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Effect of long-term supplementation with arachidonic or docosahexaenoic acids on sperm production in the broiler chicken

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The possibility was investigated that dietary supplementation of the male chicken with long-chain polyunsaturated fatty acids of the n-6 and n-3 series may prevent the decrease in sperm output that normally occurs by 60 weeks of age. From 26 weeks of age, birds were raised on wheat-based diets supplemented with either maize oil (rich in linoleic acid, 18:2n-6), arasco oil (rich in arachidonic acid, 20:4n-6) or tuna orbital oil (rich in docosahexaenoic acid, 22:6n-3). The effects of the last two oils were investigated at two levels of vitamin E supplementation (40 and 200 mg kg^{-1} feed). By 60 weeks of age, there was a small increase in the proportion of the main polyunsaturate of chicken sperm phospholipid, docosatetraenoic acid 22:4n-6, in chickens fed arasco oil diet compared with chickens given the maize oil diet, an effect that was potentiated at the higher dietary intake of vitamin E. Supplementation with tuna orbital oil significantly reduced the proportions of 20:4n-6 and 22:4n-6 in the sperm phospholipid and increased the proportion of 22:6n-3. The diet supplemented with tuna orbital oil and the lower level of vitamin E markedly depleted vitamin E from the tissues of the birds and decreased the concentration of vitamin E in the semen; these effects were largely prevented by the higher level of vitamin E in the diet. The susceptibility of semen to lipid peroxidation *in vitro* was increased in chickens fed arasco and tuna orbital oils with 40 mg vitamin E kg^{-1} feed, but was reduced when 200 mg vitamin E kg^{-1} feed was provided in the diet. The number of spermatozoa per ejaculate decreased by 50% between 26 weeks and 60 weeks of age in the birds fed the maize oil diet. This age-related decrease in the number of spermatozoa was almost completely prevented by feeding the birds with the oils enriched in either 20:4n-6 or 22:6n-3. Testis mass at 60 weeks of age was approximately 1.5 times greater in birds given of the arasco and tuna orbital oil diets compared with those given the maize oil diet.

Introduction

Phospholipids of avian spermatozoa characteristically contain very high proportions of long-chain ($C_{20–22}$) highly polyunsaturated fatty acids of the n-6 family and arachidonic (20:4n-6) and docosatetraenoic (22:4n-6) acids form the major acyl groups (Darin-Bennett *et al.*, 1974; Ravie and Lake, 1985; Surai *et al.*, 1998a). In contrast, in mammalian spermatozoa, long-chain polyunsaturated fatty acids of the n-3 family, in particular docosahexaenoic acid (22:6n-3), are predominant (Poulos *et al.*, 1973; Darin-Bennett *et al.*, 1974; Jain and Anand, 1976; Lin *et al.*, 1993; Kelso *et al.*, 1997a).

The importance of C_{22} polyunsaturates in relation to male fertility has been illustrated by studies in humans demonstrating that the amount of 22:6n-3 in spermatozoa is positively correlated with sperm motility (Nissen and Kreysel, 1983; Zalata *et al.*, 1998; Conquer *et al.*, 1999). Moreover, the reduction in the number of spermatozoa and in sperm motility in ejaculates from ageing bulls is accompanied by a decrease in the proportion of 22:6n-3 in sperm phospholipid (Kelso *et al.*, 1997a). In chickens, the decrease in spermatogenesis and semen quality (evident from 60 weeks of age) is accompanied by a decrease in the proportions of both 20:4n-6 and 22:4n-6 in the phosphatidylethanolamine fraction of the sperm phospholipids (Kelso *et al.*, 1996). Most notably, the proportion of 22:4n-6 in total sperm phospholipid showed a significant negative correlation with the age of the chicken but was positively correlated with sperm motility and fertilizing ability (Cerolini *et al.*, 1997).

These findings indicate a causal connection between a decrease in the ability of chicken testicular cells to synthesize or incorporate 22:4n-6 into the phospholipids of the developing sperm cells and the age-related decrease in the number and quality of spermatozoa (Cerolini *et al.*, 1997; Kelso *et al.*, 1997b). In the present study, dietary supplementation with polyunsaturated fatty acids was used in an

