Evaluation of Tom Fertility as Affected by Dietary Fatty Acid Composition

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Evaluation of Tom Fertility as Affected by Dietary Fatty Acid Composition

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ABSTRACT The objective of two studies was to manipulate the essential fatty acid content of turkey semen by enhancing the dietary levels of either n-3 polyunsaturated fatty acids (PUFA) or n-6 PUFA and determine the effect on fertility. In 1999 (Trial 1), and again in 2000 (Trial 2), Large White tom turkeys, 37 weeks of age, were fed one of three diets substituted with chicken fat, soybean oil, or menhaden fish oil. Chicken fat provided the industry's standard ratio of n-6 to n-3, soybean oil provided a greater ratio of n-6 to n-3, and fish oil provided a lower ratio of n-6 to n-3. Contemporary hens were inseminated weekly with semen collected from each group of toms. The effects of dietary lipids on tom body weights, fertility, motility, perivitelline layer sperm penetration percent, and live vs. dead sperm were analyzed. Whereas body weight increased linearly from 31 to 56 weeks of age (WOA), there was no effect of dietary treatment. As measured by the Accudenz® procedure, there were differences in sperm motility due to dietary treatment during 48 and 51 WOA during Trial 1. During Trial 2, sperm motility differences were observed at 53 WOA with the soybean oil-treated toms having the largest absorbance reading and the chicken fat-treated toms having the largest absorbance reading during 56 WOA. The live vs. dead sperm cells during Trial 1 revealed differences among the toms prior to treatment and post treatment. No dietary effects on percent live vs. dead sperm cells were observed during Trial 2. Once per mo,

eggs were collected for a one-week period to analyze for sperm penetration of the perivitelline layer. In Trial 1, sperm from toms fed chicken fat produced more penetrations (holes) during 36, 48, and 52 WOA. In Trial 2, sperm penetration values were lower for toms fed fish oil during 42, 47, and 51 WOA. Whereas there were significant differences in fertility, hatch of total eggs, and hatch of fertile eggs among treatments in Trial 1, a bacterial contamination on the farm during weeks seven through fourteen may have contributed to these findings. No significant differences due to treatment were found in these parameters during the second study. The fatty acid analysis of spermatozoa collected at the conclusion of Trial 2 revealed significant differences in total n-3 and total n-6 content, leading to significant differences in the ratio of total n-6 to total n-3. The mixed results indicated the fertilizing ability of domesticated turkey spermatozoa may not be affected by the n-6 to n-3 ratio in the diet of the tom.

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Table of Contents

Page

List of Tables

Table……Page

List of Figures

Figure……Page

INTRODUCTION

The phospholipids of spermatozoa of all species contain large amounts of PUFA. Manipulation of the essential fatty acid (EFA) content of turkey semen by enhancing the amounts of either n-3 PUFA or n-6 PUFA in the diet may affect reproductive performance. The n-3 and n-6 PUFA are considered to be essential due to the inability of vertebrates to synthesize them. Therefore, they must be provided in the diet. Contrary to mammalian spermatozoa, avian spermatozoa have low amounts of docosahexaenoic acid (DHA) in their phospholipid. Studies evaluating domesticated avian spermatozoa have shown arachidonic and docosatetraenoic (22:4n-6) acids to be their primary fatty acyl constituent (Howarth *et al*., 1977; Ravie and Lake, 1985; Kelso *et al.*, 1996 and 1997). The n-3 series fatty acid DHA dominates the lipid makeup of mammalian spermatozoa (Neil and Masters, 1972; Poulos *et al.*, 1973; Scott, 1973; Jain and Anand, 1976; Lin *et al.*, 1993).

The lipid profile of spermatozoa from wild birds has not yet been elucidated. However, turkey, chicken, and mammalian spermatozoa lipid has been characterized. Domesticated poultry spermatozoa appear to be characterized by high amounts of the n-6 series polyunsaturates, whereas mammalian spermatozoa lipid contains primarily the n-3 series, polyunsaturated fatty acids.

Fertility is always a major concern for turkey breeder managers. Considerable evidence has shown that reduced amounts of spermatozoan PUFA impair sperm numbers, motility, and fertilizing ability in birds (Ansah *et al.*, 1982; Kelso *et al.*, 1996) and mammals (Nissen *et al.*, 1981; Nissen and Kreysel, 1983; Sebastian *et al.*, 1987). The previous studies would seem to implicate spermatozoan PUFA as having an important role in sperm functionality and in optimizing fertility. Indeed, mammalian spermatozoa

lipid composition has been shown to play a major role in the physiochemical modifications leading to fertilization (Langlais and Roberts, 1985).

Very little is known of the influence of dietary fatty acid composition on the fertilizing ability of tom turkeys. It has been documented that phospholipid-rich tissues such as the brain and retina are very sensitive to PUFA deficiency in mammals (Neuringer *et al.,* 1988; Sanders *et al.,* 1988; Anderson *et al.,* 1989; Innis, 1991; Sardesai, 1992) and chickens (Anderson *et al.,* 1989; Cherian and Sim, 1992a,b; Anderson, 1994a,b). Commercial poultry diets typically provide a high ratio of n-6 to n-3 fatty acids (Scott, 1973; Darin-Bennet *et al.*, 1974; Kelso *et al.*, 1996). The current PUFA composition of avian spermatozoa may be an abnormality due to the PUFA composition of the diet provided.

The aim of the present study was to elucidate the effect of male dietary PUFA composition on various semen parameters. Hens were inseminated with semen from toms fed a diet that altered the ratio of n-6 to n-3 in spermatozoa.

LITERATURE REVIEW

Effects of Dietary Supplementation

Noble (1986) suggested that the fatty acid composition of diet provided to animals in captivity may differ significantly from the fatty acid compositions of diets of wild animals. In particular, commercial diets often provide a rich source of n-6 fatty acids while providing very small amounts of n-3 fatty acids. Thus, the tissue lipids of captive animals should be examined with caution when trying to interpret form and function. Kelso *et al*. (1996) questioned whether the reported predominance of n-6 PUFA in avian spermatozoa phospholipid content, in contrast to the predominance of n-3 PUFA found in

mammalian sperm, represents a true phylogenetic difference between species, or is simply due to the commercial diets provided domestic poultry.

Kelso *et al.* (1996) suggested that data obtained from the laboratory guinea-pig had caused some initial confusion among researchers due to the conflicting lipid characteristics of low proportions of DHA (22:6n-3) in the spermatozoa and high proportions of 22:5n-6 in the brain and retina (Neuringer *et al.*, 1988). Typically the mammalian brain, retina, and spermatozoa were characterized by having high concentrations of DHA (Neil and Masters, 1972; Poulos and White, 1973; Poulos *et al.*, 1973; Scott, 1973; Jain and Anand, 1976; Salem *et al.*, 1986; Neuringer *et al.*, 1988; Lin *et al.*, 1993). This initial confusion has recently been refuted by the research of Weisinger and co-workers (1995) where the guinea-pig's commercial chow was replaced with a diet enriched with n-3 fatty acids. The lipid content of 22:6n-3 was observed to increase while the 22:5n-6 diminished, reverting the tissues and spermatozoa to normal mammalian concentrations. Supporting this data, Salem *et al.* (1986) showed complete reversal of the laboratory rat's spermatozoa lipid content after replacement of its commercial chow with one rich in n-3 fatty acids. The laboratory rat's spermatozoa contained low concentrations of 22:6n-3 and correspondingly high concentrations of 22:5n-6 prior to diet manipulation, again atypical of the mammalian species. Salem's laboratory also observed greater spermatogenesis and improved testis maturation with increased 22:6n-3 in the spermatozoan. The studies by Weisinger *et al.* (1995) and Salem *et al.* (1986) may have revealed the true spermatozoa lipid content of the guinea-pig and rat by manipulating the dietary intake to represent a more wild type diet whereas contradicting early work by Neuringer *et al.* (1988) where they elucidated the spermatozoa lipid content neglecting the animal's dietary intake.

In another study by Watkins *et al.* (1991), it was observed that feeding hydrogenated soybean oil to chickens lowered the tissue concentration of arachidonic acid (AA). Fish oil (FO) has been shown to suppress lymphocyte proliferation, interleukin-2 production, natural killer cell activity, cytokine, and macrophage-mediated cytotoxicity (Calder, 1998; 1999). Also, it is thought that FO can bypass the initial metabolic process of n-3 EFA and move directly into the system as eicosapentaenoic acid (EPA). According to Watkins *et al.* (1991), the addition of soybean meal will provide a large amount of n-6 EFA to any mixed ration.

Polyunsaturated Fatty Acids

Polyunsaturated fatty acids with 20 carbon atoms are derived from dietary EFA (Tables 1 and 2). Essential fatty acids are divided into two series, the n-3 and n-6 series, derived from linoleic acid (LA) and a-linolenic acid (ALA), respectively. These EFA are the natural substrates found in body tissues used for the formation of metabolic products with important biological functions. These products include prostaglandin (PG) and other related compounds such as thromboxanes, prostacyclins, and leukotrienes. Dietary fatty acids are absorbed and deposited without significant modifications in humans and animals (Scaife *et al.*, 1994; Sardesai, 1992). Therefore, EFA concentration in body tissues can be altered by significantly increasing or decreasing the ratio of n-3 and n-6 PUFA in the diet (Draper, 1980).

The composition of the dietary fat is extremely important in the metabolism of PUFA in body tissues because each dietary fatty acid will influence the utilization of other fatty acids (Sardesai, 1992). Linoleic acid, ALA, and oleic acid (non-essential) are competitive substrates for the same sequence of desaturation enzymes. The affinity of these fatty acids for the desaturation enzymes is as follows: ALA > LA > oleic acid. Low

concentrations of ALA are very effective in suppressing the metabolism of LA. However, moderate levels of LA are necessary to inhibit the metabolism of ALA, whereas only high concentrations of oleic acid can suppress the metabolism of LA. Consequently, the metabolites of ALA and LA are normally found in higher amounts than the metabolites of oleic acid in normal body tissues and fluids. Because the dietary fat composition can be altered in favor of one of the above fatty acids, the metabolism can be shifted according to the affinity, as well as the amount, of fatty acid consumed. Therefore, dietary intake determines to a great extent the fatty acid composition of phospholipids in the plasma, tissues, and cell membranes.

Metabolism of Essential Fatty Acids

Neither 18:2n-6 nor 18:3n-3 fatty acids can be synthesized in mammalian tissues, but once consumed in the diet, additional carbons and double bonds are added to both parent EFAs, forming the n-6 and n-3 derivatives. The extent to which additional carbons and double bonds are added influences both the rate of oxidation of the fatty acids and the affinity for acyl transferases (Sanders, 1988). Oxidation of EFA will decrease when the fatty acid chain length increases. The affinity of these parent EFA for other metabolic pathways also plays a role in their conversion rate. Competitive inhibition occurs between 18:2n-6 and 18:3n-3, 20:3n-6 and 20:4n-3, and 22:4n-6 and 22:5n-3 series acids for ? 6-desaturase, ? 5-desaturase, and ? 4-desaturase respectively (Figure 1). The n-6 and n-3 derivative balance is determined by the ratio of n-6 to n-3 provided in the diet. The parent EFA conversion rate to their derivatives has been shown to be species dependent (Sanders, 1988).

