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A higher proportion of female lambs in Kurdish × Romanov ewes fed a diet rich in n-3 fatty acids and rumen undegradable protein around mating

Marziyeh Fatahi[a], Farshid Fatahnia[a]*, Hoshang Jafari[b], Golnaz Taasoli[c], Aminolah Pormalekshahi[b], Shahryar Kargar[d], Edward H. Clayton[e]

[a] Department of Animal Science, Ilam University, Ilam, I. R. Iran.
[b] Animal Science Research Department, Ilam Agricultural and Natural Resources Research and Education Centre, AREEO, Ilam, I. R. Iran
[c] Department of Animal Science, Chaharmahal and Bakhtiari Agricultural and Natural Resources Research and Education Centre, AREEO, Shahrekord 88156–89554, I. R. Iran
[d] Department of Animal Science, School of Agriculture, Shiraz University, Shiraz 71441–65186, I. R. Iran
[e] NSW Department of Primary Industries, Wagga Agricultural Institute, Wagga, NSW 2650, Australia

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ABSTRACT- An increased intake of rumen undegradable protein (RUP) or n-3 fatty acids (FA) around mating is connected with improved reproductive efficiency in ewes. This experiment was aimed to study whether the ratio of female lambs was greater when Kurdish × Romanov ewes were received a diet rich in n-3 FA and RUP together around mating. Experimental diets were supplemented with 5% (DM basis) Ca salts of saturated FA (SFA), 5% Ca salts of fish oil (as n-3 FA source; n-3FO), or a combination of 5% Ca salts of fish oil and 5% fish meal (as a RUP source; n-3FO+RUP). The results showed that the proportion of total n-3 polyunsaturated FA (PUFA) in plasma increased and the n-6:n-3 decreased at CIDR removal day in animals received the n-3FO+RUP diet ($P < 0.05$) compared with the other diets. Plasma concentrations of oestradiol, glucose, and urea nitrogen (UN) at the day of oestrus ($P < 0.05$) was also lower when ewes received the n-3FO+RUP diet compared with the other diets. The ratio of female lambs was greater when ewes were fed the n-3FO+RUP diet around mating ($P < 0.05$) and was greater than an expected 50:50 ratio (72% females, $P = 0.028$). It was concluded that feeding ewes a diet rich in n-3 FA and RUP content around mating, rather than n-3 FA alone, could skew the sex ratio of lambs toward females.

INTRODUCTION

Altering the sex ratio make a benefit for small stockholders to enhance desirable gender in the herd. For example, more rapid growth was reported in male lambs (Tatum et al., 1998).

Specific nutrients composition such as the ratio of Na + K:Ca + Mg (Arangasamy et al., 2015), total energy (Mathews et al., 2008), or glucose (Kimura et al., 2005) intake can affect offspring sex ratio in mammals.

Omega-3 fatty acids (n-3 FA) including eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and arachidonic acid (ARA) are the main FA in animal reproduction (Abayasekara and Wathes, 1999). There is strong evidence linking these FA with some reproductive factors including follicle development, oocyte maturation and quality, steroid hormone synthesis, embryo survival, pregnancy rate, and uterine prostaglandin synthesis in ruminant animals (Gulliver et al., 2012). Likewise, n-3 and n-6 FA also have connection with offspring sex ratio in mammals. For instance, significantly more male offspring were born when dogs received fish oil (Gharagozlou et al., 2016) and when ewes were supplemented with a diet reportedly rich in n-6 FA (Green et al. 2008), however, the FA profile of the diet was not reported. Several studies show other FA can skew the sex ratio towards females and feeding ewes a diet containing sunflower oil, (n-6 FA), around the time of mating altered the sex ratio towards more females (Mirzaei Alamouti et al., 2018). Likewise, the proportion of female lambs was greater in three additional studies when ewes were fed diets rich in n-6 FA (Gulliver et al., 2013; Clayton et al., 2016, 2017).

Proteins are also vital for ruminant reproduction. The reproductive performance of ewes was improved when they fed a diet containing greater rumen undegradable protein (RUP) around mating (Daghigh kia et al., 2016). However, lower fertility has been reported when dairy cows were fed excess rumen degradable protein (RDP), which was associated with higher blood urea nitrogen (UN) concentration (Butler, 1998; Rhoads et al., 2004; Hammon et al., 2005). To our knowledge, the influence of dietary protein quality and quantity and its concurrent feeding with FA source on mammalian offspring sex ratio has not previously been investigated. Hence, the objective of the this experiment was to determine whether the sex ratio of lambs altered when dams were fed diet rich in n-3 FA and RUP source together around mating.

