

**EFFECT OF DIETARY FAT SOURCE ON SUDDEN DEATH SYNDROME (SDS)
AND CARDIAC SARCOPLASMIC RETICULAR CALCIUM
TRANSPORT IN BROILER CHICKENS**

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of
Graduate Studies
The University of Manitoba
by
Hyun Cho Chung

In Partial Fulfillment of the
Requirements for the Degree
of
Master of Science
Department of Animal Science
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BY

HYUN CHO CHUNG

A thesis submitted to the Faculty of Graduate Studies of
the University of Manitoba in partial fulfillment of the requirements
of the degree of

MASTER OF SCIENCE

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ABSTRACT

Chung, Hyun Cho. M.Sc., The University of Manitoba, October, 1990. Effect of Dietary Fat Source on Sudden Death Syndrome (SDS) and Cardiac Sarcoplasmic Reticular Calcium Transport in Broiler Chickens. Major Professor; W. Guenter.

Sudden death syndrome (SDS) is one of the primary causes of mortality in rapidly growing broiler chickens. The exact etiology of the disorder is still unknown. The hypothesis that SDS is a metabolic disease or cardiac dysfunction is generally accepted.

Four experiments were designed to investigate possible nutritional and physiological aspects of SDS at subcellular levels. In the first study, small reductions in growth rate did not reduce the SDS incidence ($P > .05$). Relative organ (heart, lungs and liver) weights were greater ($P < .05$) in SDS birds compared with pen-mate control birds (MANUSCRIPT I). The incidence of SDS was reduced ($P < .05$) when birds were fed

diets supplemented with sunflower oil (SFO) instead of tallow in one experiment (MANUSCRIPT III), although only trends were observed in another experiment (MANUSCRIPT II). Weight gain and feed conversion were generally improved by the SFO diet possibly due to a better digestibility of SFO over tallow. Vitamin E supplementation of the tallow diet had deleterious effects ($P < .05$) on the incidence of SDS mortality between 3 to 6 wk of age.

Cardiac sarcoplasmic reticular (SR) membrane fluidity, Ca^{2+} uptake and $\text{Ca}^{2+} + \text{Mg}^{2+}$ -ATPase activity were not influenced ($P > .05$) by dietary fat sources, although a higher ($P < .05$) phosphatidylcholine level of heart tissue was observed in birds fed the tallow diet as opposed to the SFO diet.

Comparing with pen-mate control birds, cardiac SR Ca^{2+} uptake and $\text{Ca}^{2+} + \text{Mg}^{2+}$ -ATPase activity were depressed ($P < .05$) in SDS birds. The depressed Ca^{2+} transport characteristics of the cardiac SR of SDS birds may be in part due to reductions ($P < .05$) in levels of phosphatidylethanolamine plus phosphatidylglycerol, sphingomyelin, and total phospholipids in SDS heart.

It is suggested that SDS in broilers is a cardiac failure partially due to the defective cardiac SR function resulting from changes in membrane environment.

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FOREWARD

This thesis is written in manuscript style. All manuscripts will be submitted to Poultry Science. The authors of the first manuscript are Chung, H.C., W. Guenter, and N.E. Stanger, Department of Animal Science, University of Manitoba, Winnipeg, Manitoba, Canada, R3T 2N2, the second are Chung, H.C., W. Guenter, and G.H. Crow, Department of Animal Science, University of Manitoba, and J.L. Neufeld, Manitoba Department of Agriculture, Winnipeg, Manitoba, R3T 2N2, and the third are Chung, H.C., W. Guenter, R.G. Rotter, G.H. Crow, and N.E. Stanger, Department of Animal Science, University of Manitoba.

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ABREVIATIONS

ADS	acute death syndrome
BW	body weight
CHL	cholesterol
CP	crude protein
d	day
°C	degree celcius
FA	fatty acid
FP	fluorescence polarization
g	gram
HW	heart weight
IU	international unit
kcal	kilocalorie
kg	kilogram
m	meter
M	mole
m ²	square meter
ME	metabolizable energy
mg	milligram
ml	milliliter
mM	millimole
N	normal
PC	phosphatidylcholine

PE	phosphatidylethanolamine
PG	prostaglandin
PI	phosphatidylinositol
PL	phospholipid
SDS	sudden death syndrome
SFA	saturated fatty acid
SFO	sunflower oil
SKF	sheep kidney fat
SPH	sphingomyelin
SR	sarcoplasmic reticulum
UFA	unsaturated fatty acid
μg	microgram
μl	microliter
vit	vitamin
Wk	week
wt	weight
16:1n7	palmitoleic acid
18:1n9	oleic acid
18:2n6	linoleic acid
20:4n6	arachidonic acid
20:5n3	eicosapentaenoic acid
22:6n3	docosahexaenoic acid

GENERAL INTRODUCTION

Sudden death syndrome (SDS), also known as acute death syndrome (ADS), flip-over disease, heart attack and/or lung edema has become one of the primary causes of mortality in rapidly growing broiler chickens. The syndrome has been reported in several countries including Canada, U.S.A., England and Australia, affecting 1 to 2% of a population. Although the incidence is relatively low, it is of economic importance to find factors influencing the syndrome since it accounts for over 50% of total flock mortality.

An affected bird generally in good body condition dies quickly. SDS begins as early as 3 days of age with a peak incidence usually between 3 and 5 weeks and continues to market age of 6 weeks. However, the exact etiology or means of prevention of the disorder is still unknown.

Mollison (1983) indicated the involvement of fat metabolism in SDS mortality. Subsequent studies by Rotter (1985) suggested that dietary linoleic acid could reduce SDS and that the incidence could be related to a decrease in arachidonic acid and its metabolites in heart membrane. Julian and Bowes (1987) suggested that SDS is a metabolic disease.

To examine the hypothesis that SDS is a metabolic disorder of the heart, experiments were designed to: (1)

determine the effect of different dietary fats on SDS, (2) examine the possibility of inadequate organ capacity in SDS birds, and (3) compare cardiac membrane function of SDS birds and pen-mate controls.

LITERATURE REVIEW

1. Sudden death syndrome

1.1. Incidence

Sudden death syndrome (SDS) has been reported for over 20 years. Hemsley (1965) observed 2.04% overall mortality, while the average mortality due to SDS was .46%. Jackson et al. (1972) surveyed the mortalities from nine flocks and found that .65% of the population died "in good condition" (SDS), which accounted for 15.6% of the total mortality. Brigden and Riddell (1975) reported that the overall mortality in four broiler flocks in Western Canada was 3.80%, whereas the average mortality due to SDS was 1.13%. Steele and Edgar (1982) studied 64,000 broiler chickens in Australia. Some 6.86% of the flock died or were culled, while 2.46% died from SDS. In a survey of 51 broiler flocks in Western Canada Riddell and Springer (1985) reported that the SDS incidence ranged from .71 to 4.07% of the total flocks (average, 1.95%). More recently Gardiner et al. (1988a) compiled data from 23 experiments totalling about 90,000 male broiler chickens. The incidence of SDS ranged from 1.31 to 9.62% of flocks among experiments.

Reports on the relationship between SDS incidence and age are inconsistent. Hemsley (1965) reported that SDS mortality was more notable below 5 or above 7 weeks of age. Volk et al. (1974) observed a rapid increase in the incidence of SDS between 0 and 4 weeks of age followed by a rapid decrease to market age of 8 weeks. The time of peak mortality due to SDS is usually 3 to 5 weeks of age and then decreases until market age (Brigden and Riddell, 1975; Riddell and Springer, 1985; Gardiner et al., 1988a). Higher incidence of SDS was observed in males in the earlier study by Hemsley (1965). Many other researchers reported that on average 70 to 80% of the affected birds were males (Jackson et al., 1972; Brigden and Riddell, 1975; Steele and Edgar, 1982; Riddell and Springer, 1985; Merck and Co., 1986; Buckley et al., 1987).

Although the mortality due to SDS is relatively low, it accounts for a pronounced portion of the total mortality. A series of studies from our laboratory (Mollison, 1983; Rotter, 1985) show that it affected up to 6.7% of the population and accounted for over 60% of overall mortality.

1.2. Characteristic Observations of SDS

1.2.1. Behaviour prior to death. A major difficulty in studying SDS is the sudden onset of the disorder which happens in young, apparently healthy-looking birds without any sign of

disease. Newberry et al. (1987) monitored broiler flocks, using video cameras, for 12 hours to find any behavioural differences of SDS birds prior to death. They observed that all SDS birds exhibited normal behaviour prior to onset of typical symptoms such as loss of balance, violent wing beating and strong muscular contraction which lasted over a period of 53 seconds before death.

1.2.2. Gross Appearance. Birds dying from SDS are usually found lying on their backs or sides with neck and feet extended. Julian and Bowes (1987) reported that 70% of affected birds 'flipped over' during the convulsion and were found on their backs. The birds are in good flesh without any apparent evidence of disease. Comparing the age-matched control birds, body weights of SDS birds are not consistent. Several researchers found them at or slightly above the flock average (Brigden and Riddell, 1975; Ononiwu et al., 1979a; Hulan et al., 1980; Steele and Edgar, 1982) but Rotter (1985) observed them to be below the average.

1.2.3. Post Mortem Observations. Pulmonary congestion and edema are the most frequent findings on post mortem analysis of SDS birds, and are generally accepted as indicators of SDS (Hemsley, 1965; Jackson et al., 1972; Brigden and Riddell,

1975; Ononiwu et al., 1979a). However, Riddell and Orr (1980) reported that congestion and edema of lungs were not consistent features of birds dying from SDS and that they were absent or very slight shortly after death. These authors suggested that these conditions may be developed as a post mortem artifact after death.

Ononiwu et al. (1979a) observed several conditions at necropsy of SDS birds, including: feed in the gastrointestinal tract, generalized pulmonary congestion, enlarged hearts and contracted ventricles, blood clots in the atria, dilated intestines with pale contents, slightly enlarged liver, discolored and empty gall bladder, pale kidneys and congested thyroid, spleen and thymus. The musculature of the breast and thighs were moist and pale. Histological examinations of several tissues from 142 SDS birds were compared with those from control birds. In 70% of the cases, the hearts showed degeneration of myofibers and separation caused by edema fluid and leukocytic infiltration in the interstitium. Vascular engorgment and edema of interstitial and interlobular connective tissues were revealed in 85% of lungs from SDS birds. Steele et al. (1982) observed similar changes in the post-mortem and histopathological examination of SDS birds. Riddell and Orr (1980) conducted histological examinations of the hearts and chemical analysis of blood from SDS birds. In contrast to findings by Ononiwu et al. (1979a), no significant differences in the heart tissues between SDS and control birds were observed. However, these authors did not rule out the

possibility of ultrastructural or biochemical lesions, which could cause SDS. Marked changes in blood composition of mortalities were observed as the period of time from death to collection increased. However, there were no differences in these trends between SDS and control birds.

Biochemical studies on tissue composition of SDS birds are inconsistent. Mollison (1983) analyzed concentrations of Ca, Na, K and Cu of heart tissues and found a significantly lower Cu concentration in SDS birds compared with mortalities due to other causes. In contrast, Rotter (1985) reported no differences in Cu and Zn concentrations but higher Ca in SDS heart tissues. Rotter et al. (1985) also observed significantly higher levels of palmitic (16:0) and oleic acids (18:1n9) and lower levels of linoleic (18:2n6) and arachidonic acids (20:4n6) in the heart tissues of SDS birds compared with culled chickens. The authors also found higher levels of 18:1n9 and lower levels of 20:4n6 in the liver tissues of SDS birds. In another study (Rotter 1985), a small but significant reduction in 20:4n6 and increase in palmitoleic acid (16:1n7) were observed in SDS heart tissues compared with the pen mate controls, while no differences were found in the liver tissues. However, these findings have not been supported by Buckley et al. (1987) who reported higher levels of 20:4n6 in heart and liver tissues from SDS birds compared with controls. The contradictory results in part might be due to the differences of analytical methods. Rotter's group examined

whole heart and liver tissues, whereas Buckley's group investigated fatty acid composition of phospholipids, triacylglycerol and aliphatic carboxylic acid fractions in these tissues.

1.3. Possible causes of SDS

Although the incidence of SDS has been increasing for over 20 years since the disorder was first recognized, the exact etiology of the disease is still unknown. Several researchers suggested that it is a metabolic disease influenced by nutritional, genetic and environmental factors (Brigden and Riddell, 1975; Steele and Edgar, 1982; Julian and Bowes, 1987).

The possibility that blood clots commonly observed in SDS heart was the immediate cause of death was excluded by Cassidy et al. (1975) who failed to confirm the clots as thrombi and suggested they were of post-mortem origin. Volk et al. (1974) suggested that circulatory changes with resulting regressive changes in myocardium result in acute cardiac arrest and sudden death.

Ononiwu et al. (1979a) suggested a possible sequence of events of SDS. They proposed that when a disease is acute, pathological lesions are usually associated with vascular disturbance and that the process begins with circulatory

lesions manifested by increased permeability of the peripheral circulatory system. Physiological stress may result in even healthy capillaries becoming permeable and this permeability, caused by short term increase in blood pressure, is normally reversible. However, when the stimulus outstripped the tolerance level as in the SDS, irreversible changes occur in the vessel and the tissue it supplies. Thus death could be caused by heart damage resulting in lung edema and the bird's inability to breath. Fluid lost from the circulatory system into the lung tissue results in peripheral circulatory failure and shock.

Pulmonary congestion and edema are generally accepted as indicators of SDS. However, the conditions may be developed as post-mortem artifact due in part to the supine position of SDS birds since they were not often found upon immediate examination on death (Riddell and Orr, 1980).

Rotter (1985) observed lower levels of 18:2n6 and 20:4n6 in the heart tissue of SDS birds compared with culled or pen-mate control birds. She suggested a hypothesis that the reduced 20:4n6 levels in heart of SDS birds would reduce the synthesis of prostaglandins (PGs), which could result in failure of cardiac function. However, this hypothesis has not been supported by Buckley et al. (1987) in that the authors observed the higher levels of 20:4n6 in heart tissues from SDS birds than their controls.

Julian and Bowes (1987) indicated that SDS is related to

high carbohydrate intake which results in abnormal build up of intermediary metabolites causing cardiac arrhythmia, and that death may be caused by left ventricular fibrillation.

1.4. Factors involved in SDS

1.4.1. Sex. It is generally accepted that males are far more susceptible to SDS. However, the factors responsible for the higher incidence have not been elucidated. Bowes et al. (1989) compared biochemical profiles of 15 serum factors of male broilers with female broilers and male White Leghorns to identify metabolites or electrolytes associated with SDS. The authors confirmed that males have a much higher incidence of SDS (4%) than females (.5%) or male White Leghorns (0%) but they could not find any consistent differences in biochemical profiles between male and female broilers. Comparing with male White Leghorns, the authors observed significantly higher levels of lactate dehydrogenase and potassium in MB which might reflect the larger muscle mass or heart muscle damage. However, more research is required to determine whether these findings are related to SDS.