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attempt to increase and maintain the proportion of 22:4n-6 in the sperm phospholipid to investigate such relationships. Since oils rich in 22:4n-6 are not commercially available, male birds received an oil with a high content of 20:4n-6, which serves as a metabolic precursor of 22:4n-6. Although n-3 polyunsaturates are minor natural constituents of avian spermatozoa (Kelso *et al.*, 1997c; Surai *et al.*, 1998a), dietary supplementation of male chickens with oils rich in these fatty acids improves the fertilizing ability of semen, at least from 39 weeks to 47 weeks of age (Blesbois *et al.*, 1997a,b; Kelso *et al.*, 1997b). Thus, in the present study, the effects of inclusion of an oil rich in 22:6n-3 in the diet of male chickens were also investigated. The primary aim was to determine the consequences of long-term supplementation with either n-6 or n-3 polyunsaturates on sperm output at 60 weeks of age. In addition, the antioxidant status of the semen was investigated. The sensitivity of polyunsaturated sperm lipids to peroxidative damage (Cecil and Bakst, 1993; Alvarez and Storey, 1995; Surai *et al.*, 1997a, 1998b,c; Surai, 1999) and the reduced antioxidant capacity in the semen of chickens by 60 weeks of age (Kelso *et al.*, 1996) formed the basis for evaluating the effects of the dietary oils at two levels of vitamin E supplementation.

Materials and Methods

Dietary supplementation of chickens and semen collection

Male chickens (Ross broiler-breeder strain) were purchased at 21 weeks of age from a commercial poultry supplier and were housed individually in cages in a controlled environment under a photoperiod of 13 h light:11 h dark. The birds were provided with a standard wheat-based diet (130 g per day) containing 15.5% (w/w) crude protein and 11.5 MJ metabolizable energy kg^{-1}. From 22 weeks of age, semen was collected from the birds (Lake and Stewart, 1978); at this age all the birds had reached sexual maturity and were capable of producing semen. Three semen samples were routinely collected each week throughout the experiment. At 26 weeks of age, the chickens were randomly allocated to one of five experimental groups (ten chickens per group) and given one of the following dietary supplements: (i) maize oil (control diet); (ii) arasco oil, rich in 20:4n-6; (iii) arasco oil plus vitamin E; (iv) tuna orbital oil, rich in 22:6n-3; or (v) tuna orbital oil plus vitamin E. All oils were included at 5% (w/w) of feed. The total amount of lipid, including that present in the basal constituents, was 8% (w/w) of feed for all groups. The vitamin E was present at 200 mg kg^{-1} feed in groups (iii) and (v), whereas groups (i), (ii) and (iv) received only 40 mg vitamin E kg^{-1} feed. Arasco oil and tuna orbital oil were gifts from Scotia Pharmaceuticals Ltd, Carlisle. At 26 weeks of age, (after the birds were allocated to the experimental groups but before the consumption of the supplemented diets) semen samples were collected and ejaculate volume and sperm concentrations were determined. At 60 weeks of age, the semen samples collected were used to determine ejaculate volume, sperm concentration and fatty acid composition. All ten birds from each dietary group survived up to 60 weeks of age and contributed to both the 26 week and 60 week data. The birds were subsequently killed, and tissues and blood plasma were collected for analysis of vitamin E content.

Measurements of semen

Within 20 min of collection, ejaculate volume was measured using a graduated tube and sperm concentration was determined using a spectrophotometer (Brillard and McDaniel, 1985). For lipid analysis, fresh ejaculates were diluted with an equal volume of 0.85% (w/v) NaCl followed by centrifugation at 700 ***g*** for 20 min at 4°C to separate the seminal plasma from the cell pellet. The upper diluted plasma layer was transferred to a fresh test tube and the cell pellet was washed with 1 ml of 0.85% (w/v) NaCl and re-centrifuged as described above. The sperm cell pellet was resuspended in 2 ml of 0.85% (w/v) NaCl.

Evaluation of fertilizing ability of semen

The fertility of semen obtained from the birds at 60 weeks of age was assessed by artificial insemination of laying hens as described by Kelso *et al.* (1997b). The hens (ISA-Warren strain), also at 60 weeks of age, were maintained on a standard layer diet. Fresh ejaculates were diluted with two volumes of 0.9% (w/w) NaCl and samples from groups of three birds at 60 weeks of age were pooled to provide three replicate pooled samples per dietary treatment. Within 20 min of semen collection, each pooled semen sample was used to inseminate seven hens using a single dose of 7×10^7 spermatozoa per hen and eggs were collected over the next 7 days. The eggs were incubated for 7 days at 37.5°C and 60% relative humidity with automatic egg turning. The total number of eggs investigated was 108, 109, 105, 112 and 114 for the maize oil, arasco oil, arasco oil plus vitamin E, tuna orbital oil and tuna orbital oil plus vitamin E dietary groups, respectively. The eggs were opened and those that did not contain an embryo (live or dead) were classed as infertile.

