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Does feeding extruded linseed to dairy cows improve reproductive performance in dairy herds? An observational study

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A B S T R A C T

Feeding n-3 fatty acids (**FA**) is often cited as a promising strategy to tackle impaired reproduction in dairy cows. However, the scientific literature shows conflicting results that may be explained by the nature of n-3 FA used, the amount supplemented and the timing of supplementation. In addition, designing a proper experimental design to study n-3 FA and reproduction is subjected to other difficulties such as the choice of the control diet or gaining enough statistical power. The objective of this retrospective observational study was to quantify the average effects of supplementing extruded linseed (**EL**), a feed rich in α-linolenic acid, to dairy cows on reproductive performances under field conditions in French commercial farms. Exposure measurement to EL feeding was particularly challenging as exact cow diets are not traced in farms. Therefore, to investigate the potential dose-effect relationship, we defined a proxy of EL intake per day by using deliveries of EL based feeds from 22 companies in the study period 2008–2015 in France. An artificial insemination (**AI**) was considered exposed only if the cow was supplemented with EL from the calving until 17 days after AI. Based on recommendations for EL use on the field, 4 exposures classes were created: [1–50] (n = 14,126 AIs), [50–300] (n = 88,261 AIs), [300–600] (n = 66,136 AIs), and [600-1500] (n = 28,287 AIs) g/cow/d. The reference population was composed of cows that did not receive any EL between calving until 17 days after AI within herds that were supplied, but not continuously during the study period (n = 226,795 AIs). Mean daily EL intake in exposed population was 337 g/cow/d (±239.4). Reproductive performance was studied on 423,605 AIs from 1096 herds and 158,125 cows using Cox models for days to first AI and days to conception, and logistic regression models for risk of return-to-service, adjusted for factors likely to influence the reproductive performance and for a herd random effect. Risk of return-to-service between 18 and 78 days after first and second AI did not differ between exposed and reference populations, Nevertheless, the effect on the days to first AI was higher with the lowest EL intake (HR: 1.14; 95% CI: 1.11, 1.17) than with higher EL intake levels (HR ranging from 1.06 to 1.07; 95% CI: 1.04, 1.09). Similarly, for the effect on the time from calving to conception from the lowest EL intake (HR: 1.19; 95% CI: 1.15, 1.23) compared to the higher EL intake levels (HR ranging from 1.08 to 1.11; 95% CI: 1.06, 1.14). This original large-scale epidemiological study provides new insights into the effects of feeding EL at a commercially sustainable level to dairy cows.

1. Introduction

The deterioration of the reproductive performance of dairy cows is one of the main concerns of the modern dairy industry because it is closely linked to the profitability of the dairy farm [1]. The length and depth of negative energy balance (**NEB**) post-partum are major risk factors for poor fertility [2,3]. Improving fertility and energy status of the cow by adding fat to the diet could be a sustainable and cost-effective lever. Indeed, fat supplementation increases the energy content of the diet. However, large amounts of fat were found undesirable for the rumen function [4]. Besides, fat supplementation seldom improves the energy status of the cow [5] and could even aggravate the metabolic pressure on the cow in early post-

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partum by stimulating milk production [6]. The impact of supplementing dairy cows' diet with fat on reproduction still remains inconsistent and conflicting notably due to the nature of the supplements [6,7].

Targeting some fatty acids (**FA**) in reproductive tissues, in particular n-3 polyunsaturated fatty acids (**PUFA**), could improve reproduction in different ways such as accelerating the resumption of the post-partum ovarian cyclicity and follicle development, or by enhancing the quality of the oocyte, embryo and their environment (see reviews by Gulliver et al. [8] in sheep and cattle and by Wathes et al. [9,10] in mammals). Briefly, n-3 FA were found to be involved in reproductive mechanisms through their essential role in the composition of cell membranes, through their status of precursors of prostaglandins and modulators of the expression patterns of enzymes involved in prostaglandins metabolism and steroidogenesis.

The effect of PUFA has been shown in animals using linseed. Indeed, linseed oil contains about 55% of α-linolenic acid (**ALA**, 18:3 n-3) [11]. ALA can be converted into the eicosapentaenoic acid (**EPA**, 20:5 n-3) and docosahexaenoic acid (**DHA**, 22:6 n-3), the long chain n-3 PUFA. Supplementing EL and encapsulated flaxseed to dairy cows modified FA profiles of both the plasma and the ovarian compartments (i.e., follicular fluid, granulosa cells, cumulus-oocyte complexes) [12,13]. An increase in ALA and n-3 FA contents and a decrease in n-6:n-3 ratio were observed. Thus, linseed supplementation altered the FA profile in reproductive tissues and could improve the uterine, the oocyte and the embryo environments. Linseed also contains the plant lignan secoisolariciresinol diglucoside (SDG), which is metabolized by the rumen flora to the mammalian lignans enterolactone and enterodiol. This source of phytoestrogens could alter dairy cow reproduction by acting as estrogen-like molecules and interfering with endogenous sex hormone metabolism [14–16].

Linseed supplementation to dairy cows influences the follicular and corpus luteum developments. Unlike Petit and Twagiramungu [17], Dirandeh et al. [18] and Jahani-Moghadam et al. [19] reported a larger ovulatory follicle in cows supplemented with EL than with protected palm oil. Besides, the corpus luteum was also larger [17,18], but not in cows supplemented with rolled linseed compared with rolled sunflower seed [20]. The incidence of cystic follicles in cows supplemented with EL was lower [18,19]. However, supplementing EL or other forms of linseed scarcely improved reproductive performance in these experimental trials. The conception and pregnancy rates were not altered by linseed supplementation in comparison with saturated FA or n-6 FA supplementations [17,19–23] even if Ambrose et al. [20] observed a trend towards an increase of the conception rate at first artificial insemination (**AI**). Pregnancy loss was reduced using whole linseed [17] or rolled linseed [20]. Finally, the resumption of the ovarian activity, the number of days open and the interval from calving to pregnancy were not studied or cannot be interpreted due to the presence of estrus synchronization programs in most of the experiments.

Overall the experimental trials provide insights about the effects of PUFA on reproductive tissues but show limitations to explore the effects on reproductive performance at cow level due to their lack of statistical power [10]. Besides, difficulties are observed by researchers when balancing the treatment groups for a trial studying reproductive performance because of the numerous parameters influencing the cow fertility. Thus, an epidemiological work exploring the link between the exposition of dairy cows to EL and their reproductive performance is needed while considering the potential confounding factors under field conditions.

The objective of this epidemiological study was to quantify under field conditions the average effects of supplementing EL to dairy cows on different reproductive performance indicators, namely time to first AI, the time from calving to conception and the risk of a return to service.