Gardiner et al. (1988b) implanted 15 mg of estradiol-17 β -monopalmitate subcutaneously in male broiler chickens at 2 weeks of age to observe estradiol effect on the incidence of SDS. At 9 weeks of age, combs and testicles from hormone-

treated birds weighed as low as one quarter those from control birds. However, there was no significant difference in overall or SDS mortality between treated and control groups.

1.4.2. Growth Rate. There are conflicting reports in regard to the relationship of growth rate to the incidence of SDS. Gasperdone (1981) observed the effect of various feeding schedules on growth rate and SDS mortality. Eight hour program, feeding on alternate days and low protein starter ration, reduced growth rate of broiler chickens. However, the author found no significant differences in total and SDS mortalities on various feeding schedules, although total mortality was highest for the birds on the low protein regime (17 vs. 23%). Mollison (1983) also reported that SDS was not reduced in broiler chickens where growth rate was reduced by 10% feed restriction but the author did not rule out the possibility that a greater difference in growth rate might produce a difference in SDS mortality. Riddell and Springer (1985), from a survey of 51 broiler flocks, concluded that growth rate was not a significant factor in the incidence of SDS. However, Ononiwu et al. (1979a) reported that birds dying from SDS were slightly heavier than the flock average. Classen et al. (1982) observed that a lower nutrient density diet (2,600 vs 3,300 ME, Kcal/Kg) resulted in a reduction in growth rate, with a marginal reduction in SDS mortality. Gardiner et

al. (1988a) investigated the relationship between body weight and SDS in male broiler chickens after pooling 23 experiments. The authors found that body weights of SDS birds ranged from 1,856 to 2,578 g and that SDS rate was increased with body weight increase. To consider the magnitude of this effect, an arbitrary increase in body weight by 350 g (between the above range) would increase the SDS incidence by 4.6% (Gardiner et al. 1988a). Bowes et al. (1988) found that 25% feed restriction in broilers significantly reduced growth rate and body weight by 41%, and concurrently decreased the incidence of SDS from 3.3 to 0%.

1.4.3. Environmental Stress. Ononiwu et al. (1979b) compared the effect of continuous and intermittent lighting on the incidence of SDS. Continuous lighting resulted in significantly greater SDS mortality than the intermittent schedule. The authors postulated that a higher light intensity could result in increased stress (by inducing cannibalism, excitement, fighting and piling) and that stress thus produced a higher SDS mortality. However, the above investigations have not been supported. Riddell and Springer (1985), from a field survey, found no correlation between light intensity and the incidence of SDS. Newberry et al. (1985; 1986) also reported that light intensity had no effect on total mortality or SDS incidence.

Rotter (1985) investigated the effect of stocking

densities, 11 birds/m² vs 13 birds/m², on incidence of SDS, but found no stocking rate effect on SDS mortality.

Treatments with anti-stress drugs were observed to reduce the stress effect on broiler chickens. Proudfoot and Hulan (1983) conducted four experiments to examine the effects of dietary aspirin (acetylsalicylic acid) on the performance and SDS in broiler chickens. The aspirin supplementation had no beneficial effects on decreasing SDS and had a deliterious effect on total mortality and body weights. Gardiner and Hunt (1984) found that dietary reserpin, an anti-hypertensive and tranquilizing agent, at levels up to 3.0 mg/kg of diet reduced growth, indicating its physiological effects on birds, but that neither overall nor SDS mortality was reduced.

1.4.4. Mineral and Vitamins. Differences of several mineral contents in serum and tissue of SDS birds, compared with those from culled or control birds, were noted although the trends were not consistent (see Section 1.2.3.), indicating that the incidence of SDS is related, in part, to electrolyte imbalance. Julian (1986) conducted experiments to observe the effect of increased dietary mineral levels on SDS. The author added .2% of each Ca, P and Mg to normal commercial feed containing .9% Ca and .65% P, and found that there was no significant differences in SDS mortality between the control and increased mineral diets.

Hulan et al. (1980) observed that the addition of biotin, thiamin and pyridoxine to broiler rations tended to reduce overall and SDS mortality. Even the addition of biotin alone (.3 mg/kg of diet) resulted in significantly decreased total and SDS mortality. A biotin deficiency in SDS birds was confirmed by Buenrostro and Kratzer (1982) in that the authors found decreased biotin levels in livers from SDS birds comparing with birds that died from other causes. However, the above observations have not been supported by others. Hunt and Gardiner (1982) conducted three experiments to investigate the effects of various dietary factors (wheat-based vs corn-based diet, supplemental K and supplemental biotin, pyridoxine and thiamin) on the incidence of SDS. The authors concluded that either overall or SDS mortality was not affected by those factors. Similarly studies from our laboratory (Mollison, 1983; Rotter, 1985) indicated that SDS mortality was not reduced by dietary supplementation of biotin and/or B complex vitamins. However, dietary lipid soluble vitamins A, D and E at 2xNRC levels tended to reduce SDS (Mollison, 1983). Steele et al. (1982) supplied biotin to 64,000 commercial broiler chickens via drinking water at 20 and 100 ug/day (1.2-5x to 6-25xNRC levels depending on age) to examine the supplemental biotin effect on SDS mortality. The uptake of vitamin was confirmed by radioisotopic analysis of liver biotin status. Adequate amount of biotin was detected in livers from both SDS and normal birds, which led the authors to conclude that SDS

was not reduced by biotin supplementation.

1.4.5. Lipid Metabolism. Involvement of lipid metabolism in SDS was indicated earlier (Hulan et al., 1980; Riddell and Orr, 1980). Mollison et al. (1984) reported that the incidence of SDS increased when birds were fed a wheat-soy diet (1.9%) instead of a corn-soy diet (.95%). Subsequently, Rotter et al. (1985) demonstrated that a wheat-soy diet supplemented with sunflower oil (SFO) had a significantly lower incidence of SDS (1.07%) than birds fed the diet supplemented with tallow (2.32%), although overall mortality was not different. The authors speculated that the response was due to increased intake of 18:2n6 from SFO. Several researchers have reported that high dietary intake of 18:2n6 from SFO had beneficial effects on cardiac function in the rat (Mest et al., 1980; Charnock et al., 1985; Hoffman, 1986). Rotter et al. (1985) also observed that SDS heart tissues had lower 18:2n6 and 20:4n6 levels compared with those from culled and control birds. These authors proposed a hypothesis that the elevated levels of oleic acid (18:1n9) in SDS heart tissue competitively inhibited desaturation and chain elongation of 18:2n6. The resultant reduced levels of 20:4n6 would lead to reduction of synthesis of its metabolites, including prostaglandins (PGs), which could cause the failure of heart function. This hypothesis, however, has not been supported by

Buckley et al. (1987) in that these authors observed higher 20:4n6 levels in heart tissues from SDS birds than those from their control birds. Therefore, the hypothesis that incidence of SDS is due to the inhibition of biosynthesis of PG resulting from the reduction of their precursors remains controversial.

2. Sarcoplasmic Reticular Membrane

2.1. Membrane in general

Biological membranes consist of a lipid bilayer composed mainly of phospholipids (PLs) and cholesterol (CHL). Proteins that have important cellular functions are embedded in the lipid bilayer and maintain the structural integrity of the membrane. The dynamic state of lipids in the bilayer is based on the concept of the "fluid mosaic" model described by Singer and Nicolson (1972). Most of the lipids in cell membrane at physiological temperature are in a fluid or liquid crystalline phase which allows lateral diffusion of membrane components within the plane of the membrane (Lee et al., 1986).

The functions of cellular membrane are influenced by a number of factors which could modify membrane molecular structure and/or molecular interactions causing changes in membrane fluidity. Cholesterol plays an important role in

membrane function. Changes in membrane CHL levels (or the CHL to PL ratio) are inversely related to membrane fluidity and thus may influence membrane-related cellular activities (Cooper and Strauss, 1984; Yeagle, 1985). Phospholipid composition of the membrane is a critical parameter in determining membrane function since membrane fluidity is dependent on membrane characteristics. Phospholipids can be "fluidizers", e.g., phosphatidylcholine (PC), or "rigidifiers", e.g., sphingomyelin (SPH) (McMurchie, 1988). Thus membrane fluidity is inversely related to the SPH to PC ratio (Barenholtz, 1984). Fatty acids as components of PL can affect membrane function. In general, a decrease in fatty acyl chain length or increase in unsaturation increases the membrane fluidity (Stubbs and Smith, 1984). Several other factors such as proteins, temperature, membrane potential, pH and divalent cations, particularly Ca^{2+} could modify the membrane fluidity and function (McMurchie, 1988).

2.2. Sarcoplasmic reticulum

The sarcoplasmic reticulum (SR) is an intracellular membrane network surrounding myofibrils in muscle cells. It regulates the level of free Ca^{2+} in the cytoplasm, thereby regulating the contraction and relaxation of muscle cells.

2.2.1. Structure of Sarcoplasmic Reticulum. The SR is composed of two continuous elements, the terminal cisternae directly facing the transverse tubules (T-tubules) which are in turn invaginated by surface plasma membrane, and the longitudinal SR overlying the remainder of the myofibril (Franzini-Armstrong, 1980).

The SR membranes contain about 60% protein and 40% lipid on weight basis (Hidelgo, 1985). Calcium-magnesium-5'-adenosine triphosphase ($\text{Ca}^{2+}+\text{Mg}^{2+}\text{-ATPase}$), also known as $\text{Ca}^{2+}\text{-ATPase}$ or $\text{Ca}^{2+}\text{-stimulated Mg}^{2+}\text{-dependent ATPase}$ constitutes 65-95% of the total SR membrane protein mass (Winegrad, 1982) and is a key enzyme in SR function.

The fraction of the SR lipids is made up of PLs and CHL. Phospholipids, the main component, is characterized by the presence of highly unsaturated fatty acids (UFAs) (Fiehn and Hasselbach, 1970). The main PL species is PC, followed by phosphatidylethanolamine (PE) and phosphatidylinositol (PI) (Owens et al., 1972).

2.2.2. Function of Cardiac Sarcoplasmic Reticulum. The cardiac SR, intracellular Ca^{2+} store, plays an important role in the regulation of intracellular Ca^{2+} homeostasis which is of importance in regulation of contraction-relaxation coupling of myocardial cells.

The action potential propagates through the sarcolemma,

cardiac cell plasma membrane, and it passes through the T-tubule. Upon depolarization of the sarcolemma, the influx of extracellular Ca^{2+} into the myocardium and subsequent increase in intracellular free Ca^{2+} concentration to an activating level of 10^{-5} M by the release of free Ca^{2+} from the SR causes the removal of the inhibition of actomyosin interaction and thus contraction is ensued. During the contraction phase, the SR takes up the cytosolic free Ca^{2+} by the action of $\text{Ca}^{2+}+\text{Mg}^{2+}$ -ATPase, which decreases the intracellular free Ca^{2+} concentration to a resting level of 10^{-7} M, resulting in myocardium being relaxed (Dhalla et al., 1982).

2.2.3. Dietary Fatty Acid Effects on Cardiac Sarcoplasmic Reticulum Structure and Calcium Transport. There is accumulating evidence that dietary polyunsaturated fatty acids (PUFAs) may have beneficial effects on cardiac function. High dietary intake of omega-6 PUFA from plant oil inhibited catecholamine-induced cardiac arrhythmia (Mest et al., 1980; Charnock et al., 1985) and increased cardiac force, coronary flow rate and heart muscle function in rats (Hoffmann, 1986). Dietary fish or fish oil, an omega-3 PUFA source, has been also shown to reduce coronary ischemia (Hock et al., 1986). Some of these effects may be mediated through the regulation of cytosolic Ca^{2+} levels since intracellular Ca^{2+} levels play important roles in contractility and viability of the cardiac

myocyte (Dhalla et al., 1982).

Several researchers conducted experiments to determine the effects of dietary lipids of different FA composition on the phospholipid fatty acid profile and Ca^{2+} transport in SR vesicles. Abeywardena et al. (1984) supplied rats with diets supplemented with either SFO, high content of UFAs, or sheep kidney fat (SKF), high in saturated fatty acids (SFAs), for 9 weeks. The total SFA/UFA levels of cardiac SR were not influenced by the two dietary fats but the PLFA composition was significantly changed. Cardiac SR membrane of rats fed the SFO diet exhibited an increase in omega-6 UFAs, including 18:2n6 and 20:4n6 while those fed the SKF diet were higher in omega-3 UFAs, mainly docosahexaenoic acid (22:6n3), which results in a higher omega-6/omega-3 ratio in the rats fed the SFO diet versus the SKF diet. Fluorescence polarization, measured by diphenylhexatriene probes, indicated that cardiac SR membrane fluidity was lower in the SKF dietary group. Despite these significant changes in membrane structure and physical properties, the authors found no significant differences in the specific activity of $\text{Ca}^{2+}+\text{Mg}^{2+}$ -ATPase between rats fed the two different diets.

The above observations have been supported by Gould et al. (1987) in which the authors reported that in the SR of rabbit muscle dietary supplementation with corn or fish oil resulted in significant changes in the relative amounts of the PL. However, neither $\text{Ca}^{2+}+\text{Mg}^{2+}$ -ATPase activity nor the pattern

of Ca^{2+} uptake and release was changed by different lipid supplementation.

In the simple reconstituted membrane system, $\text{Ca}^{2+}+\text{Mg}^{2+}$ -ATPase activity is dependent on the chemical structure of the surrounding PL, with 18-carbon FA of PC supporting highest activity and PL with longer, shorter or different head-groups supporting lower activities (Lee et al., 1986; Froud et al., 1986). The uptake and release of Ca^{2+} in this system is also dependent on the PL structure (Gould et al., 1987). However, large changes in PL composition are necessary to produce significant changes in $\text{Ca}^{2+}+\text{Mg}^{2+}$ -ATPase activity and Ca^{2+} transport in SR membrane (Gould et al., 1987). The nature of PUFA and membrane fluidity are of little importance for $\text{Ca}^{2+}+\text{Mg}^{2+}$ -ATPase activity when the membrane is maintained in a liquid crystalline phase (Lee et al., 1986). Gibson et al. (1984) suggested that there is a homeostatic mechanism in biological membranes which may act to buffer membranes from environmental changes such as alterations in dietary FA intake.