Fatty acid analysis

Total lipid was extracted from the spermatozoa and seminal plasma preparations after homogenization in a suitable excess of chloroform:methanol (2:1 v/v) (Christie, 1982). The sperm lipids were separated into their major classes by thin-layer chromatography on silica gel G using a solvent system of hexane:diethyl ether:formic acid (80:20:1 v/v/v). After spraying the plate with 2,7-dichlorofluorescein (0.1% w/v in methanol), the lipid classes were visualized under UV light and the band corresponding to the phospholipid was scraped from the plate. The phospholipid was eluted from the silica gel by washing three times with 2 ml methanol and was subjected to *trans*methylation by refluxing for 30 min with methanol:toluene:sulfuric acid (20:10:1 v/v/v) in the presence of pentadecaenoic acid (15:0) standard (Christie *et al.*, 1970). The resultant fatty acid methyl esters were analysed by injecting 2 µl, via a CP9010 auto sampler (Chrompack Ltd, London), on to a Carbowax

capillary column 30 m × 0.25 mm, with a film thickness of 0.25 μm (Alltech UK Ltd, Carnforth), fitted within a Chrompack CP9001 gas chromatography instrument (Chrompack Ltd). After injection of the sample, the temperature of the capillary column was maintained at 185°C for 2 min, then increased at a rate of 5°C per min to a temperature of 230°C and maintained at 230°C for a further 24 min. The fatty acid compositions (% w/w of total fatty acids) were derived by integrating the peaks using an EZ Chrom data handling system (Speck Analytical Ltd, Alloa). The fatty acid composition of the total lipid of the seminal plasma was determined by using the same method as described for spermatozoa but with the omission of the thin-layer chromatography step.

Determination of vitamin E

α-Tocopherol was measured using the method of McMurray *et al.* (1980) as modified by Gaal *et al.* (1995). In brief, the samples were saponified with ethanolic KOH in the presence of pyrogallol and α-tocopherol was extracted from the mixture with petroleum spirit. The extract was dried under nitrogen, redissolved in methanol and injected on to a Spherisorb S30DS2, 3 μ C_{18} reverse phase HPLC column, 15.0 cm × 4.6 mm (Phase Separations, Clwyd). Chromatography was performed using a flow rate of 1.1 ml min^{-1}. Fluorescence detection and quantification of α-tocopherol used excitation and emission wavelengths of 295 and 330 nm, respectively. Calibration was performed using standard solutions of α-tocopherol in methanol.

Susceptibility of semen to lipid peroxidation

Fresh semen samples (0.2 ml) were mixed with 0.8 ml sodium phosphate buffer (0.01 mol l^{-1}, pH 7.4) containing 1.15% (w/v) KCl. The mixtures were incubated for 1 h at 37°C in the presence of $FeSO_4$ (0.01 mmol l^{-1}). In some incubations, α-tocopherol was also added to a concentration of 50 μg ml^{-1} in the incubation mixture. An aliquot of seminal plasma, prepared from the same semen sample, was sonicated with vitamin E and included in the incubation mixture as previously described (Surai *et al.*, 1997b) to incorporate α-tocopherol into the aqueous medium; inclusion of an equal amount of seminal plasma in the absence of α-tocopherol did not affect peroxidation. At the end of the incubation, butylated hydroxytoluene was added to a concentration of 0.01% (v/v). The concentration of thiobarbituric acid reactive substance (TBARS) was determined using a spectrophotometer according to the method of Ohkawa *et al.* (1979) using 1,1,3,3-tetramethoxypropane as a standard.

Statistical analysis

Statististical comparisons were based on ten replicate semen samples (each sample from an individual bird) from each dietary group, with respect to data on semen volume, the number of cells, testis mass and the vitamin E content of the tissues at slaughter. Statistical analyses of the fatty acid compositions and of the *in vitro* peroxidation susceptibilities were based on five replicate pooled samples from each dietary group and each pooled sample was obtained from two birds; pooling was necessary to provide sufficient material for these measurements. Fertility values from artificial insemination were based on three replicate pooled semen samples from each dietary group. The data were assessed by ANOVA. The fatty acid data, expressed as percentages, were arcsin transformed before ANOVA to make the variance independent of the mean (Snedecor and Cochrane, 1967).

Results

Dietary effects on fatty acid compositions of spermatozoa and seminal plasma

The fatty acid compositions of feeds supplemented with different oils (Table 1) confirm that the predominant polyunsaturates received by the birds allocated to the maize oil, arasco oil and tuna orbital oil groups were linoleic acid (18:2n-6), arachidonic acid (20:4n-6) and docosahexaenoic acid (22:6n-3), respectively. Other differences were also apparent: the diet supplemented with tuna orbital oil provided additional n-3 polyunsaturates in the form of 20:5n-3 and also contained the highest proportion of saturated fatty acids, whereas arasco oil contained the highest proportion of the mono-unsaturated oleic acid (18:1n-9). As a result of these different fatty acid profiles, the n-6:n-3 ratio and the proportion of $C_{20–22}$ polyunsaturates varied greatly among the relevant dietary groups.