MATERIALS AND METHODS

Experiment was conducted on a farm (Ilam, Iran; latitude 33° 21´E and longitude 45° 41´E) during the breeding

Corresponding Author, Professor, Department of Animal Science, Faculty of Agriculture, Ilam University, Ilam, I. R. Iran
E-mail address: f.fatahnia@ilam.ac.ir
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season (August to September). Sixty cycling Kurdish × Romanov ewes (42.3 ± 2.5 kg and 2 to 4 years of age) were randomly allocated to the experimental diets (n = 20 / diet). One month before the beginning of feeding experimental diets, all ewes were vaccinated against clostridial diseases (Razi Institute, Hesarak, Karaj, Iran) and treated with anthelmintic drugs against external and internal parasites. All animals were allowed to graze on rangeland and pasture as a single group during the daytime from 09:00 to 18:00. The space used for grazing consisted of approximately 50% pasture of alfalfa and 50% rangeland with a variety of plants, shrubs, and trees and was mainly composed of grasses and oak trees. Water was available all times. Animals were kept in indoor pens by group at night, where ewes received their experimental diets (350 g/day/hd) from 40 days before to 28 days after mating. Experimental diets were based on barley grain and soybean meal supplemented with 5% Ca salts of palm oil as saturated FA (SFA) source, 5% Ca salts of fish oil (n-3FO) as n-3 FA source, or a combination of 5% Ca salts of fish oil as n-3 FA source and 5% fish meal as RUP source (n-3FO+RUP) (Table 1).

Table 1. Ingredients and chemical composition of experimental diets

Item	Experimental diet*		
	SFA	n-3FO	n-3FO+RUP
Ingredients (g/kg of DM)			
Barley grain	700.0	700.0	700.0
Soybean meal	125.0	125.0	75.0
Wheat bran	100.0	100.0	100.0
Calcium salts of SFA **	50.0	0.0	0.0
Calcium salts of n-3 fatty acids	0.0	50.0	50.0
Fish meal	0.0	0.0	50.0
Minerals and vitamins	20.0	20.0	20.0
Salt	5.0	5.0	5.0
Chemical composition (g/kg of DM)			
Crude protein (CP)	172.0	172.0	174.0
RUP	68.8	68.8	87.0
RDP	103.2	103.2	87.0
Neutral detergent fibre (NDF)	196.0	196.0	191.0
Non fiberous carbohydrates (NFC)	517.0	517.0	508.0
Ash	65.0	65.0	71.0
Ether extract (EE)	64.0	64.0	69.0
Na	2.2	2.2	2.7
K	8.5	8.5	7.7
Ca	9.5	9.5	12.1
Mg	2.1	2.1	2.1
Metabolizable energy (MJ/kg of DM)	11.43	11.43	11.47
Na + K:Ca + Mg	0.92	0.92	0.73
Fatty Acids			
C10:0	0.08	0.01	0.45
C12:0	0.11	0.30	0.35
C14:0	1.17	0.62	0.95
C16:0	27.69	26.33	22.35
C17:0	0.22	0.11	0.33
C18:0	3.76	2.88	3.77
C20:0	0.13	0.20	0.30
C22:0	0.43	0.36	0.48
C14:1n-5	0.24	0.28	0.30
C16:1n-7	1.35	0.80	1.64
C18:1n-9	21.56	18.30	22.51
C20:1n-9	0.39	0.66	0.67
C18:2n-6	38.24	43.27	39.99
C18:3 n-3	3.58	4.11	3.26
C20:5n-3	0.00	0.09	0.30
C22:6n-3	0.00	0.52	0.69
Total saturated fatty acids (SFA)	33.59	30.81	28.98
Total mono unsaturated fatty acids (MUFA)	23.54	20.04	25.12
Total n-3 poly unsaturated acids (PUFA)	3.58	4.72	4.25
Total n-6 PUFA	38.24	43.27	39.99
Minor	1.05	1.16	1.66
n-6:n-3 Ratio	10.68	9.17	9.41
MUFA:SFA ratio	0.70	0.65	0.87
PUFA:SFA ratio	1.25	1.56	1.53

* Experimental diets, SFA = diet containing 5% calcium salts of saturated fat; n-3FO = diet containing 5% calcium salts of fish oil as n-3 source; n-3FO+RUP = diet containing 5% calcium salts of fish oil as n-3 source plus 5% fish meal as RUP source.
** Persia fat silver
*** Persia fat omega-3

Diets were formulated according to NRC (2007) and had approximately similar chemical composition (Table 1). Supplemental fats, as Ca salts of FA, were provided by a commercial company (Kimiya Danesh Alvand Co., Qom, Iran). After the end of feeding experimental diets until pregnancy and lambing, all ewes were managed and kept in the same conditions. The sequence of events and timing of feeding are shown in Fig. 1.

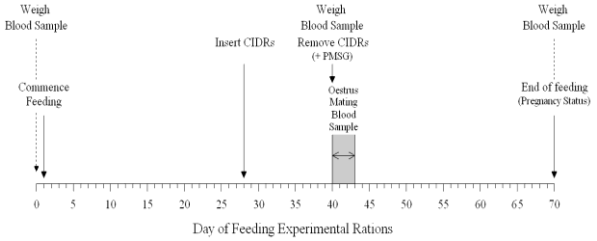

Fig. 1. Sequence of events and timing of feeding experimental diets.

Ewes synchronization

Twenty eight days after the start of feeding experimental diets, the oestrus cycle of all ewes were synchronized CIDRs intra-vaginally for 12 days, followed by PMSG (400 IU) injection immediately after CIDR removal and received an intramuscular injection of 5 mL of vitamin E and selenium solution (VitEsel, Nasr Fariman Co., Fariman, Iran) at the day of CIDR removal. Each mL of solution contained 50 mg dl-α-tocopheryl acetate and 0.5 mg sodium selenite. All ewes were mated with Romanov rams (1 ram/5 ewes) for three consecutive days. All animals were under the care of veterinarian during the experiment.