More recently, however, Swanson et al. (1989) observed that rats fed diets supplemented with 10% menhaden oil exhibited a significant reduction in cardiac SR $\text{Ca}^{2+}+\text{Mg}^{2+}$ -ATPase activity and Ca^{2+} transport as opposed to corn or olive oil supplementation. The authors speculated that the response was due to the high content of omega-3 PUFA in menhaden oil, resulting in significantly higher levels of eicosapentaenoic

acid (20:5n3) and docosahexaenoic acid (22:6n3), particularly 22:6n3, and lower levels of 18:2n6 and 20:4n6 of PL in cardiac SR membrane than was in corn or olive oil. Docosahexaenoic acid could be specifically associated with a possible membrane structure which result in reduced Ca^{2+} transport (Swanson et al., 1989; Croset et al., 1989) and $\text{Ca}^{2+}+\text{Mg}^{2+}$ -ATPase activity (Swanson et al., 1989). The authors did not rule out the possibility that 22:6n3 inhibited $\Delta 6$ -desaturase (Brenner and Peluffo, 1967), thereby reduced PL 20:4n6 levels would result in inhibition of PG synthesis which may cause abnormal cardiac functions.

3. Summary

Sudden death syndrome has been increasing in the broiler industry for the past 20 years. However, the exact etiology of the syndrome is still unknown. Several studies suggested that SDS is a metabolic disease associated with cardiac dysfunction. There is accumulating evidence that dietary PUFAs have beneficial effects on cardiac function. Therefore, it is worthwhile to investigate the effect of dietary lipids on SDS and the cardiac function of SDS birds at subcellular levels.

GENERAL MATERIALS AND METHODS

Three experiments were conducted to examine the effects of dietary lipids on the incidence of SDS and calcium transport of the cardiac sarcoplasmic reticulum. In an additional experiment, the effect of growth rate on SDS and comparison of relative organ weights between SDS and control birds were studied.

1. Birds

All studies used day old male broiler chicks vaccinated against Marek's disease. In experiments 1, 3, and 4, Peterson x Arbor Acre chicks were used, while in experiment 2, Cobb x Cobb chicks were used. The chicks were obtained from a commercial hatchery.

2. Housing

In experiments 1, 2 and 3, the birds were raised in 1.54 m x 4.31 m floor pens. Stocking density in experiments 1 and 2 was 60 birds/pen ($.11 \text{ m}^2/\text{bird}$) and for experiment 3, 70

birds/pen (.09 m²/bird). All pens, containing 5 cm of wheat straw litter, were located in an environmentally controlled barn providing continuous lighting at an intensity of about 18 lux at feeder level.

In experiments 4 and a part of 2, birds were raised in electrically heated Petersime battery brooders (Petersime Incubator Company, Gettysburg, Ohio). Each brooder contained 8 individual wire floor pens equipped with one trough feeder and one water trough per pen. Temperature and lighting schedules were similar to those in floor pens.

3. Management

In experiment 1, 2 and 3, temperature was controlled such that the chicks were exposed to 35° C for the first week, using electric brooder lights. After the first week, the temperature was gradually lowered by raising the brooders and by removing 1 or 2 of the 3 light bulbs in each brooder and eventually removing the brooder until the temperature of 21° C was reached at 3 weeks. Each pen was provided with two trough feeders during the first week. From day 7 until the end of experiment, birds were fed from 2 hanging tube feeders (40 cm diameter). Water was supplied from an automatic drinking cup (15 cm diameter). All rations, in mash form and water were supplied ad libitum.

Daily management procedures included shaking down feeders, cleaning waterers, cleaning wet spots around feeders and waterers and culling sick birds. All mortalities occurring during the first 3 days of the trials were replaced by spare birds. Subsequent mortalities were recorded and necropsied to ascertain the cause of death. Sudden death syndrome was diagnosed according to the following criteria: good body condition; well fleshed; full gastrointestinal tract; empty or small gall bladder; pulmonary edema and congestion; severe congestion of atria and ventricular systole.

4. Calculation of Data

In all experiments, average body weights for each pen were calculated by dividing the total weight of the live birds by the number of birds at the time of weighing:

$$\text{Average body wt/bird} = \frac{\text{Total live pen wt}}{\text{Total no. of live birds}}$$

Average gain per bird in each pen was calculated by subtracting the average body weight of the previous weigh date from the average body weight of the present weigh date:

$$\text{Average gain} = \text{Present average wt} - \text{previous average wt}$$

Feed:gain ratios were calculated by dividing the total pen feed consumption during the period of concern by the total weight gain of the birds, including mortalities in the same period:

$$\text{Feed:gain} = \frac{\text{Total pen feed consumption}}{\text{Total pen wt gain}}$$

5. Statistics

Data were statistically analysed using analysis of variance (ANOVA) or general linear model (GLM) procedures (SAS, 1985). A detailed method of statistical analysis is contained in each manuscript. Prior to analysis, mortality data were converted using arc-sine transformations. However, for interpretation the data were reported as percentages.

MANUSCRIPT I.

EFFECT OF NUTRIENT DENSITY ON THE INCIDENCE OF
SUDDEN DEATH SYNDROME AND ORGAN WEIGHTS.

ABSTRACT

An experiment was conducted to determine the effect of different nutrient density diets on the performance of broiler chickens and the incidence of sudden death syndrome (SDS). Comparisons of the relative organ weights between SDS and pen-mate control birds were also investigated. A total of 1,800 broiler chicks were fed wheat-soy basal diets with 3 different nutrient densities, ranging from 2,865 to 3,150 kcal ME/kg with calorie to protein ratios of 130 and 160 for the starter and finisher diets, respectively.

Birds fed the high nutrient density diet gained significantly ($P < .01$) more weight for the first 3 weeks, and showed an improved ($P < .01$) feed utilization to 6 weeks. However, body weight and weight gain during the finisher phase were not significantly ($P > .05$) different between diets. Total mortality and the incidence of SDS were not different ($P > .05$) among treatments, although a marginally higher ($P = .2$) incidence of SDS was observed in the high nutrient density diet.

No significant ($P > .05$) differences were observed in the relative organ weights among birds fed different nutrient density diets. However, compared to control birds, relative organ weights of birds dying from SDS were significantly ($P < .01$) greater. The results of this study do not support the hypothesis that SDS birds die due to organ insufficiency.

(Key words: sudden death syndrome, nutrient density, organ weights, broilers.)

INTRODUCTION

Sudden death syndrome (SDS) is a major cause of death in broiler flocks in Canada. Fast-growing broilers are susceptible and the highest incidence occurs at 3 to 5 weeks of age (Ononiwu et al., 1979a; Gardiner et al., 1988a). Affected birds die quickly, showing loss of balance, violent wing flapping and short terminal convulsions, which last less than one minute (Newberry et al., 1987). Males are more susceptible to SDS, accounting for from 70 to 80% of total SDS mortality (Proudfoot et al., 1984; Bowes et al., 1988). Rotter et al. (1985) suggested that a high intake of linoleic acid (18:2n6) can reduce the incidence of SDS. However, the exact etiology of the disease has not yet been elucidated, although nutritional or metabolic disorders have been proposed (Merck and Co., 1986; Bowes and Julian, 1988).

There are conflicting reports in regards to the effect of growth rate on the incidence of SDS. Hulan et al. (1980), Mollison et al. (1984) and Riddell and Springer (1985) reported that there was no significant relationship between growth rate and the incidence of SDS, whereas Ononiwu et al. (1979a) and Bowes et al. (1988) found a positive relationship.

Comparisons of organ weights between SDS and normal birds are not conclusive. Pass (1983) reported that heart weight/body weight ratios were higher in SDS broiler breeder hens and egg-laying strains, but this has not been supported

by Rotter et al. (1985), and Bowes and Julian (1988).

This study was conducted to determine the effect of dietary nutrient density on the performance of male broilers and the incidence of SDS. In addition, comparisons of the relative organ weights between SDS and pen-mate control birds were compared to determine whether SDS birds died because of lack of organ capacity to support the rapid growth.

MATERIALS AND METHODS

Animals and Diets. Eighteen hundred day old male broiler chicks (Peterson x Arbor Acre) vaccinated against Marek's disease, were randomly allotted, in groups of 60, to one of 30 pens (1.54 m x 4.31 m) within an environmentally controlled house provided with low intensity continuous lighting. Three different nutrient density diets (3,110-2,865 kcal ME/kg, 23.9-22.0% CP, starter; 3,150-2,948 kcal ME/kg, 19.7-18.4% CP, finisher) of the same calorie to protein ratio were randomly assigned to each pen (10 pens/treatment) and fed in mash form. Feed and water were supplied ad libitum. Starter (0 to 3 wk) and finisher (3 to 6 wk) formulations are presented in Table 1.

Postmortem examinations were carried out on all birds that died during the experiment and the cause of death was determined. Sudden death syndrome was diagnosed according to the criteria described by Rotter et al. (1985). When a bird died of SDS, heart (ventricular parts), liver, and lungs were excised and weighed. For each SDS bird sampled, a pen-mate control bird of similar weight was killed with CO₂ to prevent lung damage, and its organs were sampled like those of the SDS bird.

Traits measured were mortality in starter and finisher periods, incidence of SDS, body weight gain and feed efficiency. In addition, the relative organ weights (mg/g,

TABLE 1. Composition and calculated analysis of starter and finisher diets.

Ingredients	Starter ¹			Finisher		
	HND	MND	LND	HND	MND	LND
	(%)					
Wheat	55.1	62.5	69.9	68.8	75.0	80.0
Soybean meal(44%)	31.8	27.4	23.0	20.9	17.5	14.7
Fish meal(62%)	1.8	1.8	1.8
Tallow	6.9	3.6	.2	6.0	2.9	.3
Sunflower oil1	.42	.4
Vitamin premix ²	1.0	1.0	1.0	1.0	1.0	1.0
Mineral premix ³	.4	.4	.4	.4	.4	.4
Limestone	1.4	1.5	1.5	1.5	1.6	1.6
DiCa phosphate	1.5	1.5	1.5	1.3	1.3	1.3
DL-methionine	.1	.2	.2	.1	.1	.1
L-lysine1	.21	.1
Calculated analysis:						
ME,kcal/kg	3,110	3,015	2,865	3,150	3,039	2,948
CP,%	23.9	23.0	22.0	19.7	19.0	18.4
Linoleic acid,%	.8	.8	.8	.8	.8	.8
Lysine,%	1.4	1.4	1.4	1.0	1.0	1
Methionine,%	.5	.5	.5	.4	.4	.4
Ca,%	1.0	1.0	1.0	.9	.9	.9
Available P,%	.5	.5	.5	.4	.4	.4

¹HND, MND and LND = High nutrient density diet, medium nutrient density diet and low nutrient density diet, respectively.

²Supplies the following per kilogram of complete feed: vitamin A, 8,250 IU; vitamin D₃, 1,000 IU; vitamin E, 11 IU; vitamin K, 1.1 mg; vitamin B₁₂, 11.5 µg; riboflavin, 5.5 mg; Ca pantothenate, 11.0 mg; niacin, 53.3 mg; choline chloride, 1,020 mg; folic acid, .75 mg; biotin, .25 mg; delaquin, 125 mg; dl-methionine, 500 mg; streptomycin, 27.5 mg; penicillin, 82.6 mg; amprol, 1 g.

³Supplies the following in miligram per kilogram of complete feed: manganese, 55; zinc, 50; iron, 80; copper, 5; selenium, .1; iodine, .18; salt, 2,500.

BW⁷⁵ measured by control birds were compared.

Statistical Analysis. Performance data were analyzed as a completely randomized design, using general linear model (GLM; SAS, 1985) procedures. Mortality data were converted by arc-sine transformation but were reported as percentages. Differences among the means were determined using the Student-Newman-Keuls (SNK) test (Steel and Torrie, 1980). Data of the relative organ weights from SDS and their control birds were paired and analyzed by split-plot design (Steel and Torrie, 1980) using the GLM procedure. Differences between means were determined using least square means and their predicted differences.

RESULTS AND DISCUSSION

Performance Characteristics. During the experimental periods, birds in 3 pens from the medium nutrient density diet did not grow properly due to a water supply problem and were eliminated from the analysis. Table 2 presents the general performance of broiler chickens and the incidence of SDS. Birds fed the high nutrient density diet grew significantly ($P < .01$) faster in the starter period (0 to 3 weeks). However, in the finisher period (3 to 6 weeks), average body weight gain was not significantly ($P > .05$) different among the treatments. This appears to be due to the compensatory growth of birds fed the medium density diet and relatively faster growth for birds fed the low density diet. Classen et al. (1983), employing different nutrient density diets (2,865-3,250 kcal/kg, ME), similarly observed that differences in growth rate were more pronounced during the starter phase, with differences in average gain during the finisher phase being substantially less. Waldroup et al. (1976) reported that broilers fed the higher nutrient density diet gained more than those fed the lower density diet to 56 days of age, however, differences in nutrient densities were greater (2,970-3,740 kcal/kg) than the present experiment (2,865-3,150 kcal/kg). The higher nutrient density diet was more efficiently utilized by broiler chickens, which supports the earlier investigations by Waldroup et al. (1976) and Classen et al. (1982).

TABLE 2. Effect of different nutrient density diets on the general performance, incidence of SDS and total mortality of broiler chickens (MEAN \pm SEM).

Parameter	Dietary treatments		
	High density	Medium density	Low density
Body weight, g/bird:			
at 6 wk	1,875 \pm 56	1,802 \pm 41	1,749 \pm 14
Weight gain, g/bird:			
0 to 3 wk	574 \pm 5 ^A	486 \pm 26 ^B	507 \pm 12 ^B
3 to 6 wk	1,258 \pm 57	1,273 \pm 20	1,199 \pm 15
0 to 6 wk	1,832 \pm 56	1,758 \pm 41	1,706 \pm 14
Feed efficiency ¹ :			
0 to 3 wk	1.49 \pm .01 ^B	1.69 \pm .06 ^A	1.69 \pm .04 ^A
3 to 6 wk	2.14 \pm .10 ^{ab}	1.95 \pm .05 ^b	2.25 \pm .02 ^a
0 to 6 wk	1.91 \pm .05 ^B	1.88 \pm .05 ^B	2.07 \pm .02 ^A
Mortality, No(%):			
Total ² :			
0 to 6 wk	23(3.83 \pm 1.06)	17(4.05 \pm 1.25)	16(2.67 \pm .51)
SDS:			
0 to 3 wk	4(.67 \pm .27)	5(1.19 \pm .48)	6(1.00 \pm .44)
3 to 6 wk	15(2.56 \pm .86)	6(1.46 \pm .68)	5(.84 \pm .51)
0 to 6 wk	19(3.17 \pm .98)	11(2.62 \pm .71)	11(1.83 \pm .58)

¹Feed efficiency = Feed/gain.

²Includes culled birds.

^{AB, ab} Means within a row having no common superscripts are significantly different (AB, P<.01; ab, P<.05).

The overall mortality was not significantly ($P>.05$) affected by different nutrient density diets. Mortality due to SDS accounted for over 65% of total mortality and was somewhat higher ($P=.2$) for birds fed the high nutrient density diet during the finisher phase.

