AN ABSTRACT OF THE THESIS OF

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Title: FATTY ACIDS IN THE EGGS AND SAC FRY OF STEELHEAD

TROUT (SALMO GAIRDNERI): A DEVELOPMENTAL STUDY

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Abstract approved: _____

Developing steelhead trout eggs and sac fry were examined for wet, dry and lipid weights and for fatty acid composition over the period from fertilization until the disappearance of the yolk sac, 69 days after fertilization. The yolk sac and embryo or fry were separated shortly before hatching, and the above determinations were made on each compartment for succeeding samples.

Losses in the dry and lipid weights of the whole system were not apparent until the time of hatching. Yolk constituents decreased uniformly after hatching, while the embryo or fry gain in wet, dry and lipid weights ceased, 54 days after fertilization.

After 54 days, consumption of lipid by the fry resulted in no net deposition of lipid in the fry, implying that the amount of lipid transported out of the yolk after this time was used for production of energy.

Fatty acids were analyzed by gas chromatography. The identity of the major components was confirmed using retention times and where possible, comparison with standards. Chain length was established by hydrogenation and the number of double bonds by thin-layer chromatography on silicic acid-silver nitrate.

Gas chromatograms of the fatty acid ester mixtures showed a peak due to a polar component which proved to be cholesterol. Being unstable and with its relatively long retention time, cholesterol normally would not be expected to interfere in the analysis of fatty acid esters. This assumption was not valid in this situation.

The embryo and fry were found to have a characteristic fatty acid composition which, after hatching, differed significantly from that of the fertilized egg or yolk. A three fold increase in the relative amount of palmitic acid occurred at the time of hatching.

The embryo preferentially deposited 22:6, 18:0, and 16:0, while 18:1, 16:1, 20:1 and 22:5 were deposited in lesser proportion. This result implies a requirement in the embryo or fry for the former group of acids, and in particular for 22:6ω3. Since this acid is not made de novo in the fish, and belongs to a family of acids found to be essential to growth, and is retained under starvation and stress, a requirement for this acid in a phospholipid moiety is suggested. This requirement is apparent shortly after hatching.

The loss of fatty acids from the yolk was found to be much less selective than the deposition of fatty acids in the fry. Oleic acid was consistently retained by the yolk. Lipid mixtures transported out of the yolk were concluded to be of relatively uniform composition, with no pronounced retention or depletion of any particular class of lipids in the yolk.

Fatty Acids in the Eggs and Sac Fry of
Steelhead Trout (Salmo Gairdneri):
A Developmental Study

by

Lyle Wayne Hayes

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INTRODUCTION

Embryonic growth occurs in a metabolic system under rigorous and constant internal control. The process involves a sequence of co-ordinated events which must be accomplished in order and without interruption if the animal is to develop. The supply of nutrients to the embryo and the manner in which they are utilized offers an opportunity to examine their role in developmental events.

In teleost fish these nutrient requirements of the embryo are met by components from the yolk, a compartment distinct from the metabolically active embryo or larva. The major sources of energy are protein and fat; a trout egg loses 75% of its fat and 40% of its protein in producing a fry (Hayes, 1949, Smith, 1957).

The trout egg and sac fry comprise a convenient system for studying a developing teleost embryo for the following reasons: 1) The eggs are easily maintained in flowing water; 2) a large quantity of synchronous eggs may be obtained from one female, fertilized by one male; 3) yolk sac and embryo or fry are easily separated, per-mitting analysis of both compartments and 4) the magnitude of absorp-tion of metabolites from the environment is negligible.

Among the substances being metabolized, the lipids, and in

particular the fatty acids, are attractive subjects for examination. Until recently the role of lipids and fatty acids in the function of an organism has been considered passive. The discoveries of the rapid turnover of fatty acids of membrane phospholipids; the dependence of membrane function on unsaturation of fatty acids of phospholipid components; the derivation of prostaglandin hormones from linoleic fatty acids; the dependence of electron transport and oxidative phosphorylation in mitochondria and microsomes on the presence of particular phospholipids; the absolute dependence of isolated enzymes, such as β-hydroxy butyric dehydrogenase on particular lipid species (in this case, lecithin) (van Deenen, 1965; Sekuzu et al., 1961; Wakil et al., 1965)--all these facts point to an active role for lipids, and in particular, fatty acids in the metabolism of an organism.

The developing fish offers an attractive model for the study of some functions of fatty acids. It is an organism constructed in large part from a defined supply of lipid in the yolk, and which utilizes this supply completely and rapidly to provide both energy and tissue. The developing embryo would also be expected to show some selectivity in its use of the available fatty acids since a requirement for the linolenic acid series of fatty acids has been established for fish (Lee et al., 1967; Mead, Kayama and Reiser, 1960). This study has been designed to describe in detail the manner in which fatty acids are utilized by the developing steelhead sac fry (Salmo gardneri).

The developing fish, separated from the yolk, will be referred to as "embryo" before hatching, and as "fry" after hatching. The whole system of fry and yolk will be called "sac fry" or "alevin."

LITERATURE REVIEW

Embryonic Growth

The growth curve of a developing embryo (Fig. 1a) typically has
an "S" shape; such curves are frequently called logistic or Sachs
curves (Hayes, 1949). The S curve implies first a period of increas-
ing rate of growth, followed by a decreasing period of growth. A plot
of the time derivative of this curve (Fig. 1b) shows the maximum rate
of increase near the midpoint, indicating a maximum rate of growth.
These growth relationships are found in the Salmonid embryo and sac
fry.

The period before the inflection of the Sach's curve, which cor-
responds to the accelerating phase of embryonic growth, can often be
fitted with an equation of the form $M = K_o t^{k_1}$, where M may be wet
or dry weight (or any quantity proportional to the mass of the embryo,
such as length, volume, lipid or protein content) and K_o and k_1 are
constants, and t is elapsed time after the establishment of embryonic
axis. This restriction on the time value is made because the rate of
growth prior to the establishment of the physical form of the embryo
(i.e. in the blastular phase) is not described by the same constants
(Macdowell, Allen, and Macdowell, 1927).

A plot of the log of the mass, M, versus the log of the time

Figure 1.   Embryo growth

yields a straight line over the time of increasing growth. Taking the log of both sides of the above equation, one has: $\text{Log } M = \text{Log } K_o + k_1 \text{Log } t$, where k_1 is the slope, and $\log K_o$ = a constant, representing the log of the mass of the embryo at the time of formation from the blastula at t = 1. A plot of this nature has been made with data from salmon embryos and sac fry, with the establishment of the embryonic axis placed at nine days, by Hayes and Armstrong (1942) and a straight line relationship was attained in agreement with results for the same relationship in the chick, guinea pig, and rat (Macdowell, Allen and Macdowell, 1927).

Teleost development from egg to fish progresses in several stages. In the case of the trout this sequence of events has been described and photographed (Knight, 1963). The net result is that the fertilized egg produces a small fish, or fry from the yolk plus one viable cell. This period can be divided into two stages: the first consists of the time from fertilization until hatching; the second, the time from hatching until disappearance of the yolk sac.

The pre-hatching period finds the germ cell dividing to form first a flattened disk of cells (within a day in trout eggs at 10 °C), then a hollow sphere, or blastula. When the blastula has three layers of cells, it flattens and spreads over the yolk surface, replacing the non-cellular yolk membrane, and encloses the yolk. A localized infolding and accumulation establishes an embryonic axis on the

surface of the sac. After a week the yolk is surrounded by a sac and a well organized embryo a few millimeters in length is attached on the outside. All these events have taken place inside the chorion, a tough, transparent shell, which is separated from the yolk and embryo by the perivitelline fluid. Midway through the pre-hatching period, about two weeks after fertilization, blood constituents are formed and circulating, and the embryo begins thrashing about in its enclosure. Now the embryo begins to look like a transparent worm, and appears more and more fish-like until around the fourth week when hatching occurs, at which time the embryo with attached yolk sac is ejected from the chorion into the water as a sac fry or alevin.

The alevin gains in wet weight in the period after hatching, but loses dry weight until the yolk is gone. If the fish is denied food, wet weight decreases at the end of the yolk sac period and the rate of growth of the embryo drops to zero (Hayes, 1949).

In the period from hatching to loss of yolk sac, the alevin expends the greater portion of the energy supply of the egg. The fry or larva gains nutrients from the yolk via the vessels in the yolk sac wall.

In order that the yolk lipids reach the tissues of the embryo, they must at one time be in a form which can be transported by the blood. In the case of mobilization of mammalian adipose tissue, whose lipid is 99% triglyceride, the triglyceride molecule is broken

down by the lipases in the adipose tissue cells into glycerol, which

is transported directly, and free fatty acids, which are bound non-

covalently to albumin.

More complex lipids are transported mainly as lipoproteins

which are found in several specific varieties and compositions. In

mammalian systems, lipoproteins, with the exception of the chylo-

microns-[large; ($>.1\mu$), low density triglyceride transporting par-

ticles of dietary origin] are possibly assembled in the liver

(Frederickson et al., 1967). A review on the mobilization of

lipids is found in Masoro's book, Physiological Chemistry of Lip-

ids in Mammals (1968).

Although it would seem more than likely that the fish would

share this transport system with other vertebrates, no information

is available concerning lipoproteins in fish blood. The avian embryo

has been examined for plasma lipoproteins by Schjeide (1956). He

found chylomicrons in early embryo blood which were smaller than

those of the adult bird and contained more phospholipid and protein.

The number of chylomicrons was found to decrease abruptly at hatch-

ing. He also found less albumin (one-tenth the concentration) than in

blood of the adult bird, indicating limited free fatty acid transporting

ability. Also, the yolk contained lipoprotein species.

It is not unreasonable to assume that the trout embryo is sup-

plied with lipids via mechanisms of lipid transport such as those

described above--specifically, by the hydrolysis of triglycerides as in adipose tissue fat mobilization, and/or by forming lipoproteins.

In general, the developing vertebrate embryo has been found to possess the metabolic equipment of the adult organism, although the activities of the individual pathways may be regulated during specific periods of differentiation. For example, early cells of avian and amphibian embryos possess mitochondria with fatty acid oxidation mechanisms and Krebs cycle enzymes and electron transport system components, although the activity of these systems may show fluctuation at specific times. It has been shown that embryonic systems regulate metabolic pathways, although the mechanisms involved have not been elucidated. The pentose phosphate pathway predominates over the Embden-Meyerhof-Parnas pathway in the rapidly dividing cells, as the former is an important source of biosynthetic precursors for synthesis of nucleic acids (Papaconstantinou, 1967).

Lipid and Fatty Acid Metabolism in Fish

Lipids are typically the major dietary energy source in fish (Kayama and Mead, 1966). The aquatic diet is characterized by a high lipid content, and lipid is an efficient way of storing energy, since it has a higher energy content per gram than either protein or carbohydrate. This latter property is particularly valuable since most fish experience severe depletion of their energy reserve for a

part of each year. Their ability to cope with starvation is undoubtedly

enhanced by a low basal energy consumption (little energy is required

for physical support in the aqueous environment and body temperature

is low) and by a highly developed capability for mobilizing their body

constituents.

Specifically, starvation in fish results in mobilization of carbo-

hydrates and lipids, followed by protein after these reserves are ex-

hausted (Love, 1969, p. 256). The carbohydrate reserve can be as-

signed small importance since there is not much present at any time.

The small amount of glycogen commonly found in the liver of Salmon-

ids is rapidly lost during starvation, but blood glucose levels remain

relatively constant (Hochachka and Sinclair, 1962). It has been pos-

tulated that blood levels of glucose are maintained by gluconeogenesis

from lipids, particularly during spawning migrations (Love, 1969,

p. 252).

On starving protein catabolism is more marked at the end of

the stress in pink salmon (Parker and Vanstone, 1966). In contrast,

under extreme stress of short duration (15 to 24 hours), protein may

be utilized in preference to lipid (Krueger et al., 1968).

Migrating salmon use fat almost exclusively in their journey up

rivers to spawn. Lovern (1934) found a decrease in fat content from

14% to 1% in migrating salmon. Bilinski and Gardiner (1968) found

increased amounts of free fatty acids in rainbow trout blood during

starvation, indicating lipid utilization.

Fatty acids are oxidized in fish apparently by the β-oxidation system, as in land animals (Brown and Tappel, 1959). These authors found requirements for ATP, cytochrome C, Mg^{++}, and a member of the TCA cycle in carp liver mitochondria. These requirements are the same as those of mammalian liver. However, the liver of salmonids appears to have a storage function and a lessened oxidative capacity by comparison. A major part of the oxidative function is assumed by the dark or red muscle found along the lateral line (Brackkan, 1956). Bilinski (1963) found that dark muscle slices oxidized C-14 labelled fatty acids at a rate 10 to 40 times that found in white muscle. White muscle cells (versus red muscle cells), typically comprising a majority of the musculature, are called upon to perform fast, anaerobic contraction, using glycogen and accumulating lactate (Frasier et al., 1966). Such activity would be expected in short term action such as flight and predation. Dark cells would be called upon for continual, minimal or "cruising" activity and possibly would utilize lipid as a source of energy (Mead and Kayama, 1966; Bilinski and Jonas, 1964).

Thus, a fish enjoys an advantage over terrestrial vertebrates-- the luxury of carrying, in effect, a coiled spring of short term anaerobically derived energy in the white muscle while performing continual activity via oxidation of fat at a low rate in the dark muscle.

Conversion and Selection of Fatty Acids

In fish, fatty acid synthesis and conversion capabilities are generally similar to those of other animals. They cannot synthesize certain of the polyunsaturated fatty acids de novo and suffer deficiency symptoms when denied these essential dietary components. Klenk and Kramer (1960) found that polyunsaturated fatty acids formed in fish liver slices incubated in C-14 acetate contained all the radioactivity in the carboxyl group, indicating that the fish was merely able to elongate the precursors of these acids at the carboxyl end. This has been confirmed by Kayama and Tsuchiya (1962) who proposed a series of possible conversions of unsaturated acids in fish (Fig. 2), based on their experiments and the structures of the fatty acids. Linoleic and linolenic acids were not formed from C-14 acetate (Mead et al., 1960), but dietary $18:2\omega3$[1] was elongated.

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[1] It is customary to use the following abbreviation when referring to particular fatty acids: two number are written separated by a colon; the first number designates the number of carbon atoms in the fatty acid molecule and the second number designates the number of methylene interrupted double bonds. Polyunsaturated fatty acids may also be regarded as belonging to families in which the alkyl terminal structure is the same for each family member. To this end, the position of the ethylenic bonds along the carbon atom chains may be designated by the Greek omega (ω) and a number which refers to the number of carbon atoms separating the terminal methyl hydrogens from the nearest double bond. For example, $18:2\omega6$ stands for 9, 12, -octadecadieneoic acid (linoleic acid) and acids designated with an $\omega6$ are said to belong to the linoleic family. Similarly, endings of $\omega3$ and $\omega9$ denote the linolenic and oleic families, respectively.

DOUBLE BOND POSITION COUNTED FROM METHYL CARBON

7 PALMITOLEIC

7, 10   6, 9   4, 7

C_{16} 6, 9, 12   4, 7, 10   3, 6, 9   1, 4, 7

3, 6, 9, 12   1, 4, 7, 10

9 OLEIC

9, 12   (7, 10)   6, 9 LINOLEIC

C_{18} (7, 10, 13)   6, 9, 12   3, 6, 9 LINOLENIC

3, 6, 9, 12

CHAIN LENGTH OF FATTY ACID

9, 12

9, 12, 15   (7, 10, 13)   6, 9, 12

C_{20} (7, 10, 13, 16) 6, 9, 12, 15   3, 6, 9, 12
ARACHIDONIC

3, 6, 9, 12, 15
EICOSAPENTAENOIC

(9, 12, 15)

(9, 12, 15, 18)   6, 9, 12, 15

C_{22} 6, 9, 12, 15, 18   3, 6, 9, 12, 15

3, 6, 9, 12, 15, 18
DOCOSAHEXOENOIC

POSSIBLE CONVERSION PATHWAYS OF POLYUNSATURATED ACIDS IN FISH
FROM KAYAMA AND TSUCHIYA (1962)

Figure 2.   Possible conversion pathways of poly unsaturated fatty
acids in fish

Lee et al. (1967) have shown that ω3 or linolenic family acids are essential for maximal growth rate in rainbow trout. Also, Nicolaides and Woodall (1962) found that young chinook salmon had lower weight gain and suffered from depigmentation when placed on a fat free diet; trilineolin reversed these conditions, and the data can be interpreted as indicating a requirement for linoleate. However, a lower level of linolenate also alleviated the symptoms, and it appears certain that the linolenic family of fatty acids has much more essential fatty acid value in fish than does the linoleic family (Castell, 1970).

Thus, fish have definite structural requirements for some of the fatty acids they cannot synthesize, and suffer deficiency symptoms in their absence. An example of a structural requirement would be that of certain unsaturated acids being needed for phospholipids found in cell membranes (Menzel and Olcott, 1964). Yet at the same time phospholipid can be used for energy production in fish to a greater extent than in mammals, possibly due to the ability of the fish to utilize protein during periods of starvation--proteins which may be bound to or associated with phospholipid in tissues. In higher vertebrates, such depletion is more disruptive to the organism (Love, p. 242).