Data collection

Samples of experimental diets were collected weekly and composite samples were grounded using a Wiley mill (1-mm screen) prior to the analysis of crude protein (CP), ether extract (EE), ash (AOAC, 2007), and neutral detergent fibre (NDF) (Van Soest et al., 1991). Fatty acids profile of experimental diets was measured by gas chromatography (Folch et al., 1957).

The body weight of all ewes was measured on the day before the introduction of experimental diets (day 0), the day of CIDR removal and 30 days post-mating. Total number, sex, and birth weight of lambs were recorded. Blood samples were collected from 10 ewes/ dietary treatment group on the day before the introduction of experimental diets (day 0), on the day of CIDR removal, on the day of oestrus and mating and 30 days post-mating. Samples were collected from the same ewes on each occasion. Blood samples were taken, plasma was separated and frozen at -20 ºC. Plasma FA composition was determined by gas chromatography (Folch et al., 1957). Plasma concentrations of metabolites including glucose and UN were measured using an analyser (BT1500, Biotecnica, SRL) based on the manufacturer's protocol. Plasma insulin, progesterone, and estradiol concentrations were measured using ELISA procedure. Concentrations of minerals in experimental diets and plasma were determined by Flame Atomic Absorption Spectrometer.

Statistical analysis

Plasma fatty acids over the time was analysed as repeated measurements (SAS Institute Inc., Cary, NC). Pre-feeding concentrations of FA were examined as covariates. Hormones and blood metabolites data were analysed by MIXED procedure. The reproductive parameters were analysed by non-parametric test. Effects of ewe body weight (BW) and age were considered as covariate. Mean comparisons was performed by LSM method using the Tukey test. Significance differences among treatments were declared at $P < 0.05$.

RESULTS

Plasma FA

Dietary treatment had no significant effect on the proportion of plasma SFA and monounsaturated FA (MUFA) at the day of CIDR removal ($P \geq 0.05$) (Table 2). The proportions of C18:3n-3, C22:6n-3, and total n-3 PUFA were higher and the n-6:n-3 was lower at the day of CIDR removal when dams were fed the n-3FO+RUP diet compared with either the SFA of n-3FO diets. The proportions of C18:2n-6 and total n-6PUFA as well as the arachidonic acid to eicosapentaenoic acid ratio (ARA:EPA ratio) were lower at the day of CIDR removal when ewes were fed the n-3FO or n-3FO+RUP diets compared with the SFA diet.

Plasma hormones

Plasma concentrations of insulin and progesterone were not affected ($P \geq 0.05$) by the experimental diets at the day of CIDR removal or oestrus (Fig. 2). At the day of oestrus, the oestradiol concentration was significantly affected by the dietary treatments ($P < 0.05$). The lowest and highest concentrations were observed in ewes fed n-3FO+RUP and SFA diets, respectively, while the n-3FO had an intermediate value ($P < 0.05$). The lowest plasma oestradiol concentration at the day of oestrus was observed in ewes fed n-3FO+RUP diet ($P < 0.05$).

The plasma glucose and N-urea concentrations were lower ($P < 0.05$) at the time of oestrus when ewes received the n-3FO+RUP diet compared with the SFA or n-3FO diets (Fig. 3).

Plasma minerals

Plasma concentrations of Na, K, Ca, and Mg at the day of CIDR removal were not influenced by the experimental diets ($P \geq 0.05$), however, the plasma concentration of Na and the Na+K:Ca+Mg ratio at the day of oestrus and mating were higher ($P < 0.05$) when ewes were fed n-3FO diet compared with those fed either the SFA or n-3FO+RUP diets (Table 3).

Reproduction outcome

Experimental diets had no significant effect on the total number of lambs (Table 4). However, the female:male ratio was significantly affected ($P < 0.05$) by the dietary treatments (Table 4) with higher ratio of female lambs for dams fed the n-3FO+RUP diet compared with either the SFA or n-3FO diets (Fig. 4).

Table 2. Fatty acid composition of plasma (% of total fatty acids) of ewes were fed at Pre-feeding experimental diets (Day 0) and at CIDR removal.