Analysis of spermatozoa collected after 34 weeks of dietary supplementation (that is when the birds were 60 weeks of age) illustrates the effects of varying the fatty acid profile of the feed on the acyl composition of the sperm lipids (Table 2). Phospholipid formed 69.8 ± 3.2, 71.3 ± 5.1, 70.7 ± 2.2, 70.1 ± 3.2 and 70.0 ± 3.3 % (w/w) of total sperm lipid for birds receiving the maize oil, arasco oil, arasco oil plus vitamin E, tuna orbital oil and tuna orbital oil plus vitamin E supplements, respectively, and these proportions did not differ among the dietary groups. In the maize oil supplemented group, 22:4n-6 was the predominant fatty acid of sperm phospholipid and 20:4n-6 also made a large contribution. Dietary supplementation with arasco oil resulted in a significant but small increase in the proportion of 22:4n-6 in sperm phospholipid, an effect that was potentiated at the higher concentration of vitamin E inclusion in the feed. This increase in 22:4n-6 in sperm phospholipid was accompanied by a decrease in the proportion of 18:2n-6. The arasco oil diet, although rich in 20:4n-6, did not affect the proportion of 20:4n-6 in sperm phospholipid. Dietary supplementation with tuna orbital oil resulted in a partial remodelling of the phospholipid fatty acid profile of the spermatozoa, producing increases in the proportions of 22:6n-3 and 18:1n-9 and a commensurate decrease in the proportions of 22:4n-6 and 20:4n-6; these changes represented a marked decrease in the n-6:n-3 ratio. However, despite these changes, 22:4n-6 remained the predominate

Table 1. Fatty acid compositions of the diets supplemented with maize oil, arasco oil or tuna orbital oil

	Supplement		
Fatty acids (%, w/w)	Maize oil	Arasco oil	Tuna orbital oil
14:0	0.5	0.6	3.0
16:0	12.5	8.7	21.9
16:1n-7	0.5	0.5	4.6
18:0	1.8	6.0	5.2
18:1n-9	25.0	30.6	14.6
18:2n-6	53.4	16.0	13.8
18:3n-3	2.6	1.7	2.2
20:4n-6	0.0	28.0	1.6
20:5n-3	0.7	0.7	5.0
22:5n-3	0.2	0.9	1.1
22:6n-3	1.0	1.1	18.5
n-6:n-3 ratio	11.9	10.0	0.6
Total $C_{20–22}$ (%, w/w)	1.9	30.7	26.2

Only the major fatty acids (> 0.5%, w/w) are shown.

Table 2. Fatty acid compositions of total phospholipid in spermatozoa from chickens at 60 weeks of age on diets supplemented with maize oil, arasco oil or tuna orbital oil with or without additional vitamin E

	Dietary supplement				
Fatty acid[a]	Maize oil[b]	Arasco oil[b]	Arasco oil and vitamin E[c]	Tuna oil[b]	Tuna oil and vitamin E[c]
16:0	12.8 ± 0.5[d]	12.9 ± 0.5[d]	12.7 ± 0.3[d]	13.5 ± 0.6[d]	13.9 ± 0.6[d]
18:0	21.6 ± 0.7[d]	21.2 ± 0.6[d]	21.4 ± 0.5[d]	20.4 ± 0.6[d]	20.2 ± 0.6[d]
18:1n-9	12.6 ± 0.3[d]	13.5 ± 0.3[d]	11.7 ± 0.4[d]	16.8 ± 0.4[e]	16.7 ± 0.5[e]
18:1n-7	1.4 ± 0.1[d]	1.3 ± 0.1[d]	1.3 ± 0.1[d]	1.8 ± 0.1[e]	1.7 ± 0.2[e]
18:2n-6	2.6 ± 0.3[d]	0.8 ± 0.1[e]	1.0 ± 0.1[e]	1.2 ± 0.3[e]	1.1 ± 0.2[e]
20:1n-9	3.7 ± 0.3[d]	3.6 ± 0.3[d]	3.8 ± 0.2[d]	4.4 ± 0.4[d]	4.5 ± 0.5[d]
20:4n-6	12.3 ± 0.3[d]	12.1 ± 0.1[d]	13.4 ± 0.6[d]	8.4 ± 0.4[e]	8.3 ± 0.3[e]
22:4n-6	22.1 ± 0.3[d]	25.2 ± 0.4[e]	27.0 ± 0.4[f]	16.1 ± 0.3[g]	15.5 ± 0.3[g]
22:6n-3	4.6 ± 0.3[d]	4.8 ± 0.3[d]	3.7 ± 0.4[d]	12.2 ± 0.4[e]	13.1 ± 0.4[f]
n-6:n-3 ratio	8.4	7.9	11.4	2.2	2.0
$C_{20–22}$ (%)	40.8	42.7	44.7	38.0	38.2