2. Materials and methods

2.1. General study design and available data

A retrospective observational study was carried out based on data from French dairy herds enrolled in the official Milk Recording Scheme, where AI was used, and wherein EL was sometimes supplemented to dairy cows between January 2008 and December 2015. The reproductive performances of cows inseminated during periods of EL supplementation were compared to the ones of cows inseminated during periods of EL non-supplementation within the same herds. The deliveries of commercial feeds containing EL were obtained from companies in France selling TRADILIN® products (Tradi-Lin® Technology, Patent No. EP 1021 960 B1). TRADILIN® products are almost the only feeds with EL sold in France. The extrusion process of linseeds incorporated into these products is protected by a European patent. This ensured that cows in the control group were not supplemented with EL. Thus, the study population consisted of 4979 French dairy herds having used feeds with EL during the study period. However, the national herd identification number was needed in order to link data from deliveries to data from the official Milk Recording Scheme and AI records. The sample size was reduced to 2599 herds due to a lack of national herd identification number, and then reduced to 2250 herds due to a lack of enrollment in the official Milk Recording Scheme. Additionally, in order to obtain a sufficient number of test days exposed to EL, only 1836 herds with a minimum of 4 deliveries of feeds with EL were retained. The absence of fit between the periods of EL delivery and milk recording data, as well as missing data in deliveries reduced sample size to 1415 herds, and finally absence of AI to 1397.

Reproductive events data obtained were calving ease, date and rank of AI, bull breed, whether the semen was sexed or not. Lactation data obtained were calving date, parity, date of test day record, milk yield and milk content (fat, protein and somatic cell count) at each test-day record. Animal data obtained were breed and movements (i.e., date of arrival in and date of exit from the herd).

2.2. Estimation of exposure to extruded linseed and determination of exposure status

A cow daily exposure to EL for each delivery in each herd was calculated from the duration of TRADILIN® products delivery distribution, the quantity delivered, the products EL content, and the average number of lactating cows in the herd during the delivery distribution. We considered that the beginning of EL supplementation to dairy cows in each herd occurred immediately the day after the arrival date of feed in the farm. The daily number of cows in each herd was calculated based on movements data and test-day records. We considered that all cows (i.e., whatever lactation stage and milk yield) were supplemented with the same EL quantity within a herd. When a farmer distributed several feeds containing EL at the same time, herd daily exposures from each delivery were added. To sum up, at this step we calculated a mean EL intake by cow by herd for each day of the study period.

Exposure status was determined within a herd at the AI level. An AI was considered exposed only if the cow was supplemented with EL continuously from calving until 17 days after AI. The interval from calving to 17 d after AI was established as a cut-off in order to assess the potential benefits of EL supplementation since the early postpartum period until the moment where the embryonic implantation is normally achieved. Consequently, lactations begun before 1st January 2008 as well as AIs recorded after 14th December 2015 were excluded because of incomplete exposure sequences. Additionally, to reduce misclassification bias from potential partial exposures, data from cows inconstantly supplemented with EL from calving to 17 days after AI were excluded from the dataset. For each AI, an average daily EL exposure during the interval between calving and 17 days after AI was calculated by adding each daily exposure (from each day in this interval), estimated as described above, divided by the number of days in this interval. In practice the quantities of EL supplemented to cows are based on the expectations of the farmer in terms of improvement of milk production or its fatty acids profile according to specific commercial recommendations. Therefore, the exposure variable was categorized into several levels of EL daily intake: 0 (i.e., unexposed), [0–50], [50–300], [300–600] and [600–1500] g/cow/d based on how EL is currently used in the field. We considered that estimated EL daily intakes superior to 1500 g/cow/d (0.32% of final AI database) were inaccurate and related lactations were removed. Furthermore, in a previous work [24] this value was considered to be the upper limit of the practical range of EL supplementation to dairy cows (i.e., 600 g of fat from EL considering 40% of fat in EL). In summary, the exposure variable accounted for both the dose and period of exposure.

For each herd, all AIs recorded during the study period were considered for the study. All the AIs that were considered not exposed constituted the unexposed reference population. This enabled the comparison of reproductive performance within the same herd, thereby controlling for farming and climatic conditions [25].

2.3. Definition of reproductive performance and data selection

The effect of EL on reproductive performance was assessed using several outcome variables.

Firstly, the occurrence of a new AI (i.e., a return-to-service (**RTS**)) after a first AI (dichotomous variable, yes/no) was considered. This indicator was used in several studies quantifying the effect of a disease on fertility or embryonic losses depending on the time when the event was observed [26–30]. Three RTS were considered:

(i) an RTS between 18 and 26 days after an AI, which is likely to be associated with fertilization failure or early embryo loss (**early RTS**).
(ii) an RTS between 27 and 78 days after ab AI, which is likely to be associated with a late embryo (after the stage of maternal recognition of gestation) or fetal (after day 42 of gestation) loss (**delayed RTS**).
(iii) an RTS between 18 and 78 days after service (**overall RTS**).

Late RTS (after 78 days post-service) was not studied as no scientific literature pointed out a possible effect of EL on the risk of abortion. Only RTS after first and second AI were considered. Returns to service occurring before a given interval were excluded for the assessment of the risk of RTS during this interval (i.e., RTS before day 27 were excluded for assessing the risk of delayed RTS after AI).

Secondly, the time from calving to first AI (**DAI1**; continuous variable) was considered as a proxy to assess the resumption of cyclicity postpartum and the ability of the cow to be inseminated.

Finally, the time from calving to conception (days to AI resulting in fertilization of the oocyte **DAIF**; continuous variable) was considered. The AI was considered successful when there was no RTS between 18 and 78 days after AI. When the first AI and the AI resulting in fertilization occurred respectively after 150 or 270 days, DAI1 and DAIF were computed respectively as being 150 or 270 days.

Data from dairy herds with unusual management (i.e., very small herds, extreme primiparous cows proportion, systematic delayed first service, use of synchronization protocols) and suspected to use a breeding bull, as well as data from cows with missing data (i.e., herd identification, parity, test-day record, insemination, calving date) were excluded [26,29,31]. Furthermore, data from cows with events not considered plausible and extreme data were excluded: calving to first test day record >75 d, DAI1 <21 d or >180 d, interval between two successive AI >200 d or <3 d, AI to calving interval >297 d or <175 d, peak milk yield (expressed as the maximum at the 3 first test-day records) < 10 kg/d, milk protein content at the second test day record equal to 0. Classification bias can occur when cows are culled because their pregnancy status is uncertain. Thus cows culled within 200 days after AI were excluded from the analysis in order not to underestimate the risk of RTS. Data from nulliparous cows were excluded because of the lack of information about EL supplementation and so exposure status during their pregnancy. Only data from Holstein cows were included because of the strong effect of breed on reproductive performance [32,33]. This data selection reduced sample size to 1096 herds.