The cat, for instance, lacking ? 6-desaturase, needs to have plenty of 20:4n-6 in its diet due to the inability to synthesize 20:4n-6 from 18:2n-6 (MacDonald *et al.*, 1984).

Sanders *et al.* (1978), in studies using vegans, suggested the conversion of 18:2n-6 to 20:4n-6 occurred readily in humans at the expense of a rapid conversion of 18:3n-3 to 22:6n-3 due to the ? 4-desaturase being used up in the n-6 conversions. Additionally, Sanders and Younger (1981) found the 22:6n-3 plasma phospholipids did not increase with a dietary increase of 18:3n-3 or 20:5n-3 (Von Schacky and Weber, 1985).

The EFA that predominate in the phospholipid membrane layers such as 20:4n-6 and 22:6n-3 are not oxidized as fast as those not found in the membranes (Sanders, 1988). When an animal is fed an EFA deficient diet, synthesized oleic acid is converted to eicosatrienoic acid (20:3n-9) in place of the fatty acids. Also, the derivative EFA provided in a diet will have different effects than those derived from the parent EFA.

Dietary Deficiency

Wild birds and mammals eating a fat-free diet or lacking intake of polyunsaturated fatty acids exhibit several problems including reproductive failure, hindered growth, and organelle changes within the skin, liver, and kidneys (Burr and Burr, 1929, 1930; Holman, 1968). These problems indicate a classic EFA deficiency and are completely reversible or preventable when sufficient dietary n-6 fatty acids are provided (Holman, 1968). However n-3 fatty acids do not eliminate these problems and are not specifically required.

During an EFA deficiency, larger amounts of n-6 fatty acids are decreased in tissues, compared to n-3 fatty acids, so a specific n-3 deficiency does not occur (Anderson and Maude, 1971; Futterman *et al.*, 1971; Paoletti and Galli, 1972; Benolken *et al.*, 1973; Alling *et al.*, 1974). Two criteria, high levels of n-6 fatty acids and low levels of n-3 fatty acids, must be met for the depletion of DHA to occur. Note that this abnormally high ratio of n-6 to n-3 is seen in domesticated commercial poultry diets. The

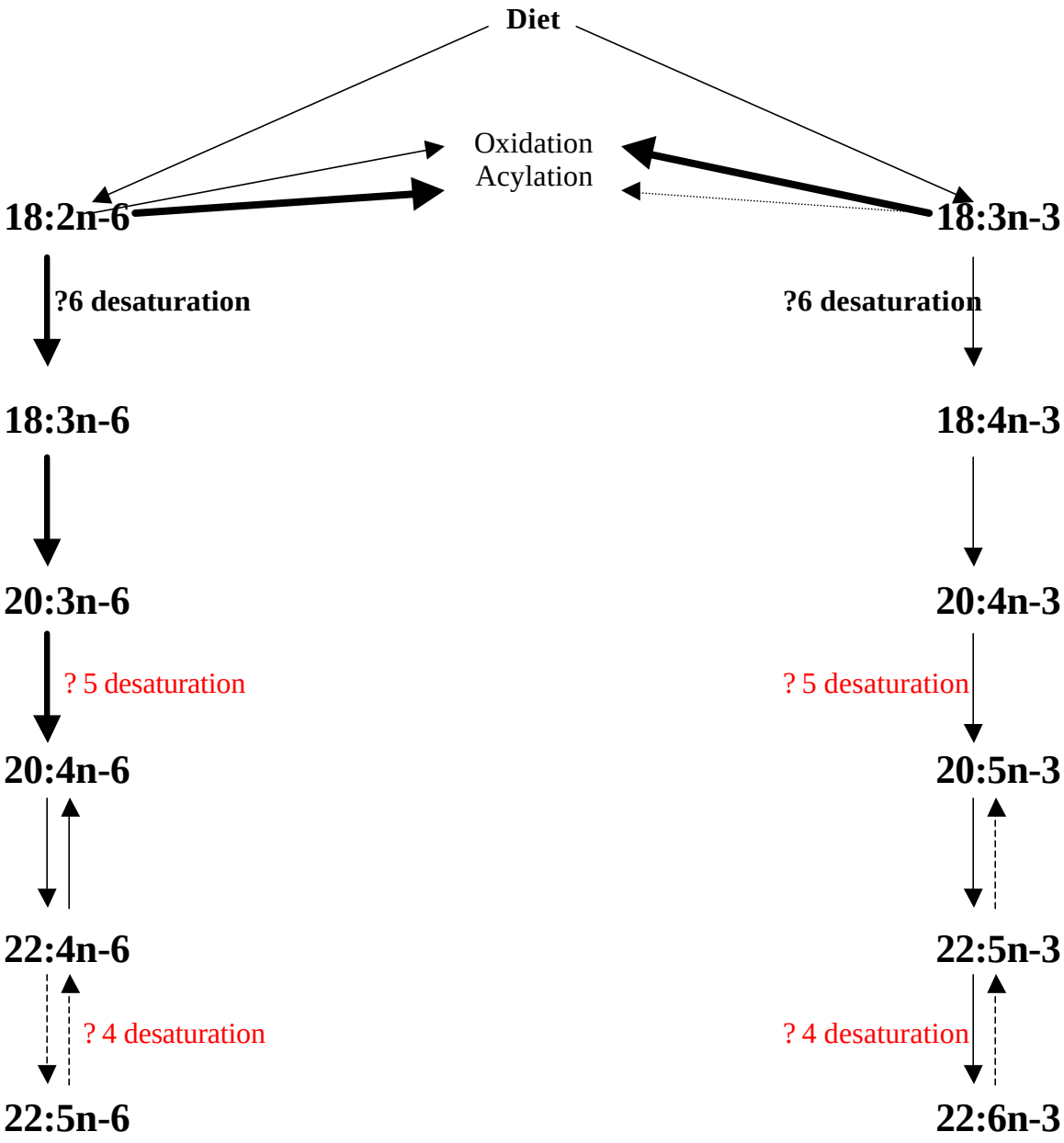

Figure 1. Metabolic scheme of the major essential fatty acids, with different arrow intensities indicating the relative predominance of the pathway under no dietary deficiency.

Innis, 1991

degree of DHA depletion is dependent on this ratio of n-6 to n-3 found in the feed fed to animals. At high levels of LA (18:2n-6), ALA (18:3n-3) metabolism to DHA is suppressed through competitive inhibition of ? 6-desaturase. Typically the use of pure linoleic acid, as when feeding safflower, sunflower, or peanut oil, will give the effect of lowering DHA levels due to the high ratio of n-6 to n-3 of 150 to 1 (Neuringer *et al.*, 1988).

Rainbow trout clearly require n-3 fatty acids in their diet. When deprived of these fatty acids, reproduction and growth are negatively influenced, feed conversion is not efficient, and a stress-induced shock syndrome that can lead to death appears (Castell *et al.*, 1972; Yu and Sinnhuber, 1972; Castledine and Buckley, 1980). Dietary n-3 fatty acids have also been implicated in normal adult emergence and wing development of some moths (Dadd, 1983; Stanley-Samuelson and Dadd, 1984).

Mammalian EFA dietary deficiencies have not shown clear negative signs and have not been easy to identify. Results from the studies of Lamptey and Walker (1976) and Tinoco *et al.* (1971), involving two to four generations of rats deprived of n-3 fatty acids did not reduce fertility, birth weight, or postnatal growth, and did not create any skin changes or organelle problems as seen with fish and insects.

One reason mammalian dietary deficiency signs have eluded researchers may be due to the bodies' ability to compensate for the EFA deficiency by replacing or converting available fatty acids. The replacement or conversion of one EFA to another may not provide the ultimate functionality nature intended, but may differ so minutely that it goes unseen in objective studies. Yu and Sinnhuber's (1972) work feeding either purified 22:6n-3 fatty acids or 18:3n-3 fatty acids to trout did not change their growth rate

or feed conversion. When substituted with 18:3n-3, only a small increase in 18:3n-3, 18:4n-3, 20:5n-3, and 22:5n-3 fatty acids was seen, as opposed to the substantial increase in 22:6n-3, indicating the bodies' ability to adapt and convert its lipid intake to the fatty acids needed.

Mohrhauer and Holman (1963) observed an increase in n-6 fatty acids, namely 22:5n-6, when DHA (22:6n-3) decreased. Moreover, the total level of PUFA did not change and the degree of polyunsaturation changed slightly (Calli *et al.,* 1971; Lamptey and Walker, 1976; Bourre *et al.,* 1984; Anding and Hwang, 1986). Typically, very small amounts of 22:5n-6 fatty acids are usually seen in animal tissue. Expanding on this work, Youyou *et al.* (1986) did the reversal by taking n-3 fatty acid deficient rats and supplementing their diet with a rich 18:3n-3 source. This feeding regimen caused DHA levels to increase by d 15 and by d 90 they were back to control values, whereas 22:5n-6 levels decreased.

Linoleic acid is thought to satisfy the EFA requirements of mammals. Although LA and ALA are needed to support growth, development, and reproduction, LA is the sole factor determining dermal integrity (Sardesai, 1992). Sinnhuber *et al.* (1972) showed a specific requirement for n-3 fatty acids, but this only applied to the fish.

Arachidonic acid (20:4n-6) and eicosapentaenoic (20:5n-3) acids are the major source of thromboxanes, cyclic endoperoxides, prostacyclins, PG, and leukotrienes found in the body. The n-3 series fatty acids have been implemented in modulating the production of active eicosanoids from the n-6 fatty acid series (Leaf and Weber, 1988). Prostaglandins can have a dramatic effect on reproductive and cardiovascular performance. The types of dietary lipids in a diet have a profound influence on the fatty acid composition of bone lipids (Alam *et al.,* 1994). Arachidonic acid concentration

fluctuates with different experimental diets. Because AA is a precursor of PGE_2, a significant reduction in its concentration may result in reduced levels of eicosanoids in the alveolar bone, leading to a reduction in PGE_2 (Alam *et al.*, 1994).

Semen contains large amounts of prostaglandins (Poyser, 1981) and has been implicated in preventing infertility of EFA deficient rats (Hafiez, 1974). Testicular degeneration occurred when Leat *et al.* (1983) fed male rats a EFA deficient diet. Also, EFA deficient females are unable to give birth to their offspring due to the inability to produce enough PGF_2. The reproductive functions of the male and female rat can be restored by feeding 18:2n-6, but not 18:3n-3, due to PGE_3 and PGF_3 inactivity, although, 18:3n-3 is able to sustain fetal growth throughout the pregnancy (Leat and Northrop, 1981).

Linoleic acid (18:2n-6) deficiency leads to decreased amounts of 20:2n-6, resulting in a diminished ability to synthesize thromboxane. Therefore, thromboxane-stimulated platelet aggregation and blood vessel vasoconstriction is inhibited, and second-phase platelet aggregation cannot occur. Dietary LA will restore normal thromboxane synthesis, function, and platelet aggregation (Sanders, 1988).

Essential fatty acid deficiencies have been shown to cause abnormalities in the QRS complex of an electrocardiograph (Hohl and Rosen, 1987). This disruption indicates impaired electrical conduction which appears to result from a deficiency of structural lipids rather than the failure to produce eicosanoids. Due to the ability to manipulate cardiac phospholipids with the diet, a dietary treatment of 18:2n-6 or 18:3n-3 fatty acids can correct the impaired electrical conduction (Hohl and Rosen, 1987).