[a]Fatty acid compositions are expressed as the percentage (w/w) of the total phospholipid fatty acids; only those fatty acids > 0.5% (w/w) are shown (values are means ± SE).
[bc]Vitamin E present at 40 and 200 mg kg^{-1} feed, respectively.
[defg]For comparison among birds on different diets, values that do not share a common letter are significantly different ($P < 0.05$).

polyunsaturate of the sperm phospholipid. There were no marked differences in these fatty acid profiles resulting from the two different levels of vitamin E supplementation in the n-3 enriched diets.

The fatty acid composition of the total lipid of the seminal plasma, obtained when the birds were 60 weeks old, was also responsive to the various dietary supplements (Table 3). The arasco oil diet produced a significant increase in the proportion of 22:4n-6 at the expense of 18:2n-6, but in this case the proportion of 20:4n-6 was also increased compared with the control (maize oil) group; this increase in 20:4n-6 content was more pronounced at the higher level of vitamin E supplementation. The tuna orbital oil diet increased the proportion of 22:6n-3 in the seminal plasma lipid, an effect that was potentiated at the higher level of vitamin E supplementation and which was accompanied by decreased proportions of 20:4n-6 and 18:2n-6.

Vitamin E and susceptibility to peroxidation

The effects of the various diets on the concentration of vitamin E in the whole semen obtained when the birds were 60 weeks of age, and in the blood plasma and tissues of the

Table 3. Fatty acid compositions of total lipid from seminal plasma from chickens at 60 weeks of age on different dietary supplements

	Dietary supplement				
Fatty acid[a]	Maize oil[b]	Arasco oil[b]	Arasco oil and vitamin E[c]	Tuna oil[b]	Tuna oil and vitamin E[c]
16:0	13.2 ± 0.6[de]	12.4 ± 0.4[e]	12.7 ± 0.3[e]	14.4 ± 0.4[d]	14.1 ± 0.4[d]
18:0	22.5 ± 1.0[d]	24.4 ± 0.6[d]	23.5 ± 0.8[d]	24.7 ± 0.8[d]	24.3 ± 0.7[d]
18:1n-9	18.7 ± 0.4[d]	16.3 ± 0.6[e]	15.2 ± 0.5[e]	20.9 ± 0.5[f]	20.1 ± 0.4[f]
18:1n-7	1.1 ± 0.1[d]	1.1 ± 0.1[d]	1.1 ± 0.1[d]	2.0 ± 0.2[e]	1.1 ± 0.1[d]
18:2n-6	9.5 ± 0.4[d]	3.6 ± 0.2[e]	3.8 ± 0.2[e]	5.1 ± 0.2[f]	4.9 ± 0.3[f]
20:1n-9	1.8 ± 0.1[d]	2.3 ± 0.3[d]	2.2 ± 0.3[d]	2.2 ± 0.3[d]	2.3 ± 0.3[d]
20:4n-6	11.2 ± 0.3[d]	14.2 ± 0.3[e]	15.7 ± 0.3[f]	8.7 ± 0.1[g]	8.5 ± 0.2[g]
22:4n-6	8.3 ± 0.2[d]	12.2 ± 0.4[e]	13.2 ±0 3[e]	7.6 ± 0.3[d]	7.7 ± 0.4[d]
22:6n-3	1.6 ± 0.1[d]	1.8 ± 0.2[d]	1.8 ± 0.2[d]	4.3 ± 0.2[e]	6.3 ± 0.2[f]
n-6:n-3 ratio	18.1	16.7	18.2	5.0	3.3
$C_{20–22}$ (%)	21.1	28.6	30.7	20.6	22.5

[a]Fatty acid compositions are expressed as the percentage (w/w) of the total phospholipid fatty acids; only those fatty acids > 0.5% (w/w) are shown (values are means ± SE).
[bc]Vitamin E present at 40 and 200 mg kg^{-1} feed, respectively.
[defg]For comparison among birds on different diets, values within a row that do not share a common letter are significantly different ($P < 0.05$).

birds at slaughter, are summarized (Table 4). The diet supplemented with arasco oil and 40 mg vitamin E kg^{-1} did not produce any significant differences in the vitamin E content of the semen, blood plasma or tissues compared with the maize oil diet which contained the same amount of vitamin E. However, the arasco oil diet supplemented with 200 mg vitamin E kg^{-1} resulted in significant increases in the tissue concentration of vitamin E and doubled the concentrations in the blood plasma and semen. The diet containing tuna orbital oil with only 40 mg vitamin E kg^{-1} resulted in a marked decrease in vitamin E in the tissues and also a decrease in blood plasma and semen compared with the maize oil diet. The presence of 200 mg vitamin E kg^{-1} in the tuna orbital oil diet partly (heart) or completely (testes, kidney, lung, blood plasma, semen) restored or over-restored (liver) the vitamin E concentrations.