FA methyl ester	Pre- Feeding (Day 0)			CIDR Out			SEM*	*P*-values		
	Experimental diets			Experimental diets						
	SFA**	n-3FO	n-3FO +RUP	SFA	n-3FO	n-3FO +RUP		D	Time	Diet × Time
Saturated fatty acids										
C10:0	3.81^{b***}	4.75^{a}	4.82^{a}	2.73^{c}	3.73^{b}	3.41^{b}	0.22	< 0.01	< 0.01	0.48
C12:0	2.57	2.82	2.70	1.91	1.87	1.86	0.17	0.85	< 0.01	0.64
C14:0	2.86	2.60	2.72	3.43	3.63	3.37	0.17	0.86	< 0.01	0.26
C16:0	16.73	16.89	16.22	16.47	16.19	16.02	0.55	0.67	0.37	0.87
C18:0	14.35	14.10	14.22	12.20	11.96	10.85	0.46	0.26	< 0.01	0.34
Minor SFA	4.93	5.56	5.98	5.15	5.40	7.49	0.98	0.28	0.47	0.61
Total SFA	45.25	46.71	46.65	41.89	42.79	43.00	0.64	0.10	< 0.01	0.91
MUFA										
C14:1n-5	0.82^{bc}	0.96^{b}	0.95^{b}	0.78^{c}	0.91^{bc}	1.25^{a}	0.05	< 0.01	0.12	< 0.01
C16:1n-7	1.52^{cd}	1.43^{d}	1.56^{cd}	1.71^{bc}	1.79^{b}	2.12^{a}	0.07	< 0.01	< 0.01	0.05
C18:1n-9	13.12^{c}	14.45^{bc}	14.59^{bc}	17.08^{a}	17.68^{a}	15.12^{b}	0.53	0.03	< 0.01	0.02
C18:1n-7t	1.36^{c}	1.36^{c}	1.47^{bc}	1.35^{c}	1.58^{b}	2.48^{a}	0.06	< 0.01	< 0.01	< 0.01
Minor MUFA	1.74	1.74	1.96	2.92	2.84	2.27	0.51	0.92	0.02	0.57
Total MUFA	18.56	19.93	20.52	23.83	24.79	23.25	0.57	0.18	< 0.01	0.05
n-3 PUFA										
C18:3n-3	1.49^{b}	1.38^{b}	1.41^{b}	1.50^{b}	1.37^{b}	1.78^{a}	0.05	< 0.01	< 0.01	0.01
C20:5n-3	0.84^{b}	0.82^{b}	0.95^{b}	0.43^{c}	1.72^{a}	1.76^{a}	0.04	< 0.01	< 0.01	< 0.01
C22:6n-3	0.33^{c}	0.39^{c}	0.39^{c}	0.12^{d}	0.81^{b}	1.30^{a}	0.03	< 0.01	< 0.01	< 0.01
Total n-3 PUFA	2.65^{c}	2.59^{c}	2.75^{c}	2.05^{d}	3.90^{b}	4.84^{a}	0.07	< 0.01	< 0.01	< 0.01
n-6 PUFA										
C18:2n-6	13.73^{b}	13.34^{b}	14.02^{b}	16.64^{a}	14.15^{b}	13.55^{b}	0.47	< 0.01	< 0.01	< 0.01
C20:4n-6	1.11	1.15	1.28	1.42	1.18	1.22	0.10	0.60	0.22	0.14
Total n-6 PUFA	14.84^{b}	14.49^{b}	15.30^{b}	18.06^{a}	15.33^{b}	14.77^{b}	0.49	< 0.01	< 0.01	< 0.01
CLA										
c9,t11CLA	0.43^{a}	0.39^{a}	0.23^{c}	0.35^{ab}	0.29^{bc}	0.31^{bc}	0.03	< 0.01	0.15	< 0.01
t10,c12CLA	0.13^{ab}	0.11^{bc}	0.16^{a}	0.05^{d}	0.08^{cd}	0.05^{d}	0.01	0.60	< 0.01	< 0.01
Total CLA	0.56^{a}	0.50^{a}	0.39^{b}	0.40^{b}	0.37^{b}	0.36^{b}	0.03	< 0.01	< 0.01	0.11
Totals and ratios										
Total identified	75.19	76.92	77.67	78.15	78.95	76.46	1.08	0.47	0.18	0.17
Total FAME	81.86	84.21	85.61	86.23	87.18	86.23	0.93	0.09	< 0.01	0.16
Minor FAME	18.14	15.79	14.39	13.78	12.83	13.77	0.93	0.09	< 0.01	0.16
n-6:n-3 ratio	5.63^{b}	5.63^{b}	5.62^{b}	8.84^{a}	3.95^{c}	3.05^{d}	0.22	< 0.01	0.05	< 0.01
ARA:EPA ratio	1.37^{b}	1.43^{b}	1.37^{b}	3.59^{a}	0.69^{c}	0.69^{c}	0.22	< 0.01	0.16	< 0.01
EFI****	1.25^{a}	1.12^{ab}	1.15^{ab}	1.10^{ab}	1.02^{b}	1.17^{a}	0.05	0.04	0.11	0.31
P:S ratio	0.45	0.43	0.46	0.56	0.53	0.56	0.01	0.09	< 0.01	0.89

* Standard error of mean. ** SFA = diet containing 5% calcium salts of saturated fat; n-3FO = diet containing 5% calcium salts of fish oil as n-3 source; n-3FO+RUP = diet containing 5% calcium salts of fish oil as n-3 source plus 5% fish meal as RUP source.
*** Means within a row with different superscripts are significantly different ($P \leqslant 0.05$).
**** Essential fatty acids index: n-3 + n-6 PUFA: n-7 + n-9 MUFA.