The effect of growth rate on the incidence of SDS in broilers is not conclusive. Hulan et al. (1980) reported that the higher incidence of SDS was not caused by the stress of rapid growth and Mollison et al. (1984) also showed that SDS was not reduced in the broiler chickens where growth rate was reduced by 10% feed restriction. From a survey of 51 broiler flocks, Riddell and Springer (1985) indicated that growth rate was not a critical factor in the incidence of SDS. However, Ononiwu et al. (1979a) reported that birds dying from SDS were slightly heavier than the flock average. Gardiner et al. (1988a) observed that SDS rate increased with body weight increase. Bowes et al. (1988) found that 25% feed restriction in broilers significantly reduced growth rate and body weight by 41%, and subsequently decreased the incidence of SDS from 3.3% to 0%. Differences in densities in this study, 8% for starter and 6% for finisher, were too small to induce significant differences in growth rate after 3 weeks and consequently no differences in the incidence of SDS was observed.

Comparisons of Relative Organ Weights. Comparisons of the relative organ weights between SDS and their pen-mate

control birds are presented in Table 3. There was no interaction between dietary treatments and bird types. Therefore, only main effects were analyzed. Dietary nutrient densities did not affect the relative organ weights. Rotter (1985) found no differences in heart weight/body weight (HW/BW) ratios of birds fed wheat-soydiets supplemented with either cornstarch or tallow, with different nutrient concentrations. However, comparing those of control birds, relative organ weights of SDS birds were significantly higher in the present study. Pass (1983) reported that ratios of HW/BW of birds dying of SDS were significantly increased comparing age-matched control broiler breeder hens and egg-laying strains. In contrast, Rotter (1985) did not find any significant differences in HW/BW ratios from SDS and culled broilers. More recently, Bowes and Julian (1988) reported that the relative organ weights of SDS broiler chickens showed great variabilities but that there were no significant differences in the relative heart, lung and intestine weights between SDS and control birds, although those of SDS birds were slightly higher. However, they observed that the relative liver weights of SDS birds were significantly higher, and indicated that SDS could be a metabolic disorders that resulted in enlargement of the liver.

In the present study the possibility that the greater relative organ weights of SDS birds were due to elapse of sampling following death can not be eliminated since sampling

TABLE 3. Relative organ weights of birds fed different nutrient density diets, and SDS and their control birds (LSM \pm SELM).

Diet and bird type	Relative organ weight ¹		
	Heart	liver	Lung
	(mg/g)		
Dietary treatments(n):			
High density(19)	23.5 \pm .4	194.1 \pm 4.9	33.3 \pm 1.0
Medium density(11)	24.1 \pm .5	188.2 \pm 6.4	35.2 \pm 1.4
Low density(11)	23.2 \pm .5	194.2 \pm 6.4	34.3 \pm 1.4
Statistic:	NS	NS	NS
SDS and Control(n):			
SDS(41)	24.3 \pm .3	198.7 \pm 4.3	38.2 \pm 1.0
Control(41)	22.9 \pm .3	185.6 \pm 4.3	30.3 \pm 1.0
Statistic:	**	*	**

¹Calculated by organ weight/metabolic body weight (BW^{.75}).

NS = Not significant

*P<.05; **P<.01.

of SDS birds was conducted on the following day but that of pen-mate controls was done immediately after death. Bowes and Julian (1988) in studying the effect of time of death to autopsy observed that the relative organ weights from normal birds during 12 hours following death were increased or decreased (increased for lungs, liver and intestine, and decreased for heart to 12 hours) possibly due to an osmotic fluid shift and that those trends were variable with ages of birds.

Sudden death syndrome has been identified as a nutritional/metabolic disease resulting in cardiac dysfunction. The results of this study indicate that a minor change in growth rate does not influence the incidence of SDS. The higher relative organ weights of SDS birds reject the hypothesis that SDS birds die due to organ insufficiency, but suggest that a higher demand was placed on the organs resulting in increased size. More research is required to determine the effect of growth rate on the incidence of SDS and compare the organs and their functions between SDS and normal birds.

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MANUSCRIPT II.

EFFECT OF DIETARY FAT SOURCE AND VITAMIN E
SUPPLEMENTATION ON THE INCIDENCE OF SUDDEN DEATH SYNDROME
(SDS) IN BROILER CHICKENS.

ABSTRACT

Two experiments were conducted to examine the effect of dietary fat sources (tallow vs sunflower oil (SFO)) and vitamin E on the general performance of broiler chickens, the incidence of sudden death syndrome (SDS) and membrane fluidity of the cardiac sarcoplasmic reticulum (SR). Growth rate and feed:gain ratio were improved when birds were fed the SFO diet vs the tallow diet, whereas vitamin E had no effect on the general performance. Sudden death syndrome was not influenced by dietary fat sources, but was increased by vitamin E supplementation during the finisher period (3 to 6 wk). Neither dietary fat sources nor vitamin E supplementation affected the cardiac SR membrane fluidity.

(Key words: dietary fat, vitamin E, sudden death syndrome, cardiac sarcoplasmic reticulum, membrane fluidity, broiler)

INTRODUCTION

Broiler growers have recognized that sudden death syndrome (SDS) is one of the main causes of mortality in rapidly growing broiler strains. Well nourished otherwise healthy birds die suddenly within 1 min with violent wing flapping and a short terminal convulsion (Newberry et al., 1987). Males are far more susceptible to SDS, causing over 75% of total SDS mortality (Proudfoot et al., 1984; Bowes et al., 1988). The exact cause of the disorder is yet to be elucidated although it has been generally known as a metabolic disease including cardiovascular dysfunction.

Rotter et al. (1985) reported that the incidence of SDS could be reduced when broilers were fed wheat-soy basal diets supplemented with sunflower oil (SFO) instead of tallow. These authors speculated that the response was due to the high level of linoleic acid (18:2n6) in SFO. Rats fed high dietary 18:2n6 from SFO supplementation showed positive effects on cardiac functions (Charnock et al., 1985; Hoffman, 1986). Some of these effects may be mediated through the changes in fatty acid composition of the cardiac membranes (Abeywardena et al., 1984) and the regulation of membrane calcium influx in the myocardial cell (Charnock et al., 1985). The cardiac sarcoplasmic reticular (SR) membrane is the internal calcium store and plays an important role in regulation of cardiac contraction and relaxation.

Abeywardena et al. (1984) demonstrated that dietary fat sources affected the fatty acid composition of SR membrane phospholipids (PLs). These changes in membrane lipids can influence the membrane fluidity and enzyme function (Spector and Yorek, 1985). Vitamin E, a lipid soluble antioxidant, prevents the formation of free radicals and peroxidative cleavage of membrane lipids (Scott et al., 1982). Vitamin E can also alter the composition as well as the physical state of membrane lipids (Curtis et al., 1984; Patel and Edwards, 1988). Biophysical studies show that as vitamin E partitions into a membrane the physical state of the membrane lipids is modified (Massey et al., 1982).

The purpose of the study was to determine the effects of dietary fat sources and vitamin E supplementation on the incidence of SDS and the fluidity of the cardiac SR membrane in broiler chickens.

MATERIALS AND METHODS

Animals and Diets. Nineteen hundred and twenty day old male broiler chicks (Cobb x Cobb), vaccinated against Marek's disease, were weighed and randomly placed in 32 floor pens (1.54 x 4.31 m) in an environmentally controlled house at a stocking rate of 60 birds per pen. Low intensity continuous lighting was supplied throughout the experiment. Eight pens were randomly assigned to each of the 4 dietary treatments (tallow, tallow/vitamin E, SFO, SFO/vitamin E). Diets, in mash form, and water were supplied ad libitum. Starter (0 to 3 wks) and finisher (3 to 6 wks) formulations are presented in Table 4. Standard management procedures were followed in starting birds. Birds dying within the first 3 days were replaced by spare birds. Subsequent mortalities were necropsied to determine the cause of death. Sudden death syndrome was determined according to the criteria described by Rotter et al. (1985).

Traits measured were body weight, body weight gain, feed efficiency, incidence of SDS and total mortality in starter and finisher periods.

To measure the fluidity of the cardiac SR membrane, 12 day old male broiler chicks were randomly placed in 4 wire pens (3 birds per pen) of a Petersime battery brooder. The brooder was located in an environmentally controlled room providing continuous lighting. Each group of 3 birds was

TABLE 4. Composition and calculated analysis of starter and finisher diets

Ingredients	Starter ¹				Finisher			
	Tal	Tal/ vit E	SFO	SFO/ vit E	Tal	Tal/ vit E	SFO	SFO/ vit E
	(%)							
Wheat	59.1	58.6	60.4	59.9	68.6	68.1	69.9	69.4
Soybean meal(44%)	28.1	28.1	28.1	28.1	19.5	19.5	19.5	19.5
Fish meal(62%)	3.0	3.0	3.0	3.0	2.1	2.1	2.1	2.1
Tallow	5.6	5.6	5.6	5.6
Sunflower oil	4.3	4.3	4.3	4.3
Vitamin premix ²	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Mineral premix ³	.4	.4	.4	.4	.4	.4	.4	.4
Limestone	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2
DiCa phosphate	1.4	1.4	1.4	1.4	1.6	1.6	1.6	1.6
DL-methionine	.2	.2	.2	.2	.1	.1	.1	.1
Vit E supplement5555
Calculated analysis:								
ME, kcal/kg	3,087	3,071	3,077	3,061	3,140	3,124	3,129	3,113
CP, %	22.5	22.4	22.7	22.6	19.5	19.4	19.7	19.6
Linolate, %	.6	.5	2.9	2.9	.6	.6	2.9	2.9
Lysine, %	1.3	1.3	1.3	1.3	1.0	1.0	1.0	1.0
Methionine, %	.5	.5	.5	.5	.4	.4	.4	.4
Ca, %	1.1	1.1	1.1	1.1	1.0	1.0	1.0	1.0
Available P, %	.5	.5	.5	.5	.6	.6	.6	.6
Vitamin E, IU/kg	24	114	38	128	25	115	40	129

¹Tal = Tallow; SFO = Sunflower oil

²Supplies the following per kilogram of complete feed:
 vitamin A, 8,250 IU; vitamin D₃, 1,000 IU; vitamin E, 111 IU;
 vitamin K, 1.1 mg; vitamin B₁₂, 11.5ug; riboflavin, 5.5 mg; Ca
 pantothenate, 11 mg; niacin 53.3 mg; choline chloride, 1.02 g;
 folic acid, .75 mg; biotin, .25 mg; delaquin, 125 mg; dl-
 methionine, 500 mg; streptomycin, 27.5 g; penicillin, 82.6 mg;
 amprol, 1 g

³Supplies the following in milligrams per kilogram of complete
 feed: manganese, 55; zinc, 50; iron, 80; selenium, .1; iodine, .18;
 copper, 5; salt, 2,500

randomly assigned to one of the 4 dietary treatments. Starter diets (Table 4) and water were supplied ad libitum for the first 4 weeks of age.

Preparation of Cardiac SR membrane. The cardiac SR membrane was isolated according to the modified method of Fernandez et al. (1980). In brief birds were killed by cervical dislocation, and the ventricular tissues were removed and placed in ice cold buffer 1 (.3-M Sucrose, .02-M Tris-Maleate, pH 7.0). The tissues were cut into small pieces and homogenized in a Polytron PT 100D Homogenizer (Brinkmann Inst., Rexdale, Ont.) for 20 seconds in 4 vols. of buffer 1. This homogenate was then centrifuged at 3,000g for 20 min to remove fat and connective tissues. The supernatant was recentrifuged at 10,000g for 20 min to remove contaminating mitochondria. The resulting supernatant was filtered through Nitex (pore size, 295 μ m) and solid KCl was added to .5 M final concentration to solublize the myofibrillar muscle proteins, and then recentrifuged at 150,000g for 30 min. The resultant sediment was suspended in buffer 1 and then recentrifuged at 150,000g for 30 min to remove the remaining KCl. The pellet, containing SR vesicles, was resuspended in buffer 2 (.01-M Tris, .9% NaCl, pH 7.4) and then recentrifuged as above. Supernatant was aspirated and the remaining pellet was dissolved in buffer 2. All isolation steps were carried out at 4° C. The protein content was determined by the assay of Bradford (1976) using bovine gamma globulin as a stock protein

solution.

Measurement of Cardiac SR Membrane Fluidity. The fluidity of the membrane is inversely related to the fluorescence polarization (FP) values of the membrane (Carlson et al., 1984). The values of FP were measured for 2 hours according to the method of Canvin (1988) as described elsewhere (Bailey, 1989), using trans-parinaric acid (tPNA; Molecular Probes Inc., Junction city, OR) as the fluorescence probe. The sample temperature was maintained at 41.5° C (for detail, see APPENDIX 1).

Statistical Analysis. Data of general performance were analyzed as a 2x2 factorial arrangement in a completely randomized design, using two-way ANOVA (Steel and Torrie, 1980). Mortality data were converted by arc-sine transformation prior to statistical analysis but were reported as percentages. Differences among the means were determined using the Student-Newman-Keuls (SNK) test (Steel and Torrie, 1980). For membrane fluidity, average FP values for 60 min from 4 groups of treatments were analyzed by split-plot design (Steel and Torrie, 1980) using a general linear model procedure (GLM; SAS, 1985). Differences among means were determined using least square means and their predicted differences.

