

Incorporation of dietary n-3 fatty acids into ovarian compartments in dairy cows and the effects on hormonal and behavioral patterns around estrus

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Abstract

The objective of this study was to examine the incorporation of dietary n-3 fatty acids (FAs) into ovarian compartments and the effects on hormonal and behavioral patterns around estrus. Multiparous 256-day pregnant cows were fed either a standard diet both prepartum and postpartum (PP) (control; $n=22$) or supplemented with extruded flaxseed (E-FLAX) providing C18:3n-3 at 172.2 and 402.5 g/day per cow prepartum and PP respectively ($n=22$). The estrous cycle was synchronized, and at day 7 of the cycle, the cows were injected with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and then subjected to 5 days of intensive examination. Compared with those in the control, in the E-FLAX group, the interval from $PGF_{2\alpha}$ injection to behavioral estrus peak tended to be longer (3.6 h; $P<0.1$), that to estradiol (E_2) peak was 6.5 h longer ($P<0.03$), and that to LH peak tended to be longer (5.3 h; $P<0.07$). The durations of behavioral estrus and E_2 surge were longer, and the area under the E_2 curve was greater in the E-FLAX cows. Afterward, 7–8 days following behavioral estrus, follicular fluids (FFs) from >7 mm follicles were aspirated. The proportions of n-3 FA increased in plasma, FF, and granulosa cells in the E-FLAX group. The concentrations of PGE_2 in the E_2 -active follicles tended to be lower in the E-FLAX cows ($P<0.06$). In conclusion, several modifications in hormonal and behavioral estrus patterns were demonstrated in cows fed n-3 FA, which might be attributed to alterations in membrane FA composition and partly mediated by lower PGE_2 synthesis.

Reproduction (2011) 141 833–840

Introduction

In the last two decades, it has become well accepted that dietary fats exert positive effects on dairy cows' reproduction and that the fatty acids (FAs) composition of the supplied fats plays a pivotal role in this effect (Mattos *et al.* 2000, Wathes *et al.* 2007, Santos *et al.* 2008). Supplementation of polyunsaturated FA (PUFA) to dairy cows has been shown to alter ovarian follicle dynamics, ovulation, corpus luteum (CL) function, and progesterone (P_4) secretion (Abayasekara & Wathes 1999, Mattos *et al.* 2000). The PUFA are essential components of all cell membranes, and the proportions of various PUFA in the tissues of the reproductive tract reflect dietary consumption (Wathes *et al.* 2007). Stoffel *et al.* (2008) have used genetically modified mice that lacked the enzyme $\Delta 6$ -FA desaturase, an enzyme that catalyzes the initial rate-limiting desaturation of C18:2n-6 and C18:3n-3 into long-chain PUFA, and found that the mice were viable but sterile. Furthermore, the $\Delta 6$ -FA desaturase knock-out mice had smaller ovaries with lower blood supply than the control mice,

and the multilayer granulosa cell syncytium, theca folliculi, and zona pellucida were absent, which demonstrated the essential role of long-chain PUFA in the development of the reproductive system (Stoffel *et al.* 2008). Dietary PUFA can influence the reproductive processes through a variety of mechanisms; they provide the precursors for prostaglandin (PG) synthesis and can modulate the expression patterns of many key enzymes involved in both PG and steroid metabolism (Wathes *et al.* 2007). Animals cannot synthesize n-6 or n-3 FAs *de novo*, therefore these need to be supplied in the diet (Wathes *et al.* 2007). The short-chain PUFA can be elongated and desaturated to long-chain eicosanoids (C20) such as the biologically active PG. The three-series PG are derived from n-3 FA and the two-series PG are generated from n-6 FA, and it has been determined that the most biologically active PGs are those of the two series (Fischer 1989). The proportions of different PUFA in the diet alter the cell membrane phospholipid composition, and this becomes quantitatively significant because the precursors of each group of PG

compete for the same enzyme systems for their synthesis (Lands 1992).

Robinson *et al.* (2002) have found higher estradiol (E_2) plasma concentrations in cows supplemented with n-3 FA than in control animals and intermediate concentrations in those supplemented with n-6 FA. Moreover, they observed a trend toward a slightly shorter cycle in cows fed n-6 and that the interval from the LH peak to ovulation was more variable in the n-6 group, and they suggested that the mechanism behind this phenomenon might be related to alterations in ovarian PG metabolism. In a previous study conducted in our laboratory, we observed longer intervals from $PGF_{2\alpha}$ injection to manifestation of estrus behavior and a delay in the beginning of the subsequent luteal phase in Israeli Holstein cows supplemented with n-3 than in those that were fed n-6 (Zachut *et al.* 2010a). Taken together, these findings suggest that dietary n-3 and n-6 FA might affect the timing and characteristics of the events around the estrus. Therefore, the objective of this study was to investigate the effects of dietary n-3 FA on the patterns of hormonal and behavioral events around estrus in dairy cows.