Membrane Lipids

Essential fatty acids are vital components of the structural lipids forming the

phospholipids found in cell membranes, mitochondria, and nuclei. Polyunsaturated fatty acids give the membrane their fluidity in addition to other properties, and are also required to maintain the structural integrity of tissues. The properties of the phospholipids rely on the fatty acid chain length and the degree to which they are unsaturated. The EFA dietary intake may change membrane component insertion, aggregation, diffusion, receptor affinity and activity, membrane permeability and transport properties, and several cellular functions (Seiler, 1971; Gill and Clark, 1980; Heron *et al.*, 1980; Mead, 1984).

The erythrocyte membrane deformability has been shown to decrease with incorporation of 20:5n-3 (Terano *et al.*, 1983). Mitochondrial membrane deformations, occurring from EFA deficiencies can be corrected upon dietary treatment with 18:2n-6, 20:4n-6 or 22:6n-3 (Houtsmuller, 1973). Some derivatives of the n-6 and n-3 series PUFA can perform several functions of the EFA, but this does not exclude the idea of each series having specific functions of their own. Phospholipid composition is known to vary within the same animal and have different specificity for certain PUFA (Sanders, 1988).

Fertility

The idea of selecting toms early in production based on their seminal traits or sperm characteristics has caused several researchers to perform turkey semen evaluation studies over the past few decades. The amount of work invested in understanding the link between the tom and fertility is easily justified when evaluating the impact poor semen quality has on turkey production. The U.S. turkey industry produces millions of turkeys annually for food consumption. Research on artificial insemination (AI) in turkeys has yielded conflicting information regarding optimum semen dosage (Parker 1946; Lorenz

1950; McCartney 1952, 1954; Brown 1974), frequency of insemination (Lorenz 1950; McCartney 1952; Brown 1974; Van Krey *et al.,* 1976), optimal time of insemination (Wyne *et al.,* 1959; Christensen and Johnson 1975), optimal depth of insemination (Biellier *et al.,* 1961; Ogasawara and Fuqua 1972; Wentworth *et al.,* 1975), and semen volume and concentration (McCartney 1956; Cherms 1968; Brown 1968; Nestor and Renner, 1968; Brown *et al.,* 1970; Kammerer *et al.,* 1972; Van Krey *et al.,* 1976).

To insure high levels of fertility in virgin turkey hens, the industry typically attempts to follow nature's increasingly high levels of mating just prior to the onset of sexual maturity. Commercially, virgin hens undergo AI 3 times in succession, within 3 to 4 days, following onset of lay, after which hens are inseminated at 7 – 14 day intervals throughout the production season (Brown 1974; Van Krey *et al.,* 1976; Ogasawara and Rooney, 1996). This procedure is thought to fill the sperm glands of the hen and prevent fertility arrest (Verma and Cherms, 1965).

The idea of selecting "superior" toms has given rise to the development of assays and semen evaluation methods that test the quality of pooled semen (Sexton, 1983). These assays and methods, typically looking at sperm viability (Bilgili and Renden, 1984; Bilgili *et al.,* 1985,1987; Bayyari *et al.,* 1990; Donoghue *et al.,* 1995), sperm plasma membrane integrity (Bakst *et al.,* 1991; Donoghue *et al.,* 1996) and sperm metabolic functions, (Cooper and Rowell, 1958; Wishart, 1982; Wishart, 1984; Cecil and Bakst, 1993; Wishart and Palmer, 1986; Chaudhuri and Wishart, 1988) have not been conducive to "in field testing".

Depth of Insemination. Variations among AI techniques, such as depth of insemination, can greatly influence the rate of fertility. Lorenz (1959) recommended deep semen deposition, whereas Rooney *et al.* (1966) found no fertility differences when

inseminating Large White and Bronze hens at 1.27 cm or 5 cm. Ogasawara *et al.* (1968) reported optimal fertility with insemination $\geq$ 5 cm in which the spermatozoa were being placed close to the storage glands. Also, Biellier *et al.* (1961) found deep insemination, 8 cm, of Broad Breasted Bronze hens produced better fertility compared to inseminations 2.5 cm deep. However, work done by Wentworth *et al.* (1975), using three different lines of turkeys, including Bronze, Large White Inbred, and Large White Hybrid, showed significantly ($P<0.01$) greater fertility following 2 cm inseminations, compared with 7 cm deep insemination. Contrary to both Large White lines, the Bronze turkeys did not have consistently superior fertility with shallow insemination. These findings may be due to the different distances of the cloaca to the shell gland in each line. Asmundson (1939) reported that the Bronze hen had a 14.1 cm long vagina, whereas Dalrymple *et al.* (1968) and Besulin and Sakhatsky (1974) reported the Large White hen to have a 2.6 – 3.9 cm long vagina.

Wentworth *et al.* (1975) stated that the depth of insemination did not effect the duration of fertility in Bronze hens, but Large White lines did obtain a longer duration of fertility with shallow inseminations. The site of deposition with both shallow and deep insemination was also evaluated using Rhodamine B dye or red latex. During shallow inseminations of Large White hens, the markers were found in the vagina independent of depth of inseminations. Deep insemination penetrated 8 out of 15 UV junctions and 2 out of 15 vaginal walls.

Duration of fertility. Lorenz (1950) reported the Broad Breasted Bronze hen duration of fertility, after an insemination of either 0.050 or 0.025 mL. of semen, to average 38 d with a maximum of 57 d, whereas Hale (1955) reported the duration of fertility to average 43 d with natural mating. Woodard *et al.* (1976) reported neither

frequency of insemination, age, dosage of semen, nor level of productivity of the tom had an effect on the duration of fertility. He went on to state that the duration of fertility was longer, 42 d vs. 37 d, with 3 or 2 inseminations of 0.030 mL rather than 1, respectively. One thing to note is that when evaluating the duration of fertility, all researchers report this number in average d, which may be easily skewed by one atypical bird. The duration of fertility seems to vary among bird lines, but they all fall in the 35 – 70 day maximum reported for turkeys (Lorenz, 1950),

Semen volume and concentration. Several researchers have studied the effect of semen volume and concentration on fertility. McCartney (1956) and Cherms (1968) found no relationship between sperm concentration and fertility. However, Kammerer *et al.* (1972) did find 1 out of 6 periods to show significance between sperm concentration and fertility. None of these reports found a significant positive correlation between fertility and semen volume. During a fertility study where turkey hens were AI with 0.025 mL semen biweekly, Nestor and Brown (1976) reported a correlation between fertility and semen volume. In addition, a significant ($P<0.05$) relationship between fertility and sperm concentration was found to exist. The significant correlation between fertility and semen volume found by Nestor and Renner's (1968) work suggested that toms could be selected for fertility midway through the breeding season based on semen volume. Also, the significant correlation found between fertility and sperm concentration late in the reproductive period is supported by work done by Kammerer *et al.* (1972) and Brown *et al.* (1970). Recently, during a mobility phenotype experiment conducted by Holsberger *et al.* (1998), no significant differences were found among the mean ejaculate volumes or sperm concentrations between high and low mobility phenotype toms.

McCartney (1952) found no fertility differences among White Holland turkey hens when inseminating with 0.025, 0.0125, or 0.010 mL of semen. Also, Van Krey *et al.* (1976) found no difference in initial fertility when inseminating with 0.025 mL vs.0.050 mL. However, differences were seen fourteen days after the last insemination. The 0.025 mL amount of semen would appear to be more than sufficient in fertilizing capacity.

Motility. Brown and Graham (1971) made the observation that hens inseminated with less motile sperm produced significantly fewer fertile eggs, compared to hens inseminated with highly motile sperm. Kammerer *et al.* (1972) found a positive correlation between motile sperm and percent fertility, and that the relationship between number of motile sperm per ejaculate and fertility was consistent and could be used as a prediction indicator.

Recently a Sperm Mobility Test was developed by Froman and McLean (1996) where they tested sperm mobility by the ability of the sperm to penetrate a 6% (wt/vol) Accudenz® layer. After differentiating toms into minimal and maximal sperm motility categories based upon the Sperm Mobility Test, Froman and McLean (1996) found the maximal motility groups had higher fertility than the minimal motility groups. These findings were consistent with the research of Froman *et al.* (1997) where the Sperm Mobility Test was used to separate semen donors from a flock of roosters. Also, Donoghue *et al.* (1998) observed a significant increase in fertility of turkey hens inseminated with high sperm mobility phenotype toms during a 16-wk fertility trial compared to hens AI with low sperm mobility phenotypes. To support the sperm mobility phenotypes use as a sire selection tool, Holsberger *et al.* (1998) tested the initial sperm mobility phenotype for time dependency. They found no significant change over a 5 mo

period in high mobility phenotype toms and no change at all among low mobility phenotype toms after the first mo.

Nissen and Kreysel (1983) demonstrated a correlation between sperm motility and sperm docosahexaenoic acid in humans and reported the relationship was not linear but could be calculated exponentially. The human ejaculates obtained with poor sperm motility contained lower amounts of docosahexaenoic acid and produced more lipid peroxides, therefore, supporting the idea that low motility leads to poor fertility.

Perivitelline Layer. The ovum descends through the infundibulum immediately following ovulation where fertilization normally takes place (Bramwell *et al.*, 1995). If successful fertilization is to occur, the sperm must penetrate the original inner perivitelline layer (PL), directly over the germinal disk (GD), which surrounds the ovum at ovulation (Romanoff, 1960). Additionally, an outer PL encloses the ovum as it nears the portion of the oviduct adjacent to the ovary. Spermatozoa must digest and penetrate the PL in order to reach and unite with the female pronucleus (Bakst and Howarth, 1977; Okamura and Nishiyama, 1978; Howarth, 1984; Koyanagi *et al.*, 1988). Bramwell and Howarth (1992a,b) have used these holes in the PL to quantify the sperm penetration in recently ovulated ova *in vitro*. Using their quantifying technique, Bramwell and Howarth (1992a) suggested that sperm preferred to penetrate directly above the GD.

Bramwell *et al.* (1995) reported a significant positive correlation between GD sperm penetration and fertility ($r = .89$, $P<0.0001$) in naturally mated birds and a similar positive correlation between GD sperm penetration and fertility in AI birds ($r = .90$, $P<0.0001$). Also, Bramwell *et al.* (1995) observed an increase in fertility in relation to sperm penetration (SP) of the GD as sperm dose increased from 25 million sperm to 100 million sperm. When inseminating hens with fresh semen or 24 h *in vitro* stored semen,

Donoghue (1996) observed large variation among treatments with 3 to 381 and 2 to 460 holes per egg, respectively. Due to the hole variation by week, no differences were observed within the treatments, therefore, the data collected over a 10-wk period were combined. Upon combination of the data, the mean number of holes in the PL was significantly greater for the fresh semen vs. 24 hr stored semen. However, there were no differences in fertility or hatchability between the fresh and stored semen when analysis for fertility and hatchability was completed.