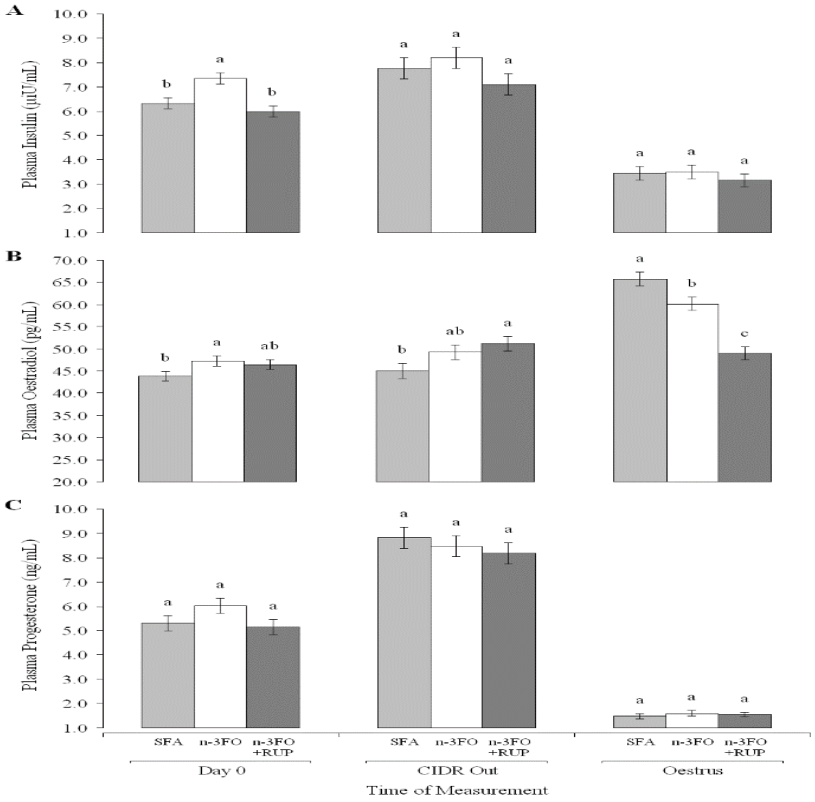

Fig. 2. Mean plasma concentrations of (A) insulin, (B) oestradiol, and (C) progesterone in relation to CIDR out and oestrus when ewes were fed experimental diets for 40 days prior to 30 days post-mating. SFA = diet containing 5% calcium salts of saturated fat; n-3FO = diet containing 5% calcium salts of fish oil as n-3 FA source; n-3FO+RUP = diet containing 5% calcium salts of fish oil as n-3 FA source plus 5% fish meal as RUP source. Mean values within each parameter and time-point with different superscripts differ significantly ($P < 0.05$).

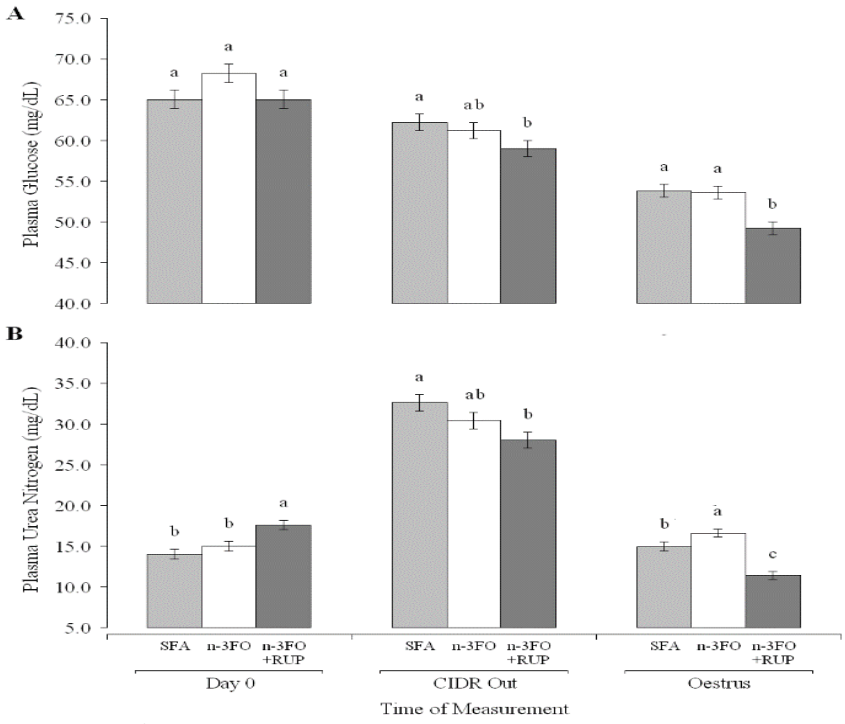

Fig. 3. Mean plasma concentrations of (A) glucose and (B) urea N in relation to CIDR removal and oestrus when ewes were fed experimental diets for 40 days prior to 30 days post-mating. SFA = diet containing 5% calcium salts of saturated fat; n-3FO = diet containing 5% calcium salts of fish oil as n-3 FA source; n-3FO+RUP = diet containing 5% calcium salts of fish oil as n-3 FA source plus 5% fish meal as RUP source. Mean values within each parameter and time-point with different superscripts differ significantly ($P < 0.05$).

Table 3. Mean plasma mineral concentrations when ewes were fed experimental diets for 40 days prior to and 30 days post-mating.