Semen from birds fed with arasco and tuna orbital oils and vitamin E at 40 mg kg^{-1} feed displayed an increased susceptibility to lipid peroxidation *in vitro* (Table 5). However, the dietary combination of these oils with vitamin E at 200 mg kg^{-1} markedly decreased this susceptibility below that observed for semen from birds on the maize oil diet. The inclusion of exogenous vitamin E in the incubations *in vitro* (50 μg α-tocopherol ml^{-1}) consistently protected all the semen samples from peroxidation.

Dietary effects on the number of spermatozoa and testis mass

For birds fed on the maize oil diet, the number of spermatozoa present in each ejaculate decreased significantly by almost 50% between 26 and 60 weeks of age (Table 6). This decrease was due to a decrease in semen volume with no significant change in sperm concentration. Most notably, the number of spermatozoa per ejaculate obtained at 60 weeks of age from the birds supplemented with arasco oil or tuna orbital oil with either amount of vitamin E was in all cases significantly higher than that observed in the birds fed the maize oil diet. As a consequence, the birds fed the diet supplemented with arasco oil or tuna orbital oil did not display an age-related decrease in number of spermatozoa. Again, this effect reflected the maintenance of semen volume with no changes in the concentration of sperm cells. At the lower level of vitamin E supplementation, tuna orbital oil was significantly more effective than arasco oil in this regard. The fresh masses of the testes at 60 weeks of age were between 1.4 and 1.8 times greater as a result of dietary supplementation with arasco oil or tuna orbital oil although the body weights of the birds were not significantly affected by the different dietary oils. Assessment of the fertilizing ability of the semen obtained from the birds at 60 weeks of age by artificial insemination of laying hens indicated that approximately 76–87% of the eggs laid had been fertilized successfully and there were no significant differences among the dietary groups.

Discussion

Previous investigations in which fertility parameters were measured throughout the reproductive lifetime of male chickens have shown that the number and the fertilizing ability of spermatozoa reach a maximum at 40–50 weeks of age and then decrease markedly towards the end of the reproductive period (Cerolini *et al.*, 1997; Kelso *et al.*, 1997b). Therefore, the present study focused on the end of the

Table 4. Vitamin E content of tissues, blood plasma and whole semen of chickens at 60 weeks of age on different dietary supplements

Tissues and body fluids	Vitamin E (μg g^{-1} fresh tissue)[a]				
	Maize oil[b]	Arasco oil[b]	Arasco oil and vitamin E[c]	Tuna oil[b]	Tuna oil and vitamin E[c]
Blood plasma	2.3 ± 0.1[d]	2.5 ± 0.2[d]	5.0 ± 0.3[e]	1.4 ± 0.1[f]	2.2 ± 0.2[d]
Heart	26.2 ± 2.1[d]	25.9 ± 2.4[d]	39.4 ± 3.1[e]	5.8 ± 0.4[f]	15.7 ± 1.3[g]
Kidney	11.3 ± 1.2[d]	12.2 ± 1.1[d]	23.9 ± 2.2[e]	2.6 ± 0.3[f]	10.4 ± 1.0[d]
Lung	14.0 ± 1.2[d]	18.4 ± 2.2[d]	47.2 ± 3.8[e]	3.3 ± 0.4[f]	14.9 ± 1.6[d]
Liver	12.0 ± 1.0[d]	15.1 ± 1.8[d]	56.5 ± 4.2[e]	7.5 ± 0.6[f]	33.6 ± 2.1[g]
Testis	8.5 ± 0.7[d]	6.5 ± 0.6[d]	16.4 ± 1.2[e]	1.8 ± 0.2[f]	8.5 ± 0.7[d]
Whole semen	1.1 ± 0.1[d]	1.2 ± 0.1[d]	2.0 ± 0.1[e]	0.7 ± 0.1[f]	1.5 ± 0.1[g]

[a]Values are means ± SE.
[bc]Vitamin E present at 40 and 200 mg kg^{-1} feed, respectively.
[defg]For comparison among birds on different diets, values within a row that do not share a common letter are significantly different ($P < 0.05$).

