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Polyunsaturated fatty acids influence offspring sex ratio in cows

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ABSTRACT

Dietary polyunsaturated fatty acids (PUFAs) can influence fertility in farm animals. Some evidence in mice and sheep have suggested that PUFAs may influence offspring sex ratio, which may have significant value for cattle production. To test this hypothesis, three groups of Holstein cows were supplemented with either 0%, 3% or 5% protected fat (PF) in the form of calcium salt of fatty acids (rich in omega-6) from 14–21 days pre-partum until conception. Proven-fertile frozen semen from the same ejaculate was used for insemination. Calf sex recorded at birth was 8/19 (42.1%) male offspring in the control group, increasing to 14/20 (70%, $P > 0.05$) and 17/20 (85%, $P < 0.05$) in 3% and 5% PF, respectively. To test if this effect was caused by a direct influence on the oocyte, we supplemented bovine cumulus oocyte complexes during *in vitro* maturation with either omega-3 alpha-linolenic acid (ALA), omega-6 linoleic acid (LA) or trans-10, cis-12 conjugated linoleic acid (CLA). Sex ratio of the produced transferable embryos was determined using PCR of SRY gene. Similar to the *in vivo* results, sex ratio was skewed to the male side in the embryos derived from LA- and CLA-treated oocytes (79% and 71%) compared to control and ALA-treated oocytes (44% and 54%, respectively). These results indicate that both dietary and *in vitro* supplementation of omega-6 PUFAs can skew the sex ratio towards the male side in cattle. Further experiments are required to confirm this effect on a larger scale and to study the mechanisms of action that might be involved.

1. Introduction

Various mechanisms of sex determination are present in amniote vertebrates, including genotypic, environmental (for example temperature in Crocodilian reptiles), or a mixture [1]. There is evidence that mammals also have the ability to skew their sex ratios in response to environmental conditions, a system which is thought to confer evolutionary benefits [2]. This would have the least cost to the mother if the sex ratio was adjusted close to the time of conception. Two main research lines have attempted to explain possible mechanisms involved [3]. The first is based on the finding that more dominant mothers produce more male offspring, possibly mediated via alterations in their testosterone levels [4]. The second has investigated associations between the body condition or diet of the mother and offspring sex. Within the latter category, alterations in sex ratio have been reported in response to pre-conceptual maternal diet [5–8], general dietary supplementation [9], changes in body condition [10] and diabetes [11]. More specifically, the dietary content of unsaturated fats [12], polyunsaturated fatty acids (PUFAs) [13,14], glucose [15] or fructose [16] have also been implicated. Results have not, however, always been consistent. For example, female mice supplemented with an n-6 rich diet gave birth to more female pups than male pups ($P < 0.001$) compared to control or n-3 fed mothers [13]. Others have shown a skew to the male side when a high fat diet was used in mice [5] or a high n-6 PUFA diet was fed to ewes [14]. In the latter study 69% males were recorded in the n-6 PUFA group fed as a protected soybean meal vs. 38% in the control.

Sex ratio is a key factor in cattle breeding. In the dairy industry a predominance of females is preferred as these are required for milk production. Whereas, beef cattle producers may use sexed semen to produce crossbred female replacements [17]. This has led to the development of semen sexing technologies which can reliably produce a 90% gender bias, but the sorting mechanism remains costly and pregnancy rates are significantly less than those using conventional semen

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[17,18]. Other potential methods of altering the sex ratio are therefore of significant interest.

Nutritional management can be a natural, effective and less expensive substitute for hormonal and medical intervention that can be a potential health hazard in milk and meat. Dietary PUFAs are already used to increase the energy density of the diet in cattle and also have some specific effects that are energy-independent. These include prostaglandin biosynthesis, steroidogenesis and transcriptional regulation, as well as regulation of genes involved in maternal immune response and tissue remodeling [19]. The predominant PUFA in most seed lipids is linoleic acid (LA; 18:2n-6), while α-linolenic acid (ALA;18:3n-3) predominates in most forage lipids and flaxseed. These two essential fatty acids can be metabolized in animal tissue to other forms of PUFAs. Dietary PUFA supplements in ruminants have to be protected against rumen bio-hydrogenation [20]. This process also results in synthesis of conjugated LA (CLA) from LA [21].