RESULTS AND DISCUSSION

Performance Characteristics. Treatment effects on the general performance of broiler chickens, incidence of SDS and total mortality are summarized in Table 5. The average weight gain of birds fed the SFO containing diet was significantly ($P < .01$) greater than the tallow diet throughout the experiment. A significant ($P < .001$) improvement in feed:gain ratio was observed for birds fed the SFO diet during the starter period (0 to 3 wks) only. The superior performance of broiler chickens fed SFO diets during the starter period could be attributed to the better digestibility and utilization of unsaturated fatty acids (UFAs) from the SFO than the saturated fatty acids (SFAs) from the tallow (Renner and Hill, 1960; Freeman, 1976), whereas the more average weight gain from the SFO diet during the finisher period (3 to 6 weeks) could be accounted for by the greater feed consumption ($P < .05$) rather than greater efficiency of utilization. Very young chicks (1 to 2 weeks of age) have limited ability to digest dietary fat due to a deficiency of bile production (Freeman, 1984), but they can digest vegetable oil to a greater extent since it is more readily emulsified (Carew et al., 1972). Rotter et al. (1985) also observed the better performance of broiler chickens fed a wheat-soy basal diet supplemented with SFO instead of tallow. No significant ($P > .05$) effect of vitamin

TABLE 5. Effect of dietary fat and vitamin E on general permance, incidence of sudden death syndrome (SDS), total mortality (MEAN \pm SEM), and fluorescence polarization value of cardiac sarcoplasmic reticulum (LSM \pm SELM)

Parameters	Dietary treatment ¹				Statistic ²		
	Tal	Tal/ vit E	SFO	SFO/ vit E	F	E	FxE
BW, g/bird:							
at 6wk	1,816 \pm 17	1,853 \pm 23	1,911 \pm 25	1,922 \pm 25	**	NS ³	NS
Weight gain, g/bird:							
0-3wk	472 \pm 8	477 \pm 7	498 \pm 10	500 \pm 10	**	NS	NS
3-6wk	1,301 \pm 13	1,333 \pm 17	1,371 \pm 16	1,380 \pm 17	**	NS	NS
0-6wk	1,774 \pm 49	1,810 \pm 66	1,869 \pm 71	1,880 \pm 71	**	NS	NS
Feed consumption, g/bird:							
0-3wk	842 \pm 9	835 \pm 17	806 \pm 20	843 \pm 9	NS	NS	NS
3-6wk	2,641 \pm 32	2,688 \pm 14	2,746 \pm 17	2,764 \pm 42	**	NS	NS
0-6wk	3,483 \pm 109	3,523 \pm 64	3,552 \pm 88	3,608 \pm 131	*	NS	NS
Feed efficiency ⁴ :							
0-3wk	1.78 \pm .03	1.75 \pm .03	1.62 \pm .03	1.69 \pm .03	***	NS	NS
3-6wk	2.03 \pm .02	2.02 \pm .03	2.00 \pm .02	2.00 \pm .04	NS	NS	NS
0-6wk	1.96 \pm .04	1.95 \pm .08	1.90 \pm .07	1.92 \pm .08	NS	NS	NS
Mortality, No(%):							
SDS:							
0-3wk	5(1.04 \pm .44)	4(.83 \pm .45)	3(.63 \pm .30)	5(1.04 \pm .44)	NS	NS	NS
3-6wk	2(.44 \pm .28)	13(2.75 \pm .63)	4(.85 \pm .45)	4(.85 \pm .45)	NS	*	*
0-6wk	7(1.46 \pm .58)	17(3.54 \pm .86)	7(1.46 \pm .49)	9(1.88 \pm .49)	NS	NS	NS
Total ⁵ :							
0-3wk	12(2.50 \pm .94)	10(2.08 \pm .69)	11(2.29 \pm .44)	11(2.29 \pm .63)	NS	NS	NS
3-6wk	3(.64 \pm .31)	16(3.38 \pm .89)	3(.64 \pm .31)	6(1.28 \pm .54)	NS	*	NS
0-6wk	15(3.13 \pm 1.02)	26(5.42 \pm .76)	14(2.92 \pm .52)	17(3.54 \pm .86)	NS	NS	NS
FP ⁶ value of cardiac sarcoplasmic reticulum:							
at 4wk	.3566 \pm .0316	.3802 \pm .0274	.4098 \pm .0316	.3781 \pm .0316	NS	NS	NS

¹Tal = Tallow; SFO = Sunflower oil

²F = Fat; E = Vitamin E; FxE = Fatxvit E

³NS = Not significant

⁴Feed efficiency = Feed/gain

⁵Includes culled birds

⁶FP = Fluorescence polarization

*P<.05; **P<.01; ***P<.001

E supplementation on the general performance was observed.

Sudden death syndrome and total mortality were not significantly ($P > .05$) affected by fat sources. In contrast, Rotter et al. (1985) reported that SDS was significantly reduced in broiler chickens fed the SFO diet compared with the tallow diet, although total mortality was not different.

Unexpectedly vitamin E supplementation of the tallow diet significantly ($P < .05$) increased the incidence of SDS during the finisher period and subsequently total mortality. There is very limited information in the literature on fat-soluble vitamins, including vitamin E, effects on SDS. Mollison (1983) indicated the lower incidence of SDS in broiler chickens fed a diet supplemented with vitamin A, D, and E at a 2xNRC requirement, but the author could not find any significant difference due to the limited number of birds in his experiment. The increase of SDS in the vitamin E supplemented tallow diet could be related to changes in membrane function.

Membrane Fluidity Characteristics. Neither different fatty acids nor vitamin E supplementation influenced the membrane fluidity of the cardiac SR (Table 5). Lipid fluidity of the biomembranes can be affected by the characteristics of the membrane PL (Spector and Yorek, 1985). Modifications of fatty acid profiles of the SR membrane PL by dietary means have been reported by several researchers (Abeywardena et al., 1984; Gould et al., 1987; Croset et al., 1989; Swanson et al., 1989). The effect of this modification on SR membrane fluidity

is not conclusive. Abeywardena et al. (1984) measured rat cardiac SR membrane fluidity using two fluorescence probes. These authors found a significantly lower fluidity in rats fed a sheep kidney fat supplemented diet instead of SFO when using the diphenylhexatriene probe technique. However, using the n-(9-anthroyloxy) fatty acid probe showed no differences between the two dietary groups. Even in the same membrane, its fluidity could be different depending on the measuring techniques and fluorescence probes used in the analysis (McMurchie, 1988).

Studies of the effect of dietary vitamin E on the cardiac SR membrane fluidity are few. Patel and Edwards (1988) conducted experiments to examine the dietary vitamin E effects on the lung microsomal lipids and membrane fluidity in rats. The authors observed significant increases in the PL, the total cholesterol and the total saturated fatty acids and decreases in total polyunsaturated fatty acid content in vitamin E deficient microsomes. These alterations in membrane composition resulted in significant decreases in the membrane fluidity of the lung microsomes and reconstituted lipid vesicles. Peroxidation of membrane lipids induced by vitamin E deficiency also decreased membrane fluidity primarily due to the disruption of the molecular organization of the membrane (Curtis et al., 1984).

In the present study, adequate or excess levels of vitamin E were supplied to young broiler chickens and no

differences in the cardiac SR membrane fluidity between the two dietary groups were found. However, limited number of observations in this experiment (3 samples) and the scant information in the literature on membrane fluidity in chicken SR prevent plausible interpretation of the results. The question remains whether an interrelationship exists between the cardiac SR fluidity and the incidence of SDS. The possibility that other mechanisms in the membrane function rather than membrane fluidity are involved in the incidence of SDS is not to be ruled out.

In summary, the incidence of SDS was not influenced by the different fat sources. However, dietary vitamin E supplementation to the tallow diet had deleterious effects on the incidence of SDS. Membrane fluidity of the cardiac SR was not modified by either different fatty acids or vitamin E supplementation. More research is required to examine the interrelationships between vitamin E and membrane function relating to the SDS incidence in broiler chickens.

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MANUSCRIPT III.

EFFECTS OF DIETARY FAT SOURCES ON SUDDEN DEATH SYNDROME
(SDS) AND CARDIAC SARCOPLASMIC RETICULAR CALCIUM
TRANSPORT IN BROILER CHICKENS.

ABSTRACT

Wheat-soy diets supplemented with either tallow or sunflower oil (SFO) were fed to broiler chicks and several parameters were examined. These parameters included the general performance of the chicks, the incidence of sudden death syndrome (SDS), and cardiac sarcoplasmic reticular (SR) calcium transport. The phospholipid content of heart tissues was also investigated.

Birds fed the SFO diet gained significantly ($P < .05$) more weight over the first 21 days of age, and had a significantly better feed:gain ratio ($P < .01$). The incidence of SDS mortality up to 39 days of age was also lower ($P < .05$) for SFO-fed birds than those fed the tallow diet. Calcium uptake and $\text{Ca}^{2+} + \text{Mg}^{2+}$ -ATPase activity were not significantly ($P > .05$) different in cardiac SR vesicles from birds fed either diet. However, compared to pen-mate controls, SDS birds showed significantly depressed calcium uptake ($P < .01$) and $\text{Ca}^{2+} + \text{Mg}^{2+}$ -ATPase activity ($P < .05$) of cardiac SR vesicles. In the cell membranes of heart tissue, phosphatidylcholine concentration in tallow-fed birds was significantly higher ($P < .05$) than in SFO-fed chicks. No differences were seen in other phospholipid constituents. SDS birds, however, had significantly ($P < .05$) less phosphatidylethanolamine plus phosphatidylglycerol and sphingomyelin in the heart tissues.

The results suggest that SDS in broilers is a cardiac

dysfunction correlated with defective cardiac SR membrane function.

(Key words: dietary fat, sudden death syndrome, cardiac sarcoplasmic reticulum, calcium transport, $\text{Ca}^{2+}+\text{Mg}^{2+}$ -ATPase, phospholipid, broiler).

INTRODUCTION

Sudden death syndrome (SDS) is the primary cause of death in rapidly growing broiler chickens, ranging from .5 to 5% mortality. It begins as early as 3 days of age with a peak incidence usually between 3 and 5 weeks of age (Ononiwu et al., 1979a; Gardiner et al., 1988a) and continues to market age. Males are far more susceptible to SDS, accounting for 70 to 80% of total SDS mortality (Brigden and Riddell, 1975; Riddell and Springer, 1985; Buckley et al., 1987). The exact etiology of SDS is still unknown, but it is hypothesized that it is a metabolic disorder influenced by nutritional, genetic, and environmental factors (Brigden and Riddell, 1975; Steele and Edgar, 1982; Julian and Bowes, 1987). Ononiwu et al. (1979a) proposed that SDS is caused by cardiac damage, associated with a failure of the cardiovascular system. Pass (1983) also suggested that this syndrome is a cardiomyopathy.

Fat metabolism has been indicated as being involved in SDS (Riddell and Orr 1980; Mollison, 1983). Rotter et al. (1985) observed a reduction in the incidence of SDS when broiler chicks were fed diets containing sunflower oil (SFO) instead of tallow. These authors speculated that the response was due to an increased intake of linoleic acid (18:2n6). A high dietary intake of 18:2n6 from SFO inhibits catecholamine-induced cardiac arrhythmias (Mest et al., 1980; Charnock et al., 1985) and increases cardiac force, coronary flow rate,

and heart muscle function from isolated rat hearts (Hoffmann, 1986). The mechanism of some of these effects may be mediated through the regulation of membrane calcium (Ca^{2+}) flux in the myocardial cell (Charnock et al., 1985), since intracellular Ca^{2+} levels are critical in both contractility and viability of the cardiac myocyte (Dhalla et al., 1982).

The cardiac sarcoplasmic reticulum (SR) plays an important role in the regulation of intracellular Ca^{2+} homeostasis which is of importance in regulation of cardiac contraction and relaxation. Upon depolarization of the myocardium, the influx of extracellular Ca^{2+} into the myocardium and subsequent increase in intracellular free Ca^{2+} concentration by the release of free Ca^{2+} from the SR initiates cardiac contraction (Dhalla et al., 1982). During the contraction phase, the SR takes up the cytosolic free Ca^{2+} by the action of calcium magnesium 5'-adenosinetriphosphatase ($\text{Ca}^{2+}+\text{Mg}^{2+}$ -ATPase), also known as Ca^{2+} -ATPase and Ca^{2+} -stimulated Mg^{2+} -dependent ATPase. This enzyme comprises 65 to 95% of the total SR membrane protein mass (Winegrad, 1982), and is a key enzyme in this regulation (Dhalla et al., 1982). The activity of SR $\text{Ca}^{2+}+\text{Mg}^{2+}$ -ATPase, reconstituted into synthetic bilayers is dependent on surrounding phospholipids (PLs), both on head groups and fatty acyl chains (Lee et al., 1986). Dietary fat sources can modify the fatty acid composition of the membrane PLs (for a review, see McMurchie, 1988), which is the major components of biomembranes, including cardiac SR membrane of

rats (Abeywardena et al., 1984; Swanson et al., 1989; Croset et al., 1989). Differences in fatty acid composition of cardiac PLs were shown between birds dying of SDS and their controls (Buckley et al., 1987).

The objectives of this study were based on the hypothesis that SDS is caused by a metabolic dysfunction of the heart. To test this, the effects of two sources of dietary fat (tallow and SFO) were examined on several parameters. These parameters included the general performance of male broiler chickens, the incidence of SDS, cardiac SR calcium transport, and cardiac phospholipid composition. Differences of the cardiac SR calcium transport and phospholipid composition between SDS and pen-mate control birds were also examined.

MATERIALS AND METHODS

Animals and diets. Nineteen hundred and sixty day-old male broiler (Peterson x Arbor Acre) chicks vaccinated against Marek's disease were randomized, weighed, and placed in 28 floor pens (1.54 x 4.31 m, 70 birds/pen) in an environmentally controlled house providing low intensity continuous lighting. The experimental diets contained either tallow or SFO (Table 6). Diets were each randomly assigned to 14 pens and fed, in mash form, as starter (0-21 days) and finisher (21-39 days) diets. Feed and water were supplied ad libitum. The flock was monitored on a continuous basis for at least 10 hours each day. All dead birds were necropsied within 30 min of death. If SDS was diagnosed as the cause of death (Rotter et al., 1985), the ventricle was excised, divided into 3 equal parts and quickly frozen and kept in liquid nitrogen until further analysis. For each SDS bird sampled, a healthy pen mate of similar weight was selected, killed by cervical dislocation and treated as described above for the SDS bird.

Weight gains and feed conversion were calculated for the experimental periods 0 to 21 days and 21 to 39 days of age.

Preparation of Cardiac Sarcoplasmic Reticulum. Frozen ventricular tissue was thawed in a medium of 1-mM Ethylenediaminetetraacetic acid (EDTA), .25-M Sucrose (pH 7.0) and the cardiac SR vesicle was isolated according to the method of Harigaya and Schwartz (1969) modified by Ganguly et

TABLE 6. Composition of starter and finisher diets.

Ingredients	Starter		Finisher	
	Tallow	SFO ¹	Tallow	SFO
	(%)			
Wheat	59.3	60.3	71.2	73.4
Soybean meal (46.4%)	25.9	27.5	15.2	14.5
Fish meal (60.5%)	3.6	2.0	2.0	2.0
Tallow	7.0	...	6.5	...
Sunflower oil	...	5.8	...	5.0
Vitamin premix ²	1.0	1.0	1.0	1.0
Mineral premix ³	.4	.4	.4	.4
Limestone	1.2	1.4	1.4	1.4
Dicalcium phosphate	1.5	1.5	1.6	1.6
DL-Methionine	.1	.1	.2	.2
Lysine1	.5	.6
Calculated analysis:				
ME, kcal/kg	3,077	3,076	3,133	3,133
CP, %	24.0	24.0	20.0	20.0
Linoleic acid, %	.8	3.4	.8	3.1
Lysine, %	1.3	1.3	1.0	1.0
Methionine, %	.5	.5	.4	.4
Ca, %	1.0	1.0	1.0	1.0
Available P, %	.5	.5	.5	.5

¹SFO = Sunflower oil.

²Supplies the following per kilograms of complete feed: vitamin A, 8250 IU; vitamin D₃, 1,000 IU; vitamin E, 11 IU; vitamin K, 1.1 mg; vitamin B₁₂, 11.5 µg; riboflavin, 5.5 mg; Ca pantothenate, 11.0 mg; niacin, 53.3 mg; choline chloride, 1,020 mg; folic acid, .75 mg; biotin, .25 mg; delaquin, 125 mg; dl-methionine, 500 mg; streptomycin, 27.5 mg; penicillin, 82.6 mg; Amprol, 1 g.