Results

Average prepartum dry matter intake was not affected by treatments, whereas the postpartum (PP) dry matter intake was 3.8% higher in extruded flaxseed (E-FLAX)

than in control cows (27.1 and 26.1 kg/day respectively, $P < 0.006$). Milk yield until 100 days in milk was 6.4% higher in E-FLAX than in control (52.9 and 49.5 kg/day respectively, $P < 0.004$) and fat content was 0.4 units lower in the E-FLAX cows than in the controls (3.23 and 3.63% respectively, $P < 0.001$). Fat yield, fat correlated milk, and milk energy output were not affected by treatments.

Composition of FA in plasma, follicular fluid, and granulosa cells

The composition of FA in plasma was considerably affected by dietary treatments. The proportions of total n-3 FA (C18:3n-3, C18:4n-3, C20:5n-3, and C22:5n-3) increased from 2.4% in the control to 10.6% in the E-FLAX group (S.E.M. = 0.60, $P < 0.0001$), and the proportions of total n-6 FA (C18:2n-6, C18:3n-6, C20:3n-6, and C20:4n-6) were higher in the controls than in the E-FLAX group (45.7 and 40.5% respectively; S.E.M. = 0.85, $P < 0.002$). The n-6/n-3 ratio decreased from 19.4 in the control to 4.0 in the E-FLAX cows (S.E.M. = 1.20, $P < 0.0001$).

The composition of FA in follicular fluid (FF) collected from the E_2 -active follicles is presented in Table 1. The percentage of C18:2n-6 was significantly lower in the E-FLAX cows than in the controls, whereas the percentage of C18:3n-3 was more than seven times higher in the E-FLAX group than in the controls. The

Table 1 Fatty acid composition of follicular fluid and granulosa cells obtained from dairy cows (g/100 g of fatty acids).

FA	Follicular fluid				Granulosa cells			
	Control	E-FLAX	S.E.M.	$P <$	Control	E-FLAX	S.E.M.	$P <$
C14:0	1.02	1.36	0.09	0.01	2.13	1.68	0.33	NS
C16:0	15.93	14.11	0.70	0.07	19.72	16.81	1.10	0.09
C16:1	0.74	0.65	0.05	NS	0.61	0.66	0.07	NS
C16:2	0.26	0.41	0.06	NS	0.05	0.22	0.10	NS
C16:3	0.76	0.85	0.14	NS	0.79	0.82	0.16	NS
C18:0	13.33	15.22	0.62	0.04	16.21	17.46	0.90	NS
C18:1n-9	6.82	7.61	0.86	NS	12.42	11.10	0.30	0.02
C18:1n-7	0.38	0.47	0.07	NS	0.47	0.76	0.15	NS
C18:2n-6	52.32	43.39	1.40	0.0003	42.53	39.78	1.53	NS
C18:3n-3	0.93	7.54	0.40	0.0001	1.36	6.11	0.33	0.0001
C18:3n-6	0.65	0.52	0.15	NS	ND	ND	–	–
C18:4n-3	0.51	0.66	0.15	NS	0.72	0.75	0.14	NS
C20:3n-6	0.22	0.33	0.17	NS	1.03	0.76	0.21	NS
C20:4n-6	2.08	0.93	0.12	0.0001	0.21	0.03	0.16	NS
C20:4n-3	1.7	1.08	0.12	0.003	0.06	0.37	0.09	0.04
C20:5n-3	0.07	0.32	0.04	0.0005	0.00	0.72	0.14	0.005
C22:5n-3	0.16	0.64	0.08	0.001	0.00	0.46	0.26	NS
C22:6n-3	1.02	1.66	0.30	0.14	0.08	0.15	0.05	NS
C24:1n-9	1.04	2.18	0.55	NS	1.27	1.97	0.65	NS
SFA	30.28	30.69	1.6	NS	38.02	35.67	1.80	NS
MUFA	10.02	12.58	1.1	0.12	14.81	14.21	1.90	NS
PUFA	58.67	55.45	1.5	0.15	46.15	48.99	0.30	NS
n-3 ^a	1.90	9.51	0.41	0.0001	1.58	7.78	1.70	0.0001
n-6 ^b	56.11	45.41	1.46	0.0001	43.84	40.51	1.70	NS
n-6/n-3	44.95	4.86	7.30	0.0009	28.99	5.24	0.45	0.0001

SFA, sum of saturated FA; MUFA, sum of mono-unsaturated FA; PUFA, sum of poly-unsaturated FA.