Fish are often found to utilize fatty acids in a selective manner during depletion or starvation. Kaneko et al. (1967) reported that the

proportion of unsaturated acids increased during starvation in <u>Salmo</u>

<u>gairdneri</u>. Krueger <u>et al</u>. (1968) reported that exercise at 52 cm/sec

water velocity for 24 hours caused a depletion in the acids 18:1, 16:0

and 16:1, while more strenuous exercise at 59 cm/sec caused a shift

to a loss of the more unsaturated acids 22:6, 18:2 and 20:4. It is

interesting to note that protein seemed to have provided a much

greater proportion of the energy supply during exercise at the higher

velocity.

Yolk Utilization

The yolk in a fish egg contains all the nutrients necessary for

the production of a juvenile fish. Hayes (1949) found that salmon

eggs had lost 76% of their fat at the time of yolk disappearance,

representing 68 calories for each egg and had lost 40% of the origi-

nal protein, representing 46 calories expended. Even though more

calories are derived from fat than from protein, fish embryos and

sac fry use proportionately more protein than the avian embryo

(Williams, 1967, p. 357). This is perhaps due to the ease of elim-

inating ammonia in the aqueous environment, as compared to the

enclosed avian system.

The developing egg or sac fry may be thought of as a two com-

partment system consisting of the yolk and the growing embryo or

fry. Several studies have been made of the changes in the nutrient

substances in this system, but these have usually used the whole system prior to hatching, and after hatching, i.e., the combined yolk and fry. Of the neutral lipids, cholesterol has been found to remain constant throughout development (Takama et al., 1969).

Glycerides are claimed by Smith (1952) to be utilized almost exclusively when the fish is starving during completion of yolk absorption. Smith used CCl_4 and encountered difficulties in homogenizing; his data is quite variable. Takama et al. (1969) concluded that glycerides decrease in parallel with embryogenesis. The discrepancy may be attributed to the different extraction procedures employed. Takama separated the complex lipids by first acetone and then chloroform-methanol extraction. By repeated extraction and recombination, the lipids were separated into acetone-soluble and -insoluble fractions. The acetone-soluble fraction was considered to be neutral lipid and the cholesterol was determined and found constant in absolute amount. The remainder of the acetone-soluble fraction was considered glyceride which appears to be a valid assumption, with the exception that free fatty acids should be included. Ando (1962) found a decrease in acetone-soluble lipid which was more pronounced after hatching than before hatching. Ando also determined that the free fatty acid content of developing rainbow trout eggs increased steadily with growth, and reached a peak at completion of yolk absorption.

In contrast, the acetone-insoluble fraction, comprised of polar lipids (mainly phospholipids), was found by Ando (1962) to decrease more before hatching than after. Takama et al. (1969) found that the polar lipid declined with development of the embryo as did non-polar lipid. The latter study seems more credible as the extraction procedures were more exhaustive. These workers also found that almost identical proportions (36% and 38%, respectively) of acetone-soluble and -insoluble lipids were lost with production of the fry. One may conclude, then, that the triglycerides and other lipids decline with embryogenesis.

Of the two predominant phospholipids present, cephalin and lecithin, the former appears to be synthesized and deposited almost exclusively in the embryo, while the latter is the major phospholipid in the yolk. This component disappears at a rate similar to that of glycerides. This conclusion is supported by the work of Takama et al. (1969) who divided the acetone-insoluble lipids into ethanol-soluble and -insoluble fractions, labeling the former lecithin and the latter cephalin. Lecithin declined with embryogenesis while cephalin began to increase before hatching and reached a maximum in the last sample, taken immediately after the yolk sac was absorbed. The avian embryo contains all the phosphotidyl serine (a cephalin) while the yolk has more phosphatidyl choline (lecithin) (Williams, p. 358).

Terner (1968), working with C^{14} labeled acetate, noted that the radioactivity was incorporated mostly into cephalin, while little was found in lecithin, further supporting the hypothesis that lecithin is provided by the yolk as an energy source for the embryo or fry, whereas cephalin is an exclusive product of the embryo. Polyglycerophosphatides (cardiolipins) were the most actively labeled of any class of lipid, an intriguing point, since they are characteristic (10%) components of mitochondrial membranes (Lehninger, 1970, p. 524) and may play a specific role in connection with electron transport and oxidative phosphorylation.

Ando (1962b) has reported selective consumption of the C-20 and C-22 polyunsaturated fatty acids in the acetone-soluble fraction of rainbow trout eggs during the last stage of the sac fry period; Takama et al. (1969) found a slight increase in polyunsaturated fatty acids in salmon eggs in the same fraction. Again, if species differences may be discounted (rainbow trout vs. salmon), Takama et al.'s work must be weighed more heavily as he used gas chromatography in obtaining this result. They also noted no overall change in percentage contribution of unsaturated fatty acids in total lipids.

At this point, the literature seems to indicate that depletion of lipid reserves may be characterized as a uniform, non-selective process. However, the cephalins appear as products of the embryo's synthesis, and free fatty acids accumulate with advancing development.

METHODS.

Steelhead Eggs and Sac Fry

Fertilized eggs of sea run rainbow trout (Salmo gairdneri) were obtained in February, 1968, at the peak of the run, from the Oregon State Fisheries Commission hatchery on the North Fork of the Alsea River. Eggs were obtained from one female and were fertilized by one male. The eggs were transported to the laboratory, in river water, within two hours, where they were kept at 10-12 C in a constant flow of spring water in two liter funnels provided with marbles in several layers at the bottom of the vessel.

At appropriate intervals, four samples, each of ten developing eggs (or fry plus yolk) were taken for wet weight determinations. Of these samples three were examined for lipid and fatty acid content. From the fourth, dry weight was determined. The first fry (without yolk) sample, taken on day 21 (fertilization day 0) consisted of 19 individuals, and was examined for wet weight, lipid and fatty acid composition. On day 28 two samples, of ten fry each, were taken for wet weight; in one, dry weight was determined, and in the other, lipid and fatty acid content. In the succeeding periods, four samples each of ten fry only and four samples including fry plus yolk were taken for the wet weight, dry weight, lipid and fatty acid determination.

Measurements of the yolk were determined by the difference between whole fish and fry without yolk. The fish were not fed at any time during the experiment, which was concluded upon disappearance of the yolk sac.

Lipids

Weighed samples of eggs or sac fry (less than 2 grams wet weight) were stored in chloroform-methanol (2:1 by volume) under nitrogen at -19C prior to lipid extraction. Lipids were obtained by the method of Bligh and Dyer (1959). The samples were homogenized in 15 ml of chloroform-methanol in a Sorvall omni-mixer for two minutes and filtered under slight vacuum on a sintered glass filter into a 35 ml screw-cap centrifuge tube. An additional 15 ml of solvent was then briefly blended to rinse the cup and blades of the omni-mixer and used to wash the residue on the filter. 6. 7 ml of water was then added; the mixture was shaken briefly and centrifuged on an International clinical centrifuge. Two phases appeared: the upper layer consisted of methanol and water; the lower was chloro-form which contained lipid. The latter was carefully pipetted into a beaker and evaporated to dryness at 50C under a stream of nitro-gen. Immediately upon evaporation of the solvent, the lipid was taken up in chloroform and transferred to a screw-cap test tube (cap lined with a teflon sealer), and adjusted to a known volume (usually 15 ml),

and stored under nitrogen at -19C in the dark.

Aliquots were subsequently taken for preparation of methyl esters of fatty acids and for determination of lipid weights. Lipid weights were measured by pipetting aliquots (in duplicate for each individual sample) into aluminum or stainless steel cups (previously dried and weighed) and were weighed on a microgram balance.

Fatty Acid Methyl Esters

Fatty acid methyl esters were prepared from aliquots of the lipid sampled by transesterification. Portions of the lipid samples containing 2 to 50 mg lipid were placed in screw-cap test tubes and taken to dryness at 50C under a stream of nitrogen. Hydrochloric acid in dry methanol and anhydrous ether, 3 ml each were added and the cap lined with teflon was screwed tight. After reacting at 80 C for 90 minutes, the solutions were cooled and two ml each of water and hexane were added. The mixtures were shaken, centrifuged briefly, and the upper layer of fatty acid methyl esters in hexane-ether was removed. A second extraction with 2 ml of hexane was added to the first, and the combined extract was taken to dryness at 50 C under nitrogen. The methyl esters were then taken up in and stored in a known volume of hexane. Weights of the methyl esters were determined as they were for lipids. Samples were stored at -19C under nitrogen until used in thin layer and gas-liquid

chromatography.

Thin Layer Chromatography

Fatty acid methyl ester mixtures were separated according to degree of unsaturation by thin layer chromatography on silver nitrate impregnated silicic acid (Privett et al., 1965). The plates were prepared by dissolving 6.25 grams of silver nitrate in 50 ml of water, adding 25 g of ether washed SilicAR TLC-7Z silicic acid (Mallinckrodt), and spreading the slurry on 8" x 8" glass plates in a layer 250 to 500 microns thick, depending on the size of the sample to be chromatographed. The fatty acids were applied in a streak by hand, using a 50μl syringe (Hamilton) and employing a plate streaking apparatus designed and built by Robert R. Lowry of the Agricultural Chemistry Department of Oregon State University. This apparatus permitted the application to be made under a stream of nitrogen, to prevent sample oxidation, and the plate was propelled slowly and in a direction parallel to the streaking line by a motor driven screw.

Plates were developed with 20% diethyl ether in hexane. Detection of the separated groups of acids was accomplished by spraying with a methanol solution of 2, 7 dichlorofluorescein and viewing under ultraviolet light. Bands formed according to degree of unsaturation: saturated fatty acid methyl esters were deposited in a band nearest to the solvent front; esters with 5 and 6 double bonds were deposited

in the band nearest the origin.

The bands were scraped off the plates and extracted with chloro-form and were either respotted and run again or analyzed directly by gas-liquid chromatography. Fractions were also saved for hydrogen-ation.

Hydrogenation

The unsaturated fatty acids obtained from thin layer silver ni-trate plates were placed in hexane and subjected to hydrogen gas in the presence of a catalyst according to the procedure of Farquhar et al. (1959). 15 mg or less of a methyl ester sample was dissolved in hexane, placed in a pyrex flask which was flushed with nitrogen gas to diminish the chance of flaming. About 20 mg Adams platinum oxide catalyst was added, the flask flushed thoroughly with hydrogen gas, and the reaction was allowed to proceed for 30 minutes with stirring.

After hydrogenation, the sample was filtered to remove the catalyst and the saturated acid esters were examined by gas chro-matography. Super dry methanol was prepared according to the method of Lund and Bjerrum as described by Weissberger (1955).

Gas-Liquid Chromatography

Gas-liquid chromatographic analysis was carried out on a Beckman GC-2 instrument specially equipped for solid sample injection (Lowry, 1964), designed such that the samples are introduced very near to the head of the colunn. The column consisted of an 1/8" OD aluminum tube packed with acid washed Chromosorb P, 80-100 mesh, coated with 15% ethylene glycol succinate. The column was run at 190 C, with helium as the carrier gas at 19 psi and 30 ml/min. Detection was by hydrogen flame ion current.

The column was periodically checked for separation and quantification of components by running standard mixtures of fatty acid methyl esters, and discarded if found deficient.

The masses of individual fatty acids are proportional to the product of the peak height and the retention time (Carrol, 1961a, b). This method of measurement is much less laborious than either triangulation or using the product of peak height and width at half-height. The recorder was provided with an F&M 50B automatic attenuator, allowing unattended runs and more precise detection of lesser components, as the larger peaks were attenuated several times.

Both saturated and unsaturated fatty acid methyl esters were tentatively identified by plotting the log of the retention time against the number of carbons in the acid (Ackman, 1963 and Evans et al.,

1962) and by comparison and spiking with Hormel Institute stand-

ards.

Solvents, Standards

All organic solvents used were redistilled from drum stock,

except diethyl ether, which was anhydrous reagent grade. Fatty acid

standards purchased from the Hormel Institute and used were 12:0,

14:0, 16:0, 18:0, 20:0, 22:0, 16:1ω7, 18:1ω9, 18:2ω6, 18:3ω3, 20:1ω9,

20:4ω6 and 22:6ω3.

Cholesterol

Cholesterol was separated from fatty acid methyl esters by

thin layer chromatography. 8" x 8" plates were prepared using 30

grams SilicAR TLC 7GZ and 55 ml water and were developed in

dichloromethanol. The cholesterol obtained in this manner was

analyzed by a Varian Mat CH7 mass spectrometer operating

70 e.v., 100 C and using direct sample introduction. An infrared

spectrum was obtained using a Beckman Microspec, with double

beam operation, and with carbontetrachloride as solvent. Gas cho-

matographic analysis was accomplished using the same equipment

and operating parameters as were used for fatty acid methyl esters.

Lieberman-Burchart reagent was prepared from sulfuric acid,

acetic acid and chloroform (1:20:50) for a spray reagent to detect

sterols on thin layer plates. Plates were dried at room temperature, sprayed lightly and heated at 100 C for 10 minutes.

RESULTS

The eggs were fertilized at day 0, hatched at day 34, and the
experiment terminated at 69 days, when the yolk sac was nearly
completely absorbed.

Gross Changes

Wet Weights

From the period shortly after fertilization[2] to hatching, days 0
to 34, the wet weights of the whole system, shown in Fig. 3 (whole
eggs, or sac plus fry) of winter run fish were found to be relatively
constant at about 140 mg. From the time of hatching (when wet weight
decreased by about 10 mg. due to the loss of the chorion and perivitel-
line fluid) until the end of embryonic growth (days 34-69), the wet
weight of the whole system increased steadily until the end of the yolk
sac period, when the values leveled off at just over 200 mg.

The wet weight of the embryo or fry (Fig.3) increased without
interruption from the first measurement at day 21, reaching 200 mg.
at the end of yolk absorption, when the fry ceased to grow. The curve
assumed an "S" shape. A concurrent decline in the wet weight of the

---

[2]When the egg is deposited in water, within one hour water is
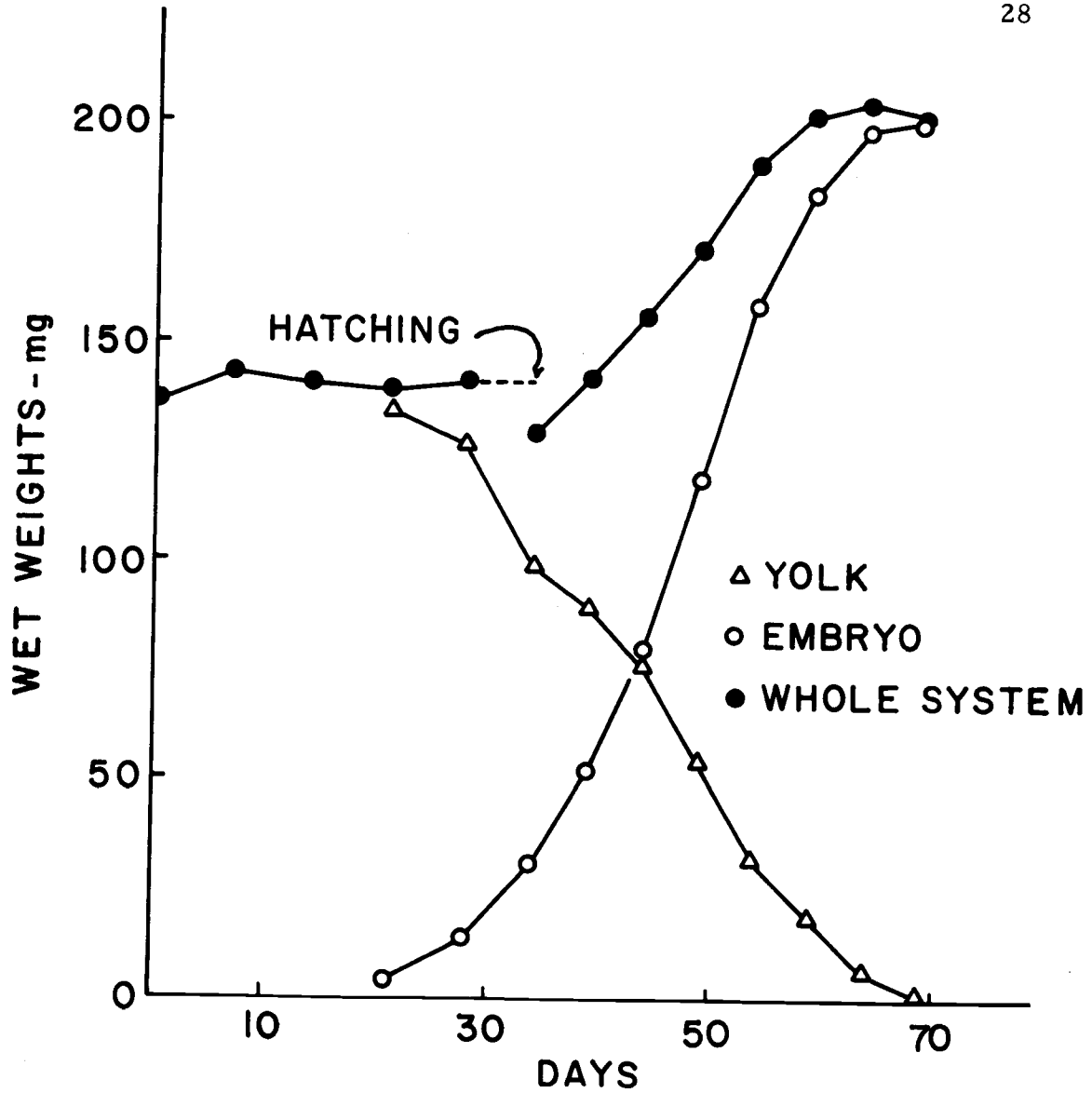taken up through the chorion and forms the perivitelline fluid.