2.4. Statistical models

The statistical unit to study RTS was the AI. The effect of EL exposure on the risk of RTS was assessed using logistic mixed regression model. To account for factors likely to influence the risk of RTS, this association was adjusted for several independent variables [26–29,31,34]: calving to AI interval (10 levels), year of AI (8 levels), month of AI (12 levels), rank of AI (1 or 2), semen from Holstein bull (yes/no), semen sexing (yes/no and one accounting for missing data), parity (4 levels), calving ease (4 levels from easy to cesarian section and one accounting for missing data), peak milk yield (7 levels), milk protein content at second test day record (7 levels), and geographical area (7 levels) (Suppl. Tables 4 and 5). A herd random effect was also added in the model to take into account in particular diseases and feeding management differences between herds:

$$Y_{ijt} \sim Bernoulli\left(p_{ijt}\right)$$

$$\ln\left(\frac{p_{ijt}}{1-p_{ijt}}\right) = \alpha + X_{ijt}\beta + \nu_j$$

$$\nu_j \sim Normal\left(0, \sigma^2\right)$$

where $Y_{ijt} = 1$ when an RTS occurred in interval t for a cow i in herd j and 0 otherwise, α = intercept, X_{ijt} = matrix of predictors including exposure status with β the vector of associated regression parameters, υ_j = herd j random effect.

Odds-ratios were converted into relative risks (RR) using the formula from Beaudeau and Fourichon [35].

The statistical units to study respectively DAI1 and DAIF were respectively the first AI and the successful AI. The effects of EL exposure on the DAI1 and DAIF were assessed using multivariable proportional hazards Cox models. The association between EL exposure and each outcome was adjusted for the same factors used to study RTS, except that the factors rank of AI, semen from Holstein bull, semen sexing and calving to AI interval for DAI1, the rank of AI and calving to AI interval for DAIF, were obviously removed from the models. A herd random effect (frailty term) assuming a gamma distribution [36] was added to the models to take into account health and management differences between herds:

$$\lambda_{ij}(t, Z_{ij}|w_j) = w_j\lambda_0(t)\exp\left(Z_{ij}^{'}\beta\right)$$

$$w_j \sim \Gamma\left(\frac{1}{\theta}, \frac{1}{\theta}\right)$$

where $\lambda_0(t)$ = baseline hazard function, Z_{ij} = matrix of predictors including exposure status with β the vector of associated regression parameters, w_j = herd j random effect.

All statistical analyses were performed in R (version 3.3.2) [37] using the function glmer from the package lme4 (version 1.1–12) and the function coxph from the package survival (version 2.40–1).

3. Results

3.1. Descriptive results

The final sample was composed of 1096 herds, 158,125 cows, and 423,605 AIs (Table 1). Almost half of the AIs were unexposed. More than 78% of the exposed AIs were at levels [50–300] or [300–600] g/cow/d whatever the reproductive outcome considered. Mean daily EL intake in exposed population was 337 (±239.4) g/cow/d. Rates of early RTS, delayed RTS, and overall RTS were respectively 22.6%, 33.3% and 48.4% in the reference population, and respectively 24.0%, 33.2% and 49.2% in the whole exposed population (Table 1). Calving-to-first AI interval and calving-to-conception interval were respectively 91 (±28.2) d and 110 (±42.0) d in the reference population, and respectively 90 (±27.9) d and 107 (±40.8) in the whole exposed population (Table 1).

3.2. Return rates were not associated with exposure to extruded linseed

Overall RTS did not differ between the reference population and the exposed population (Table 2). Very low level of EL exposure (i.e., <50 g/cow/d from calving to 17 days after AI) was not associated with early or delayed RTS. Other levels of exposure to EL were slightly associated with increased risk of early RTS (RR from 1.02 to 1.04) and with decreased risk of delayed RTS (RR from 0.95 to 0.96) (Table 2). No clear dose-dependent relationships within EL exposure levels were observed (Table 2).

3.3. Earlier days to first AI and to conception associated with exposure to extruded linseed

Exposure to EL was associated with reduced DAI1 and reduced DAIF (Table 3). Very low level of EL exposure was associated with the highest reduction in DAI1 and DAIF (HR = 1.14 and HR = 1.19) compared to other levels of exposure (Fig. 1 and Fig. 2).

3.4. Adjustment variables associated with reproductive performance

The magnitude of the association between RTS and the adjustment variables varied according to early or delayed RTS (Suppl. Table 4), but not its direction (except for parity 3). Dystocia was strongly associated with increased risk of early and delayed RTS, as well as sexing semen and Holstein semen. As expected, the increased calving-to-AI interval was positively associated with a decreased risk of RTS, whereas increased peak milk yield was positively associated with increased risk of RTS (Suppl. Table 4). Increased peak milk yield and decreased MPC at 2nd test day record were also associated with increased DAI1 and DAIF (Suppl. Table 5). Strong associations of the dystocia with DAI1 and DAIF, and sexing semen and Holstein bull with DAIF were also observed (Suppl. Table 5). Finally, inseminating in spring and early summer was associated with early and delayed RTS, as well as increased days open (Suppl. Table 4, Table 5).

4. Discussion

This observational study is to our knowledge the first one exploring the link between a feed supplementation and reproductive performance of dairy cows based on a large dataset under field conditions. This study provides further insight into supplementing EL on reproductive performance.

Exposure to EL was associated with a reduced DAIF through a

Table 1
Return-to-service (**RTS**) rates, time from calving to first AI (**DAI1**) and time from calving to conception (**DAIF**) according to extruded linseed (**EL**) exposure status in 1096 French Holstein dairy herds during the study period 2008–2015 (n = 423,605 AI from 158,125 cows).

		EL Exposure status[1]				
		Unexposed	[0–50]	[50–300]	[300–600]	[600–1500]
Herds		1064	255	915	699	372
Cows		95,083	7583	44,409	34,110	14,152
Total AI		226,795	14,126	88,261	66,136	28,287
EL (g/cow/d)	Mean	–	27.37_a	175.99_b	432.34_c	771.86_d
	SE	–	0.10	0.23	0.32	0.98
RTS rate (%)[2]						
18–26 d		22.64_a	22.30_b	23.87_c	24.17_d	24.62_e
26–78 d		33.33_a	34.23_b	33.33_c	32.78_d	33.11_e
18–78 d		48.42_a	48.90_b	49.25_c	49.03_d	49.58_e
Calving-to-first AI interval (d)[2]	n	147,377	9528	58,853	43,597	18,593
	Mean	91.27a	88.44b	90.04c	90.89ad	90.53cd
	SE	0.07	0.28	0.11	0.13	0.20
Calving-to-conception interval (d)[3]	n	116,963	7218	44,792	33,706	14,261
	Mean	109.71a	105.63b	106.74cbd	108.21e	107.33de
	SE	0.12	0.49	0.19	0.22	0.34

[1]Exposure status was defined by average daily intake of EL per cow per day during the interval from calving to 17 days after AI.
[2]*Different lowercase letters indicate significant difference determined from* Chi-square post hoc test.
[3]Mean and SE were calculated including censored AI1 with their calving-to-first AI interval fixed at 150 days.
[4]Mean and SE were calculated including censored AIF with their calving-to-conception interval fixed at 270 days.
[3–4]*Different lowercase letters indicate significant difference determined from* one-way ANOVA with Tukey's post hoc test.