^aSum of n-3 FA. ^bSum of n-6 FA.

proportions of the longer n-3 FA (C20:4n-3 and C20:5n-3) were also higher in the E-FLAX group, and the ratio of n-6/n-3 FA in FF decreased from 45.0 in the controls to 4.9 in the E-FLAX group.

The compositions of the FA in granulosa cells obtained from the E₂-active follicles are presented in Table 1. The proportions of n-3 FA were higher in granulosa cells collected from the E-FLAX cows than in those from the controls: the proportion of C18:3n-3 was 4.5 times higher and that of C20:4n-3 was six times higher. The percentage of C20:5n-3 was also higher in the E-FLAX group, and the percentages of C22:5n-3 and C22:6n-3 were numerically but not significantly higher in the E-FLAX cows than in the controls. The total n-3 FA content increased from 1.6% in the controls to 7.8% in the E-FLAX group, and the n-6/n-3 ratios were 29.0 and 5.2 respectively.

Characteristics of E₂-active follicles

A total of 23 E₂-active follicles (ten controls and 13 E-FLAX) from 20 cows (nine controls and 11 E-FLAX) were analyzed. The concentrations and content of P₄, androstenedione (A₄), and E₂ in the FF of the E₂-active follicles as well as the diameter and the volume of the follicles are presented in Table 2. As shown, no between-group differences were observed in all parameters that were examined. The concentrations of PGE₂ in the E₂-active follicles tended to be lower in the E-FLAX group than in the control ($P < 0.06$; Table 2), but the concentrations of PGF_{2 α} did not differ between groups.

Timing and characteristics of the behavioral estrus

In total, 14 control and 18 E-FLAX cows, divided into three clusters, were subjected to estrous cycle synchronization. Several cows participated in more than one cluster; therefore 23 controls and 25 E-FLAX estrous cycles were synchronized. A total of 39 successful

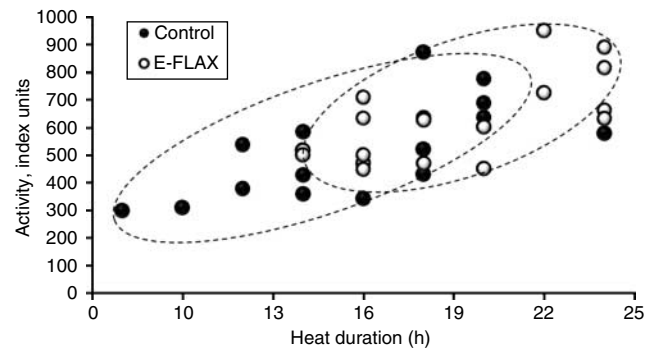


Figure 1 Activity and duration of behavioral estrus in dairy cows fed either a control diet (open circle) or supplemented with extruded flaxseed (E-FLAX; filled circle) providing C18:3n-3 at 172.2 and 402.5 g/day per cow, pre- and postpartum respectively.

estrous cycle synchronizations, involving 17 control and 22 E-FLAX cows, were statistically analyzed.

The interval from PGF_{2 α} injection to the onset of visible behavioral estrus was numerically longer in E-FLAX cows than in controls: 60.4 ± 2.3 and 56.7 ± 2.7 h respectively ($P < 0.16$). The interval from PGF_{2 α} injection to the behavioral estrus peak tended to be longer in the E-FLAX group than in the controls: 66.7 ± 1.4 and 63.1 ± 1.7 h respectively ($P < 0.1$). The duration and intensity of behavioral estrus was based on an activity index calculated from the H-tag records and is presented in Fig. 1. The cow whose activity index deviated by $\geq 50\%$ from the individual basal activity, as calculated from the measurements made 7 days before estrus, was regarded as being in behavioral estrus and its data were used for all activity calculations. The duration of behavioral estrus was longer in the E-FLAX than in the control cows: 18.6 ± 0.8 and 15.8 ± 0.9 h respectively ($P < 0.04$). Furthermore, the proportion of cows that exhibited a long behavioral estrus (> 22 h) tended to be greater in the E-FLAX group than in the controls – 30 vs 6% respectively ($P < 0.08$) – whereas the proportion of cows that exhibited a short behavioral estrus (< 16 h) tended to be higher in the control group than in the E-FLAX group: 47 vs 20% ($P < 0.09$) respectively.

To determine the behavioral estrus intensity, the deviation in activity index of each cow was calculated every 2 h, by subtracting the individual basal activity. The individual activity index deviations were summed and the resulting combined index reflects the area under curve (AUC) of activity above baseline. The total activity deviation was higher in the E-FLAX cows than in the controls: 320.6 ± 22.8 and 249.0 ± 24.5 activity index units respectively ($P < 0.03$). We have also calculated the summed activity index deviations from baseline as percentages, and this too was higher in the E-FLAX group than in the controls: 1203.8 ± 81.7 and $919.5 \pm 87.5\%$ respectively ($P < 0.02$).