Figure 3. Wet weights of whole system, embryo or fry, and yolk

yolk was associated with growth of the embryo and fry, until only about 2 mg. of separable yolk remained at 69 days.

Dry Weight

The dry weight of the whole system (Fig. 4) remained relatively constant at 50 mg. during the pre-hatching period. At hatching a gradual decline began, culminating with a weight of 35 mg. at 69 days. This constitutes a loss of only 15 mg. or 33% of the original dry mass.

The curve for dry weight of the embryo also showed an S-shape. A constant dry weight of about 33 mg. was maintained for days 64 and 69. Yolk dry weight fell precipitously and concurrently with increasing embryo weight. Less than three milligrams remained at 69 days, constituting a loss of more than 94% over the course of the experiment.

Lipid

Lipid loss for the whole system over the period of the experiment was 8.3 mg. of an original 14.0, a loss of 59%. As in the case of dry weight, the decrease was begun shortly after hatching. The decline continued through the last sampling days, and did not level off.

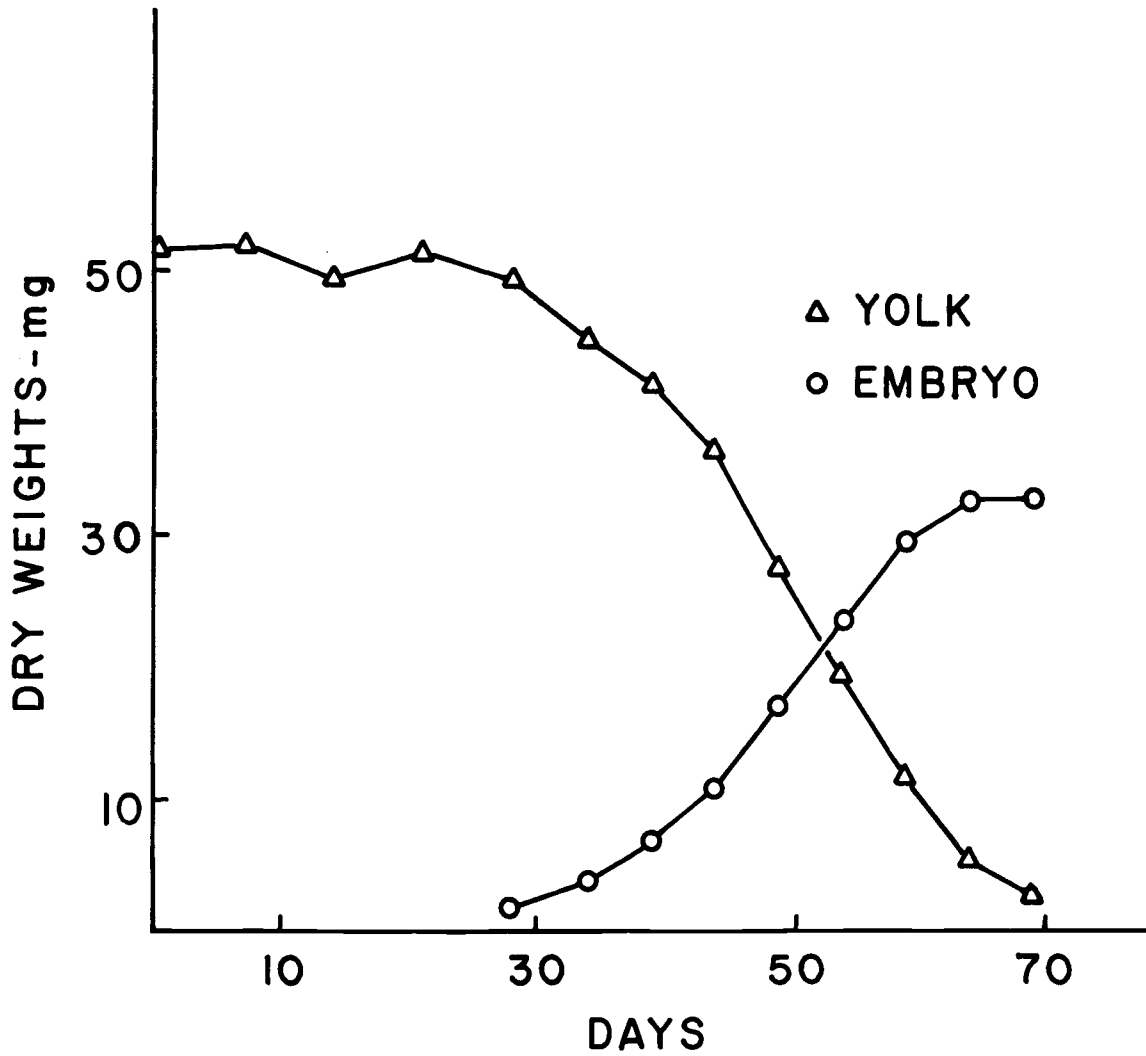Lipid content of the yolk began a rapid decline shortly after

Figure 4.   Dry weights of embryo or fry and yolk

hatching in a manner similar to yolk dry weight, and reaches a value of 1.8 mg. on day 69, compared to an original value of 13.8 mg. Thus there was a transfer of 87% (Fig. 5).

The embryo lipid increased in a manner similar to wet and dry weight, except that the maximum value of 4.2 mg. was reached at 59 days and declined slightly by day 64 (Fig. 5).

Expressed as percent of dry weight, the lipid content of the embryo (before hatching) and of the fry (after hatching), are seen to differ (Fig. 6). The embryo has a higher lipid content at 20-22%, than the fry, which has a value of 15% at hatching. A gradual decline results in a content of 12% at day 69.

The gain in lipid content of the fry as related to loss of lipid from the yolk is depicted in Figure 7; at all times the net transfer is less than complete. If the conversion were complete, the amount of lipid lost from the yolk, when plotted against the lipid deposited in the fry, would yield a 45 degree line passing through the origin. The values for the sac-fry system are not far from this line until after day 54, when a sharp break occurs, and the curve approximates a horizontal line, indicating a loss of lipid from the yolk with no net increase in fry lipid.

A plot of the logarithm of wet, dry, and lipid weights of the embryo and sac-fry against the log of time is shown in Figure 8. Time is reckoned from the establishment of the embryonic axis, at
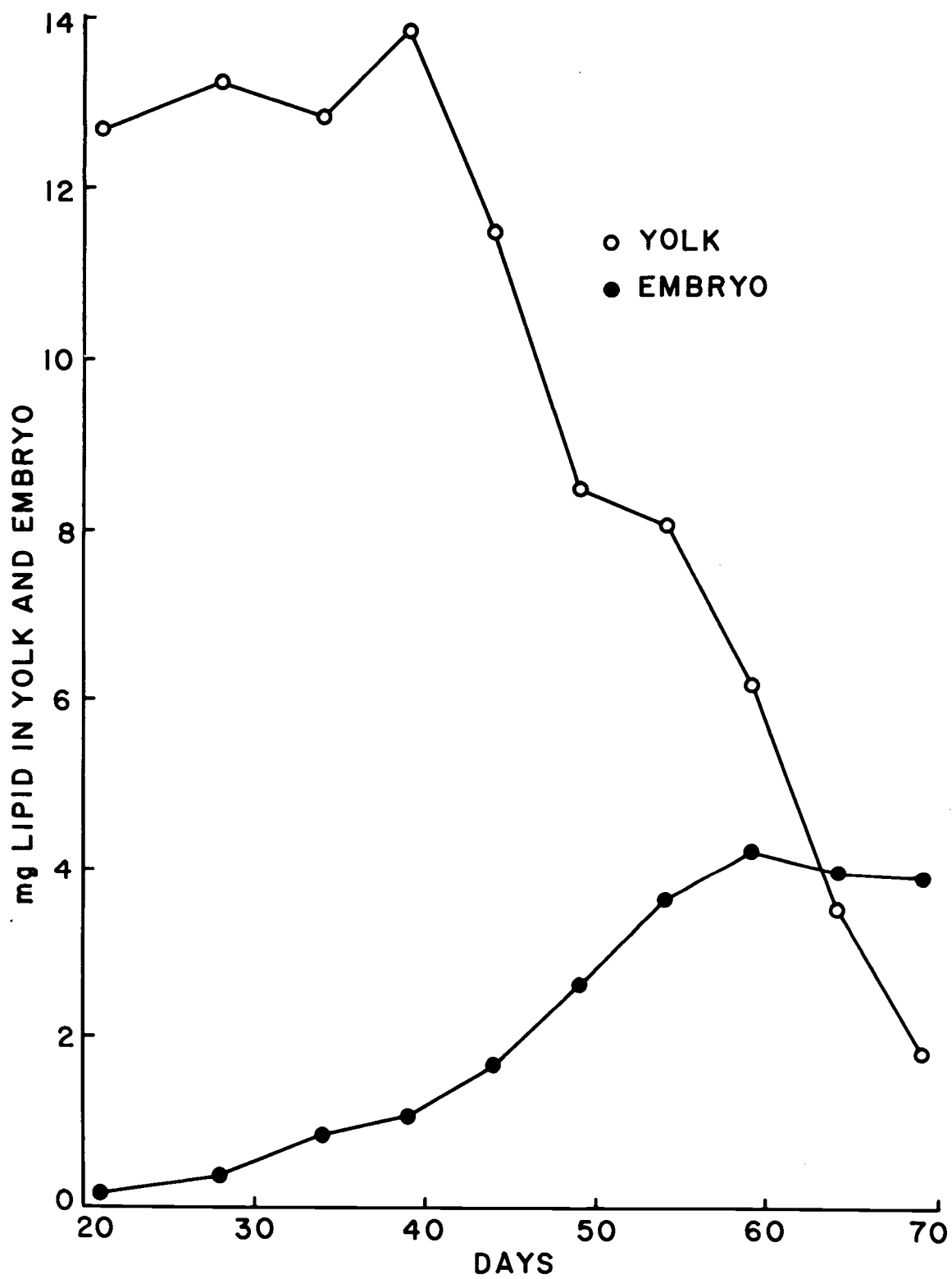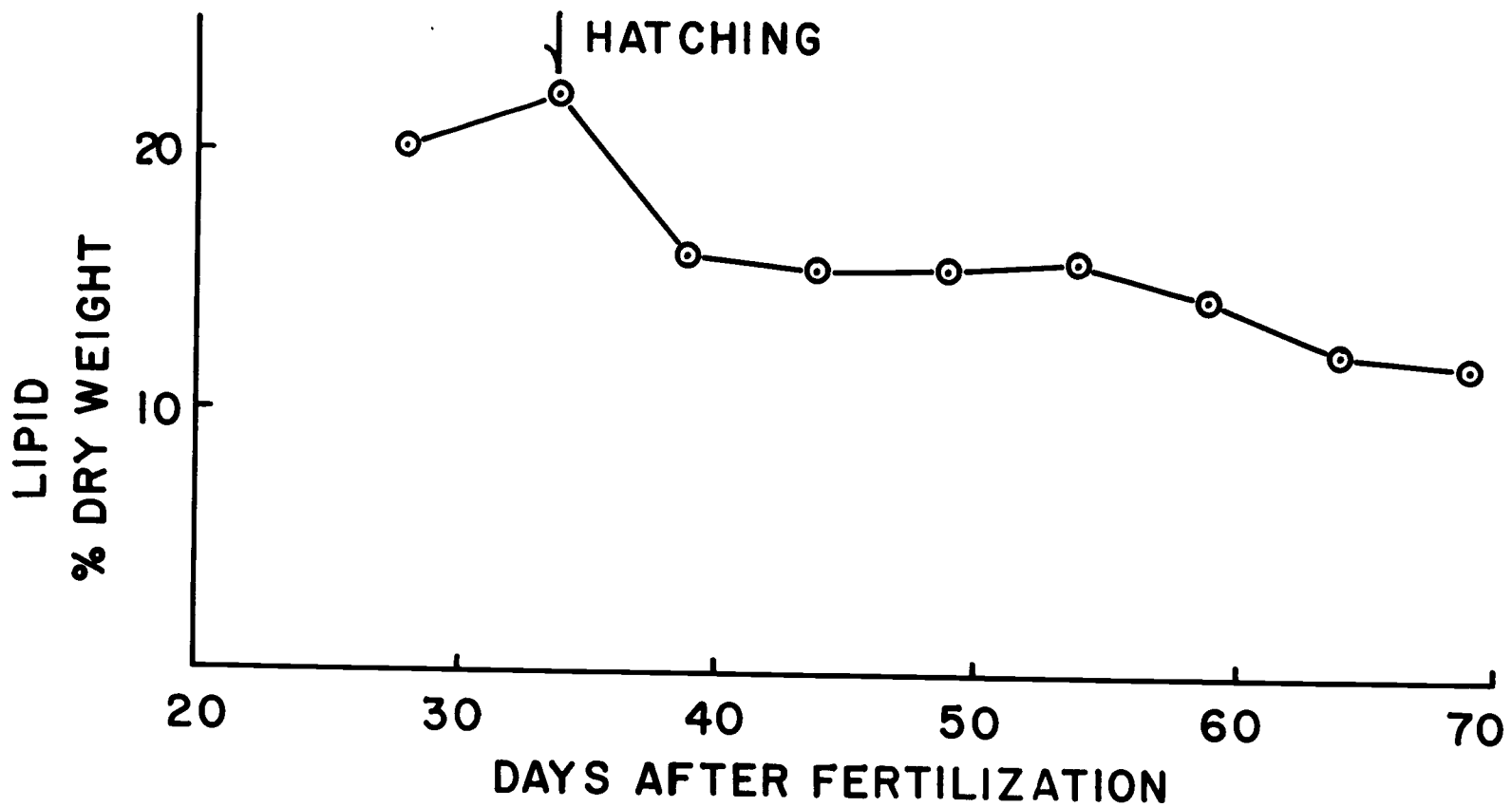
Figure 5. Lipid in yolk and embryo or fry