With respect to reproductive performance in cattle [reviewed by 22–25], some PUFA supplements were reported to have positive effects on postpartum uterine involution [26], growth rate and diameter of the ovulatory follicle [27] and *in vitro* oocyte developmental capacity [28]. Staples et al. [22] reviewed different studies examining the effect of dietary fat supplements on reproductive performance of lactating dairy cows, and reported that eleven out of twenty studies showed an average of 17% improvement in conception or pregnancy rates.

Despite the widespread use of various feeds which alter dietary PUFA concentrations and ratios, the effects of PUFAs on the sex of the offspring in cattle have not to our knowledge been investigated to date. Therefore, the aim of the present study was twofold. Firstly we examined the effect of dietary supplementation of cows with two levels of protected fat (Calcium salt of fatty acids; Magnapac) on the sex of the calves produced. Secondly we tested supplementation with either ALA, LA or CLA during bovine *in vitro* oocyte maturation media. This was to determine whether any effects on the sex ratio of the embryos produced were directly on the oocyte and to identify differential effects of different PUFAs.

2. Materials and methods

2.1. Experimental animals and feeding

The experimental work of this study was carried out at a private dairy farm in cooperation with the Animal Production Department, Faculty of Agriculture, Mansoura University. Holstein cows in late lactation, 490–540 kg LBW, 1–4 parities were individually fed according to the nutrient requirements recommended by NRC [29]. The control ration consisted of concentrate feed mixture (CFM; 19% Crude Protein), corn silage (ranged from 18–20 kg/day) and berseem hay (2 kg/day). The concentrate feed mixture (CFM) was composed of 46% yellow corn, 10% wheat bran, 10% cottonseed meal, 20% soybean, 10% horse bean, 1% NaCl, 0.1% Toxfree™ (Alfa Chemical, Mansourah, Egypt), 0.4% Premix, 1.3% sodium bicarbonate, 0.2% vitamins mixture, and 1% limestone. Roughage (silage and hay) was offered ad libitum while the CFM was offered individually for each animal twice daily before milking. This resulted in a roughage: concentrate ratio of nearly 60:40%. All animals had free access to clean drinking water and mineralized salt stone.

2.2. Experimental design

Sixty Holstein cows were stratified and randomly divided into three groups according to BW and parity. Experimental cows in group 1 (n = 20) were fed with the control diet without any fat supplementation. Groups 2 and 3 were fed with the CFM of the control diet supplemented with 3% dry matter (DM) or 5% DM respectively of a protected fat (PF) (Magnapac, Norel & Nature Comp., Madrid, Spain, a calcium salt of fatty acids). The fatty acid profile of Magnapac according to the product specifications is myristic acid (C14) 1.5%, palmitic acid (C16) 44.0%, stearic acid (C18) 5.0%, oleic acid (C18:1) 40.0% and linoleic acid (C18:2) 9.5%. The net energy for lactation (NEL) was 1.76, 1.82, and 1.84 Mcal/kg DM in control, 3% PF and 5% PF diets, respectively. The experimental feeding period started at 14–21 days pre-partum and continued up to 120 day-post-partum or conception (1–3 inseminations). The nutrient requirements were adjusted every two weeks according to changes in milk yield. Cows were inseminated by artificial insemination after detection of estrus according to the traditional am–pm rule. The same proven-fertile frozen semen source was used in all treatment groups. Cows that did not conceive at first AI were re-inseminated at the next estrous. Cows that did not return to estrus after insemination were examined transrectally by ultrasonography to confirm pregnancy at 35 days post insemination. A minimum of one and a maximum of three inseminations per conception were required. During the post-partum period, live body weight (LBW) was measured and the body condition score was estimated on a scale of 1–5 (1 = emaciated, 5 = extremely fat) by the same trained technician at 15, 30, 45 and 60 days in milk (DIM). One cow in the control group was excluded from the study due to repeat breeding. The sex of the offspring was then recorded at birth.