Parameter	Experimental diet *			SEM*	*P*-value
	SFA	n-3FO	n-3FO+RUP		
Pre-feeding (Day 0)					
Na ($mEqL^{-1}$)	261.3	267.4	258.3	12.87	0.880
K ($mEqL^{-1}$)	21.29	22.14	22.24	0.86	0.696
Mg ($mgdL^{-1}$)	2.39	2.43	2.42	0.10	0.955
Ca ($mgdL^{-1}$)	11.38	11.46	11.27	0.47	0.960
Na + K:Ca + Mg ratio	20.53	20.87	20.70	0.81	0.957
CIDR out					
Na** ($mEqL^{-1}$)	265.8	276.6	256.7	10.09	0.391
K** ($mEqL^{-1}$)	22.60	23.37	22.64	0.84	0.768
Mg ($mgdL^{-1}$)	2.45	2.53	2.48	0.09	0.807
Ca ($mgdL^{-1}$)	11.38	11.68	11.86	0.50	0.793
Na + K:Ca + Mg ratio	20.88	21.26	19.53	0.56	0.090
Oestrus and mating					
Na** ($mEqL^{-1}$)	240.2[b]	298.8[a]	237.6[b]	8.47	< 0.001
K ($mEqL^{-1}$)	21.81	22.66	23.57	0.87	0.373
Mg ($mgdL^{-1}$)	2.57	2.61	2.61	0.07	0.905
Ca ($mgdL^{-1}$)	12.03	12.02	11.88	0.53	0.975
Na + K:Ca + Mg ratio	18.04[b]	22.49[a]	18.09[b]	0.89	0.002

* Experimental diets: SFA = diet containing 5% calcium salts of saturated fat; n-3FO = diet containing 5% calcium salts of fish oil as n-3 source; n-3FO+RUP = diet containing 5% calcium salts of fish oil as n-3 source plus 5% fish meal as RUP source.
** Standard error of mean.
[a b] Means within a row with different superscripts are significantly different ($P < 0.05$).

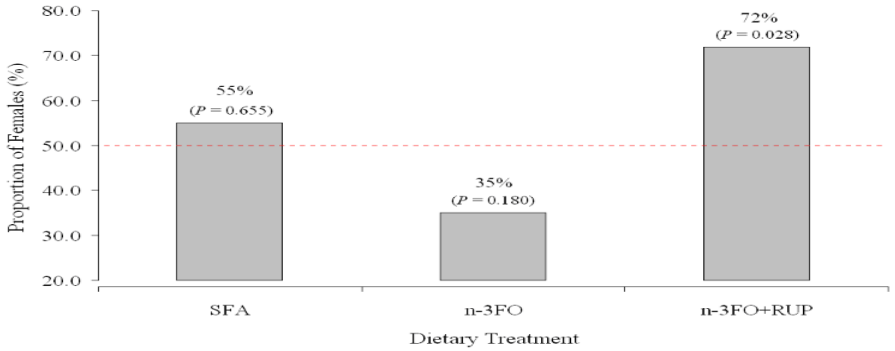

Fig. 4. Mean proportion of female lambs and difference from an expected 50:50 ratio when ewes were fed a basal diet supplemented with 5% calcium salts of saturated fatty acids (SFA), 5% calcium salts of fish oil (n-3FO) as n-3 FA source or 5% calcium salts of fish oil as n-3 FA source plus 5% fish meal (n-3FO+RUP) as RUP source.

Individual birthweight was significantly (P = 0.006) higher for lambs born from ewes that received the n-3FO diet compared with ewes that received the SFA or n-3FO+RUP diets (Table 4), whereas, the total birthweight of lambs per ewe was not affected (P = 0.079) by the diets.

For ewes from all treatments, the mean plasma ARA:EPA ratio (arachidonic acid:eicosapentaenoic acid ratio), at CIDR removal was higher (P = 0.042) when the born lamb was female (1.84 vs. 1.33 for the male). This ratio tended to be higher (Fig. 5) when the born lamb was female only for ewes fed the SFA (P = 0.087) or n-3FO (P = 0.064) diets.

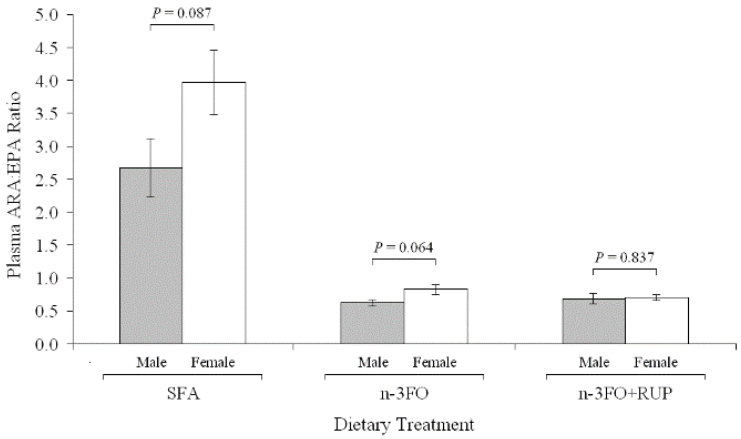

Fig. 5. Mean plasma ARA:EPA ratio at the day of CIDR out when ewes were fed a basal diet supplemented with 5% calcium salts of saturated fatty acids (SFA), 5% calcium salts of fish oil (n-3FO) as n-3 FA source, or 5% calcium salts of fish oil as n-3 FA source plus 5% fish meal (n-3FO+RUP) as RUP source and gave birth to either male or female lambs.