Figure 6.   Embryo lipid expressed as percent of dry weight of embryo or fry
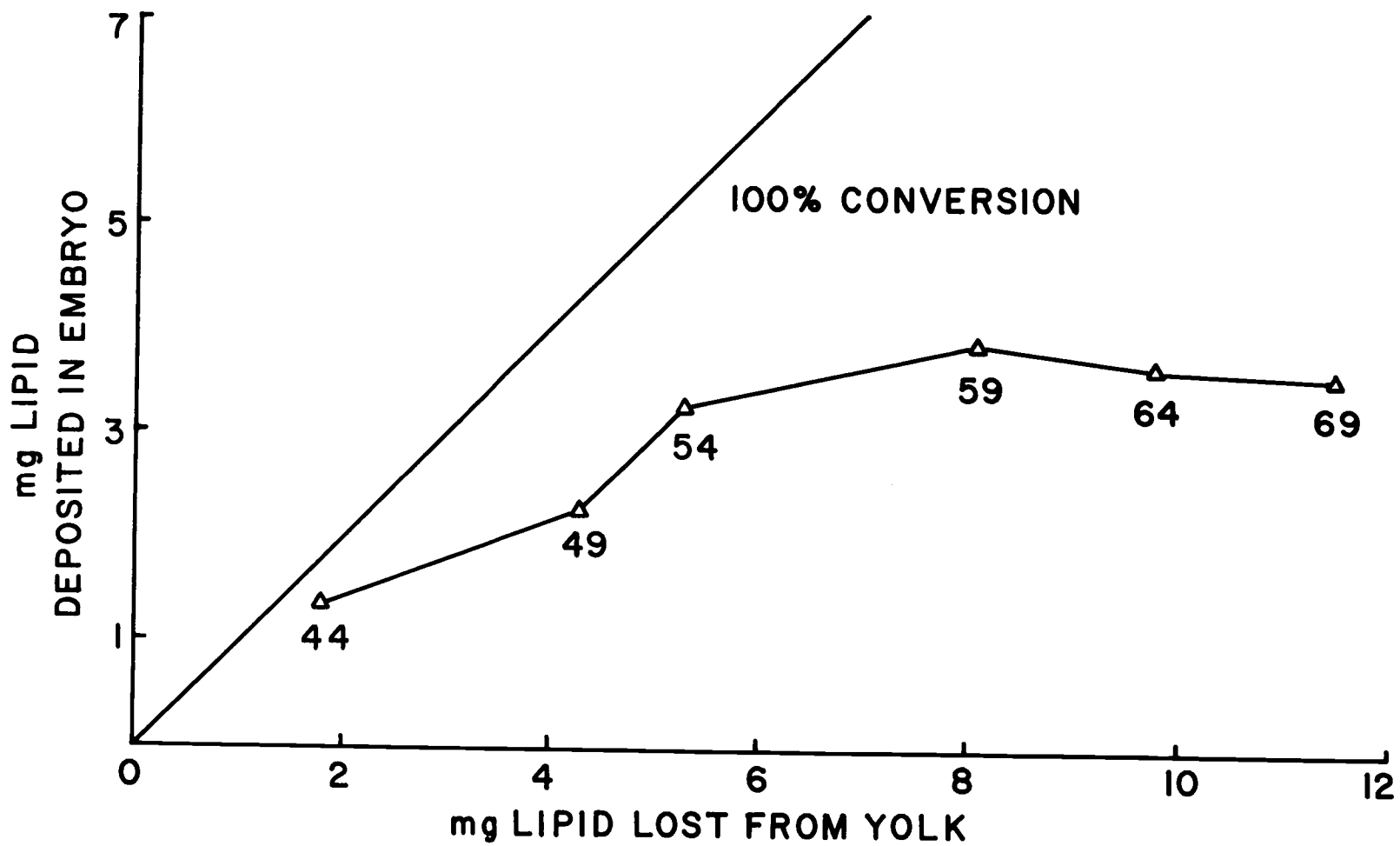
Figure 7.   Milligrams of lipid deposited in embryo or fry as a function of milligrams of lipid lost from yolk
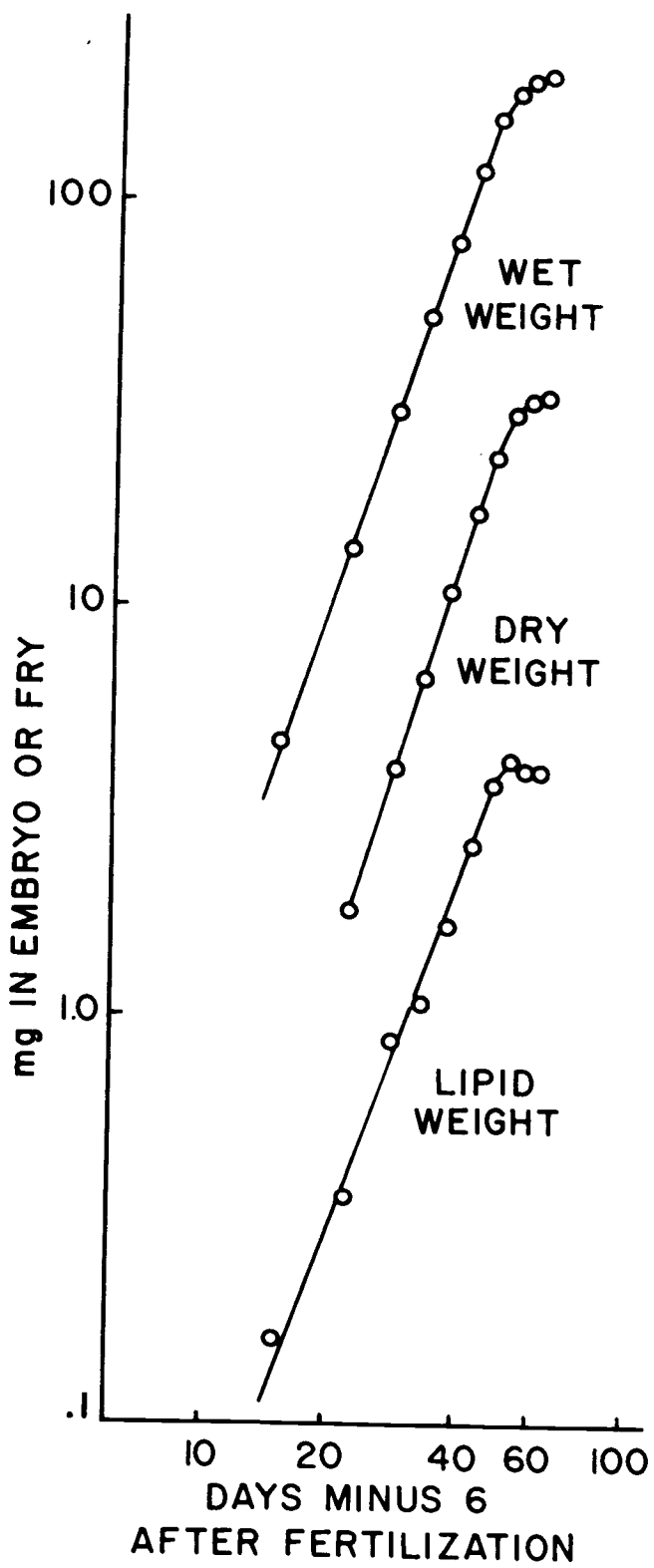
Figure 8. Logarithmic plot of wet, dry, and lipid weights in fry and embryo

about six days after fertilization. These plots yield straight lines over the interval t = 15 to t = 48 in accordance with McDowell's formula, $M = K_o t^{k_1}$; or $\log M = \log K_o + k_1 \log t$. k_1, evaluated from the slope of the line for each variable, yield values for 2.86 for wet weight, 3.28 for dry weight, and 2.94 for lipid weight. This slope is maintained in each case until the period after t = 54 days (t-6 = 48). At this point, the slope drops quickly and approaches zero at t = 69 days for wet and dry weight. The fry lipid behaves somewhat differently during this phase of decreasing growth. Instead of gradually curving over to approach zero slope as do wet and dry weight, the lipid weight curve has a negative slope, reflecting a loss of material, after day 59.

Fatty Acids

The weight of combined fatty acid methyl esters obtained from the lipid is plotted for yolk and for embryo or fry in Figure 9. The curves are similar to those obtained for total lipid in Figure 5. It should be noted that the total weight of fatty acid methyl esters of the embryo and fry increase until day 59, when the weight reaches a maximum value of 3.3 mg. The yolk fatty acid methyl esters decrease rapidly and without interruption after day 39, reaching a value of 1.5 mg. on day 69.
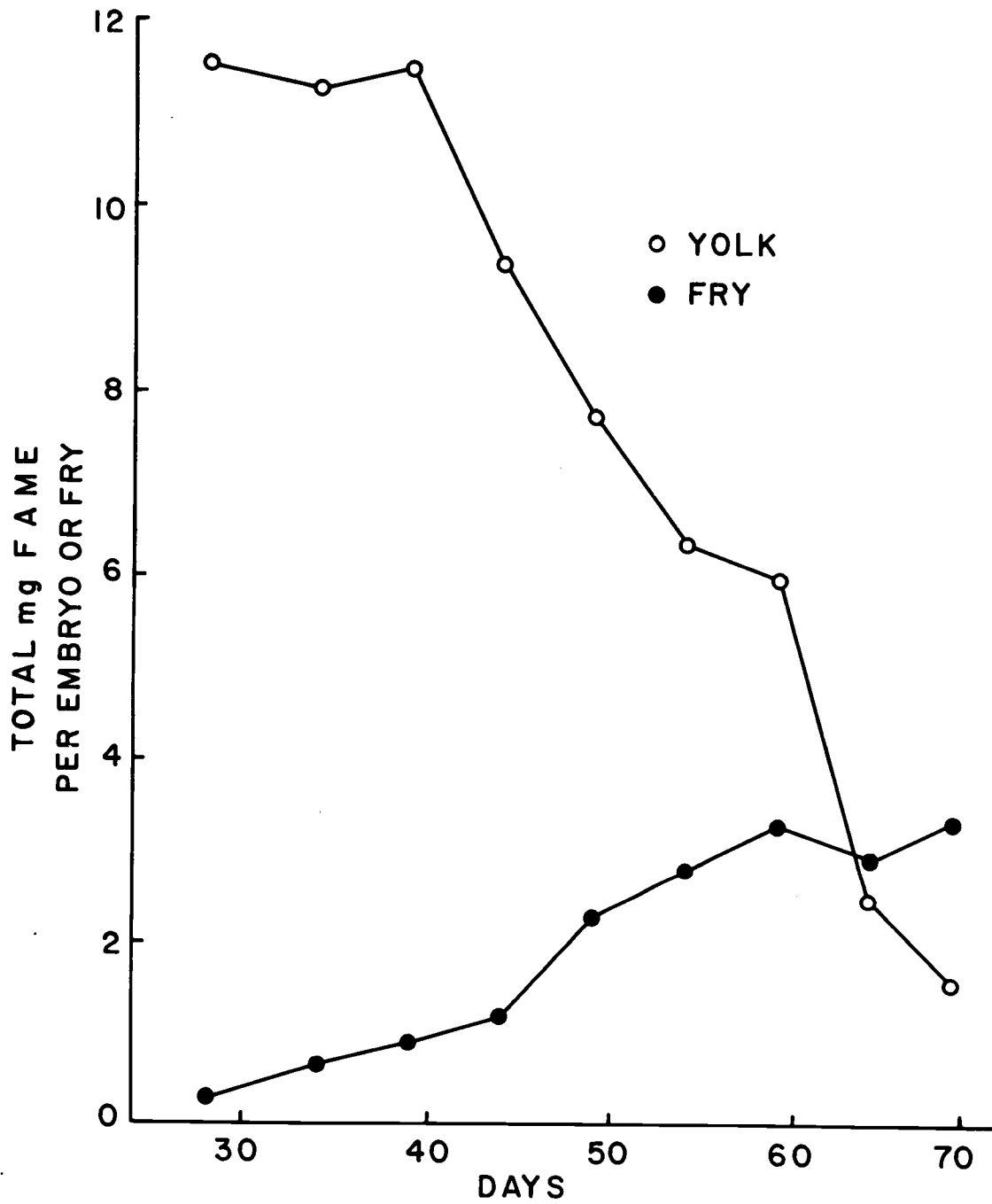
Figure 9. Milligrams fatty acid methyl esters in yolk and embryo
or fry

Identification of Components

Fatty Acids

A gas chromatogram of the fatty acid methyl esters derived from trout sac-fry lipids is shown in Figure 10.

The saturated fatty acids 14:0, 16:0, and 18:0 were identified in the sac-fry by comparison of their retention times with those of authentic standards, and by hydrogenation studies. The most mobile of the silver nitrate thin layer chromatography fractions contained these esters, and also 16:1, 18:1, and 20:1. These latter acids were found, upon hydrogenation, to add to the saturated components, 16:0, 18:0, and a new peak was found, corresponding to 20:0.

A straight line was obtained by plotting the logs of the retention times of individual saturated or hydrogenated fatty acid methyl esters against the number of carbon atoms presumed to be in their respective chains (Fig. 11). Another straight line was produced by plotting the logs of the retention times versus the presumed carbon numbers of mon-unsaturated esters (e. g. 14:1, 15:0, 15:1, 17:0, 17:1) contribute to these plots. Since they were present in the samples in small amounts (< 0.5% of the total fatty acids), they were not considered in further analysis of changes in the fatty acid methyl ester composition of the developing fish.
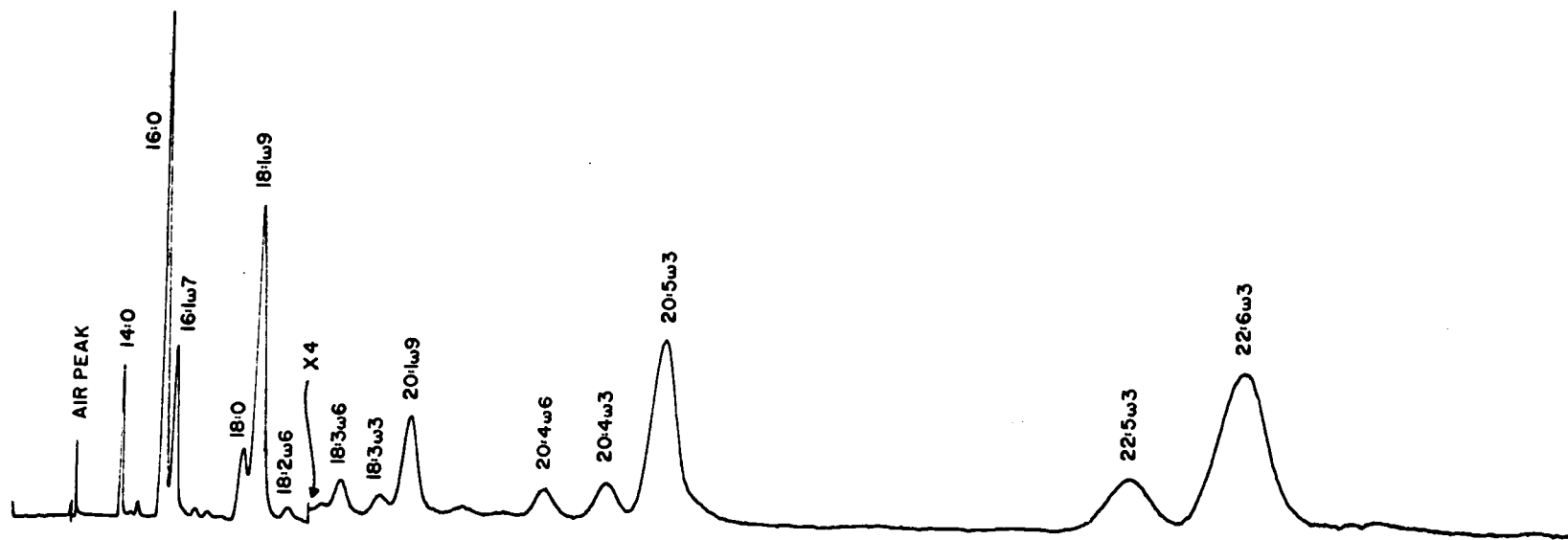
Figure 10. Gas-liquid chromatographic separation of fatty acid methyl esters of steelhead sac fry
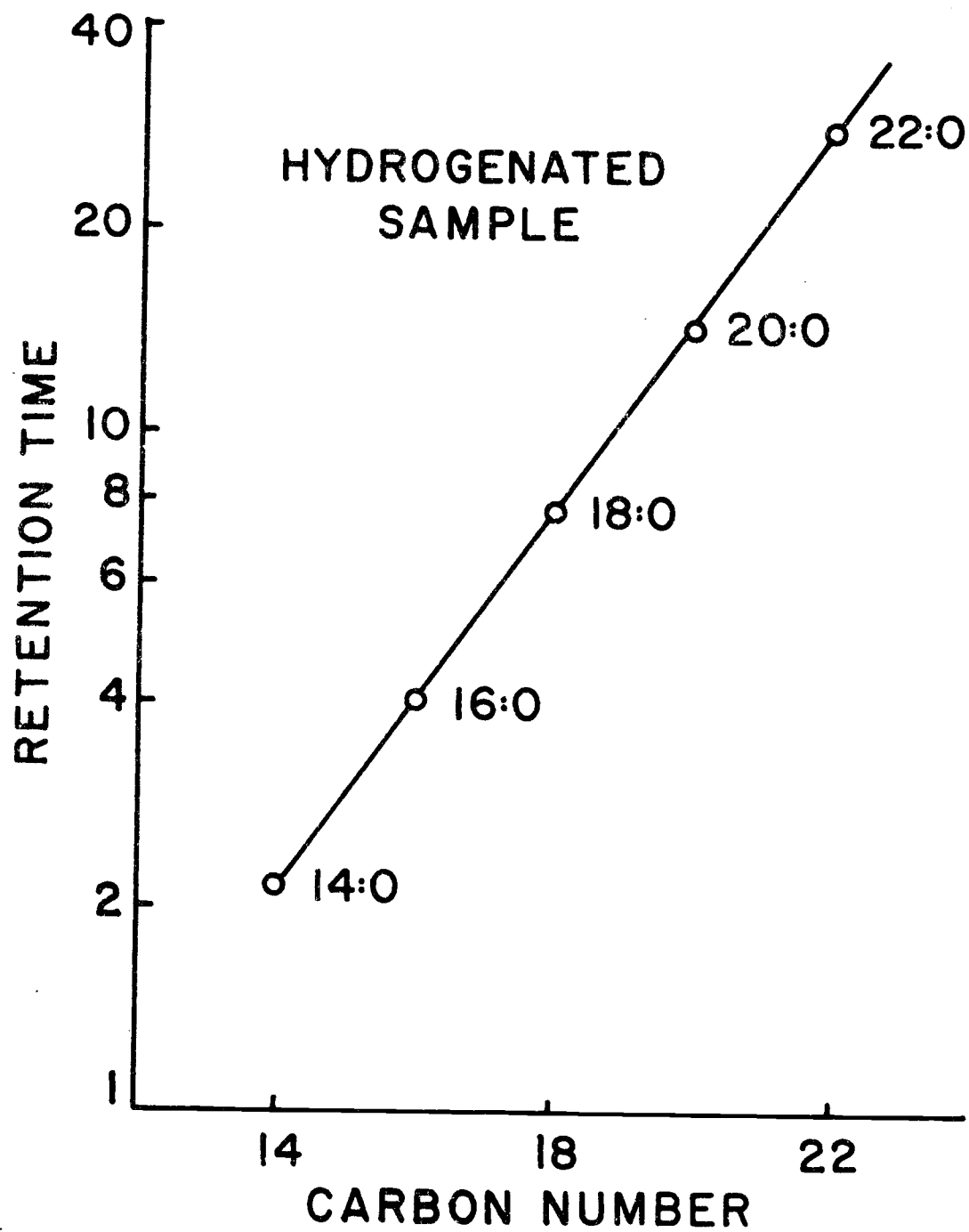
Figure 11. Semilogarithmic plot of retention times of hydrogenated fatty acid methyl esters

The fatty acids $16:1\omega7$, $18:1\omega9$, $18:2\omega6$, $18:3\omega3$, $20:1\omega9$, $20:4\omega6$, $20:5\omega3$, and $22:6\omega3$ were identified by use of authentic standards in comparisons of retention times and by spiking (adding a quantity of known ester to the mixture of fish fatty acids and observing the resulting peak reinforcement) in the complete sample.