2.3. Chemicals and reagents used for the in vitro experiment

All chemicals and reagents were purchased from Sigma Chemical Company (Poole, Dorset, UK) unless otherwise stated.

2.4. Collection of oocytes

Bovine ovaries were collected immediately after slaughter from a local abattoir and transported within 2 h to the laboratory in phosphate buffered saline (PBS) at 37 °C. Ovaries were washed in PBS and 70% ethanol. COCs were aspirated from antral follicles (3–8 mm in diameter) and only grade-I COCs were used for the experiment [30]. Serum-free TCM-199 medium supplemented with 20 mM HEPES and 0.4% (w/v) BSA was used during COC selection and washing.

2.5. In vitro maturation and PUFA supplementation

Selected COCs were cultured in four-well dishes (NUNC, Thermo Fisher Scientific, Loughborough, Leicestershire, UK) in serum-free maturation medium (TCM-199) supplemented with 10 μg/mL LH (Leutropin; Bioniche Animal Health, Belleville ON), 10 μg/mL FSH (Follitropin; Bioniche), 1 μg/mL oestradiol, 0.6% (w/v) fatty acid-free BSA, and 50 μg/mL gentamycin. Stock solutions of alpha-linolenic acid (ALA; n-3 18:3), linoleic acid (n-6; 18:2) and trans-10, cis-12 conjugated linoleic acid (CLA; n-6 18:2) were prepared in DMSO (100 mM). They were added to maturation media at a final concentration of 50 μM. Media was incubated without COCs for 2 h at 38.5 °C to allow binding of PUFAs to BSA which acts as a carrier. DMSO was added to the control group at the same concentration used with PUFAs (0.05%). We have previously shown that DMSO at this concentration has no effect on maturation and embryo development compared to DMSO-free controls [31]. COCs were incubated in maturation media containing DMSO or PUFAs for 24 h at 38.5 °C under 5% CO2 in humidified air. A total of 773 COCs were used in this experiment.

2.6. In vitro fertilization and embryo culture

In vitro matured oocytes (in the presence or absence of 50 μM ALA, LA or CLA) were fertilized with frozen semen from a single bull as previously described by Fouladi-Nashta and Campbell [32]. Briefly, swim up technique was used to select motile spermatozoa using calcium-free medium (for 45 min). Supernatant was then centrifuged at 300 × g at room temperature and spermatozoa were re-suspended (at a concentration of 1×10^6 sperm/mL) in fertilization medium (Fert-

TALP) supplemented with 0.6% (w/v) fatty acid-free BSA, 1 μg/mL heparin, 50 ng/mL hypotaurine, and 50 ng/mL epinephrine. COCs were fertilized in 4-well dishes containing 400 μl of fertilization medium with 1×10^6 sperm/mL for 18 h at 38.5 °C, in a humidified incubator of 5% CO_2 in air. Afterwards, presumptive zygotes were denuded from cumulus cells by gentle pipetting and transferred to 4-well plates containing 500 μL/well of SOFaaci (synthetic oviductal fluid medium containing amino acids, myoinositol, and sodium citrate [33]) supplemented with 0.4% (w/v) fatty acid-free BSA, then incubated at 38.5 °C in a humidified incubator with 5% O_2, 5% CO_2, and 90% N_2. The culture was continued up to Day 7. Fetal calf serum (FCS) was added at 5% to the embryo culture media starting from day 3. Blastocysts on day 7 were washed in PBS and individually snap frozen in 1.5 mL microcentrifuge tubes using liquid nitrogen. Samples were kept at −20 °C until genomic DNA extraction.

2.7. Genomic DNA extraction and conventional PCR

Genomic DNA was extracted from individual blastocyst using QIAamp DNA Micro Kit (QIAGEN Ltd, West Sussex, UK) with carrier RNA following manufacturer guidelines.

Conventional PCR amplification was performed using 5 μL purified DNA and 2 μm primers (Table 1) specific to *SRY* using a Multiplex PCR kit (Qiagen). All samples were also analyzed for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as a control. Male and female DNA samples from bull liver and uterus were used as positive and negative controls. PCR products were visualized using ethidium bromide staining on agarose gel electrophoresis.