Table 2
Relative risk of return-to-service (RTS) according to the extruded linseed exposure status in 1096 French Holstein dairy herds during the study period January 2008 to December 2015 (n = 423,605 AI from 158,125 cows).

Extruded linseed exposure status[a]	Interval of return								
	18–26 d			27–78 d			18–78 d		
	RR[b]	95% CI[c]	P[d]	RR	95% CI	P	RR	95% CI	P
Unexposed	1	Ref[e]		1	Ref		1	Ref	
[0–50]	0.98	0.94; 1.02	0.202	0.99	0.95; 1.05	0.841	0.99	0.98; 1.01	0.366
[50–300]	1.02	1.00; 1.04	0.029	0.97	0.94; 0.99	0.004	0.99	0.98; 1.01	0.274
[300–600]	1.04	1.02; 1.06	<0.001	0.95	0.92; 0.98	<0.001	0.99	0.98; 1.01	0.443
[600–1500]	1.04	1.01; 1.07	0.013	0.96	0.92; 1.00	0.040	0.99	0.98; 1.02	0.691

Herd random effect variance and standard deviation were respectively 0.08 and 0.285, 0.07 and 0.260, 0.07 and 0.273 in models respectively studying interval of return 18 to 26, 27 to 78 and 18–78 days.
[a] Exposure status was defined by average daily intake of EL per cow per day during the interval from calving to 17 days after AI.
[b] RR = relative risk adjusted for calving-to-AI interval (10 levels), year of AI (8 levels), month of AI (12 levels), rank of AI (1 or 2), semen from Holstein bull (yes/no), semen sexing (yes/no and missing data), parity (4 levels), difficulty of the last calving (5 levels including one for missing data), peak milk yield (7 levels), milk protein content at second test day record (7 levels), geographical area (7 levels) and herd random effect.
[c] CI = confidence interval.
[d] *P* = *P*-value.
[e] Ref = Reference.

reduced DAI1. Few of the experimental studies performed so far were adapted to study the effect of linseed on DAI1 and DAIF because of estrus synchronization protocols and/or fat supplementation initiated several weeks postpartum. The direction of the effect is consistent with the one found in a previous experimental trial where a reduction of 6.5 days in DAI1 was observed immediately postpartum until 40d after calving in EL supplemented group (PUFA 4.5% DM) compared to protected palm oil supplemented group (PUFA 1.6% DM) [19]. Surprisingly, no effect of EL supplementation on overall RTS was observed. Decreased risk of delayed RTS was offset by increased risk of early RTS. A decreased risk of delayed RTS is consistent with reduced pregnancy loss observed in cows supplemented with whole linseed (PUFA 10.4% DM) [17] or rolled linseed (PUFA 9% DM) [20]. Risk of early RTS reflects as well non fertilization of oocyte and early embryo mortality (before 15–17 days after AI), and so oocyte and embryo quality. Zachut et al. [12] and Moallem et al. [38] observed an improvement in embryo cleavage rate with a diet supplemented with encapsulated linseed relative to a diet supplemented with saturated FA but not relative to a diet supplemented with different sources of UFA such as sunflower oil and fish oil. Besides, Thangavelu et al. [39] found that a diet supplemented with sunflower oil or linseed enhanced embryonic development relative to a diet enriched in saturated FA. However, Petit et al. [40] found a decreased embryo quality with whole linseed supplementation relative to a commercial product rich in saturated and oleic FA supplementation. Evaluating and comparing such studies is complex because of substantial disparities between precise timing, duration, amount and nature of dietary intervention as mentioned by Leroy et al. [6] in its review on the relationship between dietary fat and oocyte and embryo quality. Besides, quantities of EL observed under field conditions were far lower than in these cited experimental trials.

In our study, overall mean of EL supplementation under field conditions was quite low: EL was supplemented at an average of 337 (±239.4) g/cow/d compared to 1181 (±742.5) g/cow/d in 29 treatment diets from 21 trials studying EL and production performance [24], and to 826 g/cow/d, 1700 g/cow/d and 1745 g/cow/d in three trials studying EL and reproductive performance [13,19,21]. Thus, we lack the knowledge to comment on the largest magnitude of the association between DAI1 or DIAF and EL exposure with the lowest intake of EL (<50 g of EL, <11 g of ALA). Such a low level of ALA supplementation was not studied previously in the literature, but two studies found huge beneficial effects of low intakes of other PUFA. Sinedino et al. [41] in a study conducted with 739 lactating cows showed a very strong effect of supplementing an algae product containing 10 g of DHA on reproductive performance with a reduction in the days-to-pregnancy interval of 22 days. De Veth et al. [42] in a multi-study analysis, predicted an optimal effect of conjugated linoleic acid on time to first ovulation (−8 d) and time to conception (−34 d) at a quantity from 8 to 10 g/d. In light of these elements, there is a need for experimental trials to focus on EL supplementation in a range observed under field conditions to

Table 3
Hazard ratios of the time from calving to first AI (**DAI1**) and the time from calving to conception (**DAIF**) expressed in days according to the extruded linseed (**EL**) exposure status in 1096 French Holstein dairy herds during the study period January 2008 to December 2015 (respectively, n = 277,948 AI1 from 156,203 cows and n = 216,940 AIF from 129,215 cows).