Table 2 Follicle concentrations of progesterone, androstenedione, estradiol (E₂), and prostaglandins of E₂-active follicles.

	Control	E-FLAX ^a	S.E.M.	P <
Cows (n)	10	11		
Number of follicles	11	13		
Diameter (mm)	14.7	15.8	1.1	0.45
Volume (ml)	1.8	2.5	0.5	0.34
Progesterone (ng/ml)	81.7	74.2	6.6	0.43
Androstenedione (ng/ml)	202.4	126.1	41.0	0.20
Estradiol (ng/ml)	1731.6	1747.8	180.7	0.95
Estradiol/progesterone	21.4	25.0	3.2	0.45
PGE ₂ (pg/ml)	155.5	25.2	46.4	0.06
PGF _{2α} (pg/ml)	215.6	272.0	133.5	0.77

^aCows were supplemented with extruded flaxseed providing C18:3n-3 at 172.2 and 402.5 g/day per cow, pre- and postpartum respectively.

Timing and characteristics of preovulatory E₂ surge

The interval from PGF_{2α} injection to E₂ peak was 6.5 h longer in the E-FLAX group than in the controls: 57.6 ± 2.2 and 51.1 ± 1.8 h respectively ($P < 0.03$). The E₂ curve around estrus is presented in Fig. 2. The AUC of the plasma E₂ curve was 1.7 times as high in the E-FLAX group as in the control: 288.5 and 166.5 pg respectively (S.E.M. = 30.1; $P < 0.02$). The average concentration of E₂ at surge peak was not different between the E-FLAX group and the controls: 26.5 ± 2.4 and 21.8 ± 2.1 pg/ml respectively ($P < 0.16$). Furthermore, the duration of the preovulatory E₂ surge was longer in the E-FLAX group than in the controls: 13.4 and 8.2 h respectively (S.E.M. = 1.6; $P < 0.04$).

Across-treatment analysis revealed a significant correlation between the duration of heat and that of the E₂ surge ($r = 0.74$, $P < 0.001$). Furthermore, the correlation between total activity during heat and the length of the E₂ surge was also significant ($r = 0.50$, $P < 0.05$).

Timing of the preovulatory LH peak and subsequent P₄ secretion

The interval from PGF_{2α} injection to preovulatory LH peak was 5.3 h longer in the E-FLAX cows than in the controls: 67.0 ± 1.8 and 61.7 ± 2.1 h respectively ($P < 0.07$). Furthermore, the interval from the onset of behavioral estrus to LH peak tended to be longer in E-FLAX cows than in the controls: 7.7 ± 0.5 and 6.4 ± 0.6 h respectively ($P < 0.1$). The average LH concentrations at peak did not differ between groups.

A delay in the commencement of the increase in plasma P₄ (from PGF_{2α} injection) was found in the E-FLAX cows as compared with controls (6.0 ± 0.1 and 5.5 ± 0.1 days respectively, $P < 0.02$). Furthermore, the

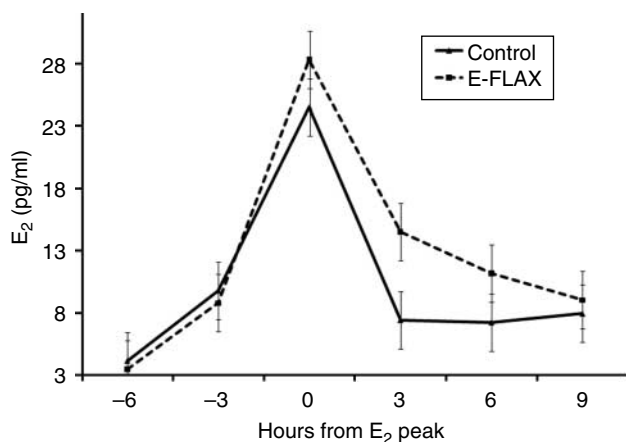


Figure 2 Average concentrations of estradiol (E₂) around estrus in plasma of dairy cows fed either a control diet (solid line) or supplemented with extruded flaxseed (E-FLAX; broken line), providing C18:3n-3 at 172.2 and 402.5 g/day per cow, pre- and postpartum respectively.

day in which plasma P₄ concentration reached ≥ 1 ng/ml tended to be later in the E-FLAX cows than in controls (7.8 ± 0.2 and 7.3 ± 0.2 days respectively, $P < 0.1$).