³Supplies the following in miligram per kilogram of complete feed: manganese, 55; zinc, 50; iron, 80; copper, 5; selenium, .1; iodine, .18; salt, 2,500.

al. (1983) (for detail, see APPENDIX 2A).

Measurement of Ca^{2+} Transport and $\text{Ca}^{2+}+\text{Mg}^{2+}$ -ATPase Activity. Calcium uptake by cardiac SR was measured using a modified Millipore filtration technique (Ganguly et al., 1983). The modifications included: (1) SR vesicles were incubated at 37.5°C ; (2) the reaction of Ca^{2+} uptake was initiated by addition of 5-mM Tris-ATP (pH 6.8) and terminated by filtering the aliquot (100 μl) of the incubation mixture through a .45 μm Millipore filter (Millipore Co., Bedford, MA) and washed with 4 ml ice cold deionized distilled water (for detail, see APPENDIX 2B). The initial rates of Ca^{2+} uptake were determined from the slopes of the linear portions of the curve relating Ca^{2+} uptake to time. The maximum Ca^{2+} uptake was determined from the asymptote of the curve. The cardiac SR $\text{Ca}^{2+}+\text{Mg}^{2+}$ -ATPase activity was measured by the method of Ganguly et al. (1983) (for detail, see APPENDIX 2C). The protein content of SR vesicles was determined using bovine gamma globulin as a protein reference solution (Bradford, 1976).

Measurement of Phospholipid Content of Heart Tissue. A portion of ventricular tissues was lyophilized in a freeze-dryer (Virtis Research Equipment, Gardiner, NY) and then extracted using a modification of the method of Bligh and Dyer (1959). Twenty five ml of a methanol:chloroform (2:1) mixture was added to approximately 300 mg of freeze-dried tissue and then homogenized using a Polytron PT 100D Homogenizer (Brinkmann Inst., Rexdale, Ont.) for 2 min. The homogenate was

filtered through Whatman No. 4 filter paper, and rinsed twice with 40 ml chloroform into a 100 ml graduated cylinder with a ground glass top. A volume of 18 ml .1-N NaCl was added, the cylinder sealed, shaken and left over night to allow for complete separation of the layers. The water/methanol (upper) layer was aspirated off and the chloroform (lower) layer was transferred to a round bottom flask and evaporated using a rotary evaporator (Rotavapor, Brinkmann Inst.). The residue was reconstituted in 3 ml chloroform, transferred to a screw top vial and dried under nitrogen gas. The residue was stored at -70° C until subjected to thin-layer chromatography (TLC) to separate the phospholipids. For TLC, the samples were reconstituted in 500 μ l methanol:chloroform (1:2) mixture from which a 50 μ l subsample was applied to Whatman K5 Silica gel plates. All samples were spotted in triplicate. Spotted plates were developed using a chloroform:methanol:acetic acid:water (70:30:4:2) mixture. Phospholipids were visualized using iodine vapors and identified utilizing standards obtained from Sigma Chemical Company (St. Louis, MO). Each PL area was completely scraped, and placed into siliconized (Repel-Silane, LKB-Produkter AB, Bromma, Sweden) tubes. Each tube was extracted three times using a methanol:chloroform (1:2) mixture and then air-dried in a heated water bath (70° C). The phospholipid concentration of each tube was determined by quantitating inorganic phosphorus (Raheja et al., 1973).

Statistical Analysis. The experiment was set up as a

completely randomized design, and was analyzed using the one-way ANOVA (SAS, 1985). Mortality data were converted using arc-sine transformation before statistical analysis. For interpretation purposes the values were presented as percentages. Differences between means were determined using the student T-test (Steel and Torrie, 1980). Calcium uptake data over incubation time and over free Ca^{2+} concentration were fitted into the lines using nonlinear regression (NLIN) and stepwise regression models, respectively (SAS, 1985). Values estimated from the above lines and all other data from the rations were analyzed as a split-plot design using the general linear model (GLM) procedure. Data from SDS and their pen mate controls were paired and analyzed by the same method used for data from rations. Differences between means were determined using least square means (LSM) and their predicted differences (Steel and Torrie, 1980).

RESULTS AND DISCUSSION

Performance Characteristics. Performance data and the incidence of SDS are presented in Table 7. The average weight gain of SFO-fed birds was significantly ($P < .05$) greater than for those fed tallow for the starter period (0 to 21d) only. This observed difference could be attributed to differences in the digestibilities of the two fat supplements. Renner and Hill (1960) and Freeman (1976) reported that the digestibility of animal fat, tallow in particular, is poor initially and increases gradually to 8 weeks of age in broiler chickens. The effects of age on digestibility of vegetable oil are less pronounced. During the first one or two weeks, the chick's ability to assimilate dietary fat is impaired due to a deficiency of bile production (Freeman, 1984). Vegetable oil, however, can be digested to a greater extent because it is more readily emulsified (Carew et al., 1972).

Average feed:gain ratios were superior for birds fed SFO diets throughout the experiment. This may have been due to a better utilization of the unsaturated fatty acids (UFAs) in the SFO diet than the saturated fatty acids (SFAs) in the tallow diet (Renner and Hill, 1960). Renner and Hill (1961) reported that when tallow, lard and soybean oil are fed to broiler chicks, the absorption of SFAs, such as palmitic and stearic acids present in mixtures of UFAs increases as the proportion of UFAs increases. In the gut lumen, UFAs readily

TABLE 7. Effect of dietary tallow and sunflower oil on the general performance, the incidence of SDS and total mortality (MEAN \pm SEM).

Parameter	Dietary treatment		Statistic ($<$)
	Tallow	Sunflower oil	
BW,g/bird/39 d	1,853 \pm 8.1	1,866 \pm 9.8	NS
Weight gain,g/bird:			
0 to 21 d	574 \pm 4.4	587 \pm 4.9	.05
21 to 39 d	1,235 \pm 6.0	1,235 \pm 6.8	NS
0 to 39 d	1,808 \pm 8.1	1,821 \pm 9.8	NS
Feed efficiency ¹ :			
0 to 21 d	1.52 \pm .004	1.45 \pm .008	.01
21 to 39 d	1.85 \pm .006	1.83 \pm .006	.05
0 to 39 d	1.74 \pm .003	1.70 \pm .005	.01
Mortality,No(%):			
Total ² :			
0 to 39 d	60(6.12 \pm .826)	40(4.08 \pm .667)	NS
SDS:			
0 to 21 d	17(1.73 \pm .454)	11(1.12 \pm .372)	NS
21 to 39 d	19(2.01 \pm .452)	10(1.05 \pm .365)	NS
0 to 39 d	36(3.67 \pm .534)	21(2.14 \pm .631)	.02

¹Feed efficiency = Feed/gain.

²Includes culled birds.

NS = Not significant.

form micelles with bile salts, while SFAs, due to their non-polarity do not. Once micelles have formed, they will themselves solubilize substantial amounts of SFAs. This fatty acid "synergism" accounts for the increased availability of SFA to the chicken (Leeson and Summers, 1976). The results of average weight gain and feed efficiency from this study support the earlier investigations (Rotter, 1985).

Although overall mortality was not affected, SDS mortality was significantly ($P < .05$) reduced in birds fed the SFO containing diet. Rotter et al. (1985) observed the same trend in SDS mortality in chickens fed similar rations. They speculated that the major factor which accounted for the reduction of SDS in birds fed the SFO diet was the higher content of 18:2n6 in SFO. These authors also found significantly lower 18:2n6 and 20:4n6 in the heart tissues from SDS birds than those from birds culled due to leg problems (Rotter et al., 1985) and pen-mate control birds (Rotter, 1985). They proposed a hypothesis that the elevated levels of oleic acid (18:1n9) in heart tissues from SDS birds competitively inhibited desaturation and elongation of 18:2n6, thereby reducing 20:4n6 levels in heart tissue of SDS birds which would reduce the synthesis of prostaglandins (PGs) in the heart. This reduction in PG could lead to the failure of heart function, including cardiac fibrillation and/or arrhythmia. In contrast, Buckley et al. (1987) observed the levels of 20:4n6 in heart tissues of SDS birds as compared to

controls. Therefore, the hypothesis that SDS is due to a reduction in the biosynthesis of PGs as a result of the inhibition of their precursors remains controversial. It is possible that the conversion of 20:4n6 was inhibited in SDS heart resulting in higher concentration of 20:4n6 as observed by Buckley's group.

Calcium Transport Characteristics. Plots of calcium uptake and $\text{Ca}^{2+}+\text{Mg}^{2+}$ -ATPase activity of the cardiac SR vesicles are presented. Isolated cardiac SR vesicles actively accumulated Ca^{2+} from a medium containing Ca^{2+} and ATP. Measurements made early in the progress curve are linear and provide a measure of the initial rate of Ca^{2+} transport. At the point where the Ca^{2+} uptake has reached a steady state (asymtote) a measure of the maximum Ca^{2+} uptake is provided. No significant ($P>.05$) differences were observed in Ca^{2+} uptake of the cardiac SR vesicles from birds fed either diet over all incubation times (Figure 1) or free Ca^{2+} concentrations (Figure 2). Table 8 presents the initial rate and maximum Ca^{2+} uptake. No significant ($P>.05$) differences were observed between the two dietary groups. Like Ca^{2+} uptake, there were no significant differences either in Mg^{2+} -ATPase or in $\text{Ca}^{2+}+\text{Mg}^{2+}$ -ATPase activity (Table 8). This would be expected since ATP hydrolysis and Ca^{2+} transport are coupled events in intact SR membranes (Winegrad, 1982). The results of this study support the findings of Chung and Guenter (1989).

Table 9 shows the influence of dietary fat source

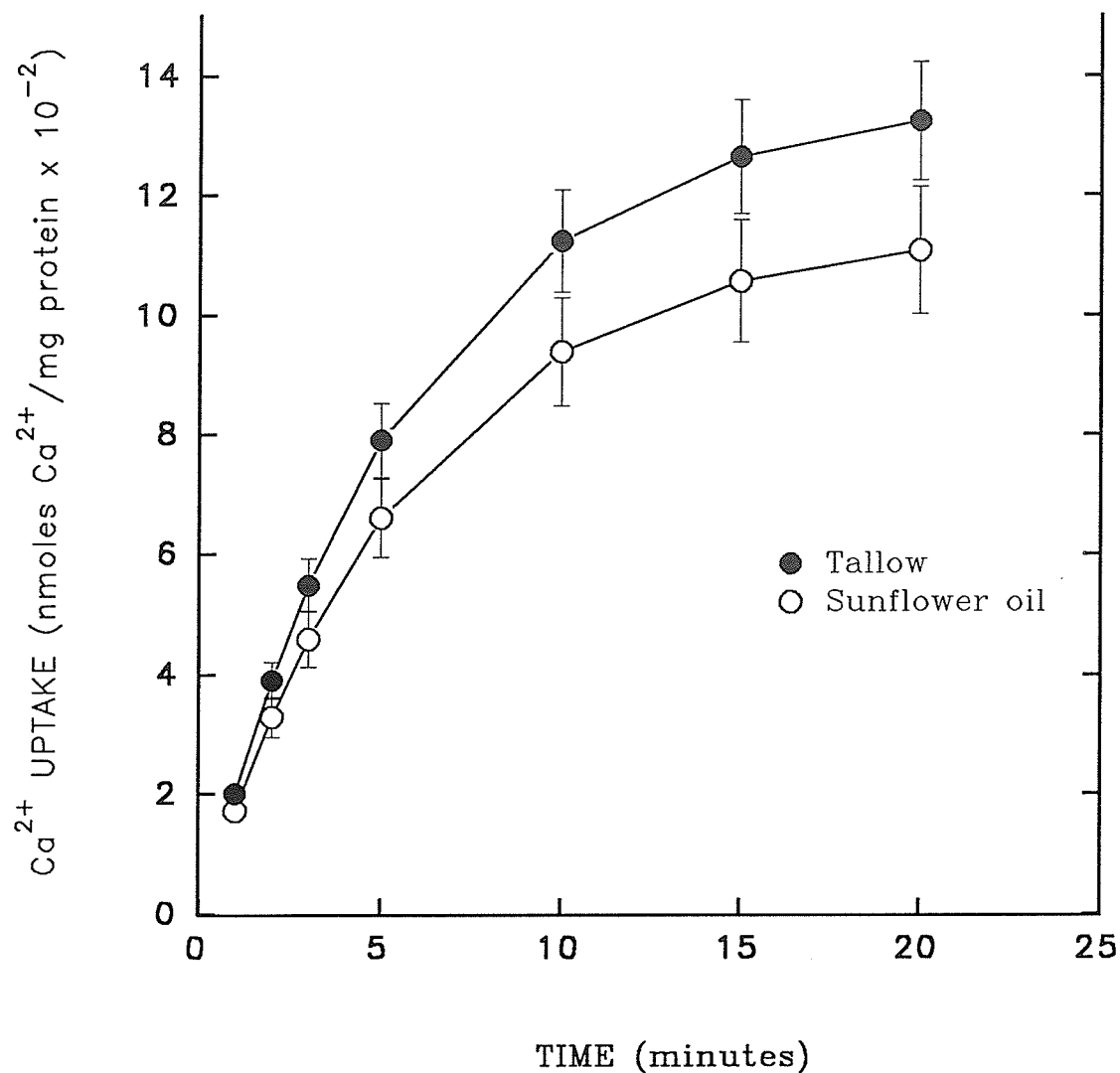


FIGURE 1. Effect of dietary fat source on cardiac SR calcium uptake during incubation. The curves are expressed as in the function, $Y=A-B(\text{EXP})^{-kt}$, A is the asymptote, B is A minus the value at t, k is the slope of the curve and t is the time of incubation in minutes. Values $(\text{LSM} \pm \text{SELM})_2$ at each incubation time represent the cardiac SR Ca^{2+} uptake of 16 birds fed tallow or 14 birds fed sunflower oil diets.