The fatty acid methyl ester which was located upon gas chromatography between $20:4\omega6$ and $20:5\omega3$ was tentatively identified as $20:4\omega3$, since it was isolated in one band on silver nitrate thin layer chromatography with $20:4\omega6$ as the only other major component, and both peaks, when hydrogenated merged at the retention time of $20:0$.

The acid designated $22:5$ was ascertained to have 22 carbon atoms by separation on silver nitrate silicic acid thin layer plates, and subsequent hydrogenation. Double bond number was assigned on a basis of retention time versus carbon number plot (Fig. 13).

Cholesterol

In the preparation of FAME from steelhead eggs and sac fry, a component was encountered which possessed a long relative retention time (8. 6 with respect to Me-stearate) and had not been encountered previously in this lab in sufficient quantity for identification (the present sample contained this component at a level of 2-5% of total FAME). Neither had a similar component been reported in the fatty acids of Salmo gairdnerii (Lee et al., 1967, Knipprath and
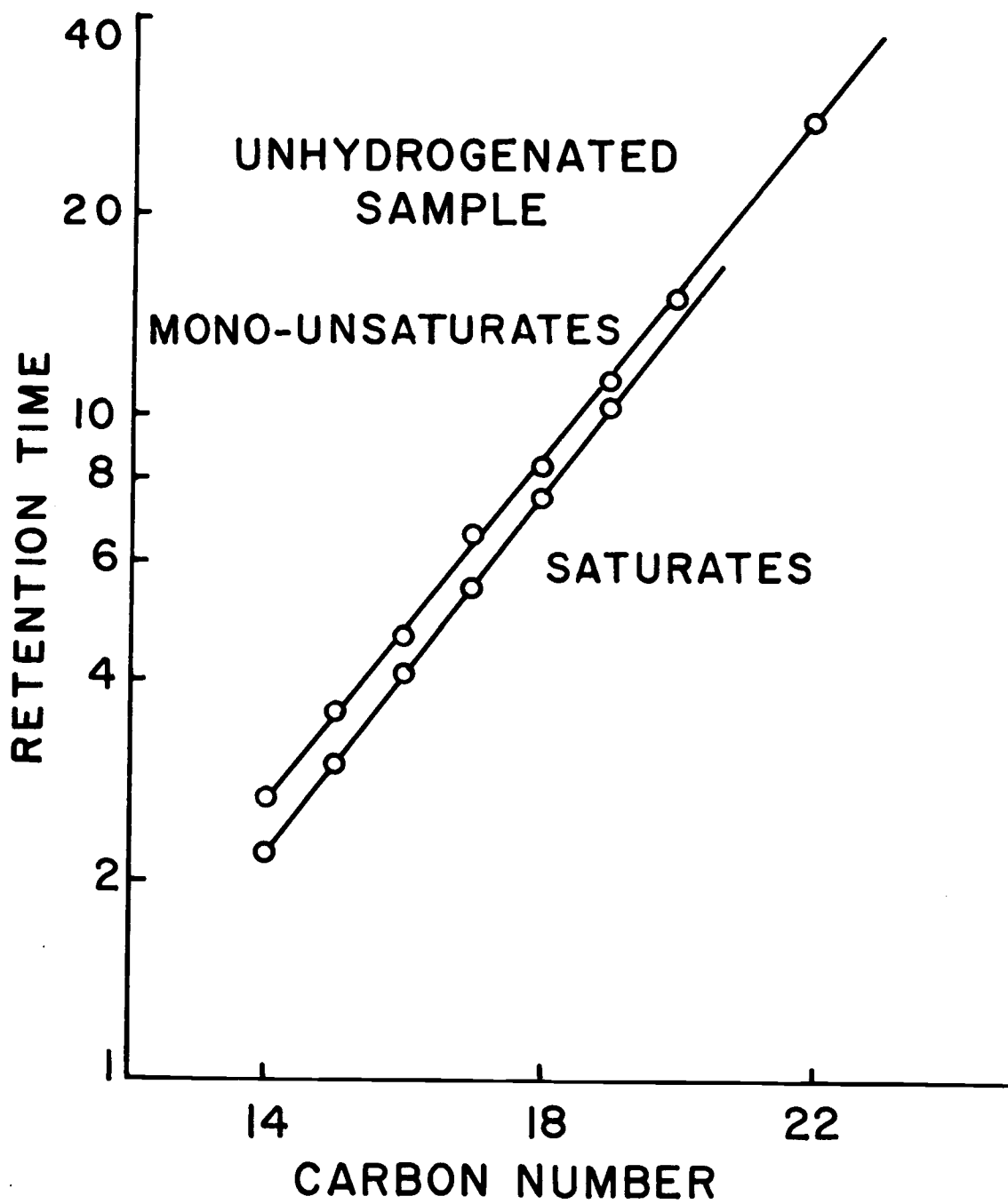
Figure 12. Semilogrythmic plot of retention times of mono
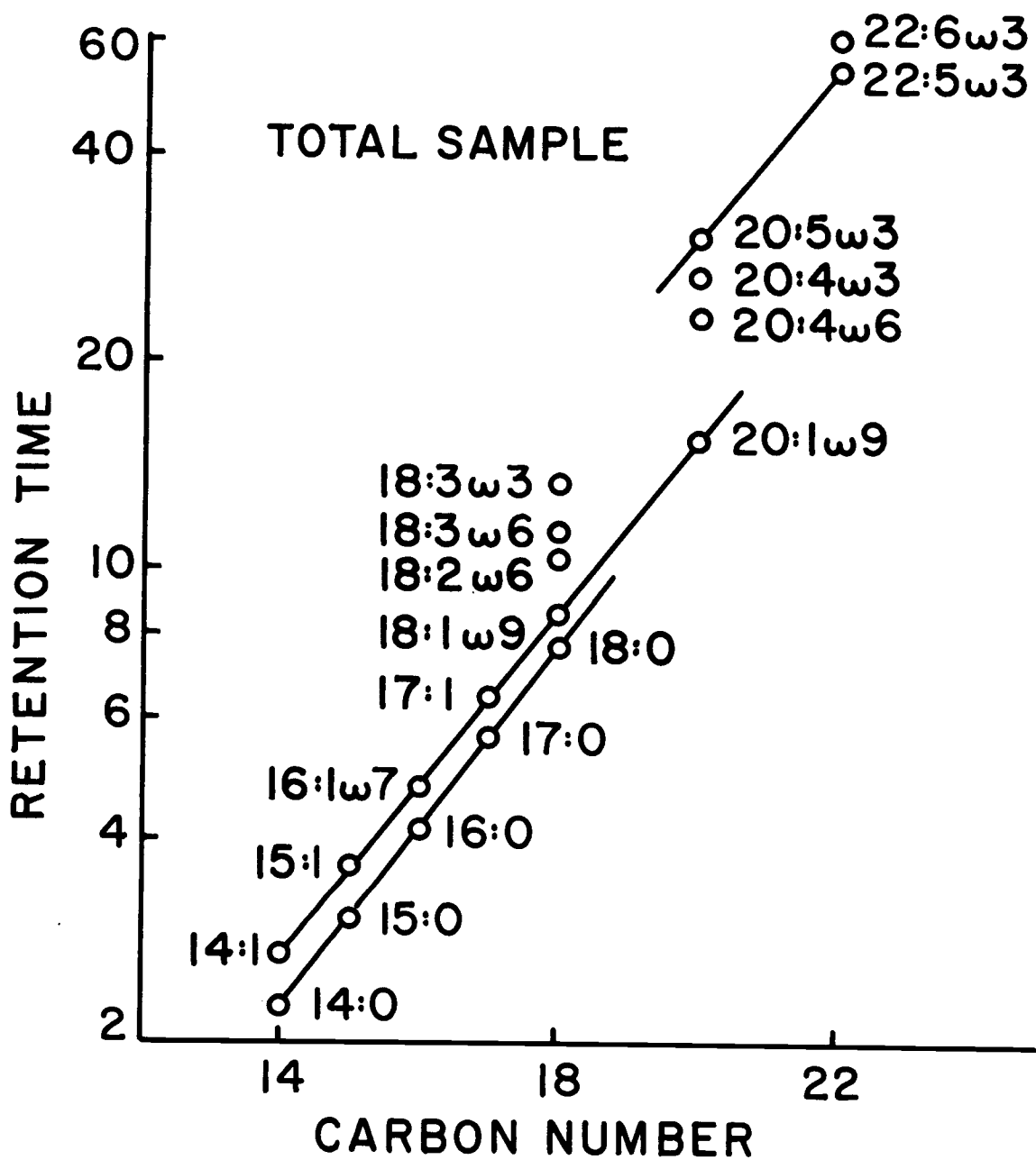unsaturated and saturated fatty acid methyl esters

Figure 13. Semilogrythmic plot of retention times of fatty acid methyl esters of steelhead sac fry

Mead, 1964).

Although several investigators have reported 24-carbon fatty

acids in the fat of marine and fresh water fish (Grager, Nelson and

Stansby, 1964, Ito and Fukuzumi, 1963, Ackman and Sipos, 1964),

the most commonly reported acid being 24:1, it became apparent

that the component isolated here was not an ordinary fatty acid.

Upon hydrogenation the retention time became shorter, but did not

correspond to either 24:0 or 23:0. Separation from the other compo-

nents of the FAME sample was easily accomplished by thin layer

chromatography (TLC) on silica gel G, using dichloromethane as

solvent. Rf for the unknown was .3, similar to that of hydroxy-fatty

acids, while the remainder of the FAME moved with the solvent front.

In this manner, sufficient quantity was obtained for further analysis.

The IR spectrum gave major absorptions at 2.8, 3.4, 6.8, 7.2,

and 9.5μ. Conspicuously absent was an ester absorption at 5.8 μ.

Mass spectral analysis indicated a molecule-ion peak at m/e 386,

with a distribution of m/e signals very similar to that reported for

cholesterol (Friedland et al, 1959) and which corresponded very

closely to that of a sample of cholesterol run on the same machine

The chromatographic behavior of the unknown and of cholesterol

were identical on TLC and GLC. TLC on silica gel G, developed

with 40% ether in hexane, produced identical Rf values for both

compounds. Upon spraying with Liebermann-Burchard reagent and heating at 100 C for 10 minutes both standard and unknown exhibited a characteristic deep rose color. GLC on a 6' x 1/8" OD aluminum column of 15% diethylene glycol succinate on AW Chromosorb P, 80-100 mesh at 190 C, 19 psi and 30 ml/min., utilizing solid sample injection (Lowry, 1964), resulted in identical retention times for the unknown and the cholesterol standard.

It is assumed that the inclusion of steroids as contaminants in fatty acid methyl ester preparations does not interfere with their analysis by gas-liquid chromatography (Holman and Rahm, 1966). This assumption is based on the long retention times and thermo-labile nature of steroids. A large dead space between the injection site and column inlet promotes decomposition, particularly when the sample is injected in solution. That cholesterol interference was encountered in this investigation may be due in part to the use of a solid sample injector which provides "on column" injection and minimizes dead space. In addition, those conditions which give shorter retention times and better peak definition for polyunsaturated fatty acids may also favor the appearance of a peak due to cholesterol. This interference may be avoided by the addition of a saponification step, or elution of the ester sample from a silicic acid column with 3% ether in hexane.

Fatty Acid Composition

Eggs or Whole Fish

The relative amount of each fatty acid of the whole egg or sac-
fry is plotted throughout the development and growth period in Fig.
14. The percent composition of the fatty acid methyl esters is sur-
prisingly uniform. The two acids present in greatest amounts were
oleic (18:1) and docosahexaneoic (22.6), each comprising about 20%
of the esters. The only other acids present in amounts greater than
6% were palmitic (16:0) and eicosapentaneoic (22:5), each averaging
between 10 and 15 percent. The percentage of remaining acids, dis-
tributed between one and six percent, exhibit very little fluctuation
or overall change over the course of the experiment.

The fatty acid methyl ester showing the most change in relative
percent is 22:6, which increases from 20 percent at day 59 to 24
percent of the total in the last two samples on days 64 and 69.

Palmitic acid 16:0 also increases in the last sample. There is
no apparent shift in the overall level of unsaturation of fatty acids
of the whole fish.

Percent in Embryo and Fry

The percent composition of fatty acids in the embryo or fry
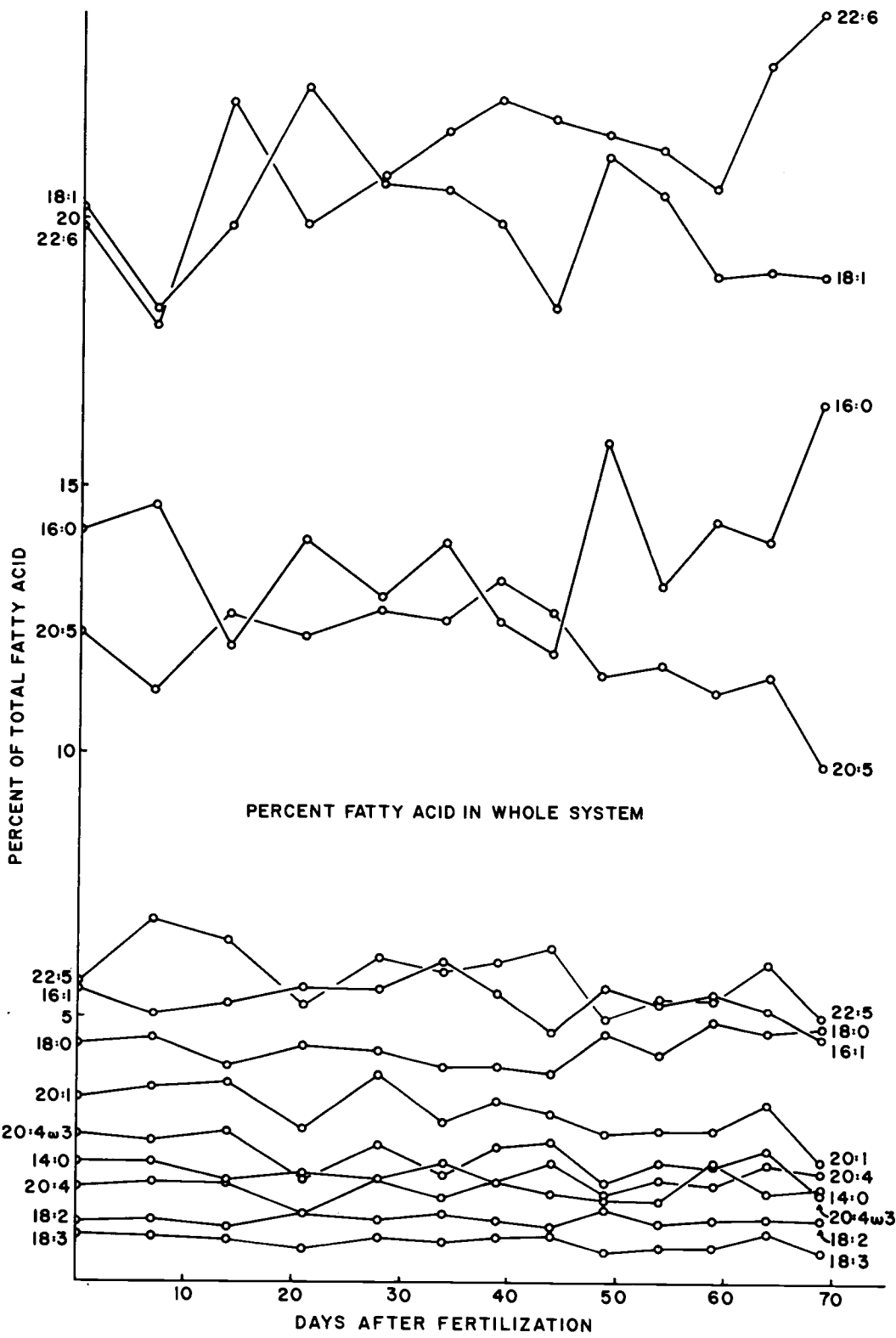taken separately from the yolk had wider fluctuations at the time of

Figure 14. Individual fatty acid methyl esters as a percentage of total fatty acid methyl ester sample of whole system

hatching, on day 34, than those of the whole system (Fig. 15). The most striking change is that of 16:0, which jumps from 6 percent to over 21 percent at the time of hatching, day 34, and then falls back to remain between 15 and 20 percent for the remainder of the experiment. The only other acids increasing at the time of hatching are 14:0 which moves from .09 percent to .90 percent at hatching, and 16:1, which moves from .90 to 3.11 percent. The remaining acids either change only slightly or decrease moderately. Those which fall at hatching are 22:6, 22:5, 20:4ω6, and 20:1.