Discussion

In this study, dietary fat rich in C18:3n-3 increased individual and total n-3 FA and reduced the n-6/n-3 ratio in FF and granulosa cells aspirated *in vivo* from preovulatory follicles of dairy cows. Several modifications in the hormonal and behavioral patterns around estrus were observed in cows that were supplemented with C18:3n-3.

Composition of plasma, FF, and granulosa cells

The effects of feeding E-FLAX on the composition of plasma, as found in this study, were consistent with the findings of previous studies in which flaxseed was fed to dairy cows (Petit *et al.* 2004, Gonthier *et al.* 2005). Supplementation of C18:3n-3 at 242.2 g/day per cow in our previous study resulted in 7.98% C18:3n-3 in plasma (Zachut *et al.* 2010a), whereas supplementation at 402.5 g/day per cow in this study resulted in 9.53% C18:3n-3 in plasma. This demonstrates that large amounts of C18:3n-3 supplementation are effectively transferred into the plasma and that its incorporation was proportional to the rate of feeding. The n-6/n-3 ratio in plasma of the E-FLAX cows in this study was lower by a factor of 5 in the E-FLAX group than in the controls, and this was similar to our previous results (Zachut *et al.* 2010a).

The proportion of n-3 FA in FF of E₂-active follicles was five times as high in E-FLAX cows as in controls, which matches our previous findings (Zachut *et al.* 2010a). In FF obtained from follicles collected at an abattoir, Childs *et al.* (2008) have observed enhanced proportions of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in those from heifers fed fish oil at 2.08 or 4.15% of the diet; these results imply that the dietary FA are considerably infiltrated into FF.

In this study, similarly to FF, composition of FA in granulosa cells was altered by dietary treatments, and the proportion of n-3 FA was 4.9 times as high in the E-FLAX group as in the controls. Effects of dietary n-3 on the FA profiles of granulosa cells have been demonstrated in ewes and dairy cows elsewhere (Wonnacott *et al.* 2010, Zachut *et al.* 2010a). Specific FA dietary supplementation altered the FA profile in ovarian compartments, and this might impact physiological and functional aspects.

Effects of n-3 FA on estrus behavior pattern

A low estrus detection rate has been identified as an important factor affecting reproductive efficiency, and the duration and intensity of estrous behavior in the

modern high-yielding dairy cow were found to be considerably lower than that in cows of a few decades ago (Lopez *et al.* 2004). In this study, greater length and intensity of behavioral estrus, accompanied by a different plasma E_2 profile, were observed in cows fed n-3 FA than in controls. Boken *et al.* (2005) found that feeding cows in pasture with soybean oil product, rich in C18:2n-6, increased the number of mounts during estrus compared with the control, which also demonstrates the potential of dietary PUFA to influence behavioral estrus characteristics. E_2 plays a pivotal role in the induction of estrous behavior (Pfaff 2005, Boer *et al.* 2010), and it was demonstrated in sheep that the duration of estrous behavior mostly depend on the duration of E_2 presence rather than on its maximum concentration (Fabre-Nys *et al.* 1993). This agrees with our present findings that the AUC and duration of the E_2 surge, rather than E_2 peak concentrations, were higher in E-FLAX cows than in controls and that the former animals also exhibited longer duration and greater intensity of behavioral estrus. Moreover, across-treatment analysis revealed a positive correlation of the duration of behavioral estrus with that of the E_2 surge, but not with maximum E_2 concentration.

Concentrations of E_2 in plasma

In this study, we observed enhanced levels of E_2 in the plasma of cows fed C18:3n-3, as also reported by Robinson *et al.* (2002). During pro-estrus, the elevated E_2 levels led to increased secretion of GnRH, which, together with direct effects of E_2 on the pituitary, triggers the LH surge (Glidewell-Kenney *et al.* 2007). The elevated plasma E_2 levels that were observed in this study could be attributed either to increased ovarian steroidogenesis or to decreased metabolism of steroids in the liver. Supplementing cows with PUFA was hypothesized to enhance plasma steroid concentrations by increasing steroid hormone production through various mechanisms, including increased availability of lipoprotein-cholesterol, modulation of PG synthesis, or direct stimulation of ovarian steroidogenesis (Grummer & Carroll 1991, Staples *et al.* 1998, Williams & Stanko 2000). However, this hypothesis is not supported by our present findings; we did not observe higher E_2 concentrations in FF of preovulatory follicles in cows fed n-3 FA than in those in controls. Alternatively, the steroid concentrations in plasma could be regulated by changes in the rate of metabolism of steroids in the liver (Sangsritavong *et al.* 2002, Wiltbank *et al.* 2006). Recently, Piccinato *et al.* (2010) found that addition of C18:3n-3 to bovine liver slices increased the half-life of P_4 and E_2 by ~60%, and this was the most profound increase compared with other FA (C16:0, C16:1, and C18:1). Indeed, PUFA have been demonstrated to have inhibitory effects on a variety of CYP (cytochrome P450) enzymes, which metabolize steroids in the liver (Hirunpanich *et al.* 2006, Yao *et al.* 2006). These findings

might indicate that C18:3n-3 could inhibit the metabolism of steroid hormones in the liver, which could lead to greater and long-lasting elevation of E_2 levels in cows consuming n-3 FA than in controls.