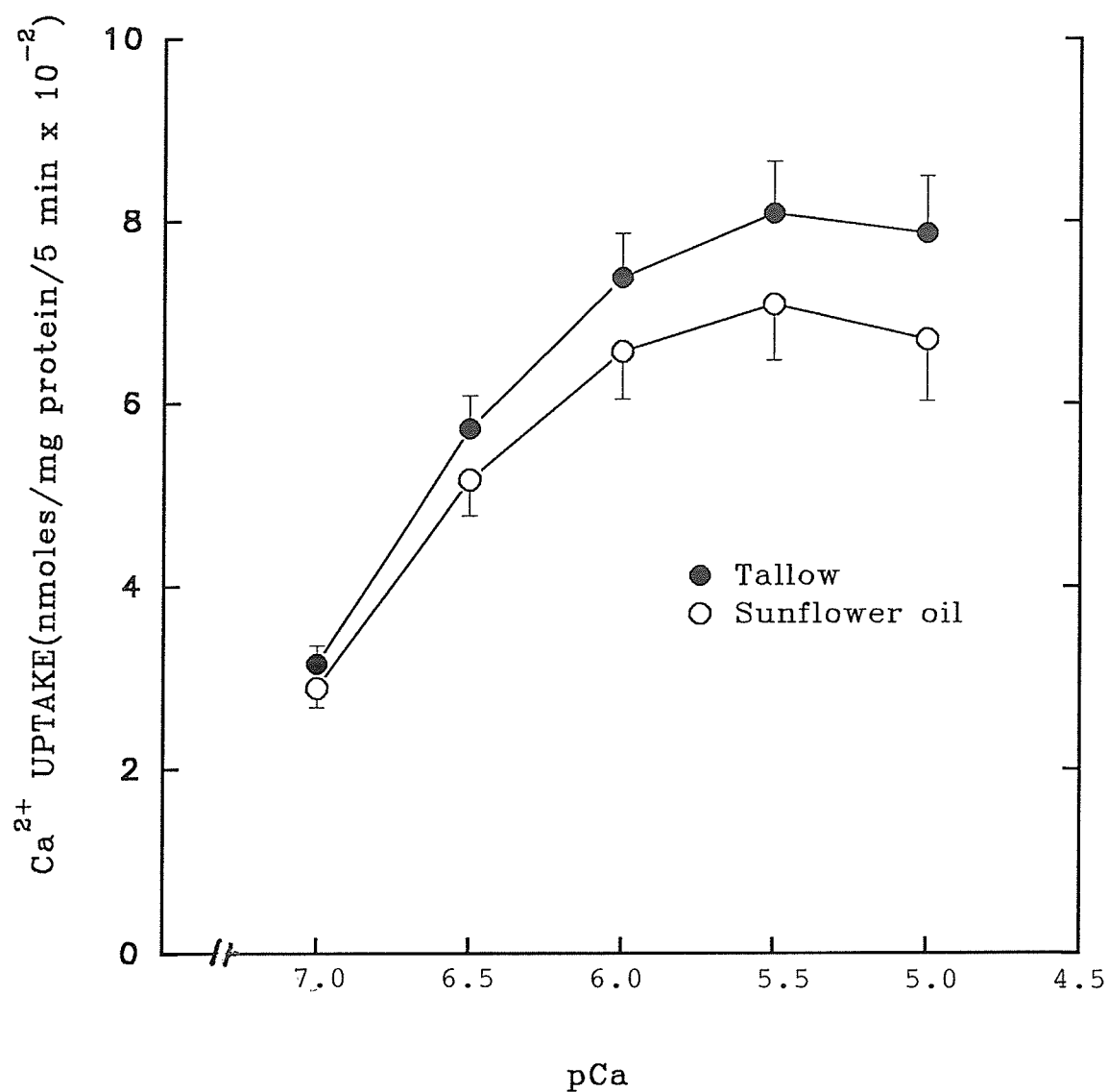


FIGURE 2. Effect of dietary fat₂₊ source on the cardiac SR calcium uptake at varying free Ca^{2+} concentrations. The curves are expressed as in the function, $Y=A+Bx+Cx^2$, x is the free Ca^{2+} concentration. Transformation of pCa is described in MATERIALS AND METHODS. Values (LSM \pm SELM) represent the cardiac SR Ca^{2+} uptake of 16 birds fed the tallow or 14 birds fed the sunflower oil diets.

TABLE 8. Effect of dietary fat source on initial rate and maximum uptake of calcium, and ATPase activities from cardiac SR of male broilers fed tallow or SFO diets (LSM \pm SELM).

Parameter	Dietary treatment		Statistic
	Tallow (n=16)	Sunflower oil (n=14)	
Initial rate ^{1,2}	183 \pm 14.6	153 \pm 15.6	NS
Maximum uptake ³	1,234 \pm 99.5	1,108 \pm 106.4	NS
Mg ²⁺ -ATPase ⁴	.84 \pm .078	.74 \pm .083	NS
Ca ²⁺ +Mg ²⁺ -ATPase ⁴	1.00 \pm .105	1.08 \pm .112	NS

¹Initial rate was measured from 0 to 3 min.

²Unit = n moles Ca²⁺/mg of SR protein/min.

³Unit = n moles Ca²⁺/mg of SR protein/20min.

⁴Unit = μ moles Pi/mg of SR protein/5min.

NS = Not significant.

TABLE 9. Effect of dietary fat source on phospholipid composition of the heart tissue of male broilers fed tallow or SFO diets (LSM \pm SELM).

Parameter	Dietary treatment		Statistic ($<$)
	Tallow (n=12)	SFO ¹ (n=10)	
	(μ g P/g freeze-dried tissue)		
Phosphatidylethanolamine + phosphatidylglycerol	16.9 \pm 1.20	13.4 \pm 1.31	NS
Lysophosphatidylethanolamine	.1 \pm .04	.1 \pm .05	NS
Phosphatidylcholine	13.8 \pm .85	10.6 \pm .93	.03
Sphingomyelin	1.3 \pm .14	1.5 \pm .15	NS
Lysophosphatidylcholine	.2 \pm .08	.1 \pm .09	NS

¹SFO = Sunflower oil.

NS = Not significant.

on phospholipid (PL) composition of the heart tissue. Only phosphatidylcholine (PC) concentration was significantly ($P < .05$) higher for tallow-fed birds. Gould et al. (1987) demonstrated that Ca^{2+} uptake and $\text{Ca}^{2+} + \text{Mg}^{2+}$ -ATPase activity were not in response to dietary-induced changes in PL composition of skeletal muscle SR from rabbits. Abeywardena et al. (1984) also reported that moderate modifications in the fatty acid composition of the SR membrane PL that occurred after feeding sheep kidney fat vs SFO to rats did not influence the $\text{Ca}^{2+} + \text{Mg}^{2+}$ -ATPase activity of cardiac microsomes.

The major protein found in the SR membrane is the $\text{Ca}^{2+} + \text{Mg}^{2+}$ -ATPase (Winegrad, 1982). The activity of this enzyme is dependent on the chemical structure of the surrounding PLs of the membrane, which, in turn, affects the membrane function (Stubbs and Smith, 1984). However, large changes in PL composition seem to be required to produce significant changes in ATPase activity (East and Lee, 1982).

Calcium transport characteristics between SDS and control birds are shown in Figures 3 and 4, and Table 10. Calcium uptake values of the cardiac SR from SDS birds were significantly ($P < .01$) depressed both over the incubation times and over the free Ca^{2+} concentrations. The initial rate and maximal Ca^{2+} uptake of SDS birds accounted for 70 and 74% of control birds, respectively (Table 10). The decrease in Ca^{2+} uptake by SR vesicles from SDS birds is probably due to the depression in $\text{Ca}^{2+} + \text{Mg}^{2+}$ -ATPase activity (Table 10). This

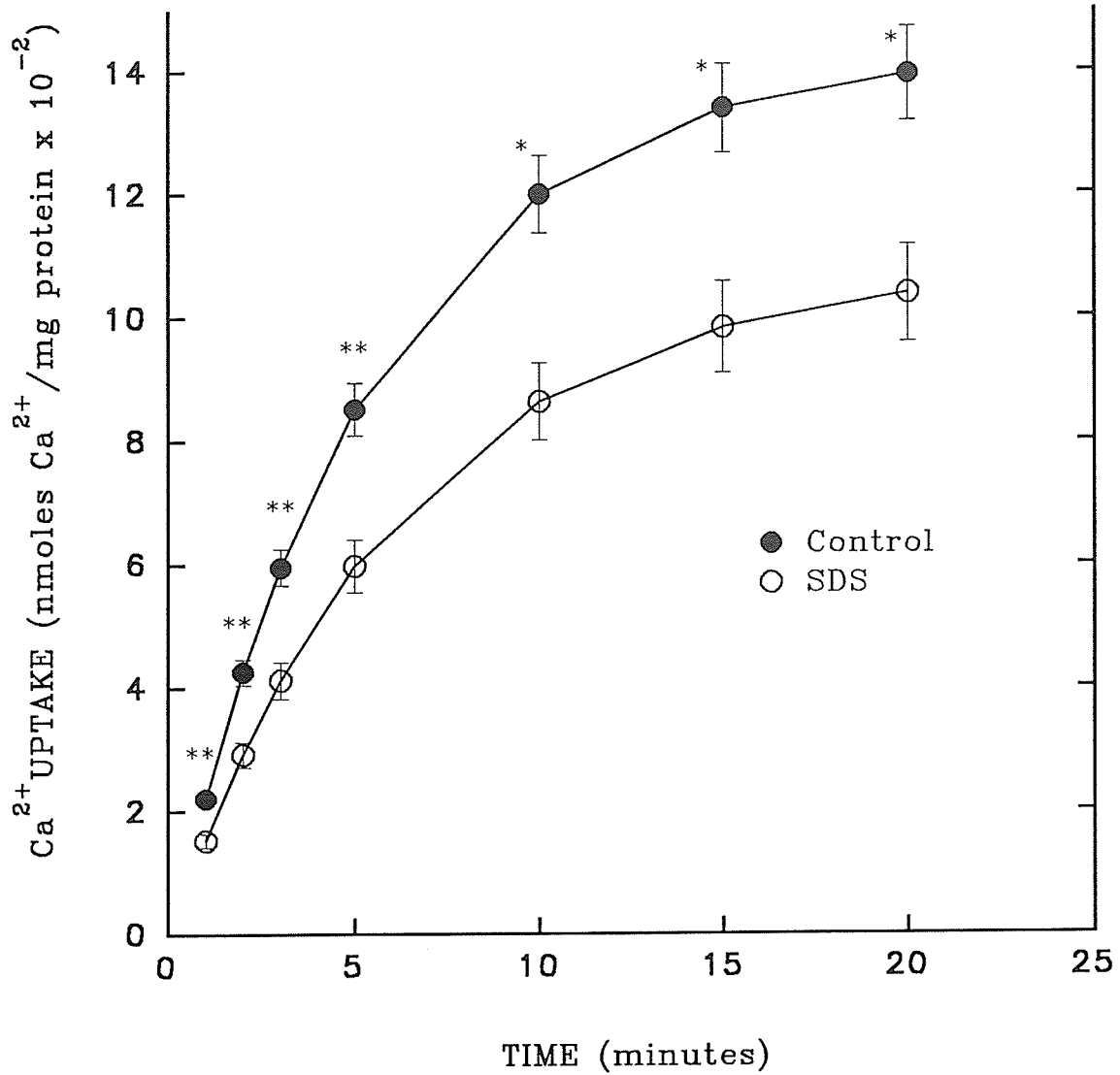


FIGURE 3. Calcium uptake of the cardiac SR from SDS and control birds. The curves are expressed as in the function $Y=A-B(\text{EXP})^{-kt}$. For description, see FIGURE 1. Values (LSM \pm SELM) represent the cardiac SR Ca₂₊ uptake of 15 birds. Notations for significant differences: * P < 0.01; ** P < 0.001.

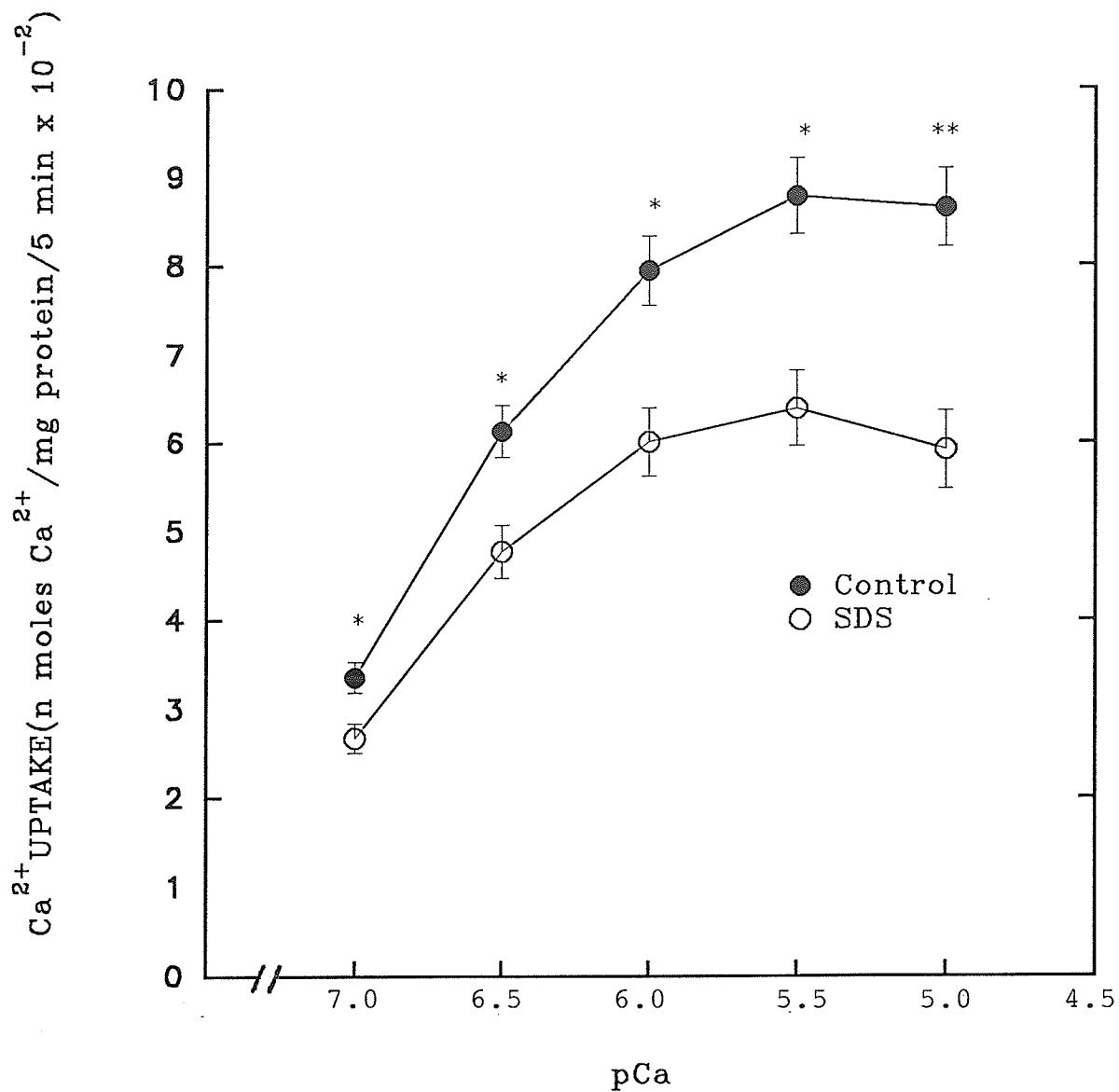


FIGURE 4. Calcium uptake of the cardiac SR from SDS and control birds at varying free Ca^{2+} concentrations. The curves are expressed as in the function, $Y=A+Bx+Cx^2$. For description, see FIGURE 2. Values (LSM \pm SELM) represent the cardiac SR Ca^{2+} uptake of 15 birds. Notations for significant differences: * $P < .01$; ** $P < .001$.