Table 5. Susceptibility to peroxidation *in vitro* of whole semen obtained from chickens at 60 weeks of age on different dietary supplements

Exogenous vitamin E[b]	Malondialdehyde formed (μg min^{-1} ml^{-1} initial semen)[a]				
	Maize oil[c]	Arasco oil[c]	Arasco oil and vitamin E[d]	Tuna oil[c]	Tuna oil and vitamin E[d]
Absent	1.6 ± 0.1[e]	2.2 ± 0.2[f]	0.6 ± 0.1[g]	2.4 ± 0.3[f]	0.8 ± 0.1[g]
Present	0.5 ± 0.1[e*]	0.6 ± 0.1[e*]	0.5 ± 0.1[e]	0.7 ± 0.1[e*]	0.5 ± 0.1[e]

[a]Values are means ± SE.
[b]Addition of vitamin E to a concentration of 50 μg ml^{-1} in the incubation.
[cd]Vitamin E present at 40 and 200 mg kg^{-1} feed, respectively.
[efg]For comparison among birds on different diets, values within a row that do not share a common letter are significantly different ($P < 0.05$).
*Significant difference ($P < 0.05$) between values in the presence and absence of exogenous vitamin E.

reproductive period. The results indicate that the decrease in spermatogenesis, normally evident in male domestic chickens by 60 weeks of age, is almost completely inhibited by dietary provision of oils rich in either 20:4n-6 or 22:6n-3. The explanation for this finding is not clear, particularly as the two oils produce very different and in some ways opposing effects on the fatty acid composition of the sperm phospholipid. The characteristic polyunsaturate of avian spermatozoa (22:4n-6), in chickens is normally derived from 18:2n-6 by sequential desaturation and elongation steps (Watkins and Kratzer, 1987; Watkins, 1995; Lenzi *et al.*, 1996; Walzem, 1996). The pathways of synthesis of n-6 and n-3 $C_{20–22}$ polyunsaturates from C_{18} precursor in rat testicular cells have been elucidated in some detail (Retterstol *et al.*, 1998). Commercial poultry diets are usually a rich source of 18:2n-6 (Speake *et al.*, 1998), especially when supplemented with maize oil; therefore, failure to reach an optimal concentration of 22:4n-6 in sperm lipids cannot be ascribed to inadequate provision of its C_{18} precursor. The increase in the proportion of 22:4n-6 in sperm phospholipid brought about by the dietary intake of arasco oil probably reflects the fact that 20:4n-6 is the immediate precursor of 22:4n-6 and that this one-step conversion bypasses the rate-limiting δ6-desaturase reaction. However, after 34 weeks of a diet supplemented with high levels of arasco oil, only a small increase in the proportion of 22:4n-6 in sperm phospholipid was evident and there was no difference in the concentration of 20:4n-6 in these cells. Thus, the fatty acid profiles of spermatozoa result from a high degree of selectivity, presumably to maintain the appropriate biophysical properties of the cell membrane, and is not simply a passive reflection of dietary intake. The differential responsiveness of the fatty acid profiles of the seminal plasma and sperm lipids to dietary supplementation is consistent with this view.

One hypothesis to explain the increase in sperm output as a result of dietary arasco oil is that the supply of 22:4n-6 may be a limiting factor in the production of viable spermatozoa and that the enzymatic ability to synthesize or incorporate this fatty acid may decrease during ageing. However, this explanation does not encompass the observation that dietary tuna orbital oil, rich in n-3 fatty acids, is as effective as arasco oil in maintaining sperm output at 60 weeks of age despite causing a significant decrease in the sperm content of 22:4n-6 concomitant with an increase in 22:6n-3. Such changes, although representing a partial shift towards a 'mammalian' composition, nevertheless may be compatible with the promotion of appropriate biophysical properties in the sperm

Table 6. Effects of dietary supplementation with polyunsaturated fatty acids on semen characteristics of chickens

	Dietary supplement				
	Maize oil[b]	Arasco oil[b]	Arasco oil and vitamin E[c]	Tuna oil[b]	Tuna oil and vitamin E[c]
26 weeks of age[a]					
Semen volume (ml)	0.44 ± 0.02[g]	0.44 ± 0.02[g]	0.45 ± 0.02[g]	0.43 ± 0.02[g]	0.45 ± 0.02[g]
Sperm concentration[d]	3.34 ± 0.12[g]	3.32 ± 0.18[g]	3.26 ± 0.14[g]	3.27 ± 0.17[g]	3.34 ± 0.19[g]
Number of spermatozoa[e]	1.46 ± 0.0.9[g]	1.46 ± 0.18[g]	1.45 ± 0.10[g]	1.41 ± 0.10[g]	1.50 ± 0.10[g]
60 weeks of age					
Semen volume (ml)	0.25 ± 0.01[g*]	0.38 ± 0.02[h]	0.41 ± 0.01[hi]	0.45 ± 0.03[i]	0.40 ± 0.02[hi]
Sperm concentration[d]	3.05 ± 0.14[g]	3.01 ± 0.12[g]	2.99 ± 0.15[g]	3.11 ± 0.14[g]	3.01 ± 0.19[g]
Number of spermatozoa[e]	0.75 ± 0.04[g*]	1.14 ± 0.11[h*]	1.23 ± 0.06[hi]	1.39 ± 0.09[i]	1.21 ± 0.11[hi]
Fertility (%)[f]	80.5 ± 2.4[g]	75.7 ± 6.4[g]	80.8 ± 5.0[g]	84.1 ± 7.7[g]	86.7 ± 5.5[g]
Testis mass (g)	20.96 ± 1.9[g]	32.44 ± 1.31[h]	36.52 ± 4.26[h]	30.11 ± 2.42[h]	37.14 ± 2.49[h]
Body weight (kg)	5.37 ± 0.30[g]	5.44 ± 0.44[g]	5.42 ± 0.40[g]	5.69 ± 0.12[g]	5.89 ± 0.31[g]