Timing of LH peak

In this study, a tendency for a delay in LH peak was observed in cows fed E-FLAX. This could be explained by the delay in E_2 surge, which was followed by a delay in the positive feedback of E_2 on hypothalamus and pituitary (Glidewell-Kenney *et al.* 2007). Alternatively, dietary PUFA could influence ovulation timing via alterations in PG-mediated changes in LHRH release; for example, PGE_2 was shown to enhance the release of LHRH from the hypothalamus (Ojeda *et al.* 1979, Kim & Ramirez 1986). PGE_2 , which is synthesized from its precursor C20:4n-6 in the preovulatory follicle by the granulosa cells, is known to be crucial for ovulation (Mattos *et al.* 2000). Peters *et al.* (2004) have injected a selective inhibitor of PTGS2 (also known as cyclooxygenase-2, COX-2) – a key regulator of PG synthesis – into preovulatory follicles and observed a considerable reduction in FF PGE_2 concentration, which was followed by inhibition of the follicle rupture and ovulation process. In this study, we found a tendency to lower PGE_2 concentrations in the preovulatory follicles of E-FLAX cows, which may imply a reduction in two-series PG synthesis. This reduction might have occurred also in hypothalamic membranes, in which case it could influence the release of LHRH and thereby partly account for the delay of the LH peak in cows fed n-3 FA.

The effects of dietary n-3 FA on ovulation in rats were recently demonstrated by Broughton *et al.* (2009), who found that dietary EPA reduced ova release without altering PTGS2 expression. However, previous work in rats showed that feeding a large amount of EPA + DHA increased ova release (Trujillo & Broughton 1995). In this study, cows were provided with C18:3n-3, which can be elongated to EPA, and indeed, the proportion of EPA was higher in FF and granulosa cells of the E-FLAX cows than in those of controls. Therefore, it might be that the larger proportion of EPA found in the E-FLAX cows than in the controls was associated with the delay in hormonal patterns around estrus and perhaps timing of ovulation. Moreover, the delay in commencement of the increase in plasma P_4 in the E-FLAX cows in this study supports the possibility of delayed ovulation. Indeed, Robinson *et al.* (2002) suggested that delayed luteal development in PUFA-fed cows might be caused by a delay in ovulation in these cows. Although ovulation *per se* was not determined in our study, modified hormonal patterns that were observed in cows fed n-3 might result in delayed ovulation, which would be consistent with the findings of Robinson *et al.* (2002).

In conclusion, the proportions of specific and total n-3 FA were greater in plasma, FF, and granulosa cells

in the E-FLAX group than in the controls. Delays in several hormonal and behavioral indices around estrus were observed in the E-FLAX group; an extended interval from $\text{PGF}_{2\alpha}$ injection to E_2 surge and a tendency to a delay in timing of LH peak in the E-FLAX group than in the controls. Also, the durations of behavioral estrus and E_2 surge were longer, and the area under the E_2 curve was greater in the E-FLAX group. The modifications in hormonal and behavioral patterns around estrus in the E-FLAX cows might be attributed to alterations in membrane FA composition, which could be followed by functional consequences. However, further research is required to establish the mechanism by which n-3 FA affect the cascade of events around estrus.

Materials and Methods

Cows and diets

The Volcani Center Animal Care Committee approved all the procedures involving animals. The study was conducted from September through May to avoid impacts of heat stress. In total, 44 multiparous Israeli Holstein 256-day pregnant dry cows at the Volcani Center experimental farm (Bet Dagan, Israel) were stratified randomly within stratum and strata defined according to the following parameters: previous lactation milk and fat yields, parity, body weight, and body condition score. The dietary treatments continued until 100 days in lactation and were as follows: 1) controls ($n=22$) were fed standard diets both prepartum and PP according to NRC (2001) recommendations. 2) E-FLAX ($n=22$) were fed prepartum with a dry cow diet supplemented with 1 kg/day per cow (7.9% dry matter) E-FLAX (Valomega-160; Valorex, Combourtille, France), which provided C18:3n-3 at 172.2 g/day per cow; they received a lactating-cow diet PP that included 9.2% (dry matter basis) of the same supplement, which provided C18:3n-3 at an average of 402.5 g/day per cow. The compositions and contents of the diets were described in Zachut *et al.* (2010b). The extruded supplement contained flaxseed and wheat bran at 700 and 300 g/kg, respectively, and contained 21.7% crude protein and 30.4% ether extracts. Cows were individually fed once a day at 1100 h and fat supplements were hand-mixed into the total mixed ration.

