary for the formation of spermatozoa (Hess and Meyer, 1963). Meyer, Hess and Beerman (1961) found crystalline structures suggestive of a metabolic block and noted that nuclear structures were missing in the spermatocytes of males lacking a Y chromosome.

Genes necessary for the utilization of choline in the development of spermatozoa may be located in the Y chromosome. It is possible that some of the seven heterochromatic genes essential for the fertility of *D. melanogaster* males (Brosseau, 1960) may be active in the utilization of dietary choline. This, of course, is only speculation, but it is a possibility that warrants investigation.

I am most grateful to Mr. Richard Dates and Mr. John Maguire for their technical assistance and to Mr. William Dolph, Jr., for his critical examination of the manuscript.

Summary

1. Adult *D. melanogaster* raised on a carnitine-supplemented diet fail to reproduce unless choline is included in their diet. The sterility is due primarily to a lack of motile sperm but carnitine-raised adults also mate much less readily than choline-raised adults. Carnitine-raised females are fertile, however, when inseminated by choline-raised males. Supplementation of the diet with 5.7×10^{-4} *M* choline for 5 days will correct the sterility of carnitine-raised males provided 7 additional days elapse before the fertility test. All males possess some motile sperm by day 8 following the initial choline meal but females mated to test males do not lay eggs that hatch at the optimal level until day 12. Thus, 5 days of feeding are required for the accumulation of sufficient choline for optimal fertility but it is not until 7 days after the choline feeding period that a maximum number of motile sperm are formed. A choline meal of less than 5 days in duration results in less than optimal male fertility, whereas a feeding period longer than 5 days is required for optimal fertility if choline is fed at a concentration less than 5.7×10^{-4} *M*. The choline requirement for the development of motile sperm is very specific; betaine, homocholine, sulfocholine, diethylcholine, monoethylcholine, carnitine, β-methylcholine, 2-dimethylaminoethanol, and 2-methylaminoethanol failed to substitute for choline.

2. Choline may be required for the synthesis of phospholipid needed as an energy source for sperm motility. This requirement would be similar to the requirements of many vertebrate and invertebrate spermatozoa for a choline-containing phospholipid as an endogenous energy source for motility. Since *D. melanogaster* can not synthesize choline, the choline needed for sperm motility must be supplied by the diet.

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