Table 4. Reproduction outcomes when ewes were fed experimental diets for 40 days prior to and 30 days post-mating.

Reproduction measure*	Experimental diet**			*P*-value
	SFA	n-3FO	n-3FO+RUP	
Total number of lambs	20	20	25	0.119
Proportion of females (%)	55.0^{ab}	35.0^{b}	72.0^{a}	0.046
Birthweight (kg)				
Individual lambs	3.97^{b} (± 0.15)	4.61^{a} (± 0.15)	4.06^{b} (± 0.13)	0.006
Per ewe (total weight)	4.67 (± 0.30)	5.12 (± 0.29)	5.64 (± 0.29)	0.079

* Values are proportions (percentages) or least squares means ± standard errors of the least squares means.
** Experimental diets: SFA = diet containing 5% calcium salts of saturated fat; n-3FO = diet containing 5% calcium salts of fish oil as n-3 FA source; n-3FO+RUP = diet containing 5% calcium salts of fish oil as n-3 FA source plus 5% fish meal as RUP source.
a b Means within a row with different superscripts are significantly different ($P < 0.05$).

DISCUSSION

In the current study a higher proportion of female lambs was observed when dams were received a diet rich in n-3 FA and RUP together (n-3FO+RUP) compared with those who received a diet rich in n-3 FA (n-3FO) or saturated FA (SFA) without RUP supplement around mating. These results confirmed previous observations that maternal nutrition around mating is an important factor affecting the sex ratio of offspring in different mammalian systems (Rosenfeld and Roberts, 2004).

There is substantial evidence that the nutrient composition of the diet may be more important than feed availability itself in the manipulation of offspring sex ratios (Navara, 2018). Supplemental FA may be associated with offspring sex ratio, however, the directions of the sex ratio skews depend on the type of FA. The greater proportion of male lambs in ewes fed the n-3FO diet around mating was in line with results reported in ewes fed diets supplemented with n-3 FA or when ewes were fed a diet of lucerne rich in n-3 FA (Robertson et al., 2014). Conversely, the results of some studies (Gulliver et al., 2013; Clayton et al., 2016, 2017; Mirzaei Alamouti et al., 2018) showed that the proportion of female lambs increased in animals recieved diets rich in n-6 FA around the time of conception. In some of the above mentioned researches (Gulliver et al., 2013; Clayton et al., 2016, 2017), experimental diets differed in several components other than FAs alone, therefore, these differences in sex ratio of lambs might have been related to other nutrients in the diet or the interaction with FA composition rather than dietary FA alone. In the current study, the highest proportion of female lambs was observed when the experimental diets varied in both n-3FA and RUP, which also means the observed effects could be due to the dietary interactions rather than individual components.

One an important source of absorbable amino acid to the animal is RUP (Bach et al., 2005), which has positive effects on the reproductive performance of ewes. For example, the proportion of pregnant ewes and twining rate were higher, while blood urea nitrogen concentration was lower in ewes fed flushing diets containing higher RUP content from 3 weeks before to 2 weeks after mating compared with those fed a diet lower in RUP content (Daghigh kia et al., 2016). In contrast, excess CP or RDP intake in dairy cows was associated with higher

circulating BUN and lower pregnancy rate (Butler, 1998).

The proportion of female lambs decreased when ewes were fed the n-3FO diet and greater when ewes were fed the n-3FO+RUP diet (Table 4). Alterations in the sex ratio of lambs were not different when ewes were previously fed n-6 FA just pre-mating alone compared with those fed from 40 days before to 17 days after mating (Clayton et al. 2016), which confirms that the sex ratio of lambs may be influenced by events at or before conception rather than events post-conception. The detailed mechanisms linking dietary nutrient content with sex ratio does not completely discovered. Factors like modulation of the ability of either the X or Y sperm to reach or pass the oocyte, chemical composition of vaginal secretions, eicosanoids synthesis, and plasma hormone and metabolite concentrations (Navara, 2018) may be involved. The plasma ARA:EPA ratio was lower in ewes fed the n-3FO diet compared with the SFA diet (Table 2), which may be related to the lower proportion of female lambs (Table 4) similar to previously observed effects (Clayton et al., 2016). Although not statistically significant, the ARA:EPA ratio was also higher when ewes gave birth to female compared with male lambs after receiving the SFA or n-3FO diets (Fig. 5). Previous authors (Clayton et al., 2016) have proposed that the sex ratio of lambs may be changed by the effect of the ARA:EPA ratio on the ratio of series-2 to series-3 prostaglandins, since these prostaglandins play different roles in several aspects of reproductive processes (Gulliver et al., 2012) and their effects on sex ratio of offspring need to be clarified in future studies. Our date showed that, the ARA:EPA ratio was similar in ewes fed the n-3FO and n-3FO+RUP diets (Table 2), however, the proportion of female and male lambs was different. These results indicate that offspring sex ratio may be altered by factors other than plasma ARA:EPA ratio alone.