Spermatozoa lipid content. When comparing young (25 wk) to old naked neck broiler breeders (60 wk) of the same genetic stock, Kelso *et al.* (1996) observed many differences in their semen characteristics. The spermatozoa phospholipids, free cholesterol, and free fatty acids increased in the older birds whereas triacylglycerols and cholesterol esters decreased. The older bird's sperm concentration was significantly lower than the younger bird's sperm concentration (1.70 ± 0.57 vs. 2.08 ± 0.41). The live spermatozoa percent dropped significantly in the 60-wk birds compared to the 25-wk birds. While the glutathione peroxidase dropped significantly in the older group to just 9%, the younger age group's expression of superoxide dismutase stabilized. Also, a 75% drop in the metabolic activity of the old birds was evident, when compared to the younger birds. A combination of the spermatozoa lipid changes observed with old toms may play a significant role in the decreased fertility observed with older toms.

In addition to confirming the results of Kelso *et al.* (1996), Cerolini *et al.* (1997) also observed fertility changes they believed to be linked with the increased proportion of total sperm phospholipids that reached its maximum at 39 wk. Cerolini *et al.* (1997) reported semen concentrations increased from 24 to 39 WOA, remained steady at 39 to 54 WOA, and then dropped significantly at 72 WOA. The sperm motility also followed this trend, being highest at

24 to 39 WOA and dropping significantly during the second half of the reproductive period. Fertility remained unchanged, reaching its peak at 39 wk of age and gradually decreasing from this point. Supporting this data, Zalata *et al.* (1998) showed infertile human spermatozoa to have significantly less DHA, PUFA, n-3 monounsaturated fatty acids, total n-3 series fatty acids, and a lower double bonded index.

TABLE 1. The primary sights of deposition and dietary sources of fatty acids

Fatty acid	Deposition	Diet sources
a -Linolenic acid (18:3n-3)	Tissues (minor component)	Leafy vegetables & vegetable oils
Eicosapentaenoic acid (20:5n-3)	Tissues (minor component)	Shellfish and fish
Docosahexaenoic acid (22:6n-3)	Membrane phospholipids (major comp.): sperm, testes, retinal photoreceptors, and cerebral gray matter	Shellfish and fish
Linoleic acid (18:2n-6)	Most tissues	Vegetable oils (not all)
Arachidonic acid (20:4n-6)	Most membrane phospholipids (major component)	Meat, liver, brain
Docosapentaenoic acid (22:5n-6)	Most tissues (low amounts) Note: replaces 22:6n-3 during n-3 dietary deficiency	
Oleic acid (18: ln-9)	Tissues (major component)	Animal and vegetable fats
Eicosatrienoic acid (20:3n-9)	Accumulates during EFA deficiency	

(Neuringer, 1988)

TABLE 2. Fatty acid composition of selected vegetable oils (% of fatty acids)

Oil	14:0	16:0	18:0	18:1	18:2n-6	18:3n-3	20:0	20:1	20:5
Soybean		10	4	25	54	7			
Safflower		7	2	14	76	0.5			
Sunflower		7	5	19	68	1			
Corn		11	4	24	54	1			
Cottonseed	1	22	3	19	54	1			
Linseed		5	4	21	16	54			
Menhaden	9.5	20.3	3.6	14.4	1.2	1	0.7	1.8	18.1

(Innis, 1996;1991)

EVALUATION OF TOM FERTILITY AS AFFECTED BY DIETARY FATTY ACID COMPOSITION

OBJECTIVE

The objective of two experiments was to determine the effects of enhancing the levels of either n-3 or n-6 PUFA in the diet on tom turkey fertility. The effects of dietary lipids on hatchability, motility, perivitelline layer sperm penetration, live vs. dead sperm cells, sperm lipid content, and tom body weights also were determined.

MATERIALS AND METHODS

Animals and Diets

Large White turkeys of a commercial Nicholas strain where hatched and maintained under standard husbandry practices at the Virginia Polytechnic Institute and State University Turkey Center. Trial 1 and Trial 2, unless stated otherwise, where conducted under the same conditions during 1999 and 2000, respectively. Birds were hatched in the fall and trials were conducted during the fallowing summer and fall mo.

The birds were housed in floor pens in a controlled environment, with feed and water available for *ad libitum* consumption. Table 3 shows the dietary schedule provided. All poults were exposed to constant light until 17 WOA. The photoperiod was then reduced to 14 h of light (L) and 10 h of darkness (D) and continued until 20 WOA. The photoperiod was further decreased to 7L:17D and remained there until 27 WOA. Toms were then photostimulated by increasing the photoperiod to 14L:10D and the hens were increased to 9L:15D. The hens, weighing approximately 10 kg, were photostimulated by increasing the lights at 31 WOA to 14L:10D. Day length was increased an additional 1 h at 42 WOA to reduce broodiness and improve production.

Trial 1. At 31 WOA, female turkeys (90) where weighed, wing-banded, and randomly assigned to 1 of 9 floor pens (10 birds per pen) with 4 trap nests. Male turkeys

(27) where weighed, wing-banded, and randomly assigned to 1 of 3 floor pens with 9 birds per pen.

Hens and toms were fed breeder diets at 31 WOA. Diet compositions are shown in Table 4. At 37 WOA toms where provided either a control diet containing 5% (wt/wt) CF, a diet substituted with 5% (wt/wt) FO rich in DHA acid (22:6n-3), or one substituted with 5% (wt/wt) soybean oil (SBO) rich in LA (18:2n-6). Fatty acid compositions are shown in Table 5. The 6-wk prior to switching the toms to diets containing various lipids (37 WOA) provided a preliminary period with which to compare the remaining results. Trial 1 had 9 toms per treatment and ended at 54 WOA, whereas Trial 2 had 10 toms per treatment and ended at 56 WOA. Also, at 49 WOA toms were taken off their substituted diet and placed back on their initial diet during Trial 1. Throughout the duration of the experiments respective feeds were made biweekly and stored at room temperature.

Trial 2. During Trial 2 more pens were provided, giving the turkeys more room. At 31 WOA, female turkeys (84) where weighed, banded, and randomly assigned to 1 of 12 floor pens (7 birds per pen) with 4 trap nests. Male turkeys (30) where weighed, banded, and randomly assigned to 1 of 6 floor pens with 5 birds per pen.

Fatty Acid composition

Near the completion of Trial 2, three semen samples, pooled from each tom, were collected during 55 and 56 WOA enabling sufficient material for lipid analysis. Semen samples were extracted, and analyzed for their fatty acid content.

Semen Extraction. Semen samples were pipetted into 12x75 mm test tubes, with equal amounts of .85% (w/v) NaCl solution, vortexed, and centrifuged at 700 g for 20 min at 4ºC. The supernatant, containing seminal fluid, was discarded and the wash procedure was repeated with 1 mL .85% NaCl solution. Final pellet was resuspended in

2 mL .85% NaCl and transferred to 50 mL glass test tubes. Each sample had 15 mL 2:1 chloroform:methanol added, vortexed, and allowed to stand at room temperature for 1 to 2 h. Tubes were then centrifuged for 5 min allowing separation and aspiration of the aqueous layer. The residual solvent layer was filtered through Whatman 42 filter paper and washed with 7 mL 2:1 chloroform:methanol. Four mL double distilled water was added to each tube then capped and shaken horizontally at high speeds for 10 min after which they were centrifuged at 3000 rpm for 5 min. The upper water layer was aspirated and the solvent was evaporated under nitrogen at 60ºC in an N-Evap until approximately 2 to 4 mL remained. The remaining solvent was transferred to 16x100 mm screw capped test tubes. Extraction tubes were washed twice with 2 mL amounts of 2:1 chloroform:methanol, evaporated under nitrogen at 60ºC in N-Evap, capped tightly, and stored at -20ºC.

Methylation. Each tube had 200 µL dichloromethane, 500 µL hexane containing 120 µg undecenoic acid as an internal standard, and 2 mL .5N NaOH in methanol added. Tubes were capped tightly, vortexed and heated for 10 min at 90 to 95ºC and then allowed to cool to room temperature. Two mL 14% boron triflouride in methanol was added to each tube and again heated for 10 min at 90 to 95ºC. After cooling to room temperature, 2 mL double distilled deionized water and 1 mL hexane was added. Capped tubes were shaken horizontally at high speeds for 10 min then centrifuged at 200 g for 5 min. Upper hexane layers containing the methyl esters of the fatty acids were transferred to chromatography vials containing sodium sulfate. Vials were sealed with crimp tops and stored at -80ºC until further analysis.

Chromatographic Conditions. A Hewlett Packard 5890 gas chromatograph fitted with a 7673 autosampler and ChemData Station with 8.0-aquisition software was used.

The column was a capillary CP-Sil 88 for FAME, 100 m x .25 mm ID x .2 μm film thickness.[1] Ultra high purity hydrogen was used as carrier gas with a flow rate of 23.0 mL/min and a linear velocity of 23.1 cm/s at 175ºC. A flame ionization detector (275ºC) was used. Injection volume was 1.5 to 2.5 μL using 1:30 or 1:25 split ratios (injector temperature = 250ºC). The column temperature was gradually increased 5ºC/min from 70ºC (held 1 min) to 100ºC. After 2 min at 100ºC the temperature was increased 10ºC/min to 175ºC (held 40 min) and finally increased to 250ºC at 5ºC/min (hold 15 min).

Measurements

Sperm mobility, fertilizing ability, concentration, live-dead staining, and holes made in the perivitelline layer of laid eggs were analyzed monthly in order to help elucidate any effect of diet on spermatozoa. Individual bird weights were recorded monthly to the nearest 0.01 kg.. Feed consumption per bird was determined monthly, and daily egg production per hen was recorded.

Insemination and Egg Handling. The semen was collected by abdominal massage twice before 31 WOA (Burrows and Quinn, 1937). Starting at 31 WOA, semen was collected twice per wk, once for artificial insemination and another to collect semen for analysis. Semen samples (n = 4) were pooled by dietary treatment, providing enough semen to inseminate 30 hens previously assigned to the treatment group. The pooled semen samples were diluted 1:1 with Beltsville poultry semen extender – II, and hens were inseminated, 1 cm depth, within 25 min of collection with 0.050 mL semen.[2] Three pens of hens were inseminated per male dietary treatment during Trial 1, and 4 pens of hens were inseminated during Trial 2.

[1] Varian Associates, Palo Alto, CA
[2] Continental Plastic Corp., P.O. Box 902, Delavan, WI 53115-0902

Egg collection started at first egg (33 WOA) and continued throughout the trial. Eggs were collected daily and stored at 12ºC and 75% relative humidity until a full wk of eggs could be set. The eggs were incubated at 37.5ºC and 60% relative humidity and were turned automatically for 25 d. The eggs were then transferred to a hatcher with a temperature of 37.5ºC and 70% relative humidity for 3 d. The poults were euthanized and recorded as hatched. Eggs that did not hatch were broken open and recorded as infertile, early-dead, mid-dead, late-dead, or pipped. Those containing milky white albumen, no embryo, or brownish albumen were considered infertile. Embryos without visible formation of eyes were classified as early-dead. Mid-dead were classified as embryos with large black eyes, but lacking feather formation. The late-dead embryos were those having feather formation.