The fatty acids present in highest amount are 22:6, varying from 20-30 percent of the sample. After hatching, 16:0 is next highest, at 15-20 percent, followed by 18:1 at 15 percent. 20:5 is the lone inhabitant of the 10-12 percent range, followed by 18:0 and 22:5 at 4-6 percent. The remaining acids are present in proportions less than 4 percent, with 14:0 the least abundant acid of those measured in the embryo and fry.

Yolk Percentage Composition

Fatty acid composition values for yolk are quite uniform over the greater portion of sampling dates (Fig. 16), with only small fluctuations being observed from sample to sample. The most striking changes in the fatty acid composition occur after day 59, when the percentage of 18:1 rises from 20 to 30 percent of the
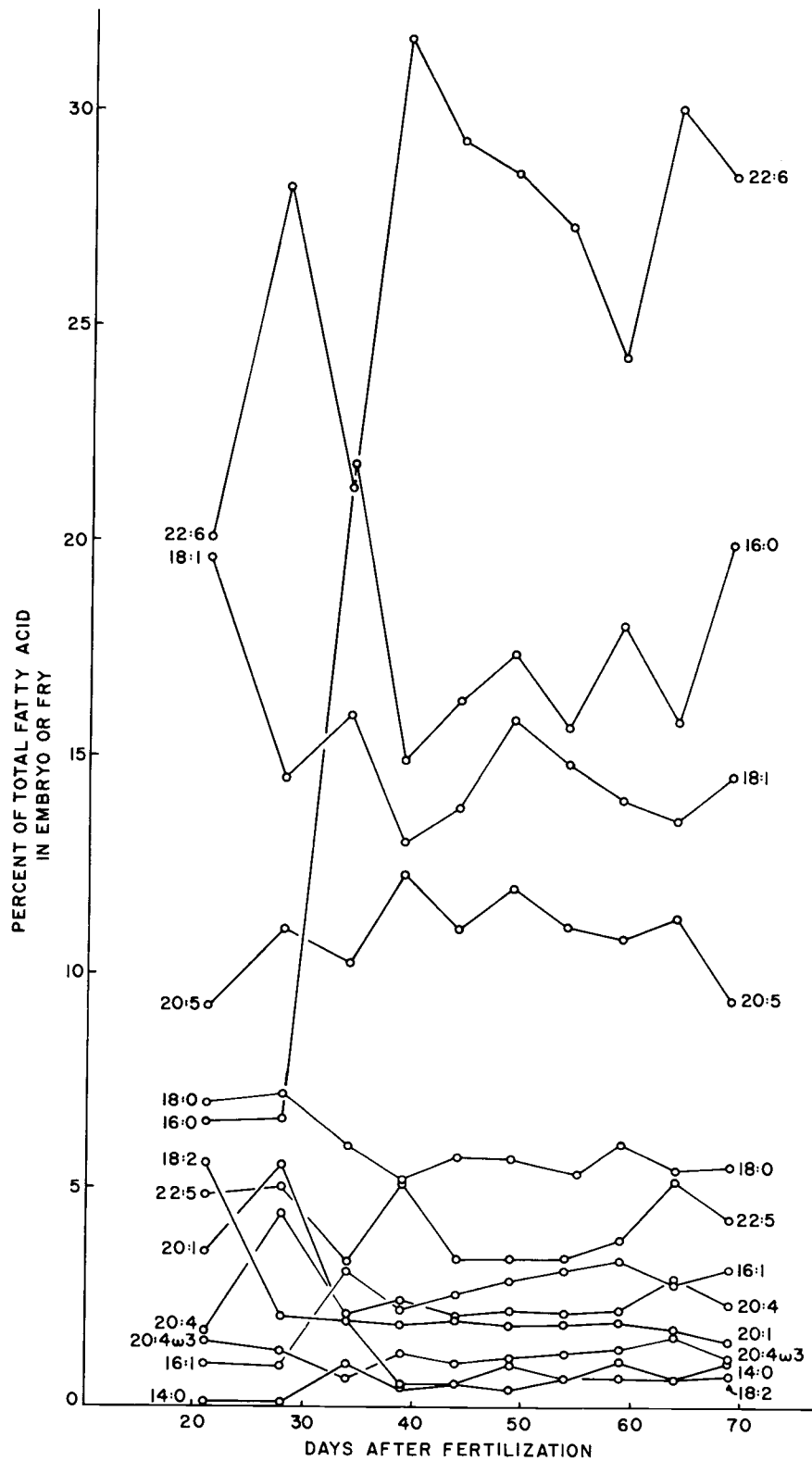
Figure 15. Individual fatty acid methyl esters as a percentage of total fatty acid methyl ester sample of embryo or fry
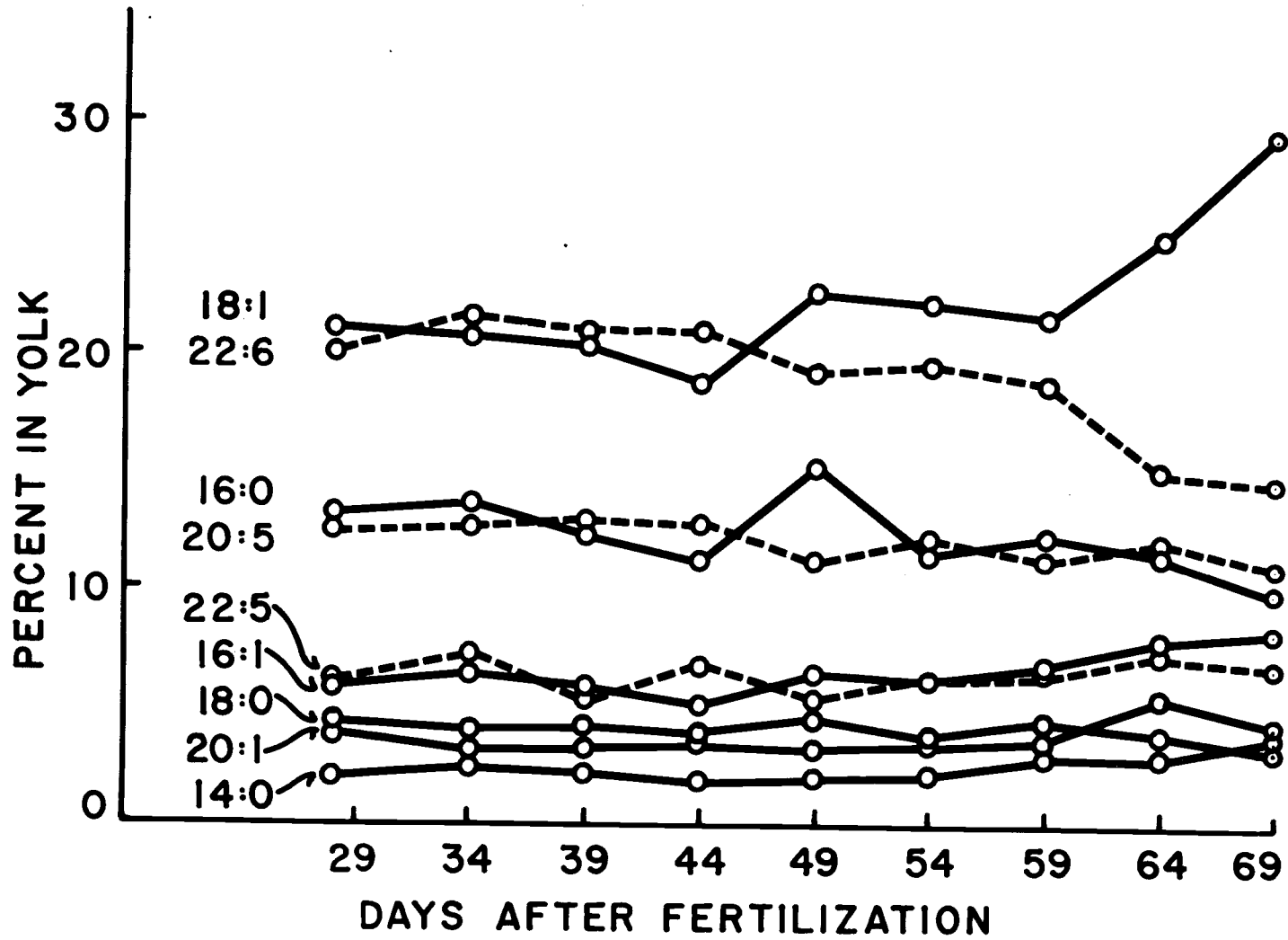
Figure 16. Individual fatty acid methyl esters as a percentage of total fatty acid methyl ester sample of yolk

total, accompanied by a decrease in 22:6 from 20 to 14 percent.

These large changes are accompanied by similar but smaller changes

in several acids: 22:5 and 20:5 decline slightly, while 14:0, 16:0, and

16:1 increase by small amounts.

Before hatching, and for most of the sac-fry period, the fatty

acid composition of the yolk is very similar to that of the whole sys-

tem. At the end of the yolk-sac period, the percentage of 18:1 is far

above that of the whole system or the fry, while a relative amount of

22:6 is very much less than that found in either fry or the whole sys-

tem.

Individual Acids

A plot of the percent of palmitic acid 16:0 in the whole fish, fry

and yolk is shown in Fig. 17. The value for the fry in the two days

before hatching, days 21 and 28, is far below that for the whole fish.

At hatching the value jumps from 7 percent to 22 percent, and re-

mains consistently above that for yolk or whole fish for the remainder

of the experiment. It should also be noted that over the same time

period the percent of 16:0 in yolk remains constant at 14 percent.

A similar plot for 22:6 (Fig. 18) again gives values for the

embryo or fry which are above those for the whole fish and the yolk,

with the exception of days 21 and 34, when the percent falls among

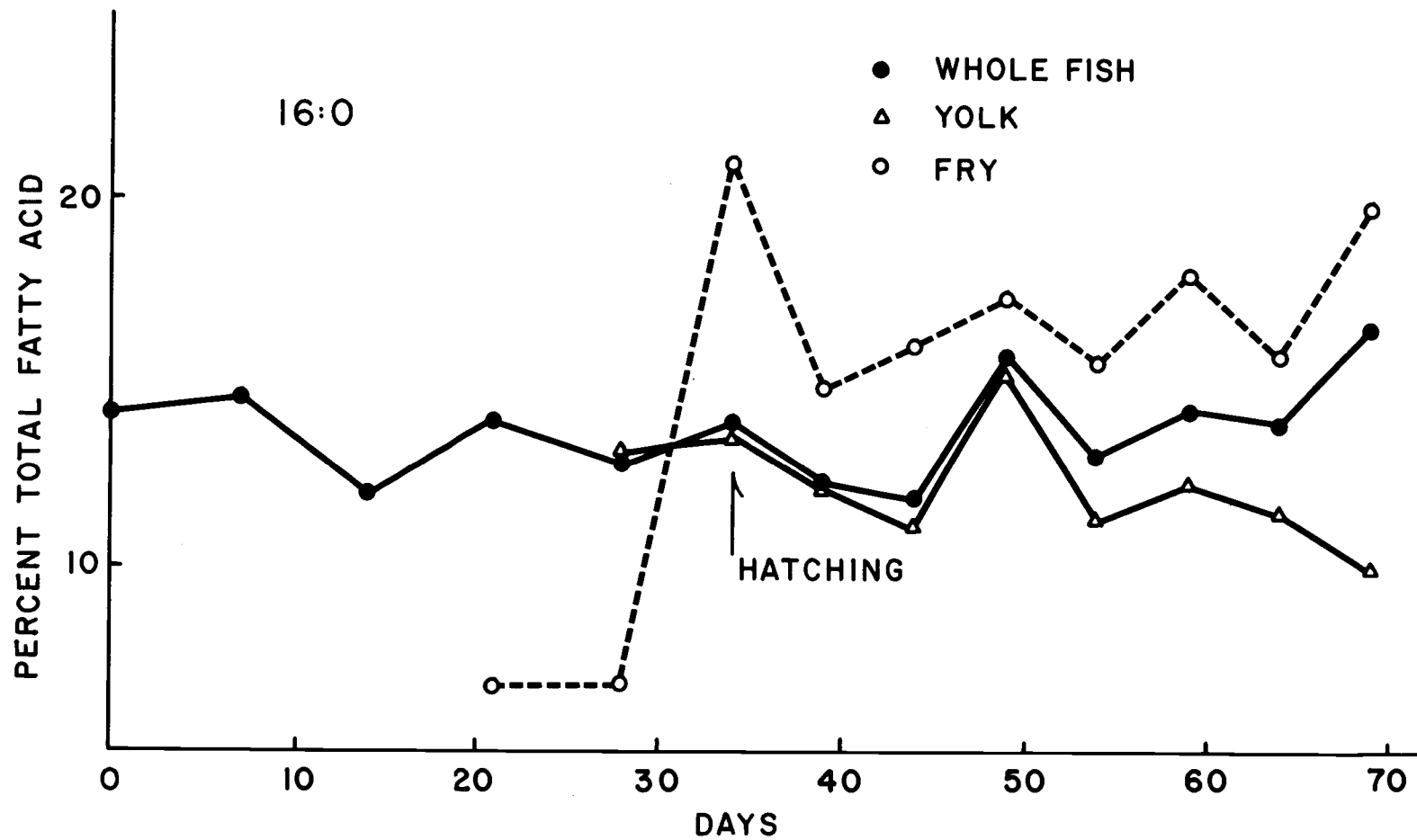the values for yolk and whole fish, at 20 percent. At the end of the

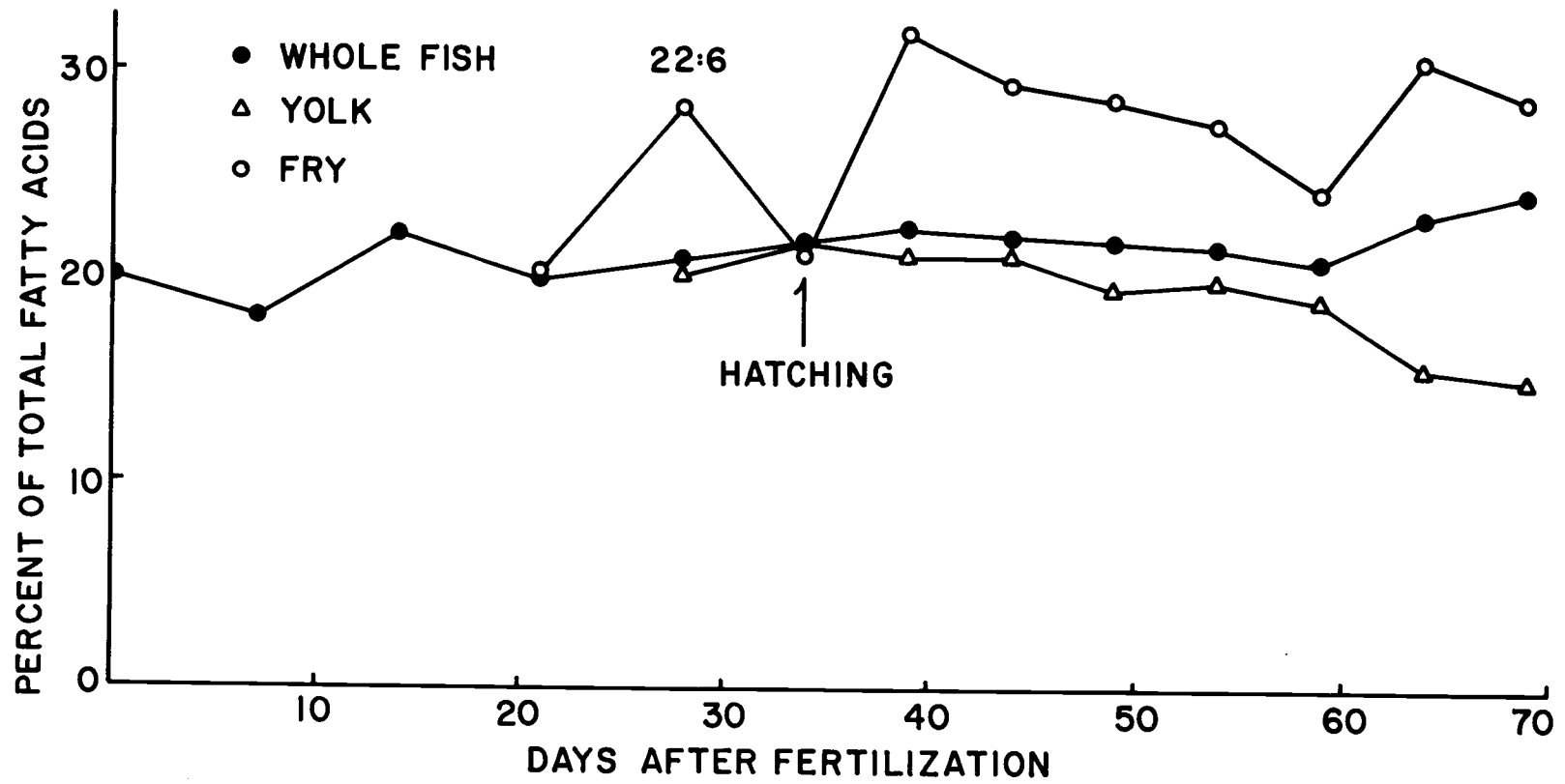Figure 17. Percentage of 16:0 in whole sample, yolk, and embryo or fry

Figure 18. Percentage of 22:6 in whole sample, yolk, and embryo or fry

yolk-sac period, the yolk decreases from 20 to 14 percent content of this acid. At the same time the percent in the fry is around 30 percent, higher than the 24 percent in the whole fish.