TABLE 10. Initial rate and maximum uptake of calcium, and ATPase activities from cardiac SR of SDS and control birds (LSM).

Parameter	Cardiac SR			Statistic (<)
	SDS (n=15)	Control (n=15)	SELM	
Initial rate ^{1,2}	137	198	± 10.0	.01
Maximum uptake ³	1,037	1,394	± 77.7	.01
Mg ²⁺ -ATPase ⁴	.80	.79	± .058	NS
Ca ²⁺ +Mg ²⁺ -ATPase ⁴	.96	1.13	± .055	.04

¹Initial rate was measured from 0 to 3 min.

²Unit = n moles Ca²⁺/mg of SR protein/min.

³Unit = n moles Ca²⁺/mg of SR protein/20min.

⁴Unit = μ moles Pi/mg of SR protein/5min.

NS = Not significant.

appears to be a specific effect because the basal Mg^{2+} -ATPase activity was not changed in SDS birds. Depressed cardiac Ca^{2+} uptake associated with a decreased $Ca^{2+}+Mg^{2+}$ -ATPase activity has been shown for chemically-induced diabetic rats with cardiomyopathy (Penpargkul et al., 1981; Ganguly et al., 1983). Whitmer et al. (1988) also observed a decrease in Ca^{2+} uptake function, but not in ATPase activity in cardiac SR of genetically-induced cardiomyopathic Syrian hamsters. It was postulated that the major defect in Ca^{2+} uptake in the cardiomyopathic hamsters was due to a decrease in volume and/or number of SR Ca^{2+} transport sites.

Table 11 presents the phospholipid composition of heart tissues from SDS and control birds. The concentrations of phosphatidylethanolamine (PE) plus phosphatidylglycerol and sphingomyelin were significantly ($P<.05$) reduced in SDS hearts. The level of PE plus phosphatidylglycerol for SDS birds was reduced to 50% of the value for control birds. In addition, the total amount of PL in SDS birds was less than that for control birds. The reduced levels of PLs could be related to change in the membrane function. Mrak (1985) showed that decreased levels of PLs in microsomes of muscular dystrophic chickens were related to a reduced calcium transport rate and a decrease in $Ca^{2+}+Mg^{2+}$ -ATPase activity.

In summary, the incidence of SDS was reduced when male broiler chickens were fed high level of dietary linoleic acid. Compared to control birds, the SDS birds showed depressed

TABLE 11. Phospholipid composition of heart from SDS and control male broilers (LSM).

Parameter	Heart tissue			Sta- tis- tic ($<$)
	SDS (n=11)	Control (n=11)	SELM	
	(μ g P/g freeze-dried tissue)			
Phosphatidylethanolamine + phosphatidylglycerol	10.3	20.1	\pm 2.57	.02
Lysophosphatidylethanolamine	.1	.1	\pm .03	NS
Phosphatidylcholine	11.5	12.9	\pm 1.75	NS
Sphingomyelin	1.0	1.8	\pm .22	.02
Lysophosphatidylcholine	.1	.2	\pm .08	NS
Total	22.9	35.1	\pm 2.81	.02

NS = Not significant.

calcium uptake, $\text{Ca}^{2+}+\text{Mg}^{2+}$ -ATPase activity and lower phospholipid concentrations. Therefore, it is suggested that SDS is a cardiac dysfunction partially due to the defective cardiac SR function, caused by changes in membrane environment.

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GENERAL DISCUSSION

Sudden death syndrome (SDS) is the major cause of mortality in rapidly growing broiler chickens. A number of factors have been reported to be associated with SDS, including sex (Jackson et al., 1972), growth rate (Mollison, 1983; Gardiner et al., 1988a), vitamins (Hulan et al., 1980), electrolytes (Julian, 1986), environmental stresses (Newberry et al., 1986) and fat metabolism (Mollison, 1983; Rotter, 1985). However, the exact etiology of the disorder is still unknown. The hypothesis that SDS is a metabolic disease or cardiac dysfunction is generally accepted. The current research was designed to examine this hypothesis at subcellular levels. This study was based on the findings by Rotter (1985).

Small reductions in growth rate induced by different dietary nutrient densities were found not to reduce the SDS incidence. There are conflicting reports in regards to the relationship between growth rate and the incidence of SDS. Mollison et al. (1984) and Riddell and Springer (1985) observed no significant effect of growth rate on the incidence of SDS, while Gardiner et al. (1988a) and Bowes et al. (1988) found a positive relationship. The possibility that greater differences in growth rate may affect SDS mortality is not to

be ruled out. The relative organ (heart, liver and lungs) weights of SDS birds were significantly ($P < .05$) higher than those of pen-mate control birds, although they were not influenced by dietary nutrient densities or growth rates. Bowes and Julian (1988) found higher relative liver weights in SDS birds than control birds, while Rotter (1985) found no differences in the relative heart weights between SDS and culled broiler chickens. This finding does not support the hypothesis that SDS birds died due to organ insufficiency, but suggests that a higher demand was placed on the organs resulting in increased size.

The relationship between cardiac membrane function and the incidence of SDS was investigated in the current project. As far as we know, this was the first study in this area. Birds were fed wheat-soy diets supplemented either with tallow or sunflower oil (SFO). Effects of dietary fat sources on incidence of SDS and membrane function were examined.

The incidence of SDS was significantly ($P < .05$) reduced when birds were fed the SFO diet instead of the tallow diet in one experiment (Manuscript III) although only trends were observed in another experiment (Manuscript II). These results support the findings by Rotter (1985). Vitamin E supplementation of the tallow diet significantly ($P < .05$) increased SDS mortality between 3 to 6 wk of age. However, membrane fluidity, Ca^{2+} uptake and $\text{Ca}^{2+} + \text{Mg}^{2+}$ -ATPase activity of the cardiac SR of birds were not influenced by dietary fat

source, although significantly ($P < .05$) lower PC level of heart tissues was observed for the birds fed the SFO diets as opposed to the tallow diets. Dietary fatty acids can influence the PL fatty acid profile and thus the function of the cardiac SR membrane of rat (Abeywardena et al., 1984; Croset et al., 1989; Swanson et al., 1989), but dramatic changes in PL composition seem to be required to induce significant changes in membrane function (Gould et al., 1987). Gibson et al. (1984) indicated that a homeostatic mechanism may exist in the biomembrane to buffer against environmental changes.

Compared to pen-mate controls, cardiac SR Ca^{2+} uptake and $\text{Ca}^{2+} + \text{Mg}^{2+}$ -ATPase activity were significantly ($P < .05$) depressed in SDS birds. Similar depressed cardiac SR Ca^{2+} uptake and $\text{Ca}^{2+} + \text{Mg}^{2+}$ -ATPase activity were observed in cardiomyopathic rats (Ganguly et al., 1983; Whitmer et al., 1988). In this regard Pass (1983) suggested that SDS in adult hens was a cardiomyopathy. In the current experiments the changes observed in the hearts of SDS birds with regard to levels of PE plus phosphatidylglycerol, sphingomyelin, and total PL may have influenced cardiac SR Ca^{2+} uptake characteristics of these birds. Mrak (1985) observed decreased PL levels in microsomes of muscular dystrophic chickens, and concurrently noted reductions in Ca^{2+} uptake and $\text{Ca}^{2+} + \text{Mg}^{2+}$ -ATPase activities.

Based on the current findings, it is suggested that SDS in broiler chickens is a cardiac failure partially due to the defective cardiac SR function resulting from changes in

membrane environment. Dietary unsaturated fatty acids would be expected to act as a buffer against the changes and could explain the lower incidence of SDS among birds fed the SFO diets in comparison to those fed the tallow diets.

SUMMARY AND CONCLUSION

Four experiments were conducted to investigate possible nutritional and physiological aspects of sudden death syndrome (SDS).

From the experiments the following conclusions can be drawn:

1. Sudden death syndrome may affect up to 3.7% of the population and account for over 80% of total mortality in male broiler chickens.

2. Small differences in growth rate did not influence SDS mortality.

3. Relative organ weights (heart, lung and liver) of SDS birds were higher than those of pen-mate control birds.

4. Sunflower oil containing diets were beneficial in reducing the SDS incidence in male broilers compared with the tallow diets (MANUSCRIPT III). A similar trend was shown in MANUSCRIPT II.

5. Calcium uptake and $\text{Ca}^{2+}+\text{Mg}^{2+}$ -ATPase activity of cardiac SR were depressed in SDS birds.

6. Reductions in levels of phosphatidylethanolamine plus phosphatidylglycerol, sphingomyelin and total phospholipids were observed in heart tissues of SDS birds.

The following aspects of SDS could be included for

further investigations:

1. Fatty acid compositions of cardiac sarcoplasmic reticular (SR) membrane phospholipids (PLs) should be compared between SDS and control birds. To overcome limited quantity of sample, gas chromatography (GC) and high performance liquid chromatography (HPLC) techniques may be employed.

2. Lipid fluidity of cardiac SR membrane were measured using trans-parinaric acid (tPNA) in the current study. Other fluorescence probes such as cis-parinaric acid, diphenylhexatriene, n-(9-anthroxyloxy) fatty acid should be used to examine the membrane fluidity in detail.

3. Differences in membrane fluidity, if any, between SDS and control birds could be related to Ca^{2+} transport characteristics and $\text{Ca}^{2+}+\text{Mg}^{2+}$ -ATPase activity of cardiac SR. This relationship, in turn, should be explained by possible changes in fatty acid profiles of the membrane PLs mentioned in 1.

4. Kinetic studies of cardiac SR $\text{Ca}^{2+}+\text{Mg}^{2+}$ -ATPase should be investigated in SDS and control birds.

5. Functions of cardiac sarcolemmal and mitochondrial membranes could be examined since these organelles are also involved in cardiac Ca^{2+} homeostasis.

6. Dietary omega-3 polyunsaturated fatty acids (PUFAs) could be used to reduce the incidence of SDS since there is cumulating evidence that these PUFAs have beneficial effects on the cardiac functions.

7. Metabolic or biochemical markers should be developed to validate SDS as cause of death.

8. It is necessary to set up the method(s) to standardize sampling time for SDS and pen-mate controls.

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APPENDICES

APPENDIX 1. Method for Measurement of Fluidity of Cardiac Sarcoplasmic Reticular Membrane (MANUSCRIPT II)

The membrane fluidity is inversely related to the fluorescence polarization values of the membrane. The isolated cardiac sarcoplasmic reticular (SR) membrane (see MATERIALS AND METHODS in MANUSCRIPT II) was subjected to fluorescence analysis in an LS5 Perkin-Elmer Spectrofluorimeter fitted with a polarizing accessory (Perkin-Elmer, Oak Brook, IL). Excitation slit and emission slit widths were set at 5 and 10 nm, respectively.

The fluorescence probe, trans-parinaric acid (tpNA; Molecular Probes Inc., Junction City, OR) was used to measure viscosity of the membrane. A tpNA stock solution of 2 mM in 95% Ethanol was stored in the dark under N₂ gas at -20° C. The stock solution was diluted 1,000-fold in a medium containing 10-mM Tris, .9% NaCl (pH, 7.4). The diluted probe solution (1.5 ml) was added to 1.5 ml of SR (100 ug/ml) in a quartz cuvette.

The cuvette holder was connected to a Lauda RC3 circulating waterbath and fluorescence measurements were undertaken for two hours. The sample temperature was maintained at 41.5° C.

Fluorescence intensities were converted to polarization values using the Perrin equation (Sklar et al., 1979, J. Biol. Chem. 254:9583-9591):

$$\text{Polarization value} = \frac{IV_{\text{para}} - IV_{\text{perp}} \times G}{IV_{\text{para}} + IV_{\text{perp}} \times G}$$

$$\text{where } G = \frac{IH_{\text{para}}}{IV_{\text{perp}}}$$

I = Intensity of the exciting light

V = Vertically polarized exciting light

H = Horizontally polarized exciting light

Para = The emission light parallel to the
vertical component

Perp = The emission light perpendicular to
the vertical component.

**APPENDIX 2. Methods for Measurements of Ca²⁺ Transport and
Ca²⁺+Mg²⁺-ATPase Activity.**

A. Isolation of Cardiac Sarcoplasmic Reticulum (SR).

Ventricular tissue was homogenized in 10 vols. of medium containing 10-mM NaHCO₃, 5-mM NaN₃, 15-mM Tris HCl (pH, 6.8) in a Waring Blender for 25 s at high speed. The homogenate was filtered through one layer of gauze and then centrifuged at 10,000g for 20 min. The supernatant was centrifuged at 40,000g for 45 min. The resultant sediment was suspended in 8 vols of .6-M KCl, 20-mM Tris HCl (pH, 6.8) and then recentrifuged at 40,000g for 45 min. The final pellet was suspended in a medium of .25-M Sucrose, 20-mM Tris HCl (pH, 6.8) as a sample solution (.05-.10 mg/ml).

B. Measurement of Ca²⁺ Uptake

Calcium uptake of SR was determined using the Millipore filtration technique. The cardiac SR membrane (.05-.10 mg/ml) was preincubated for 3 min at 37.5° C in 100-mM KCl, 20-mM Tris HCl (pH, 6.8), 5-mM MgCl₂, 5-mM NaN₃, 5-mM K-Oxalate, and ⁴⁵CaCl₂ (total vol., 1 ml). The reaction was initiated by the addition of 5-mM Tris ATP (pH, 6.8). The free Ca²⁺ concentration was maintained by addition of ethylene glycol-

bis-(β -aminoethylether)-N,N'-tetraacetate (EGTA), and the present free Ca^{2+} concentrations were calculated by Katz et al. (1970, *Biochim. et Biophys. Acta* 205:473-490). The reaction was terminated by filtering the aliquots through Millipore filters (pore size, $.45 \mu\text{m}$, Millipore Co., Bedford, MA) and washed with 4 ml of ice cold deionized distilled water. The radioactivity of the filters were counted using liquid scintillation counting techniques.

C. Measurement of ATPase Activity

The SR membrane (.03-.06 mg/ml) was preincubated for 3 min at 37.5°C in a medium similar to that used for Ca^{2+} uptake. When total ATPase was determined, CaCl_2 was used, and when Mg^{2+} -ATPase was measured, Ca^{2+} was omitted and .2-mM EGTA was added. The reaction was initiated by the addition of 5-mM Tris ATP (pH, 6.8) and was terminated after 5 min by 1 ml 12% (wt/vol) of cold trichloroacetic acid (TCA). The samples were centrifuged at 1,000g for 10 min and inorganic phosphate in protein free supernatant was measured using the method of Taussky and Shorr (1953, *J. Biol. Chem.* 202:675-685). The $\text{Ca}^{2+}+\text{Mg}^{2+}$ -ATPase activity is the difference between total and Mg^{2+} -ATPase activities.