Estrous cycle synchronization, estrus detection, and blood sampling

Cows were divided into three clusters for estrous synchronization, according to time from calving. At 40 days PP, all cows were monitored twice (5 days apart) by ultrasonography with a 5 MHz linear array transducer (Aquila; Pie Medical, Maastricht, the Netherlands) to evaluate the cyclic status of the ovaries, and those with a large (>24 mm) persistent follicle or smooth ovaries in both screenings were not included in the estrous synchronization procedure. A schematic schedule of the estrous synchronization is presented in Fig. 3. Cows were injected with 0.02 mg of busserelin (Receptal; Intervet

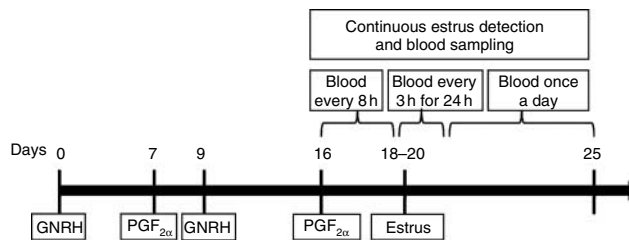


Figure 3 Schematic representation of synchronization and estrus detection protocol. Cows were given GNRH on day 0 and prostaglandin $\text{F}_{2\alpha}$ ($\text{PGF}_{2\alpha}$) on day 7. On day 16, they were re-injected with $\text{PGF}_{2\alpha}$ and subjected to a 5-day period of intensive examination.

International B.V., Boxmeer, The Netherlands), a GNRH analog, on day 0. On day 7, cows were monitored by ultrasonography to ensure the presence of a CL and then were injected with $\text{PGF}_{2\alpha}$ as 2.5 ml Estrumate (Coopers Animal Health Ltd, Berkhamsted, UK). On day 9, cows were injected again with GNRH. On day 16 (day 7 after GNRH injection), the ovaries were monitored for the presence of CL, and cows with CL were injected with $\text{PGF}_{2\alpha}$ and a 5-day period of intensive examinations began. Estrus was visually monitored continuously (24 h/day during 5 days) by two people. Exact onset of estrus was determined by recording the time of the first standing event, providing that the cow subsequently manifested several standing events and continually exhibited other behavioral signs typical of a cow in estrus.

For activity measurements, the cows were fitted with collar-mounted tags (H-tag; SCR Engineers Ltd, Hadarim, Netanya, Israel) with the aid of a genuine developed and manufactured three-dimensional accelerometer. Neck movements were analyzed and filtered by using complex algorithms in an on-board central processing unit (CPU). The result was a dimensionless 'activity index' that was stored in 12 2-h memory cells inside the H-tag. Whenever the cow passed under external readers, the H-tag transmitted the contents of these cells to the connected PC.

Blood samples for E_2 determination were collected every 8 h from the second $\text{PGF}_{2\alpha}$ injection until the onset of estrus, after which samples were collected every 3 h for 24 h, for LH and E_2 determination. Blood samples for P_4 determination were collected once daily from $\text{PGF}_{2\alpha}$ injection for 10 days. Blood samples were collected from the jugular vein into vacuum tubes containing lithium heparin (Becton Dickinson Systems, Cowley, UK), and plasma was immediately separated from blood samples and stored at -18°C pending analysis.

Aspiration of large follicles

Following the above procedure, on days 7–8 after an observed behavioral estrus, cows received a $\text{PGF}_{2\alpha}$ injection, and after a further 48 h period, FF from follicles >7 mm in diameter was aspirated as described previously (Zachut *et al.* 2010a). Follicles were aspirated separately with the aid of an ultrasound scanner (Pie Medical, Maastricht, The Netherlands) connected to a 7.5 MHz vaginal sector transducer equipped with a needle guide and connected to a suction pump (MP86; Biometra,

Goettingen, Germany) set at a flow rate of 25–30 ml/min. The needles used were 18 gauge and were changed between follicles. After collection, FF was centrifuged for 15 min at 3000 g, and the sediment, which contained the granulosa cells, was separated from fluids, and both the fractions were frozen at –18 °C pending analysis.