The acid 18:1 contributes a smaller percent to the total fatty acid mass in the fry than in either the yolk or the combined system (Fig. 19). The largest percent is in yolk and the smallest in fry, with the disparity accentuated at the end of the yolk-sac period, when the percent in the yolk rises to 30, and that of the fry is 14 percent. This is in contrast to observations made on 16:0 and 22:6.

Mass Changes

To examine these changes in a less facile, but hopefully more elucidating manner, let us turn to Figure 20, which shows the amount of each fatty acid which is gained by the embryo over the course of growth as a function of the amount present in the yolk at the beginning of growth. On the same graph, the amount of each fatty acid lost by the yolk over this period is also shown as a function of the amount present in the yolk at the beginning of embryo growth (also the starting point of measurable yolk depletion). To this end, the weights, in milligrams, of each fatty acid present in the yolk at day 28 is plotted on the abscissa. From this point, the number of milligrams found to be gained by the fry from day 28 to day 64, is plotted upward on the positive ordinate, and the number of milligrams lost from the

Figure 19. Percentage of 18:1 in whole sample, yolk, and embryo or fry

Figure 20. Individual fatty acid methyl ester loss from yolk and
gain by embryo or fry from day 28 to day 64, as a
function of the amount present in yolk on day 28

yolk is plotted downward. A similar plot is made for the period from
28 to 69 days after fertilization (Fig. 21).

The amount of each fatty acid found in the fry at day 64 or day
69 is not a regular function of the amount available in the yolk at day
28. Acids which have higher retention by the fry are 22:6, 16:0, 18:0,
and 20:4ω6. Those with lower values are 18:1, 20:5, 22:5, and 20:1.
In this respect, then, the acids are treated differently.

A plot of the milligrams gained by the embryo plotted against
the mg. lost from the yolk over the period from day 28 to day 59 is
shown in Fig. 22. A 45 degree line passing through the origin would
represent equal amounts lost by the yolk and gained by the embryo.
None of the acids lie above this line. The acids whose points lie
closest to this line are 18:0, 16:0, and 22:6. Those lying at an angle
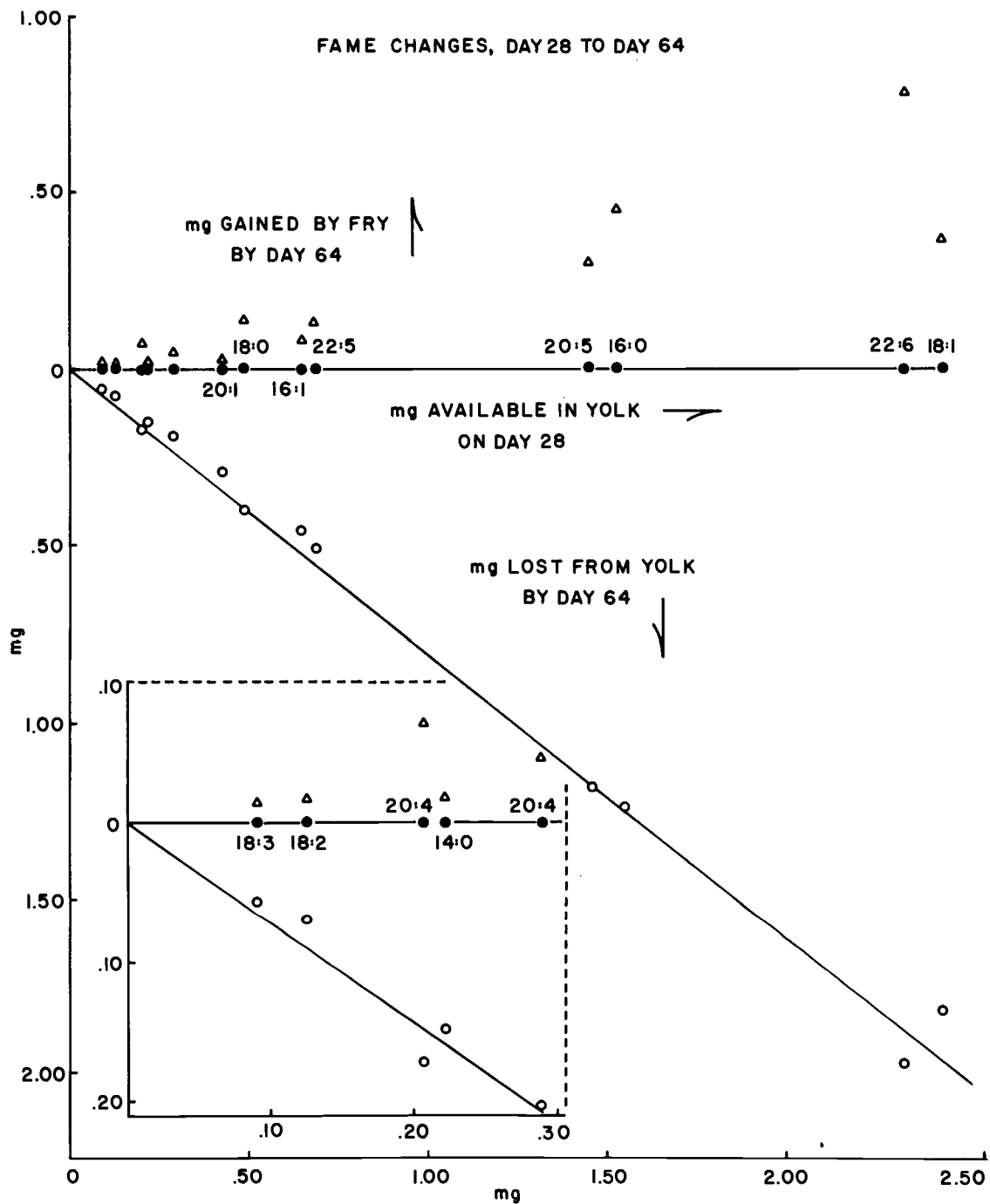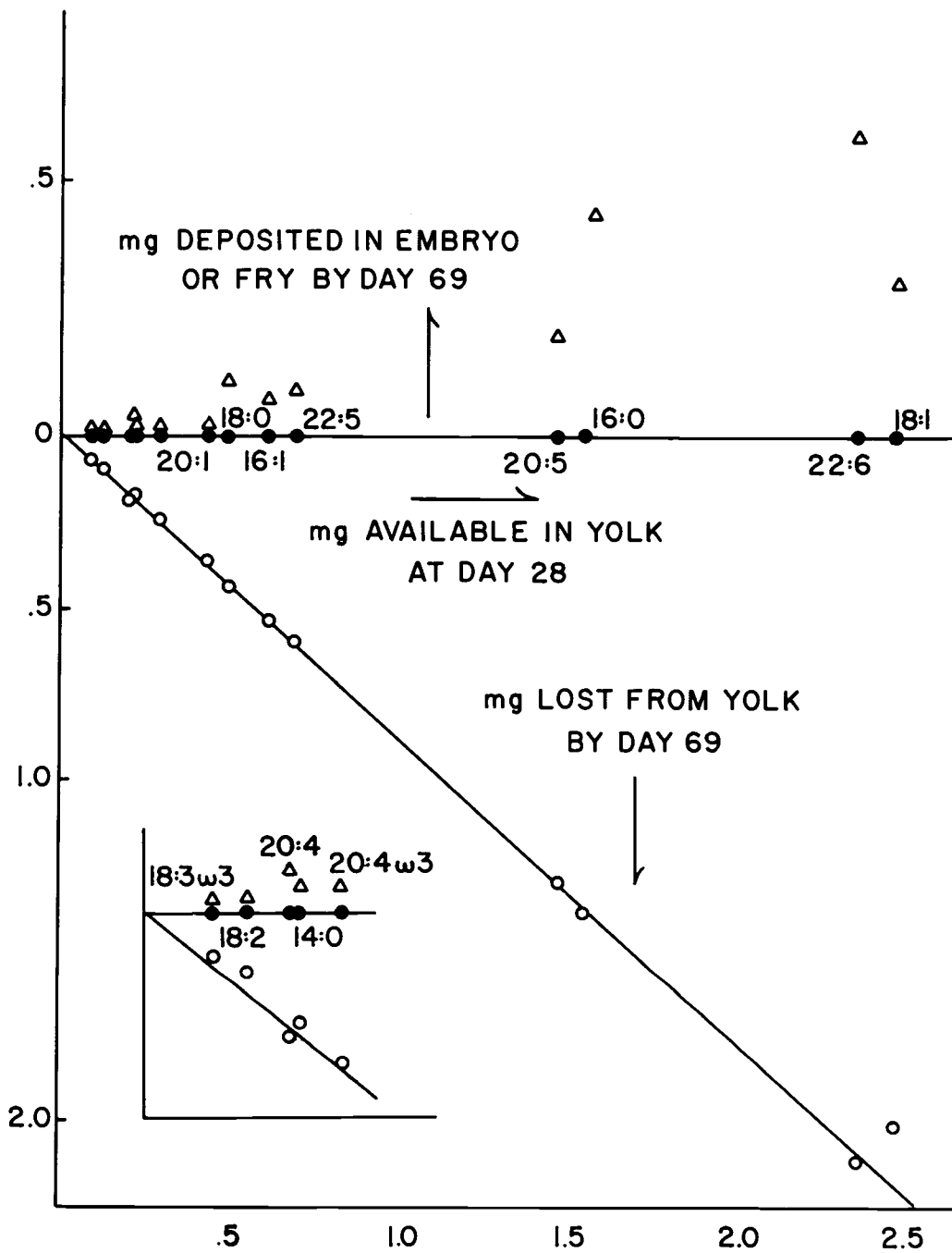furthest from the line are 20:1, 22:5, and 18:1.

Figure 21. Individual fatty acid methyl ester loss from yolk and
gain by embryo or fry from day 28 to day 69, as a
function of the amount present in yolk on day 28

Figure 22. Milligrams of each fatty acid gained by fry as a function of the milligrams of each lost by yolk, from day 28 to day 59

DISCUSSION

Gross Changes in Trout Development

In order to interpret the changes in the levels of fatty acids in the developing sac fry and its parts, it is necessary first to consider the more general changes in the physical condition of the system.

Major changes in the magnitude of wet weight, dry weight and lipid of the developing system were not evident until the fry with attached yolk sac was expelled from the chorion capsule into the aqueous medium. At this point the embryo was growing at a rapid rate, and gained freedom of movement outside the chorion. This new capability of expending energy and also increasing growth are the apparent factors which initiate the decreases in mass within the whole system and in the yolk constituents except water.

The decline probably represents the loss of compounds through combustion for energy to be used by the embryo in growth and muscular activities. Although the fertilized (and even unfertilized) egg has been shown to consume oxygen and simple substrates (Terner, 1968) the level of this metabolism has been found to double at hatching. Thus the measurable changes in the amounts of embryonic constituents are delayed until the time of hatching.

A second period of interest, after day 54, occurs towards the

end of the yolk absorption. The sac fry is forced, in this experiment,

to survive only on the substances contained in the yolk sac and its

own protoplasm, whereas the sac fry in the native state would have

begun to feed midway through the stage, or shortly before day 54 in

this study. Thus the fry may be placed under a stress of mild starva-

tion in the final part of the study.

Whole System

The wet and dry weight and lipid content of the whole system

began to decrease in the period shortly after hatching. Lipid was

lost at a more rapid rate than dry weight, culminating in an overall

loss of 58 percent of the original lipid and 31 percent of the original

dry weight. Since dry weight includes lipid, protein, nucleic acids,

carbohydrates and inorganic salts, with lipid and protein accounting

for the greater portion of the weight, a rough estimate of the protein

in a given sample may be made by subtracting the lipid weight from

the dry weight. This operation indicates a loss of 8.1 of an original

38.0 milligrams, or 21 percent of the original protein. These values

compare roughly to those of Hayes (1949) who found a 75 percent loss

in lipid and 36 percent loss in protein with salmon sac fry. In the

studies described by Hayes (1949), the experiment was continued

until the yolk was completely gone. A smaller proportion of each

was lost in the present experiment because observations were not

continued after the yolk sac became only a bulge on the ventral area of the fry.

Yolk

Hatching also initiated yolk losses in wet, dry, and lipid weight which continued throughout the developmental period. Towards the end of the experimental period, the rate of loss of wet and dry weight tended to decrease in contrast to that of the lipid which continued unabated. All three components for yolk were still decreasing at the end of the yolk sac period, although the fry had stopped growing as indicated by fry weights on days 64 and 69. This would indicate that at this stage the material lost was consumed primarily for the production of energy to be used in maintenance and movement, rather than growth.

Embryo and Fry

The lipid content of the embryo and fry, expressed as percent of dry weight, fell from 20 percent to 15 percent after hatching. This effect may be associated with the change in environment resulting from expulsion from the chorion. The fry would no longer be surrounded by perivitelline fluid, nor pressed into intimate contact with the yolk nutrients, and thus may have experienced a change in the availability of lipids from the yolk. Also the alevin are growing so

that distance from the nutrients is continually increasing and transport is becoming a more difficult problem.

The changes which occurred at the time of final yolk depletion at the end of the experiment indicate a continuing expenditure of energy, with termination of growth. The loss of yolk constituents continues after the growth stops after day 59 (Fig. 4). The fry was found to have weights of 32. 7 and 32.9 milligrams on days 64 and 69, respectively. This leveling of the growth curve is also apparent to a lesser extent in the case of wet weight, which gives values which approach a zero slope if the curve is extrapolated beyond day 69. The decline of growth is shown most clearly in the curve for the lipid content for the embryo and fry, Fig. 5, which reaches a maximum value at day 59 and declines slightly on days 64 and 69. The same response is illustrated in Fig. 8.

Fatty Acid Utilization

Whole System

The fatty acid composition of the whole system shows few marked changes throughout development. At the end of the experiment, on days 64 and 69, the acids 22:6 and 16:0 are present in greater proportion, as might be expected, since these acids will be seen to be characteristic of the fry, which constitutes most of

the whole system in these two samples. There appears to be no

general change in proportion of saturated to unsaturated acids, in

aggeement with the generalization of Takama et al. (1969). Through-

out the entire period of growth, it should be noted that the absolute

amount of each fatty acid decreases, in agreement with the observed

loss of lipid from the system (Fig. 9).

Yolk

The percentage composition of fatty acids in the yolk (Fig. 16)

remained quite constant over the period from the first measurement

at day 28 until after day 59. This result is to be expected for the

period from day 28 to day 44, when only 18 percent of the fatty acids

had been lost from the yolk. During the period from day 44 to day 59,

however, about 40 percent of the fatty acids were lost, and the lack

of any pronounced change in the percentage composition would argue

against a strong selection among the individual fatty acids for trans-

port out of the yolk. During the later stages of yolk utilization (Days

59 to 69) the loss of 22:6 and 16:0 and the accumulation of 18:1 in the

yolk became apparent.

A more sensitive measure of the loss of individual fatty acids

from the yolk is found in the plots of milligrams of each fatty acid

lost from the yolk versus the amount originally present at day 28.

After day 44, some selection became apparent in the loss of

fatty acids from the yolk. On day 54 (Fig. 23) 16:0 had been lost

in greater proportion, while 18:1 was selectively retained. On day

64 (Fig. 20), 18:1 was again lost in lesser proportion while a slight

excess of 22:6 was lost. On day 69 (Fig. 21), only 18:1 deviated

from the average amount lost, as once again it was selectively re-

tained by the yolk.

Thus the loss of fatty acids from the yolk demonstrated a degree

of selection, which is not as marked as that for deposition in the

embryo, as will be seen in the next section.

Different lipid classes (i. e., phospholipids and glycerides)

in marine animals are characterized by different fatty acid compo-

sition (Brockerhoff et al., 1968, Menzel and Olcott, 1964) and any

transport process which varied in lipid composition would result in

a change in the fatty acid composition of the yolk and the transported

lipid.