2.8. Statistical analysis

Mean body condition score and live body weight (LBW, as log transformed data) were compared using ANOVA followed by Bonferroni correction for multiple comparison. Sex ratio was analyzed using binomial analyses in SPSS against the expected proportion of 0.5 (1:1). Differences of P values ≤0.05 were considered as significant.

3. Results

3.1. Effect of protected fat dietary supplementation on the live body weight (LBW) and body condition score of the dam

In the control group, a gradual loss of live body weight resulted in an overall reduction of 28 ± 16.3 kg within 45 days postpartum. Weight loss was less marked in cows receiving 3% PF ($P > 0.05$ compared to control) while, 5% PF supplementation prevented weight loss and resulted in an average body weight gain of 8 ± 7.6 kg during the same period (Table 2). This was also observed in body condition score.

3.2. Effect of protected fat dietary supplementation on sex ratio of the offspring

The sex ratio of calves produced by cows in the different dietary treatment groups (0%, 3% and 5% PF) during treatment period is presented in Table 3. Cows in both fat-supplemented treatment groups produced significantly more male than female calves ($P < 0.05$), while the number of males in the 5% PF group was relatively higher than that in the 3% PF group.

Table 1
Oligonucleotide primer sequence and amplicon size used for PCR.

Gene	Sequence	Accession number
SRY	Forward: AAA CAG TGC AGT CGT ATG CTT CTG Reverse: GCC TTT GTT AGC GAG AGT AAG GAA	EU294189.1
GADPH	Forward: CCA ACG TGT CTG TTG TGG ATC TGA Reverse: GAG CTT GAC AAA GTG GTC GTT GAG	NM_001034034.2

Table 2
Effect of dietary protected fat on live body weight (LBW) and body condition score (BCS) of dairy cows during early postpartum period.

	Control	3% PF	5% PF
LBW at 15 DIM, kg	557.4 ± 38.13	560.0 ± 38.56	531.4 ± 39.94
LBW at 30 DIM, kg	530.6 ± 27.99	540.8 ± 39.65	536.4 ± 41.54
LBW at 45 DIM, kg	532.2 ± 23.86	554.4 ± 39.12	535.0 ± 39.10
LBW at 60 DIM, kg	529.0 ± 29.94	544.0 ± 35.66	539.2 ± 37.44
Overall change in LBW, kg	−28 ± 16.3[a]	−16 ± 6.2[a]	8 ± 7.6[b]
Average change in LBW, g/d	−631 ± 361.5[a]	−356 ± 137.9[a]	173 ± 169.4[b]
BCS at 15 DIM	3.3 ± 0.27	3.3 ± 0.18	3.1 ± 0.14
BCS at 30 DIM	2.9 ± 0.13	3.0 ± 0.20	3.1 ± 0.20
BCS at 45 DIM	2.8 ± 0.21	3.1 ± 0.17	3.1 ± 0.17
BCS at 60 DIM	2.7 ± 0.20	3 ± 0.14	3.2 ± 0.17
Overall change in BCS	−0.6 ± 0.12[a]	−0.3 ± 0.08[b]	0.1 ± 0.1[c]

a-c, means with different superscripts are significantly different at $P < 0.05$. DIM, days in milk.

Table 3
Sex ratio of offspring produced from cows fed different experimental diets during the postpartum period.

Treatment group	Male (%)
Control	8/19 (42.1%)[a]
PF 3%	14/20 (70%)[a,b]
PF 5%	17/20 (85%)[b]

Proportions with different superscripts are significantly different at $P < 0.05$.

3.3. Effect of PUFA supplementation during IVM on the sex of embryos produced

Morulae and blastocysts produced *in vitro* following exposure to free fatty acids were individually analyzed by PCR to determine their sex (Fig. 1). In COCs treated with n-6 LA or CLA fatty acids during maturation, the sex ratio was skewed toward males ($P < 0.05$), whereas in control and ALA groups the sex ratio was not significantly different from the expected proportion of 50% (Table 4).