Sperm Mobility. Semen was collected from the toms once per mo to evaluate sperm mobility using the Sperm Mobility Test (SMT; Froman and McLean, 1996; Donoghue *et al*., 1998). Ejaculates were diluted to 5 X 10^8 sperm/mL in 111 mM NaCl buffered with 50 mM N-tris-[hydroxymethyl]methyl-2-amino-ethanesulfonic acid (TES), pH 7.4, containing 25 mM glucose and 4 mM $CaCl_2$. A 60-µL sample of each sperm suspension was overlaid onto 600 µL of prewarmed 6% (wt/vol) Accudenz® solution in a semi-micro polystyrene disposable cuvette. Cuvettes were incubated for 5 min in a 41ºC water bath, placed in a spectrophotometer, and absorbance measured at 550 nm. Percentage of light transmission was recorded after 1 min (Holsberger *et al*., 1998).

Sperm Concentration. A hemacytometer was used to determined sperm concentrations of the semen samples used in the Sperm Mobility Test. A 1:100 dilution of the semen was prepared by mixing 10 µL of semen with 990 µl of 2% (wt/vol) acetic acid. Both chambers of the hemacytometer were loaded with the dilute semen sample

and allowed to settle for 3 min. Using a 40X objective microscope, the numbers of sperm cells in four large squares (64 small squares) were counted.

Live-Dead staining. Semen was collected from toms once per mo to perform live- dead spermatozoa counts. A drop of sperm was added to a drop of eosin B-aniline blue dye and placed on a glass slide (Shafter and Almquist, 1948; Casarett, 1953). A smear was made and the slide quickly dried with a blow dryer and then coverslipped. Using a 4X objective microscope fitted with a 10X eyepiece, the slides were examined for the number of live vs. dead sperm in one field of view, counting up to 300 sperm per slide. The live vs. dead sperm are reported as a percent of the total sperm counted per slide.

Perivitelline layer. The perivitelline-hole assay was used as an indicator of the effects of male diet on the toms' fertilizing ability (Bramwell *et al*., 1995; Donoghue, 1996). Eggs collected for 1 wk during each mo of production were used for the perivitelline-hole assay, and the ovum was separated from the albumen. The chalazae was cut away using small dissecting scissors. The ovum was submerged in 1% (w/v) NaCl for 25 s. A section of the PL, ~ 15 X 25 mm, was then cut so as to include the portion directly over the GD. The PL section was held with fine forceps and gently washed with phosphate-buffered saline (PBS), removing the remnants of the yolk. The PL section was then mounted on a glass microscope slide, fixed with four drops of 3% (w/v) paraformaldehyde (PFA), and allowed to stand for 15 s until the slide was tipped to drain the PFA. One small drop of undiluted Schiffs' Reagent was allowed to stay on the slide until purple coloration appeared (several seconds).[3] The slide was then tipped to drain excess stain. Under a bright field microscope using a 10X objective, fitted with a 10X eyepiece, the circular unstained holes caused by sperm digestion were easily

detected. One field of view of holes created in the PL due to SP was counted (Donoghue *et al*., 1998).

Statistical analysis

Unless stated otherwise, statistical significance was declared at $P \leq 0.05$. Data were analyzed using the Univariate Mixed Model procedure of SAS® (SAS Institute, 1988). Dietary treatments were FO and SBO, with CF serving as the control. Sperm mobility, fatty acid composition, tom body weight, and live-dead stains were based on analysis of individual birds within each treatment group. Akaike's information criterion fit statistics were used to uncover the best correlation model for the repeated measures command used for every analysis preformed. All percentage data were arcsin transformed prior to analysis. Lsmeans procedure of SAS® was used to obtain the equivalent of Tukey's honestly significant difference comparisons (SAS Institute, 1988). Means, standard error, minimum, maximum, and coefficient of variation of the means were calculated using the Mean procedure of SAS® (SAS Institute, 1988).

[3] Sigma Chemical Co., St. Louis, Missouri

TABLE 3. Diets[1] provided

	Feeding regimens			
Age[2]	HF	CF	FO	SBO
31-36[3]	TB2	TBD	TBD	TBD
37-56[4,5]	TB2	DT	DT	DT

[1] TB2 = Turkey Female Breeder; TBD = Turkey Male Breeder; DT = Dietary treatment applied.

[2] Birds age in wk.

[3] Birds feed breeder diets for 6 wk;

[4] Toms TBD fat sources were substituted with chicken fat, fish oil, or soybean oil, respective to treatment group at 37 wk of age.

[5] Toms of the 1999 trial were switched back to TBD at wk 49 and the trial ended at 53 wk of age.

TABLE 4. Composition of diets

Ingredient	Diets				
	T1	T2	T3	TB2[1]	TBD
			(%)		
Ground yellow corn	42.20	58.95	64.78	68.80	69.95
Stabilized Fat[2]	3.50	0.18	0.25	2.00	----
Gluten	----	----	----	----	9.00
Wheat middlings	----	----	----	----	9.00
Fat[3]	----	----	----	----	5.00
Dicalcium phosphate	1.00	1.00	1.00	1.00	1.96
Dehulled soybean meal	38.00	33.25	28.50	8.75	2.32
Menhanden fish meal	7.50	5.63	3.75	5.00	----
Meat and bone scrap	5.00	5.00	5.00	5.00	----
Dehydrated alfalfa meal	1.25	1.25	1.25	2.50	----
Dried whey	----	----	----	2.50	----
Ground limestone	0.50	0.50	0.50	3.50	1.73
Iodized salt	0.50	0.50	0.50	0.40	0.35
L-lysine	0.11	0.09	0.14	----	----
Copper sulfate	0.05	0.05	0.05	----	----
Trace mineral mix[4]	0.05	0.05	0.05	0.05	0.10
Vitamin mix[5]	0.50	0.50	0.50	0.50	0.50
Dietary Analysis:					
Crude protein (%)	29.60	26.70	23.80	17.00	10.21
Metabolizable energy (kcal/kg)	2,970	3,047	3,124	3,036	3,180

[1] Calcium min = 2.25%, max = 2.75%, and available phosphorus = 0.60%

[2] A commercially available, stabilized animal/vegetable blend.

[3] Three fat sources: Chicken Fat, Fish Oil, or Soy Bean Oil

[4] Trace mineral mix: Cobalt 0.45 mg; Copper 5 mg; Iodine 2 mg; Manganese 120 mg; Zinc 120 mg; Iron 40 mg; Calcium carbonate as a diluent.

[5] Vitamin mix supplied the following per kilogram of diet: Vitamin A 2,273IU; Vitamin D_3 682 ICU; Vitamin E 7 IU; Menadione sodium bisulfite 0.24 mg; Thiamine HCl 0.20 mg; Riboflavin 1.4 mg; Calcium D-pantothenate 3 mg; Niacin 9 mg; Choline chloride 67 mg; Vitamin B_{12} 0.003 mg; Vitamin B_6 0.19 mg; Folic acid 0.23 mg; Biotin 0.011 mg; Selenium 0.09 ppm; Ethoxyquin 249.45 mg (as a preservative).

TABLE 5. Fatty acid composition of fat substituted in diets

Fatty acid	Chicken Fat	Soybean Oil	Menhaden Fish Oil
		µg/g	
18:1n-9	16,086	8,827	2,305
18:2n-6	7,154	18,135	241
18:3n-6	0	0	115
18:3n-3	0	0	227
20:1n-9	0	98	361
20:4n-6	0	0	74
20:5n-3	0	0	730
22:5n-3	0	0	130
22:6n-3	0	0	775
Total n-6	7,154	18,135	430
Total n-3	0	0	1,862
Ratio:			
Total n-6/n-3			0.23

RESULTS AND DISCUSSION

Fatty Acid Composition of Spermatozoa

While the data regarding spermatozoa fatty acid content may not be conclusive, due to the small sample size, the fatty acid analysis of spermatozoa collected at the conclusion of Trial 2 revealed a significant difference in total n-3 and n-6 content. This led to a significant difference in the ratio of total n-6 to n-3 (Table 6). Among the dietary treatments, the toms provided CF or SBO (n-6 rich diets) contained significantly higher amounts of n-6 PUFA in their spermatozoa. Additionally, the FO treated toms spermatozoa contained significantly higher amounts of n-3 PUFA.

Body Weight

Figure 2 shows tom BW from 31 through 54 WOA during Trial 1. The decline in BW at the beginning of the trial can be attributed to stress that was encountered when placing several new toms together in a pen. A new social hierarchy was established during which time the toms fought with each other. Once the fighting had diminished, feed consumption was observed to increase simultaneously with BW. No significant differences existed between dietary treatments at any age. Although toms in Trial 2 started approximately 1 kg heavier than Trial 1, a similar trend was seen in their BW gains (Figure 3). Again, no significant differences existed between BW and treatment when compared weekly. It is apparent that the dietary fat supplementation with CF, SBO, or FO did not have a significant effect on tom BW; therefore, male BW was not a factor contributing to any fertility differences in these studies.

Live-Dead staining

Percent live-dead spermatozoa for Trial 1 and 2 are shown in Tables 7 and 8, respectively. In Trial 1 there was a significant difference in the percent live sperm

between treatments at 36 WOA, which was prior to providing the dietary treatments (Table 7). After dietary treatment application, at 37 WOA, the differences disappeared and were not seen again until 53 WOA, where a significance ($P<0.05$) was seen 4 wk after dietary treatments had been removed at 49 WOA. Although all toms used in both trials were of the same genetic stock, individual traits may have been the cause of these significant findings.

The differences seen at wk 36 may be attributed to faulty technique because these were the first slides made using this technique. Wilson *et al.* (1968) observed significantly ($P<0.01$) higher cock spermatozoa death when preparing slides with nigrosin-eosin stain vs. trypan blue stain. When preparing the slides during the present studies, the stain had to be dried very quickly, via a hair dryer, or all the spermatozoa would appear dead with a purple to pink coloration. The percent live spermatozoa in Trial 2 did not differ among dietary treatments (Table 8). Therefore, dietary fat appeared to have no effect on sperm viability.

Many experiments have been conducted in attempts to find a correlation between sperm viability and fertility. Whereas several researchers have found a low correlation between percent viable sperm and fertility (Sexton, 1988; Bakst *et al*., 1991), more recently Donoghue *et al*. (1996) and Bakst *et al*. (1991) found no differences in viable sperm between low or high mobility phenotype tom turkeys. Because mobility is strongly correlated to fertility (Holsberger *et al*., 1998), it may be a better indicator of fertility than sperm viability. Due to their poor predictive ability of avian fertility, the traditional measures of semen quality such as sperm concentration, viability, and semen volume are being used less frequently (Donoghue, 1999).

Mobility

In Trial 1, dietary supplementation had no significant effect on sperm motility until 48 WOA (Figure 4). At 48 WOA, mobility of sperm from toms fed SBO was significantly lower than that of the remaining groups ($P<0.01$). The toms were all switched back to their initial diet at 49 WOA. At 51 WOA, the mobility of the sperm from toms previously provided FO and SBO was higher than that of toms fed CF. Motility in the second trial was not significantly different until 53 WOA (Figure 5). At 53 WOA, mobility of sperm from toms fed SBO was significantly higher than that of the CF and FO groups ($P<0.01$). At 56 WOA, the mobility of toms fed FO was significantly lower than that of the other 2 groups ($P<0.05$).