Extruded linseed exposure status[a]	DAI1 (d)			DAIF (d)		
	HR[b]	95% CI[c]	P[d]	HR	95% CI	P
Unexposed	1	Ref[e]		1	Ref	
[0–50]	1.14	1.11; 1.17	<0.001	1.19	1.15; 1.23	<0.001
[50–300]	1.06	1.04; 1.07	<0.001	1.10	1.08; 1.11	<0.001
[300–600]	1.06	1.05; 1.08	<0.001	1.08	1.06; 1.10	<0.001
[600–1500]	1.07	1.05; 1.09	<0.001	1.11	1.08; 1.14	<0.001

Number of events was 263,859 and 216,066 respectively for DAI1 and DAIF. Herd random effect variance was respectively 0.62 and 0.28.
[a] Exposure status was defined by average daily intake of EL per cow per day during the interval from calving to 17 days after AI.
[b] HR = hazard ratio adjusted for year of AI (8 levels), month of AI (12 levels), parity (4 levels), difficulty of the last calving (5 levels including one for missing data), peak milk yield (7 levels), milk protein content at second test day record (7 levels), geographical area (7 levels) and a herd random effect, plus semen from Holstein bull (yes/no), semen sexing (yes/no and missing data) for DAIF.
[c] CI = confidence interval.
[d] *P* = *P*-value.
[e] Ref = Reference.

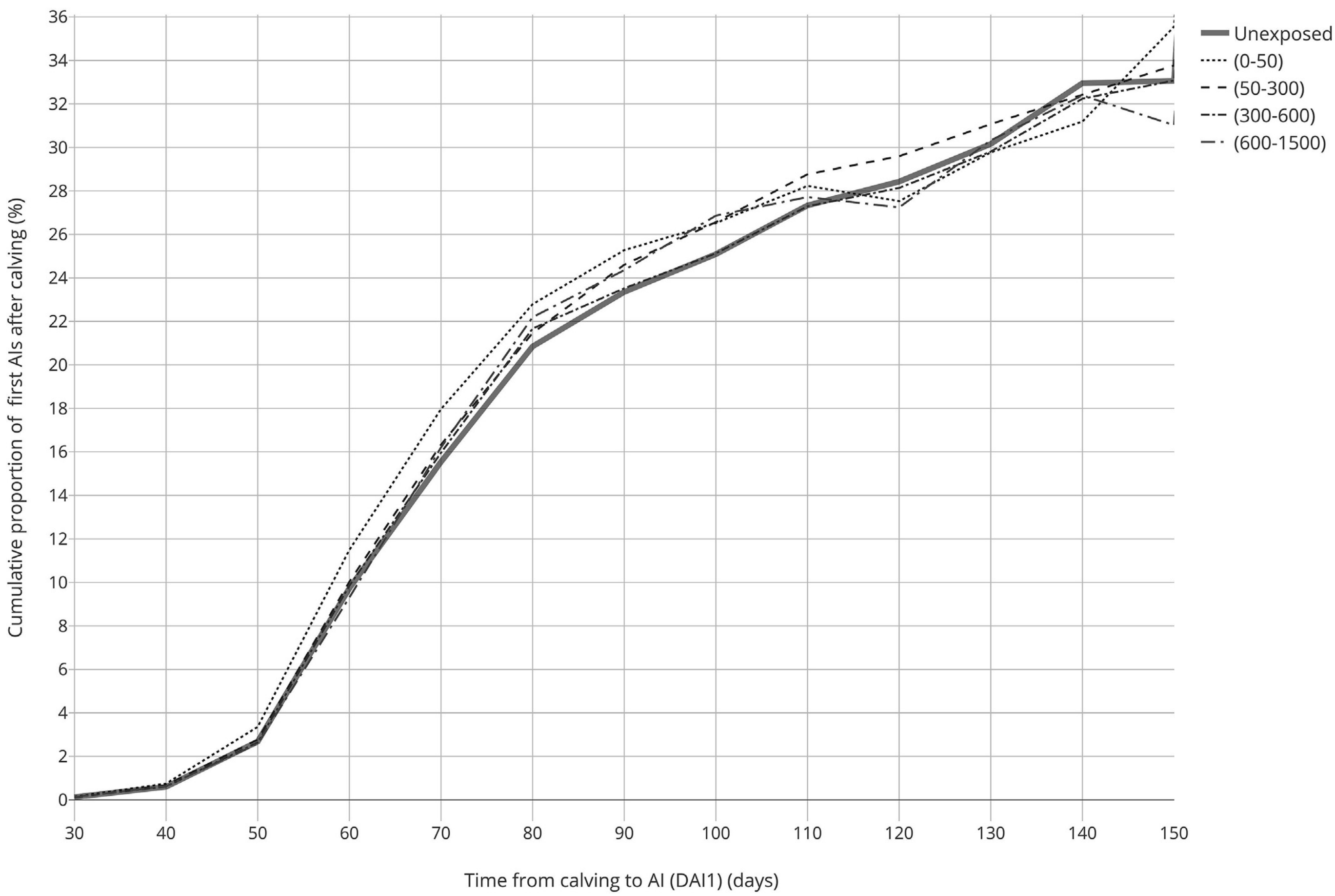

Fig. 1. Cumulative proportions of first AI across the time from calving to first AI (**DAI1**) according to the extruded linseed (**EL**) exposure status in 1096 French Holstein dairy herds during the study period January 2008 to December 2015 (n = 277,948 AI1 from 156,203 cows fixed at 150 days).

strengthen our results obtained with low EL supplementation.

Our study was, to our knowledge, the first one exploring a dose-dependent association of reproductive performance with EL or n-3 FA. In the present study, the dose-dependent relationship was far from being linear: no effect was demonstrated on the risk of RTS and a quite constant positive effect was observed on DAI1 and DAIF. Besides, contrary to other exposure levels, the lowest level of EL intake was not associated with early and delayed RTS, whereas its association with reduced DAI1 or DAIF was of larger magnitude than other exposure levels. Yet, linear dose-dependent associations between intake of EL and other dairy cows production traits were reported: milk FA profile [24], enteric methane emission [43], and milk yield and milk contents (Meignan et al., unpublished data). Interestingly, this latter study was based on the same initial dataset than the one used in the present study. Estimated daily milk yield increased with increased estimated EL daily intake compared to the daily milk yield of the reference population: respectively +0.00, +0.59, +0.90 and + 1.13 kg/d with an EL intake of [0–50], [50–300], [300–600] and [600–1500] g/cow/d considering a second parity Holstein cow. Milk yield and negative energy balance (**NEB**) are known to negatively influence the return to ovarian cyclicity and the estrous behavior [44–46], and the oocyte and embryo quality [47]. Therefore, milk yield could act as a confounding factor and/or an explanatory factor on the association between EL exposure and reproductive performances. Here, the estimates of reproductive performances were adjusted for peak milk yield in order to take into account both milk yield and level of NEB. High peak milk yield was associated with an increased risk of overall RTS and increased DAI1 and DAIF. However, the way we adjusted for milk yield (using a discrete variable with categories of 5kg-range) did not allow to fully account for the concomitant association between milk yield and EL supplementation (of 0.6–1.1 kg/d) while estimating the effects on reproductive performances. Another explanation for this non-linear effect of the EL supplementation could be that the antagonistic biological effect of PUFA depending on their concentration. For example, long-chain n-3 FA could act rather as pro- or anti-oxidant agents depending on the level used [48]. Finally, we have to keep in mind that EL contains not only PUFA, but also phytoestrogens that could interfere with the reproductive function [14–16].