Increasing the proportion of female lambs in dams fed the n-3FO+RUP diet was associated with a lower plasma urea concentration. Urea, the final product of nitrogen metabolism, is a relatively small molecule that can freely move between cell membranes, therefore, circulation of urea between blood and other tissues such as the uterine wall is especially high (Elrod and Butler, 1993). The low reproductive performance in response to higher dietary RDP content may be related to toxic effects of ammonium ions and urea on sperm and oocyte quality as well as uterine electrolyte balance (Butler, 1998). The exact mechanisms linking dietary protein content on offspring sex ratios has not previously been determined, however, alterations to uterine environment characteristics like pH (Rhoads et al., 2004) or expression of endometrial genes associated with tissue turnover, immune function, inflammation, and lipid metabolism (Cheng et al., 2015) may be involved. Uterine pH was lower when dairy cows were infused with urea leading to a short-term increase in urea (Rhoads et al., 2004) and X- and Y-bearing sperm may be differentially susceptible to very small differences in uterine pH (Navara, 2018). Essential amino acids supply from RUP may also affect female and male embryo development (Sturmey et al., 2010).

Other effects of diet, including the balance of individual cations, may be involved with alterations in sex ratio. Researches showed that the ionic balance of the diet at the time of conception may influence the ability of the X- or a Y-bearing sperm to successfully reach and fertilize the egg. In particular, a higher ratio of Na + K to Ca + Mg in the diet at the time of conception appeared to benefit Y-bearing sperm to alter offspring sex ratio towards males (Alhimaidi et al., 2021). The potential mechanisms of the ionic balance for altering sex ratio could be attributed to changing the charge balance in the reproductive tract or the egg itself, which could differentially affect survival of X- versus Y-bearing sperm or the ability to penetrate the egg (Arangasamy et al., 2015). The lower proportion of female lambs in ewes fed n-3FO diet compared with those fed the SFA or n-3FO+RUP diets was associated with a higher plasma Na + K to Ca + Mg ratio at the day of oestrus (Table 3), which was in agreement with a previous study in rodents (Arangasamy et al., 2015).

Circulating concentrations of glucose is an important factor that may determine the sex ratio of lambs. In our results, increasing proportion of female lambs when dams received the n-3FO+RUP diet was accompanied by lower plasma glucose concentrations at the day of CIDR removal and oestrus. Male and female blastocysts have different glucose requirements and high circulating levels of glucose in field voles around conception resulted in male-biased litters (Helle et al., 2008). Future experiments may investigate the glucose concentrations over a shorter period, rather than once per day, in order to show its impact on blastocyst survival.

Previous study indicating that offspring sex ratio in mammalian species may be influenced by altering circulating concentrations of reproductive hormones (James, 1996). In our experiment, the lower proportion of male lambs in ewes fed n-3FO+RUP was also associated with a lower plasma oestradiol concentration at the day of oestrus (Fig. 2). The exact mechanism linking oestradiol with altering offspring sex ratio does not completely determined and better binding and fertilization ability of Y-bearing sperm may be responsible (Navara, 2018). Emadi et al. (2014) also concluded that oestradiol might change the timing between ovulation and fertilization. It has been suggested that alterations in the timing of mating and ovulation may have been involved with the observed effects of dietary FA on the sex ratio of lambs (Gulliver et al., 2013).

CONCLUSION

This data indicate that the sex ratio of lambs is skewed towards females when ewes were received a diet rich in calcium salts of fish oil as n-3 FA source together with fish meal as a RUP source from 40 days before to 28 days after mating compared with those fed Ca salts of n-3FA or SFA without a source of RUP. In addition, the proportion of male lambs was higher when ewes received a diet containing n-3FO alone. These findings also, indicate that supplementing a premating flushing diet with a combination of n-3 FA source from fish oil and fish meal as RUP source could be beneficial to sheep enterprises requiring more breeding females, while those

enterprises that require more males may benefit from feeding diets high in n-3FA alone around mating. The effects of further combinations of dietary FA and RUP sources on reproductive performance and the sex ratio of lambs should be confirmed in future researches with a larger number of ewes.

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CRediT AUTHORSHIP CONTRIBUTION STATEMENT

Conceptualization: Farshid Fatahnia. Methodology: Marziyeh Fatahi. Software: Hoshang Jafari. Formal analysis: Farshid Fatahnia, Hoshang Jafari, and Edward H.Clayton. Resources: Farshid Fatahnia. Investigation: Marzieh Fatahi, Hoshang Jafari, and Aminolah Pormalekshahi. Data curation: Marzieh Fatahi and Hoshang Jafari. Writing—original draft preparation: Farshid Fatahnia and Golnaz Taasoli. Writing—review and editing: Golnaz Taasoli and Shahryar Kargar. Supervision: Farshid Fatahnia, Hoshang Jafari, Golnaz Taasoli, and Aminolah Pormalekshahi. Project administration: Farshid Fatahnia and Hoshang Jafari. Funding acquisition: Farshid Fatahnia.

DATA AVAILABILITY

Data will be made available upon reasonable request.

DECLARATION OF COMPETING INTEREST

None.

ETHICAL STATEMENT

This study was done according to the Iranian Council of Animal Care (1995).

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