Nissen and Kreysel (1983) found that poorly motile human spermatozoa had lower DHA compared to spermatozoa with good motility. Also, they found immobile or poorly motile spermatozoa having more lipid peroxides than fully motile spermatozoa. More recently Cerolini *et al*. (1997) revealed a negative correlation between motility and the levels of the 16:0, 18:0, and 18:1n-9 fatty acids, whereas the 22:4n-6 and 22:6n-3 were positively correlated. An increase in motility was seen with an increase in sperm 22 PUFA (Cerolini *et al*., 1997). Both n-6 and n-3 EFA have been implicated in motility and overall fertility. The ratio of these n-6 and n-3 EFA may be the factor of main importance when trying to assess avian semen quality, and more importantly, the motility that can be expected from a given ratio of n-6 to n-3. Blesbois et al. (1997) observed consistently higher fertility rates during a 4-wk trial with a low 2.8 ratio of n-6 to n-3 compared to a high ratio of 154. Recently Kelso *et al*. (1997) observed no differences in sperm motility during a 49-wk experiment when feeding a rich n-6 or n-3 diet.

During the present studies we followed a similar protocol used for chickens while using the SMT, unaware of the slight change in the 6% (wt/vol) Accudenz® layer required when using the SMT for turkey semen (Froman and McLean, 1996). The SMT has been found to be effective with turkey semen when using a 4% (wt/vol) Accudenz® layer. Even with this Accudenz® change, the turkey sperm will penetrate the 4% (wt/vol) Accudenz® layer slowly, as compared to chicken sperm using the 6% (wt/vol) Accudenz® layer. The rooster's greatest rate of SP occurs within the first 5 min, the theoretical turkey semen's rate of penetration would seem to be greatest during the initial 10 min of incubation in a 41 ºC water bath. The different rate of penetration of the Accudenz® layer may have led to inconsistent low absorbency readings.

Perivitelline layer

The range of holes due to SP observed in the PL of individual eggs varied among all three dietary treatments during both trials. The PL penetration ranges seen over time for CF, FO, and SBO were 0 to 234, 0 to 149, and 0 to 150, respectively for Trial 1. The mean SP values for the PL sections removed from ovipositioned eggs throughout Trial 1 were significantly different at 36, 48, and 52 WOA ($P<0.01$) (Figure 6). At 44 WOA the eggs were not analyzed due to unforeseen complications. The average number of hens (eggs/hen n = 3.5) observed for toms dietary treatment of CF (n = 48.8), SBO (n = 40.4), and FO (n = 53.6) varied considerably.

In Trial 1 the holes in the perivitelline membrane were generally lower for sperm collected from toms fed FO or SBO (Figure 6). The range of SP holes observed for CF treated toms were almost 64% greater than the SBO and FO treated toms during the mo of significant differences. No significant differences were observed during 40 or 54 WOA. The reason for the decrease seen for CF treated toms during 40 and 54 WOA has

yet to be elucidated. While all the toms briefly started to molt during the mo of August, it does not appear to have affected the CF substituted toms, as this effect would be consistent across treatments.

Similarly, the range of holes found to penetrate through the PL of individual eggs varied among all three dietary treatments during Trial 2 (Figure 7). The PL penetration ranges seen during Trial 2 were 0 to 145, 0 to 44, and 0 to 216 for CF, FO, and SBO substituted toms, respectively. The mean SP values for the PL in Trial 2 were significantly different during 42, 47, and 51 WOA ($P<0.01$). At 38 WOA the eggs were unable to be analyzed due to faulty processing (Figure 7). The average number of hens (eggs/hen n = 3.5) observed for tom dietary treatment of CF (n = 18.7), SBO (n = 20.0), FO (n = 22.7) varied considerably. Throughout the entire experiment the range of SP holes in the perivitelline membrane were lower for sperm collected from toms fed FO.

The large ranges of SP holes per egg may be attributed to the large dose (0.050mL) of semen used during AI of the hens. Other researchers have also experienced large ranges in SP of the PL when using large doses of semen for AI. Donoghue (1996), when observing the effects of fresh vs. stored semen on PL and SP, reported a range of 2 to 460 and 3 to 381 holes per egg, respectively, when inseminating with 100×10^6 viable sperm. Also, during the same trial, Donoghue inseminated with 10×10^6 viable fresh or stored semen and reported a smaller range of 0 to 53 and 1 to 42 holes per egg, respectively. Likewise Bramwell *et al.* (1995) reported SP values as high as 632 holes in the PL directly over the GD in naturally mated chickens. When hens were AI with 100, 50, or 25 million sperm, the mean SP holes for the GD PL area was 40.2, 19.5, and 14.1, respectively. Although Bramwell *et al.* (1995) did not report the ranges, the same trend

of increased SP holes can be seen among the hens that where inseminated with a larger dose of semen.

Using a smaller dose of semen during the AI process in the present studies would have led to a smaller range of PL holes and may have proven to be more beneficial for analysis. The PL holes may have been decreased with a smaller semen dose.

While the fertility of males decreases with age, a change in the proportion of spermatozoal phospholipids is also observed (Kelso *et al*., 1996; Cerolini *et al*., 1997; Kelso *et al*., 1997a). It is also well known that the lipid composition determines the membrane fluidity; making sperm lipid composition a very important factor in sperm motility (Stubbs and Smith, 1984; Salem *et al*., 1986). Recently Hazary *et al*. (2000) observed the number of holes hydrolyzed through the PL and fertility declined with age. They found approximately 200 SP holes in the PL corresponding to 94% fertility at the peak of production with a decline to 20 SP holes and 79% fertility at 55 WOA. As mentioned before, motility is correlated to fertility, and as theorized before, motility may be a function of the ratio of n-6 to n-3 in sperm. Therefore the variability of SP holes observed in the PL may be a direct response due to tom dietary treatment.

Fertility, Embryonic Mortality, and Hatchability

In Trial 1, there were no weekly differences in fertility, hatch of total eggs, hatch of fertile eggs, pipped eggs, early dead, mid dead, or late dead embryos. Therefore, data for all 20 sets of the study were combined for both fertility trials. Fertility was higher in hens inseminated with semen from toms fed CF compared to either FO or SBO (Table 9).

The observations made by Wishart *et al.* (1995) using the PL hole assay to predict fertility support the results of Trial 1. He observed 50% fertility when only 3 SP holes were found around the GD and 100% fertility when $\geq$ 6 SP were found. Other

investigators have also found a correlation between the number of holes created by sperm in the PL around the GD and fertility (Bramwell *et al.*, 1995; Donoghue, 1996).

In Trial 1, fertility, hatchability of total eggs, and hatch of fertile eggs was higher for the CF-substituted toms than for the FO-substituted or SBO-substituted toms (Table 9). Although embryonic mortality was high due to hatchery bacterial contamination, no differences between treatments were observed in percent early dead, mid dead, or late dead (Table 9). Also, during this time there were no differences found among shell pipped embryos of the different treatments (Table 9).

In Trial 2, there were no dietary treatment effects on percent fertility, hatch of total eggs, hatch of fertile eggs, pipped eggs, early dead, mid dead, or late dead embryos (Table 10). Contrary to Trial 1, fertility was greater than 96% in all treatments resulting in no differences between treatments (Table 10). It would appear that the differences in the PL SP holes during Trial 2 (Figure 7) did not correlate with changes in fertility. The observation made by Wishart *et al.* (1995) where 3 SP the area around the GD led to 50% fertility and 6 SP the PL located around the GD led to 100% fertility still applies, but the fertility was so high in all treatments no differences were observed.

The results of the present studies indicate some differences due to dietary treatments, but do not clearly correspond with each other. These inconsistent findings may have resulted from the availability of both n-6 and n-3 fatty acids in all diets. The 5% dietary fat substitution may have provided adequate amounts of both n-6 and n-3 fatty acids, confounding the evaluation of the effects of a single fatty acid source on the fertilizing capacity of turkey toms. Although the results do indicate some differences among treatments, the diets may well need to be purified in order to properly evaluate any effects dietary EFA may have on fertility.

Other researchers have had similar problems detecting significant differences among avian spermatozoa when feeding rich n-6 or n-3 sources. Blesbois *et al.* (1997) reported higher fertility rates in chickens when feeding salmon oil vs. corn oil. The salmon oil provided long chain PUFA of the n-3 series with a total n-6 to n-3 ratio of 2.3 compared to the corn oil ratio of 7.6. In agreement with this data, Kelso *et al*. (1997a) found that dietary supplementation with n-3 rich linseed oil to chickens produced a significant improvement in fertility only during wk 39 of a 49-wk dietary trial, when compared to a diet substituted with SBO rich in n-6. During this 49-wk dietary trial the chicken spermatozoan lipid composition did not contain large amounts of n-3 fatty acids although substituted with a rich n-3 diet.

These findings both refute and support the present finding. The work of Blesbois *et al.* (1997) and Kelso *et al.* (1997a) contradict the first findings of the experiment of improved fertility with CF, an n-6 source. The present results, in which there was no difference between SBO and FO, contradicts the results of studies in which significant differences between SBO and FO were found. During the present study the dietary FO may have contained sufficient amounts of both n-6 and n-3 PUFA, whereby masking the significant differences observed in other studies. If one excludes the 1 wk of significance found by Kelso *et al*. (1997a), their results would support Trial 2 where no significance was observed between dietary substitution of CF, SBO or FO.

Despite the inconsistent reports, both Blesbois *et al.* (1997) and Kelso *et al.* (1997a) agree that avian sperm seem to have a unique fatty acid composition, and the precise balance ratio of n-6 to n-3 may be crucial to optimizing avian fertility. Moreover, Kelso *et al.* (1997b) observed that the n-6 content of chicken spermatozoan with dietary fish oil substituted, and the resultant n-6 proportions were not higher than those found in

mammalian semen. Kelso *et al.* (1997b) stated the dietary supplementation of birds with n-3 fatty acids does not induce a dramatic conversion of the characteristic fatty acid profile of the chicken spermatozoa into that of the typical mammalian profile, but rather produces a somewhat hybrid composition. This is supported by a study (Kelso *et al.* 1997a) that showed no increase in 22:6n-3 in cockerels spermatozoan while being fed a diet containing linseed oil (rich in 18:3n-3).

IMPLICATIONS

The present studies may provide improvements in our understanding of the manner in which turkey fertilization capacity responds to diet composition. As a consequence of the inconsistencies between studies conducted in consecutive years, further investigation of the effects of the dietary ratio of n-6 to n-3 on fertilization is warranted.