This retrospective observational study presented different strengths and weaknesses. To adequately detect even small effects of a practical range of EL supplementation on reproductive performance, the present study was designed to be carried out on a large dataset. The reference population was composed of AIs recorded in herds that have been supplemented with EL, but during periods of EL non-supplementation, in order to limit potential confounding factors due to different herd managements between herds. Nevertheless, the within-herd management may have evolved during this long study period. Therefore, a random herd effect was added to account for possible disparities between herds.

In this study the true intake of EL for each cow could have been under or overestimated as we hypothesized that all lactating cows within a herd were supplemented with the same quantity of EL whatever their days in milk. In practice, cows in late lactation are less likely to be fed as much EL than cows in early and mid-

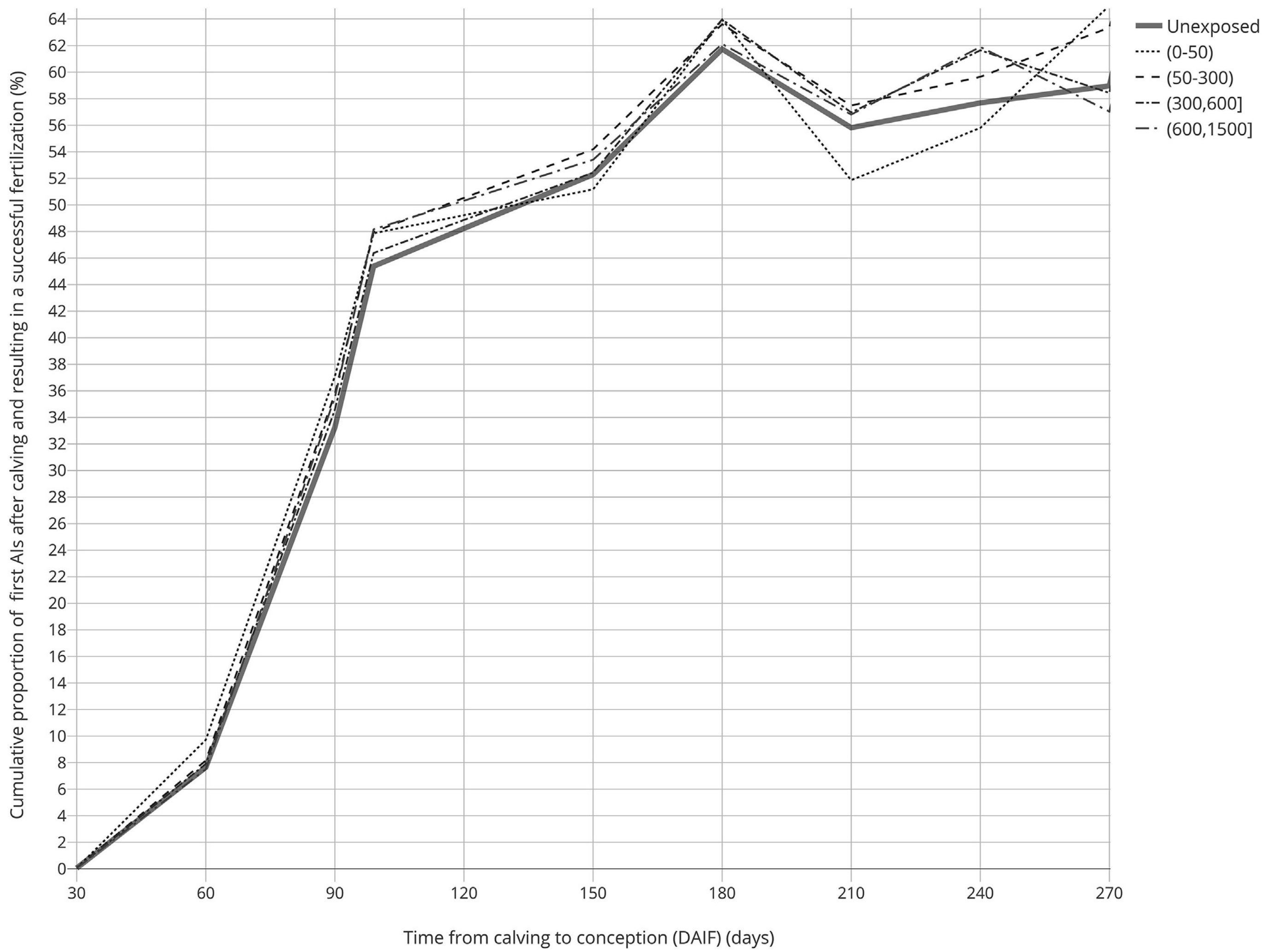

Fig. 2. Cumulative proportions of first AI across time from calving to conception (**DAIF**) according to the extruded linseed (**EL**) exposure status in 1096 French Holstein dairy herds during the study period January 2008 to December 2015 (n = 216,940 AIF from 129,215 cows.

lactation. Therefore, to minimize this misclassification bias, different exposure levels with broad ranges were defined, so that a cow was unlikely changing between two categories during the time sequence comprised in the IA studied. In addition, as the results show an absence of dose-dependent effect of EL on reproductive performance, the misclassification bias within the exposure levels did not affect our results.

To reduce potential intake-related bias, a prospective study designed to record diet composition could increase precision on EL exposure. Likewise, biomarkers already implemented in human nutritional epidemiological studies such as the milk ALA content could be used to enhance the precision of the exposure intake measures [49,50]. However, the milk ALA content is closely dependent on ruminal biohydrogenation and modulated by other dietary components as grass or alfalfa [51] and its measurement by mid-infrared spectroscopy still lacking precision [52,53]. Therefore, the current options for increasing reliability on EL exposure are represented by prospective designs which may be difficult to carry out because of their cost and lack of practical feasibility.

5. Conclusions

Under field conditions, supplementing EL, even at low level, to dairy cows was associated with a reduced number of days to first AI and days to conception but was not associated with overall risk of return-to-service. To our knowledge, this is the first time that an association between cow nutrition and reproductive performances was assessed by a large-scale retrospective observational study. Further experimental trials using low levels of EL and at proper timing are still needed to fully understand underlying biological mechanisms associated with EL compounds (ALA, phytoestrogens, antioxidants) and dairy cow reproduction.