[a]Semen characteristics at 26 weeks of age before commencement of dietary supplementation.
[bc]Vitamin E present at 40 and 200 mg kg^{-1} feed, respectively.
[de]Sperm concentration: 10^9 cells ml^{-1} semen; number of spermatozoa: 10^9 cells per ejaculate, respectively.
[f]Fertility is percentage of eggs that contained an embryo after artificial insemination.
[ghi]For comparison among birds in different dietary groups, values within a row that do not share a common letter are significantly different ($P < 0.05$).
[*]Significant difference ($P < 0.05$) between values at 26 and 60 weeks of age.

membrane. This view is supported by reports that the fertilizing ability of chicken semen is improved by dietary supplementation with n-3 fatty acids (Blesbois *et al.*, 1997a,b; Kelso *et al.*, 1997b).

The effects of the various dietary oils on the recipient birds are not confined to changes in the fatty acid composition of the tissues (Surai *et al.*, 1999) and semen (current study). Feeding birds with the tuna orbital oil diet without supplementation with excess vitamin E resulted in a marked depletion of vitamin E from the tissues. Presumably this depletion is due to the increased peroxidative susceptibility of tissues enriched with the highly polyunsaturated 22:6n-3, which places excessive demands on the antioxidant capacity of the bird. It is also evident that the antioxidant capacity of the semen can be compromised by the highly polyunsaturated dietary oils, as indicated by the reduction in vitamin E concentration in semen of the birds fed the tuna orbital oil (low vitamin E) diet. Moreover, the susceptibility of semen to lipid peroxidation *in vitro* was increased as a result of feeding the oils rich in 20:4n-6 or 22:6n-3 with the lower level of vitamin E, an effect that was effectively prevented by the inclusion of supplementary vitamin E in the diet. However, the dietary effects on the peroxidative susceptibility of the semen did not produce significant differences in fertilizing ability as assessed by artificial insemination.

Among the multifarious consequences of feeding chickens with oils that provide diverse polyunsaturate profiles, the generation of increased concentrations and disparate compositions of eicosanoids may be relevant to the changes in sperm output. Arasco oil provides large amounts of 20:4n-6 for potential conversion to type 2 prostaglandins and type 4 leukotrienes, whereas tuna orbital oil provides substrate for the synthesis of type 3 prostaglandins and type 5 leukotrienes after the retroconversion of 22:6n-3 to 20:5n-3 (Lands, 1979; Fischer, 1989; Smith, 1989; Sardesai, 1992). Although prostaglandins in seminal plasma regulate various aspects of sperm function (Gottlieb and Bygdeman, 1988), their relevance to the present findings is unclear, especially since a range of prostaglandins has been shown to inhibit spermatogenesis in rodents (Abbatiello *et al.*, 1976). Other possible mechanisms whereby dietary fatty acids could promote spermatogenesis, such as the regulation of gene expression (Jump and Clarke, 1999), remain to be examined. An important consideration is the potential interaction of polyunsaturated fatty acids or their derived eicosanoids with the hypothalamo–pituitary–gonadal axis and the hormonal control of spermatogenesis (De Kretser, 1990; Etches, 1996). Thus, the effects of dietary polyunsaturates on the secretion of GnRH, LH, FSH and testosterone, and on the responsiveness of the relevant types of cells to these hormones, may be worthy of investigation.

Irrespective of the underlying mechanism, the present study indicates that dietary polyunsaturated fatty acids of both the n-6 and n-3 series can prevent the age-related decrease in sperm output in chickens, at least up to 60 weeks of age, and also emphasizes the importance of adequate dietary vitamin E in preventing peroxidative reactions in semen. It would be useful in future work to identify the stage during the reproductive lifetime when such differences in sperm output between the control and supplemented groups first becomes evident.

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