4. Discussion

Our results revealed a significant shift to a more male sex ratio in offspring of cows supplemented with a Ca salt of fatty acids that is rich in LA. Furthermore, oocytes matured *in vitro* in the presence of the omega-6 PUFAs, LA or CLA, were more likely to produce a male embryo compared to those supplemented with the omega-3 ALA or to FA-free controls. This may have very important implications in cattle production systems as such feeds are currently used for their potential to improve fertility. Positive impact of PF on some fertility outcome parameters of the cows used in this study (n = 5 per group) namely: duration of placental drop, uterine involution, and conception rate have been previously reported [34]. Similarly, dairy cows (n = 511) fed with Ca-soap of long chain FAs enriched with LA had higher pregnancy rates at 27 days ($P = 0.07$) and 41 days ($P = 0.05$) after insemination, compared to cows fed Ca-Palm oil [35]. This FAs supplementation also reduced incidence of metritis during the early postpartum period.

Several factors have been proposed to modulate the sex ratio of mammalian offspring. Most relevant to the present study are the effects of maternal diet, type and quantity as reviewed by Rosenfeld [36]. Among all studies that investigated the effect of maternal nutrition on

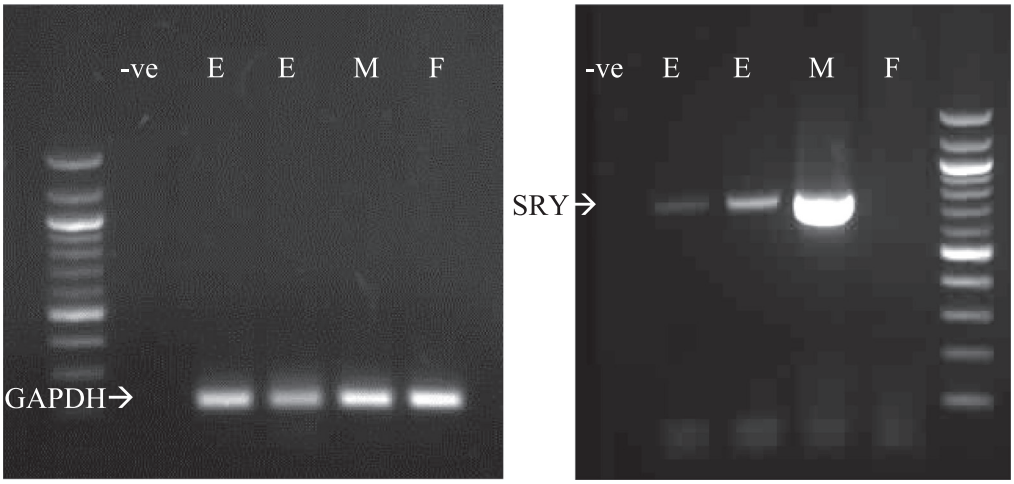

Fig. 1. Agarose gel electrophoresis showing PCR products of *GAPDH* and *SRY* genes in genomic DNA extracted from E, embryo; M, liver of a bull; F: uterus; -ve, no template control. 100 bp ladder was also loaded for reference.

Table 4
Proportion of male and female embryos produced from COCs matured in the presence or absence of 50 μM of ALA, LA or CLA. The control group was supplemented with 0.05% DMSO equivalent to that used to dissolve fatty acids. *P* values are generated by comparing treatment group proportions to the expected sex ratio of 0.5.

	n	Female	Male	Male ratio	*P* value
Control	18	10	8	0.44	0.8
ALA	26	12	14	0.54	0.8
LA	42	9	33	0.79	0.001
CLA	31	9	22	0.71	0.03

sex ratio of offspring, the underlying mechanism favouring the dominance of one sex over the other remains unknown. The sex allocation hypothesis proposed by Trivers and Willard [37] predicted that females with higher body condition score (BCS) would be better in producing male offspring, allowing the genes of these fitter females to be propagated in the gene pool of following generations. In the present study the BCS of the females supplemented with protected fat was higher than in the controls. This is also in line with the effect of maternal caloric intake in mice on the sex ratio of the offspring. Female mice supplemented with very high dietary fat (55%) resulted in a high male to female ratio of up to 71% [5,6]. On the contrary, calorie restriction resulted in 1:3 male to female ratio in mice compared to 1:1 ratio in controls [38,39]. A similar positive correlation between the socioeconomic patterns of food availability and male births have been reported in humans [40].