Conflicts of interest

T.M. is affiliated with Valorex for the sake of transparency as Valorex is the official PhD employer in the official financing arrangement Conventions Industrielles de Formation par la Recherche (CIFRE). Two employees of Valorex, Guillaume Chesneau and Vincent Chatellier, were observers during the study. Data collection of feed deliveries of feeds containing extruded linseed was performed by Valorex and 21 companies selling TRADILIN® products. The funders had no role in study design, data analysis, decision to publish and preparation of the manuscript. The funding agreement allowed us to independently publish our findings whatever the nature of the results.

T.M., A.M., F.B., and N.B. designed the study. T.M. and A.M. analyzed the data. T.M., A.M., F.B., J.M.A., C.L., and N.B interpreted results. T.M. J.M.A., and N.B. drafted the manuscript. All authors revised critically the manuscript for important intellectual content and approved the final version to be published.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.theriogenology.2018.11.020.

References

[1] Inchaisri C, Jorritsma R, Vos PLAM, van der Weijden GC, Hogeveen H. Economic consequences of reproductive performance in dairy cattle. Theriogenology 2010;74:835–46. https://doi.org/10.1016/j.theriogenology.2010.04.008.
[2] Butler WR. Energy balance relationships with follicular development ovulation and fertility in postpartum dairy cows. Livest Prod Sci 2003;83:211–8. https://doi.org/10.1016/S0301-6226(03)00112-X.
[3] Walsh SW, Williams EJ, Evans ACO. A review of the causes of poor fertility in high milk producing dairy cows. Anim Reprod Sci 2011;123:127–38. https://doi.org/10.1016/j.anireprosci.2010.12.001.
[4] Palmquist DL, Jenkins TC. Fat in lactation rations: review. J Dairy Sci 1980;63:1–14. https://doi.org/10.3168/jds.S0022-0302(80)82881-5.
[5] Staples CR, Burke JM, Thatcher WW. Influence of supplemental fats on reproductive tissues and performance of lactating cows. J Dairy Sci 1998;81:856–71. https://doi.org/10.3168/jds.S0022-0302(98)75644-9.
[6] Leroy J, Sturmey R, Van Hoeck V, De Bie J, McKeegan P, Bols P. Dietary fat supplementation and the consequences for oocyte and embryo quality: hype or significant benefit for dairy cow reproduction? Reprod Domest Anim 2014;49:353–61. https://doi.org/10.1111/rda.12308.
[7] Santos J, Bilby T, Thatcher W, Staples C, Silvestre F. Long chain fatty acids of diet as factors influencing reproduction in cattle. Reprod Domest Anim 2008;43:23–30. https://doi.org/10.1111/j.1439-0531.2008.01139.x.
[8] Gulliver CE, Friend M a, King BJ, Clayton EH. The role of omega-3 polyunsaturated fatty acids in reproduction of sheep and cattle. Anim Reprod Sci 2012;131:9–22. https://doi.org/10.1016/j.anireprosci.2012.02.002.
[9] Wathes DC, Abayasekara DRE, Aitken RJ. Polyunsaturated fatty acids in male and female reproduction. Biol Reprod 2007;77:190–201. https://doi.org/10.1095/biolreprod.107.060558.
[10] Wathes D, Cheng Z, Marei W, Fouladi-Nashta A. Polyunsaturated fatty acids and fertility in female mammals: an update. CAB Rev Perspect Agric Vet Sci Nutr Nat Resour 2013;8:1–14. https://doi.org/10.1079/PAVSNNR20138041.
[11] Petit HV. Review: feed intake, milk production and milk composition of dairy cows fed flaxseed. Can J Anim Sci 2010;90:115–27. https://doi.org/10.4141/CJAS09040.
[12] Zachut M, Dekel I, Lehrer H, Arieli A, Arav A, Livshitz L, et al. Effects of dietary fats differing in n-6:n-3 ratio fed to high-yielding dairy cows on fatty acid composition of ovarian compartments, follicular status, and oocyte quality. J Dairy Sci 2010;93:529–45. https://doi.org/10.3168/jds.2009-2167.
[13] Zachut M, Arieli A, Moallem U. Incorporation of dietary n-3 fatty acids into ovarian compartments in dairy cows and the effects on hormonal and behavioral patterns around estrus. Reproduction 2011;141:833–40. https://doi.org/10.1530/REP-10-0518.
[14] Landete JM. Plant and mammalian lignans: a review of source, intake, metabolism, intestinal bacteria and health. Food Res Int 2012;46:410–24. https://doi.org/10.1016/j.foodres.2011.12.023.
[15] Pierson LM, Ferkin MH. The impact of phytoestrogens on sexual behavior and cognition in rodents. Mamm Biol 2015;80:148–54. https://doi.org/10.1016/j.mambio.2014.11.006.
[16] Kiyama R. Biological effects induced by estrogenic activity of lignans. Trends Food Sci Technol 2016;54:186–96. https://doi.org/10.1016/j.tifs.2016.06.007.
[17] Petit HV, Twagiramungu H. Conception rate and reproductive function of dairy cows fed different fat sources. Theriogenology 2006;66:1316–24. https://doi.org/10.1016/j.theriogenology.2006.04.029.
[18] Dirandeh E, Towhidi A, Ansari Pirsaraei Z, Adib Hashemi F, Ganjkhanlou M, Zeinoaldini S, et al. Plasma concentrations of PGFM and uterine and ovarian responses in early lactation dairy cows fed omega-3 and omega-6 fatty acids. Theriogenology 2013;80:131–7. https://doi.org/10.1016/j.theriogenology.2013.03.012.
[19] Jahani-Moghadam M, Mahjoubi E, Dirandeh E. Effect of linseed feeding on blood metabolites, incidence of cystic follicles, and productive and reproductive performance in fresh Holstein dairy cows. J Dairy Sci 2015;98:1828–35. https://doi.org/10.3168/jds.2014-8789.
[20] Ambrose DJ, Kastelic JP, Corbett R, Pitney PA, Petit HV, Small JA, et al. Lower pregnancy losses in lactating dairy cows fed a diet enriched in α-linolenic acid. J Dairy Sci 2006;89:3066–74. https://doi.org/10.3168/jds.S0022-0302(06)72581-4.
[21] Fuentes MC, Calsamiglia S, Sánchez C, González A, Newbold JR, Santos JEP, et al. Effect of extruded linseed on productive and reproductive performance of lactating dairy cows. Livest Sci 2008;113:144–54. https://doi.org/10.1016/j.livsci.2007.03.005.
[22] Bork NR, Schroeder JW, Lardy GP, Vonnahme KA, Bauer ML, Buchanan DS, et al. Effect of feeding rolled flaxseed on milk fatty acid profiles and reproductive performance of dairy cows. J Anim Sci 2010;88:3739–48. https://doi.org/10.2527/jas.2010-2841.
[23] Dirandeh E, Towhidi A, Zeinoaldini S, Ganjkhanlou M, Ansari Pirsaraei Z, Fouladi-Nashta A. Effects of different polyunsaturated fatty acid supplementations during the postpartum periods of early lactating dairy cows on milk yield, metabolic responses, and reproductive performances. J Anim Sci 2013;91:713–21. https://doi.org/10.2527/jas.2012-5359.
[24] Meignan T, Lechartier C, Chesneau G, Bareille N. Effects of feeding extruded linseed on production performance and milk fatty acid profile in dairy cows: a meta-analysis. J Dairy Sci 2017;100:4394–408. https://doi.org/10.3168/jds.2016-11850.
[25] Nusinovici S, Souty C, Seegers H, Beaudeau F, Fourichon C. Decrease in milk yield associated with exposure to bluetongue virus serotype 8 in cattle herds. J Dairy Sci 2013;96:877–88. https://doi.org/10.3168/jds.2012-5800.
[26] Robert A, Beaudeau F, Seegers H, Joly A, Philipot J. Large scale assessment of the effect associated with bovine viral diarrhoea virus infection on fertility of dairy cows in 6149 dairy herds in Brittany (Western France). Theriogenology 2004;61:117–27. https://doi.org/10.1016/S0093-691X(03)00182-1.
[27] Malher X, Beaudeau F, Philipot JM. Effects of sire and dam genotype for complex vertebral malformation (CVM) on risk of return-to-service in Holstein dairy cows and heifers. Theriogenology 2006;65:1215–25. https://doi.org/10.1016/j.theriogenology.2005.08.003.
[28] Marcé C, Beaudeau F, Bareille N, Seegers H, Fourichon C. Higher non-return rate associated with Mycobacterium avium subspecies paratuberculosis infection at early stage in Holstein dairy cows. Theriogenology 2009;71:807–16. https://doi.org/10.1016/j.theriogenology.2008.10.017.
[29] Nusinovici S, Seegers H, Joly A, Beaudeau F, Fourichon C. Quantification and at-risk period of decreased fertility associated with exposure to bluetongue virus serotype 8 in naïve dairy herds. J Dairy Sci 2012;95:3008–20. https://doi.org/10.3168/jds.2011-4799.
[30] Marceau A, Madouasse A, Lehébel A, van Schaik G, Veldhuis A, Van der Stede Y, et al. Can routinely recorded reproductive events be used as indicators of disease emergence in dairy cattle? An evaluation of 5 indicators during the emergence of bluetongue virus in France in 2007 and 2008. J Dairy Sci 2014;97:6135–50. https://doi.org/10.3168/jds.2013-7346.
[31] Seegers H, Coulon R, Beaudeau F, Fouchet M, Quillet JM. Factors associated with variation in occurence of different types of return-inseminations in dairy herds. Rencontres autour des Rech. sur les ruminants 2001;8:357–60. Paris.
[32] Bedere N, Disenhaus C, Ducrocq V, Leurent-Colette S, Delaby L. Ability of dairy cows to ensure pregnancy according to breed and genetic merit for production traits under contrasted pasture-based systems. J Dairy Sci 2017;100:2812–27. https://doi.org/10.3168/jds.2016-11588.
[33] Bedere N, Disenhaus C, Ducrocq V, Leurent-Colette S, Delaby L. Ability of dairy cows to be inseminated according to breed and genetic merit for production traits under contrasting pasture-based feeding systems. Animal 2017;11:826–35. https://doi.org/10.1017/S1751731116002111.
[34] Gröhn YT, Rajala-Schultz PJ. Epidemiology of reproductive performance in dairy cows. Anim Reprod Sci 2000;60–61:605–14. https://doi.org/10.1016/S0378-4320(00)00085-3.
[35] Beaudeau F, Fourichon C. Estimating relative risk of disease from outputs of logistic regression when the disease is not rare. Prev Vet Med 1998;36:243–56. https://doi.org/10.1016/S0167-5877(98)00095-6.
[36] Ducrocq V, Casella G. A Bayesian analysis of mixed survival models. Genet Sel Evol 1996;28:505–29. https://doi.org/10.1186/1297-9686-28-6-505.
[37] R Core Team R. A language and environment for statistical computing. 2017.
[38] Moallem U, Shafran A, Zachut M, Dekel I, Portnick Y, Arieli A. Dietary -linolenic acid from flaxseed oil improved folliculogenesis and IVF performance in dairy cows, similar to eicosapentaenoic and docosahexaenoic acids from fish oil. Reproduction 2013;146:603–14. https://doi.org/10.1530/REP-13-0244.
[39] Thangavelu G, Colazo MG, Ambrose DJ, Oba M, Okine EK, Dyck MK. Diets enriched in unsaturated fatty acids enhance early embryonic development in lactating Holstein cows. Theriogenology 2007;68:949–57. https://doi.org/10.1016/j.theriogenology.2007.07.002.
[40] Petit HV, Cavalieri FB, Santos GTD, Morgan J, Sharpe P. Quality of embryos produced from dairy cows fed whole flaxseed and the success of embryo