Chemical analysis

The concentrations of E₂ in plasma were determined by RIA after extraction of 1 ml plasma by elution with methanol according to Shore *et al.* (1998), on C-18 solid-phase columns (Bond Elut-C18, 500 mg 3 ml; Varian, Lake Forest, CA, USA). The antibody for estrogen bonded 100% to 17β-E₂ and 50% to estrone. The assay sensitivity was 2 pg/ml, and the intra- and interassay variations were 11 and 15% respectively. Plasma LH concentrations were measured with the LH Detect enzyme immunoassay kit (INRA, Nouzilly, France).

The concentrations of P₄ and E₂ in FF and plasma P₄ were determined by RIA (Diagnostic Products, Los Angeles, CA, USA), as were FF A₄ concentrations (Diagnostic Systems Laboratories, Webster, TX, USA). All FF samples were diluted 100-, 500-, and 30-fold for P₄, E₂, and A₄ assays respectively, to match the detected range. The minimal detectable amounts were 0.2, 20, and 0.1 ng/ml for P₄, E₂, and A₄ respectively. The intra- and interassay coefficients of variation for the P₄ assay were 9.2 and 8.5% respectively; those for the E₂ assay were 4.1 and 3.6%; and those for the A₄ assay were 6.1 and 4.5% respectively. The follicles were regarded as E₂ active when the E₂/P₄ ratio was >1, and these follicles were then subjected to further analysis. The concentrations of PGE₂ and PGF_{2α} in FF were determined with the EIA kits (Cayman Chemical Company, Ann Arbor, MI, USA).

FA composition of plasma, FF, and granulosa cells

FAs in plasma, FF, and granulosa cells were extracted according to Moallem *et al.* (1999). FA methyl esters were analyzed with a model 7890N gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) equipped with a DB-23 capillary column (60 m × 0.25 mm, 0.25 μm; Agilent Technologies, Santa Clara, CA, USA) and a flame-iodizing detector. The initial temperature of the column was set at 130 °C, increased at 6.5 °C/min to 170 °C, and then at 2.75 °C/min to 215 °C, at which it was held for 18 min. Then, the temperature increased to 230 °C at 40 °C/min for the remainder of the analysis. The carrier gas was hydrogen, flowing at a linear velocity of 1.6 m/min; injection volume was 1 μl.

Statistical analysis

Milk and dry matter intake were analyzed as repeated measurements with the MIXED procedure version 9.2 of SAS (2002). Dry matter intake was analyzed separately for prepartum and PP periods. Each production variable was analyzed, with the specific data of the 100 days in milk (DIM) of the previous lactation used as covariable.

The final model used was:

$$Y_{ijklm} = \mu + T_i + L_j + C(T \times L)_{ijk} + DIM_{ijkl} + DIM_{ijkl} \times DIM_{ijkl} + E_{ijklm}$$

where μ, overall mean; T_i, treatment effect; L_j, parity, j=2 or >2; C(T×L)_{ijk}, cow_k nested in treatment_i and cow nested in parity_j; DIM_{ijkl}, DIM as continuous variable; E_{ijklm}, random residual.

FA profiles in plasma, FF, and granulosa cells and hormones in FF were analyzed with the MIXED procedure, version 9.2 of SAS (2002).

The data of E₂ plasma concentrations was transformed to AUC against time (hours) by the trapezoid method. The concentrations of hormones in FF, and of P₄, E₂, E₂ AUC, and LH in plasma, activity and timing were analyzed as repeated measurements with the MIXED procedure, version 9.2 of SAS (2002). The effects of treatment, cow, days in lactation and cluster were tested.

$$Y_{ijklm} = \mu + T_i + L_j + C(T \times L)_{ijk} + DIL_{ijkl} + E_{ijklm}$$

where μ, overall mean; T_i, treatment effect i=1 or 2; L_j=cluster, j=1 to 3; C(T×L)_{ijk}, cow_k nested in treatment i and cow_k nested in cluster j; DIL_{ijkl}, days in lactation as continuous variable; E_{ijklm}, random residual.

The autoregressive order 1 (AR 1) was used as a covariance structure in the model.

Distributions of cows with differing estrus durations and intervals from PGF_{2α} injection to LH peak were analyzed with the χ² procedure of SAS (2002).

Least squares means and adjusted s.e.m. are presented and the significance level was P<0.05 unless stated.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This research did not receive any specific grant from any funding agency in the public, commercial or not-for-profit sector.

Acknowledgements

The authors are grateful to Mr Pierre Weill and VALOREX company (Combourtille, France) for the kind donation of the extruded flaxseed supplement (Valomega-160). We also wish to thank the entire staff of the experimental dairy farm at the Volcani Center (Bet Dagan, Israel) for their assistance with animal care.

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Received 16 December 2010
 First decision 27 January 2011
 Accepted 9 March 2011