It was suggested that the dietary effects on sex ratio are mainly maternal since exposing the breeder male mice to high fat diets failed to alter sex ratio and resulted in a normal percentage of X: Y spermatozoa [12]. We therefore hypothesized that the effect of protected fat supplementation mentioned above is mediated by a direct effect on the oocyte. Many studies have clearly shown that the metabolic changes in blood serum are reflected in the follicular fluid composition and may thus directly influence cumulus cells and oocyte composition and quality [41–43]. PUFAs constitute the major portion of the fatty acid content of bovine follicular fluid, with linoleic acid (18:2) contributing about one third of the total fatty acid composition [44]. Fatty acid composition of lipids in the oocytes and their surrounding cumulus cells was found to vary according to many factors including quality of the oocyte [45], the season [46], as well as dietary fatty acid composition [47] and may have direct effects on oocyte cellular functions and developmental potential. For example, feeding encapsulated sunflower (E-SUN) or flaxseed (E-FLAX) oils to Holstein dairy cows markedly increased the corresponding fatty acid content in the COCs [47]. The percentage of LA in the COCs was 54% higher in E-SUN vs. E-FLAX and was 2.4-fold higher than the controls fed no fat supplement [47]. This clearly shows that the FA composition of the oocyte can be manipulated by diet. In sheep, n-3 (salmon oil) and n-6 (sunflower oil) enriched diets also resulted in significant increases in the n-3 and n-6 content of the granulosa cells and oocytes, respectively [43].

We also tested the individual effects of ALA, LA and CLA, the most predominant PUFAs in dietary supplements, during oocyte maturation on the sex ratio of *in vitro* produced embryos. We found that the omega-6 LA and CLA, but not the omega-3 ALA, skewed the sex ratio towards male embryos. We have previously shown that supplementation of PUFAs (LA and ALA) during oocyte maturation significantly increase prostaglandin production as detected in the spent media [31,48]. This proves that the treated COCs could incorporate and use the supplemented fatty acids. Fatty acid incorporation into the cellular structure may affect various signaling molecules as well as cell membrane properties [24].

In a recent review examining sex ratio after assisted reproduction in humans, four suggestions were made to account for skewed sex ratios in 4–8 cell embryos produced by IVF [49]. These were: (i) male embryos have a developmental advantage after fertilization; (ii) the sperm preparation technique may favour Y-bearing spermatozoa; (iii) the composition of the zona pellucida may increase the chances of Y spermatozoa fertilizing the oocyte or (iv) Y bearing spermatozoa may have higher fertilizing ability. Of these, the effects on the zona pellucida were considered most likely although Zuccotti et al. [50] reported that mammalian oocytes are not selective towards X- or Y-bearing spermatozoa. Another suggestion was that culture conditions could influence the process of X-inactivation, which normally occurs in female embryos via interference with DNA methylation, chromatin modification or histone deacetylation [49]. Interestingly, a recent study found that knockout of Annexin A1, a glucocorticoid-regulated anti-inflammatory protein, in mice resulted in a significantly higher percentage of female pups, without affecting the proportion of Y and X chromosomes in the males [51]. Annexin A1 inhibits phospholipase A2 inhibitory activity, needed for the biosynthesis of inflammatory mediators such as prostaglandins [52]. Omega 3 PUFAs (EPA and DHA) can differentially affect the expression patterns of cell-associated Annexin A2 in cultured human umbilical vein cells [53], however effects on Annexin A1 on sex ratio have not been reported.

5. Conclusions

In conclusion, this study is the first to report effects of omega 6 PUFAs on the sex ratio of the offspring in cattle. Dietary protected fat supplementation increased the male: female ratio. Our data also suggest

that this might be mediated, at least in part, via a direct effect of PUFAs on the oocyte. Further research is required to determine the associated structural and molecular mechanisms involved.

Competing interests

The authors have no conflict of interest to declare.

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