TABLE 6. Percent of total fatty acid composition of tom semen

Fatty acid	Chicken[1]	Fish	Soybean	SEM[2]
		%		
14:0	0.57[a]	0.44[a]	1.10[b]	0.17
15:0	0.42	0.46	0.44	0.11
16:0	11.83[ab]	10.99[a]	13.39[b]	0.69
16:1	0.56[ab]	0.43[a]	0.79[b]	0.10
17:0	0.15[ab]	0.13[a]	0.26[b]	0.04
18:0	20.55[a]	19.60[ab]	18.90[b]	0.47
t6 or t7 18:1	0.02	0.00	0.01	0.01
t9 18:1	0.23[ab]	0.21[a]	0.31[b]	0.03
t10 18:1	0.14[a]	0.12[a]	0.28[b]	0.04
t11 18:1	0.11	0.10	0.17	0.02
t12 or c7 18:1	0.14[a]	0.25[b]	0.30[b]	0.04
t13 or c6 18:1	0.09	0.00	0.00	0.01
c9 18:1	6.20[a]	6.75[ab]	7.64[b]	0.47
c11 18:1	1.41	1.51	1.40	0.08
c12 18:1	0.11[a]	0.01[b]	0.03[b]	0.02
c13 18:1	0.14[ab]	0.09[a]	0.27[b]	0.06
t9 or t12 18:2	0.10	0.02	0.14	0.05
c9 or t12 18:2	0.17	0.09	0.14	0.03
t9 or c12 18:2	0.19[ab]	0.12[a]	0.28[b]	0.04
c9 or c11 18:2	0.21	0.20	0.13	0.04
c11 or c13 18:2	0.01[a]	0.00[a]	0.18[b]	0.04
t11 or t13 18:2	0.16	0.20	0.21	0.06
Other t18:2	0.11[a]	0.01[b]	0.21[a]	0.03
18:2n-6	4.90[a]	3.54[b]	5.44[a]	0.35
18:3n-3	12.56[a]	11.63[ab]	10.18[b]	0.65
20:0	0.90	0.89	0.85	0.05
20:3n-3	0.93[a]	0.96[a]	0.74[b]	0.06
20:4n-6	12.88[a]	8.28[b]	11.60[a]	0.47
20:5n-3	0.68	0.79	2.61	0.77
22:1	2.26[a]	1.88[a]	3.73[b]	0.50
22:4n-6	18.94[a]	8.57[b]	16.08[c]	0.87
22:5n-3	0.76[a]	6.61[b]	0.82[a]	0.12
22:6n-3	1.60[a]	15.13[b]	1.40[a]	0.51
Total n-3	16.51[a]	35.11[b]	15.75[a]	1.46
Total n-6	36.71[a]	20.39[b]	33.12[a]	1.35
Ratio:				
Total n-6/n-3	2.22[a]	0.58[b]	2.10[a]	0.14

[1] Means within a row lacking a common superscript are significantly different ($P<0.05$).

[2] Pooled standard error mean.

TABLE 7. Percent[1] Live Sperm for Trial 1

	Weeks of Age[3,4]					
Treatment[2]	36	43	47	50	53	SEM[5]
	(%)					
Chicken Fat	84.15[x]	91.15	88.05	91.15	82.88[x]	.0531
Fish Oil	73.18[x]	88.80	79.92	89.88	86.24[xy]	.0437
Soybean Oil	54.63[y]	96.70	83.88	89.03	93.63[y]	.0445

[1] Percent live cells calculated: live cells/ (live cells + dead cells).
[2] Male diets substituted with chicken fat, fish oil, or soybean oil.
[3] Toms age.
[4] Means within a column lacking a common superscript are significantly different ($P<0.05$).
[5] Pooled standard error mean.

TABLE 8. Percent[1] Live Sperm for Trial 2

Treatment[2]	Weeks of Age[3] 39	44	49	53	56	SEM[4]
			(%)			
Chicken Fat	89.03	97.19	92.22	80.40	92.11	.0773
Fish Oil	87.09	94.51	82.49	84.95	83.16	.0843
Soybean Oil	84.20	88.15	92.53	79.68	83.66	.0877

[1] Percent live cells calculated: live cells/ (live cells + dead cells).
[2] Male diets substituted with chicken fat, fish oil, or soybean oil.
[3] Toms age.

TABLE 9. Fertility, hatchability, and embryonic mortality of turkey hen's embryos by tom's dietary treatment[1] in Trial 1

Factor[2]	Treatment[3]	SET[4] 1 – 20	SEM[5]
		(%)	
Fertility	Chicken	98.45^{X}	.0751
	Fish	93.51^{Y}	.0756
	Soy	94.23^{Y}	.0738
Hatch of total eggs	Chicken	83.61^{X}	.0958
	Fish	69.66^{Y}	.0966
	Soy	73.50^{Y}	.0942
Hatch of fertile eggs	Chicken	87.91^{x}	.0960
	Fish	80.49^{y}	.0994
	Soy	82.87^{xy}	.0965
Early dead	Chicken	15.23	.0619
	Fish	20.51	.0624
	Soy	16.74	.0609
Mid dead	Chicken	5.05	.0366
	Fish	5.50	.0369
	Soy	5.66	.0361
Late dead	Chicken	15.51	.0608
	Fish	16.02	.0613
	Soy	15.21	.0599
Pipped eggs	Chicken	7.27	.0410
	Fish	6.41	.0413
	Soy	9.89	.0403

[1] Means within a factor lacking a common upper case superscript are significantly different (P<0.01) and lower case superscript are significantly different (P<0.05).
[2] Early dead = embryo died during day 1 – 6 of incubation.
Mid dead = embryo died during day 7 – 17 of incubation.
Late dead = embryo died during day 18 – 28 of incubation.
Pipped eggs= embryo pipped shell, but did not hatch.
[3] Tom's dietary treatment.
[4] Values represent the average mean over 20 sets of eggs.
[5] Pooled standard error mean.

TABLE 10. Fertility, hatchability, and embryonic mortality of turkey hen's embryos by tom's dietary treatment in Trial 2

Factor[1]	Treatment[2]	SET[3] 1 – 20	SEM[4]
		(%)	
Fertility	Chicken	96.04	.0782
	Fish	97.38	.0757
	Soy	96.62	.0741
Hatch of total eggs	Chicken	87.71	.0975
	Fish	84.47	.0943
	Soy	84.74	.0924
Hatch of fertile eggs	Chicken	94.64	.0927
	Fish	90.73	.0877
	Soy	92.16	.0868
Early dead	Chicken	5.49	.0426
	Fish	8.01	.0409
	Soy	7.50	.0402
Mid dead	Chicken	0.81	.0150
	Fish	1.33	.0144
	Soy	1.40	.0142
Late dead	Chicken	14.76	.0635
	Fish	18.21	.0612
	Soy	14.82	.0601
Pipped eggs	Chicken	8.01	.0489
	Fish	11.63	.0475
	Soy	12.66	.0461

[1] Early dead = embryo died during day 1 – 6 of incubation.
Mid dead = embryo died during day 7 – 17 of incubation.
Late dead = embryo died during day 18 – 28 of incubation.
Pipped eggs= embryo pipped shell, but did not hatch.
[2] Tom's dietary treatment.
[3] Values represent the average mean over 20 sets of eggs.
[4] Pooled standard error mean.

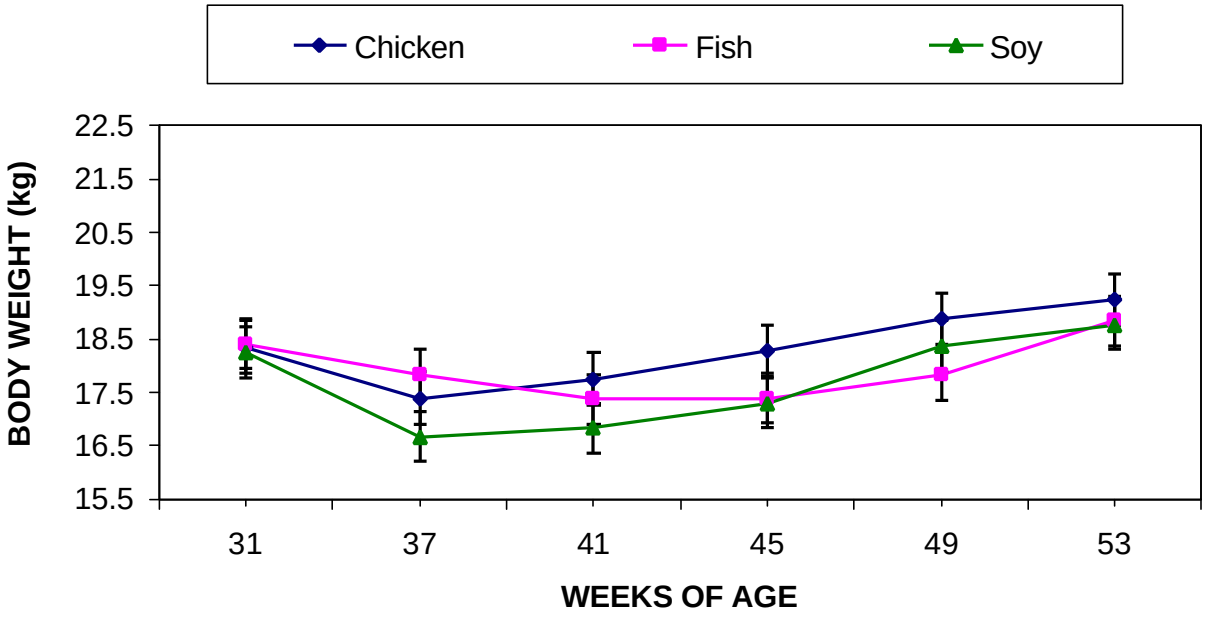

Figure 2. Male body weights in Trial 1 from 31 to 53 weeks of age as affected by dietary treatment.

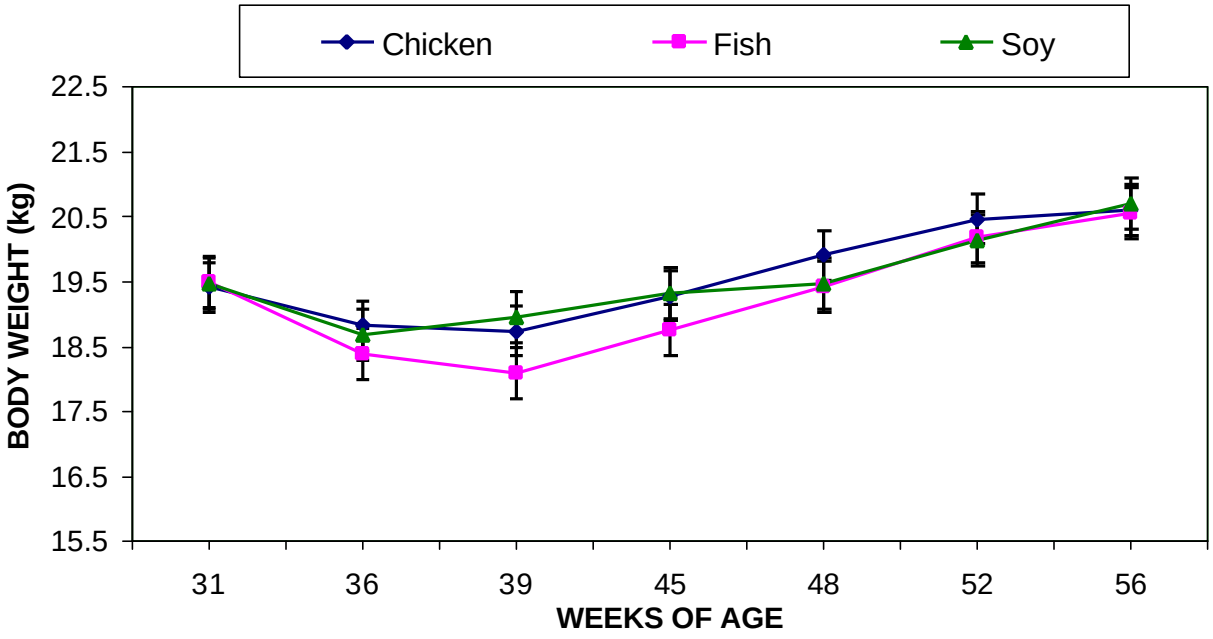

Figure 3. Male body weights in Trial 2 from 31 to 56 weeks of age as affected by dietary treatment.