These small differences in proportion of each fatty acid lost

from the yolk strongly argue against the suggestion of Smith

(1952) and Ando (1962). These workers postulate that a large loss

of non-polar lipid occurs during the latter stages of yolk utilization.

The observation that 18:1 is preferentially retained in the yolk

during the latter stages of yolk utilization would also argue against

this suggestion since this fatty acid has been found to be a major

component of triglycerides in trout (Menzel and Olcott, 1964).

Embryo

The fatty acid composition of the embryo at the first two samp-
ling periods was quite similar to the fatty acid composition of the
yolk. At this time the small fish was still enclosed in the chorion.
The two major fatty acids were 22:6 and 18:1 each comprising about
20 percent of the total fatty acids. At this stage the level of 16:0 in
the embryo was somewhat lower than that observed in the yolk (6%
in the embryo versus 13% in the yolk), while the reverse situation
existed for 18:2 (Fig. 15).

A dramatic change in the fatty acid composition of the embryo
occurred at hatching. The proportion of 16:0 increased from 6 percent
to over 21 percent while the proportion of 22:6, 14:0, and 16:1 also
increased. Increases in the proportions of 16:0, 16:1 and 14:0 could
be associated with the appearance of a fatty acid synthetase activity
in the embryo whose major product was 16:0. The increase in the
proportion of 14:0 might also be due to synthesis. In fatty acid
synthetase systems of avian and mammalian species specificity for
chain termination is highest for 16:0, however significant levels of
14:0 can also be produced under appropriate conditions (Carey, Dils
and Hansen, 1970). An increase in the level of 16:1 could result if
larger quantities of 16:0 became available for desatuation. However,
synthesis of fatty acids, de novo by the embryo would not seem to be

a particularly efficient process energetically.

At hatching the embyo begins to expend energy at an increased rate and a sudden demand is imposed on the system by the change in environment. New membranes would be required which would in turn have requirements for complex lipids. The changes in fatty acid composition at this transition could also involve such factors as these.

When the amount of each fatty acid deposited in the embryo or fry is examined in a similar manner to that applied to yolk loss, an entirely different relationship is evident, as seen in Figs. 20-24. The embryo does not deposit the acids in its tissues in proportion to either the amount present in the yolk or to the amount lost from the yolk. Rather, there is a consistent and unique selection of particular fatty acids for depostion. On this basis, it is possible to divide the fatty acids present in larger quantity into two groups; those which are selectively retained to a greater extent and those which are not. 22:6, 16:0, and 18:0 are of the former group, and in the latter group are found the acids 18:1, 20:5, 22:5 and 16:1. This result is borne out in simultaneous plots of the relative percents in whole system, fry, and yolk of each of the three most abundant acids, as seen in Figs. 17, 18, and 19. Two of these acids, 22:6 and 16:0 are present in greater proportion in the embryo and fry. Oleic acid, 18:1, is found in lesser percentage in the embryo than in either the
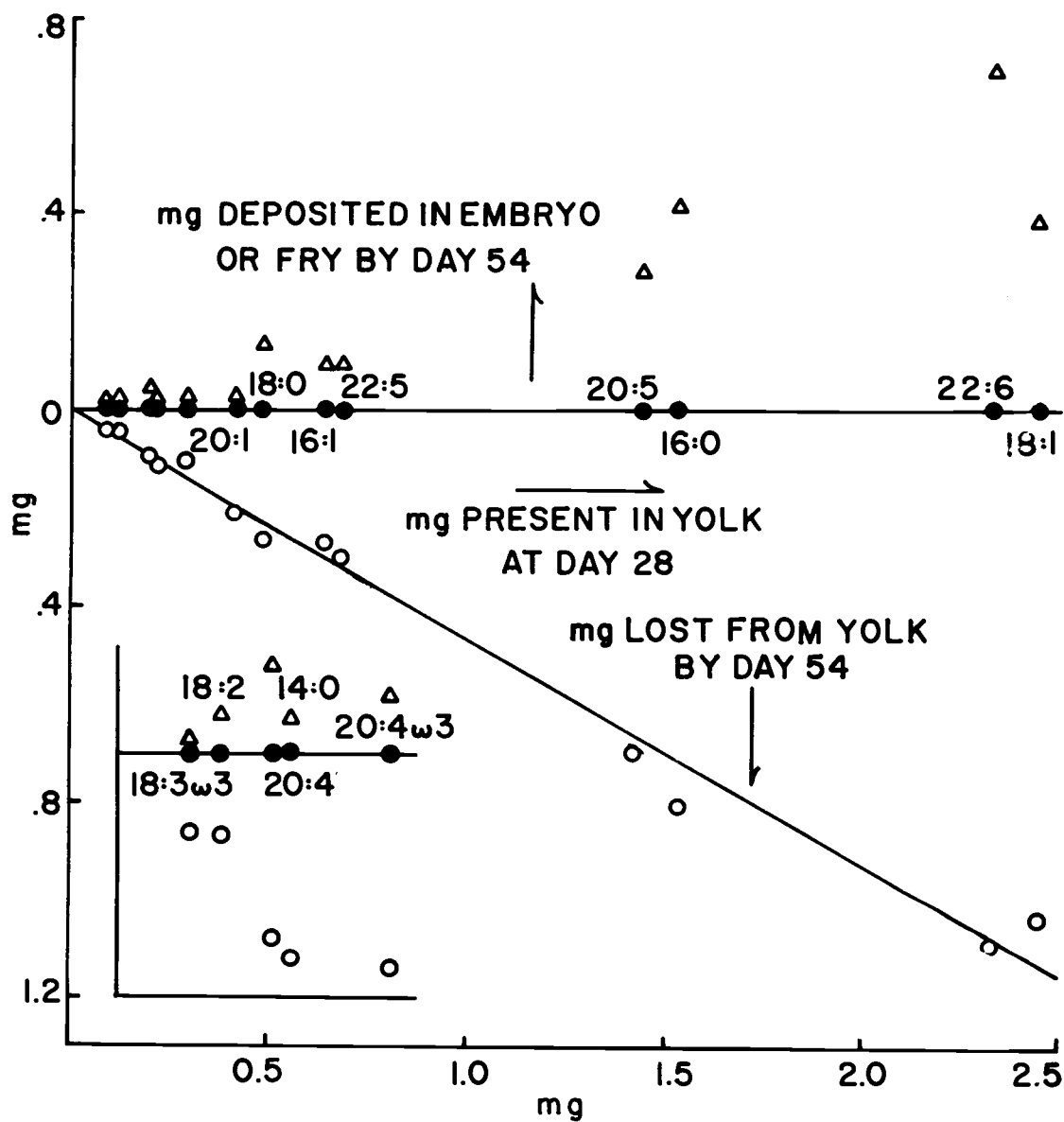
Figure 23. Individual fatty acid methyl ester loss from yolk and gain by embryo or fry from day 28 to day 54, as a function of the amount present in yolk on day 28
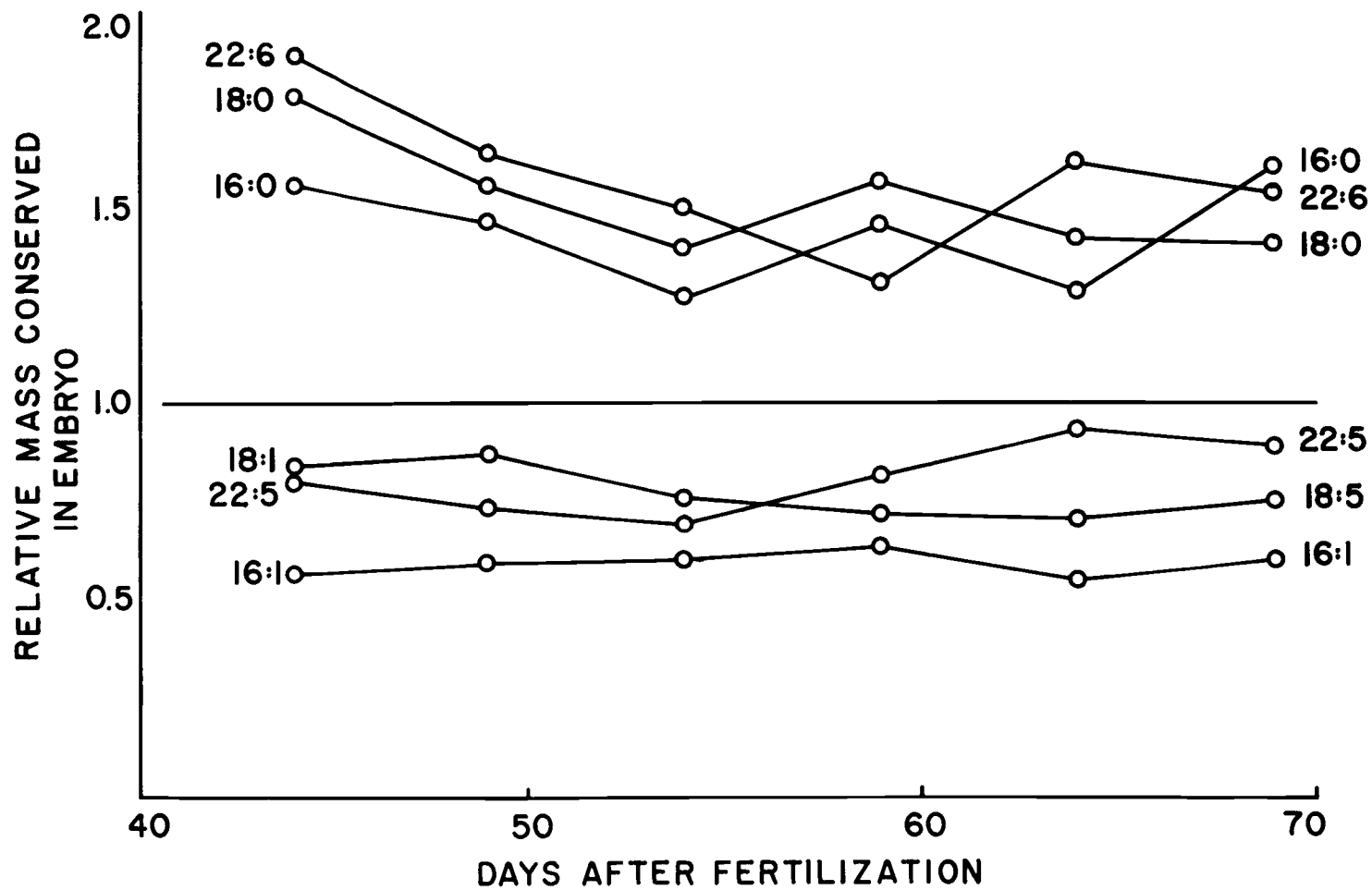
Figure 24. Percentage of each acid in the yolk at day 28 which is found
in the fry at days 44, 49, 54, 59, 64, and 69, expressed as
a proportion of the average percent for the total mass of
fatty and methyl esters

yolk or the whole system.

By averaging the quantities of individual fatty acids present in the yolk at day 28 and in the embryo at a specific sampling period one can derive a relationship relating the amount of individual fatty acids incorporated into the embryo to the quantities available in the yolk. The deviation of individual acids from the average value is given in Fig. 24 for a series of sampling times. The division of acids is also displayed in the plot of milligrams of each fatty acid gained by the embryo of fry versus the amount lost, over the period from day 28 to day 59 (Fig. 22). This selection of preferred and less preferred fatty acids is continuous for the period from day 44 to day 69.

These data would suggest a rather unique role for $22:6\omega3$ in the developing steelhead embryo which observation would support other observations made in this laboratory. This fatty acid is conserved when salmon are starved (Saddler et al.,1971) or allowed to exercise at velocities which the fish can tolerate for extended periods (Krueger et al., 1968). Hanes et al. (1968) have shown that this fatty acid is retained to a much greater extent than other fatty acids in salmon fed restricted amounts of tubificid worms. This selectivity is lost in salmon poisoned with pentachlorophenol (Hanes et al., 1968) or when salmon are exercised at velocities higher than that normally sustained by the fish (Krueger et al., 1968). It is significant that this fatty acid is the major acid of the linolenic acid series which

has been shown to be required for normal development of the rainbow

trout (Lee et al, 1967).

A parallel for this result may be found in the observations of

Coots (1969) who examined the metabolism of arachidonate in the rat.

In this animal, 20:4ω6 is an essential fatty acid, and Coots observed

a slow turnover of ^{14}C labeled arachidonate in phospholipids--signifi-

cantly slower than any of the other fatty acids. He also demonstrated

that the phospholipids containing this acid turned over at a rate slower

than phospholipids containing linolenate or palmitate, due to a reduced

oxidation rate. This slower turnover rate for arachidonate was sug-

gested to indicate a structural requirement for phospholipids con-

taining this acid, and was proposed to be a reflection of the "essen-

tial" nature of this polyunsaturated acid in the rat. Collins (1963)

found that stearoyl arachidonoyl lecithin comprised about a third

of the total lecithin in rat liver, yet he calculated from ^{32}P labeling

studies that this lecithin species was turning over at a rate much

slower than the bulk of the lecithin fraction.

Thus it might be proposed that those acids in the developing

embryo which are retained in greater proportion in embryo or fry

tissues have a structural role to fulfill as in the form of a specific

phospholipid, as seems to be the case for fatty acids in rat liver

lecithin. Since Lee et al. (1966) reported a growth stimulating effect

for ω3 fatty acids in juvenile trout, one might expect the

docosohexaenoate to fulfill in trout a role analogous to that of arachidonate in the rat. Neither of these acids can be synthesized de novo in the respective animals. Furthermore, since the α position in phospholipids is characteristically a saturated acid, while the β position is unsaturated, the inclusion of 16:0 and 18:0 in the preferred group may not be unexpected, with 16:0 predominating.

It is interesting to note that the work of Takama et al. (1969) and Ando (1962) indicates the presence of a cephalin fraction in developing trout sac fry that is synthesized and deposited almost exclusively in the embryo or fry. Turner (1968) also noted that cephalin was one of the most actively labeled phospholipids in trout ova incubated in ^{14}C acetate. This could be the basis of a requirement and role for certain of the retained or perferred fatty acids described in this work.

SUMMARY

The input of nutrients for a developing steelhead sac fry is defined by losses from the yolk. With this information, metabolic characteristics of the developing system can be derived from observations of the manner in which various components are incorporated into the sac fry. Thus the utilization of fatty acids by the sac fry has been studied by monitoring the fatty acid content of the appropriate compartments during the developmental period.

Detectable losses in dry weight and lipid content of the whole system occurred only after hatching. Subsequent to this time the yolk showed continuing losses in wet weight, dry weight and lipid content. The growth of the embryo represented either by wet weight, or dry weight or lipid content could be represented by the expression $M = K_o t^{k_1}$, where M is the mass of the embryo or fry, K_o and k_1 are constants and t is time after establishment of the embryonic axis.

Fatty acids were analyzed by gas chromatography. The identity of the major components was confirmed using retention times and where possible, comparison with standards. Chain length was established by hydrogenation and the number of double bonds by thin-layer chromatography or silicic acid impregnated with silver nitrate.

Gas chromatographic analysis of the fatty acid ester mixtures showed a peak due to a polar component which proved to be

cholesterol. Cholesterol would not normally be expected to interfere in the analysis of fatty acid esters, since it is relatively unstable and has a long retention time with respect to fatty acid esters. This assumption was not valid in this case.

The most pronounced change in the fatty acid composition of the embryo and sac fry occurred at hatching when a marked increase in the level of 16:0 was observed. Prior to hatching the fatty acid composition of the embryo was more like that of the yolk lipids.

By considering the quantities of different fatty acids incorporat-ed in the sac fry with reference to the amount of each fatty acid avail-able it was possible to draw some conclusions as to the degree of selection exhibited by the sac fry. Consistently higher proportions of available 16:0, 18:0 and 22:6 were incorporated in the sac fry. This was in contrast to 16:1, 18:1, 20:5 and 22:5 which were incor-porated in the sac fry to a smaller degree. These characteristics were observable by day 49, and continued throughout the experiment period indicating that the control processes by which the selection was accomplished were established early in the development of the sac fry.

That 16:0, 18:0 and 22:6 are preferentially incorporated into the sac fry would imply a structural requirement for these acids. The inclusion of 22:6 in this group is significant substantiating other observations of the unique manner in which this particular fatty acid

is utilized by the fish. It is suggested that the essential character

of the linolenic acid series of fatty acids for fish could be due to the

requirement for 22:6.

The loss of fatty acids from the yolk was found to be much less

selective than the depostion of fatty acids in the sac fry. No conclu-

sions can be drawn in the early stages of the developmental process

since only small proportions of the yolk lipid has been lost. During

the intermediate stages the fatty acid composition of the yolk lipids

remain, relatively constant precluding the exclusive transport of

any one class of lipid molecules. The data indicated that 18:1 had

a tendency to be retained in the yolk lipids to a greater extent than

any other fatty acids. This response was more pronounced toward

the end of the experimental period.

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