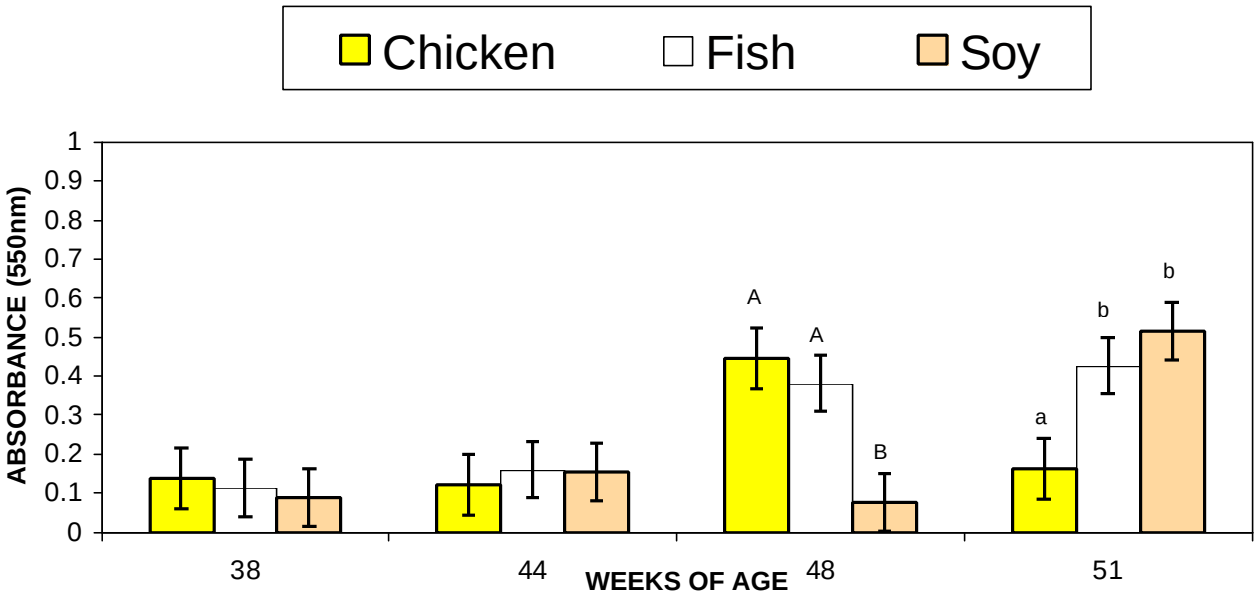

Figure 4. Sperm mobility in Trial 1. Absorbance after a 60 µL of sperm suspension, containing 5 X 10^8 sperm/mL, was overlaid onto 600-µL of prewarmed 6% (wt/vol) Accudenz® solution and allowed to incubate in 41 ºC water bath for 5 to 6 min. Means within a week of age lacking a common upper case superscript are significantly different (P<0.01) and lower case superscript are significantly different (P<0.05).

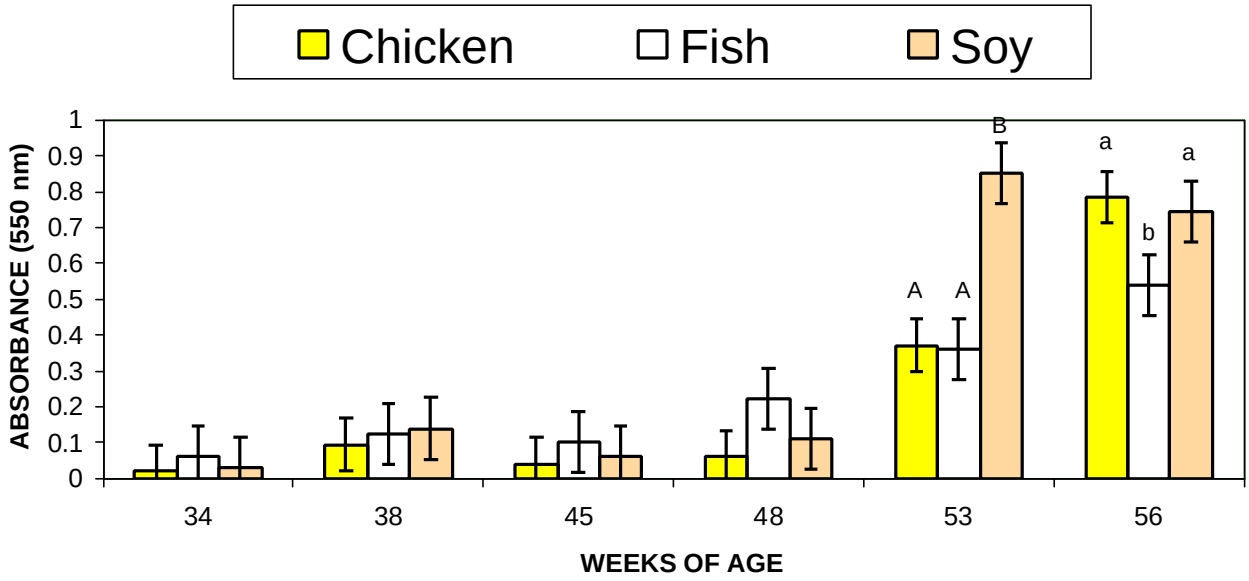

Figure 5. Sperm mobility in Trial 2. Absorbance after a 60 µL of sperm suspension, containing 5 X 10^8 sperm/mL, was overlaid onto 600-µL of prewarmed 6% (wt/vol) Accudenz® solution and allowed to incubate in 41 ºC water bath for 5 to 6 min. Means within a week of age lacking a common upper case superscript are significantly different (P<0.01) and lower case superscript are significantly different (P<0.05).

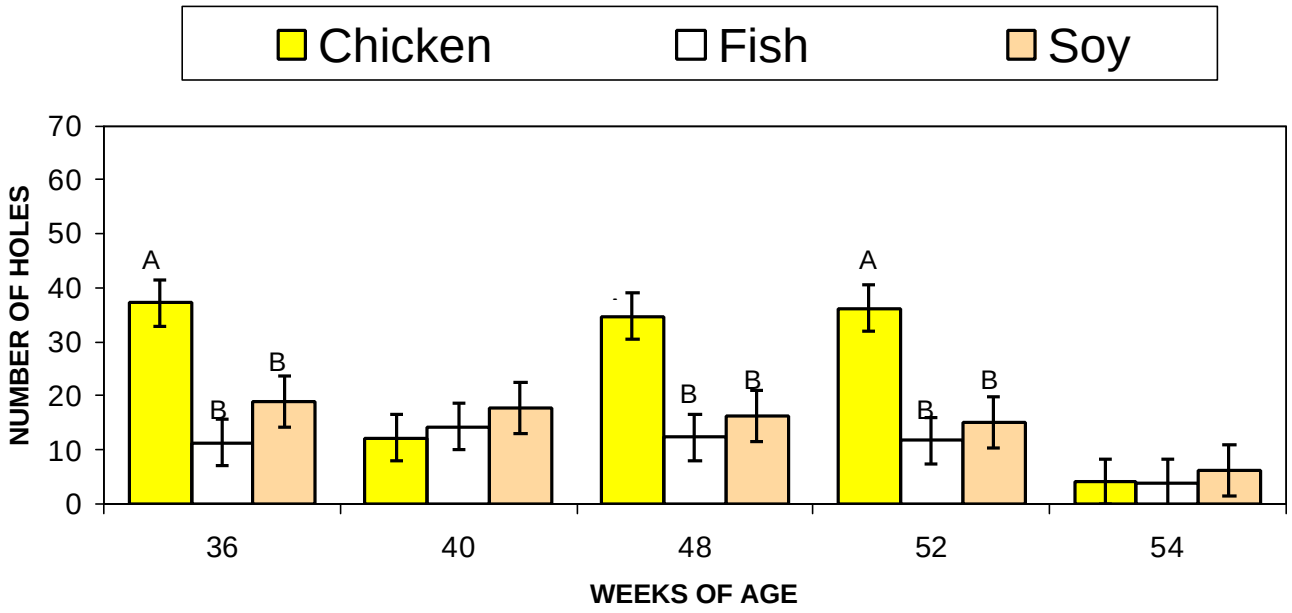

Figure 6. Observed mean number of sperm penetration holes in perivitelline layer of eggs collected from hens artificially inseminated weekly with 0.050mL freshly polled semen in Trial 1. Means within a week of age lacking a common superscript are significantly different (P<0.01).

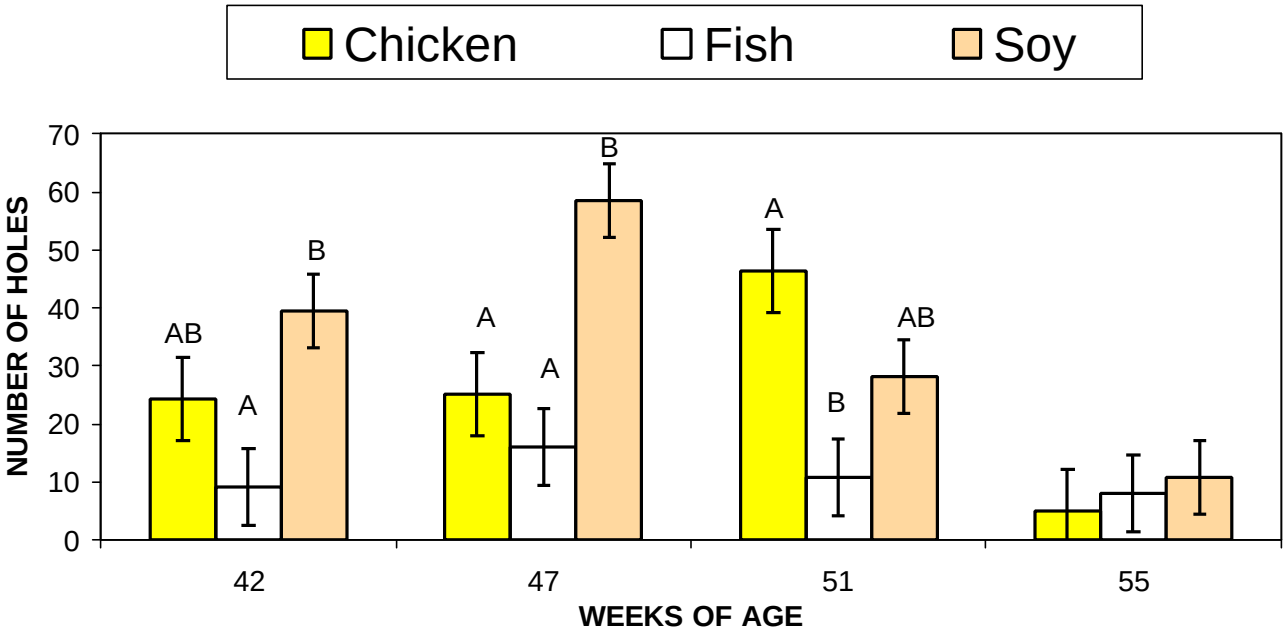

Figure 7. Observed mean number of sperm penetration holes in perivitelline layer of eggs collected from hens artificially inseminated weekly with 0.050mL freshly polled semen in Trial 2. Means within a week of age lacking a common superscript are significantly different (P<0.01).

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