transfer. J Dairy Sci 2008;91:1786–90. https://doi.org/10.3168/jds.2007-0782.
[41] Sinedino LDP, Honda PM, Souza LRL, Lock AL, Boland MP, Staples CR, et al. Effects of supplementation with docosahexaenoic acid on reproduction of dairy cows. Reproduction 2017;153:707–23. https://doi.org/10.1530/REP-16-0642.
[42] de Veth MJ, Bauman DE, Koch W, Mann GE, Pfeiffer AM, Butler WR. Efficacy of conjugated linoleic acid for improving reproduction: a multi-study analysis in early-lactation dairy cows. J Dairy Sci 2009;92:2662–9. https://doi.org/10.3168/jds.2008-1845.
[43] Martin C, Ferlay A, Mosoni P, Rochette Y, Chilliard Y, Doreau M. Increasing linseed supply in dairy cow diets based on hay or corn silage: effect on enteric methane emission, rumen microbial fermentation, and digestion. J Dairy Sci 2016;99:3445–56. https://doi.org/10.3168/jds.2015-10110.
[44] Lopez H, Satter L, Wiltbank M. Relationship between level of milk production and estrous behavior of lactating dairy cows. Anim Reprod Sci 2004;81:209–23. https://doi.org/10.1016/j.anireprosci.2003.10.009.
[45] Dobson H, Smith R, Royal M, Knight C, Sheldon I. The high-producing dairy cow and its reproductive performance. Reprod Domest Anim 2007;42:17–23. https://doi.org/10.1111/j.1439-0531.2007.00906.x.
[46] Cutullic E, Delaby L, Causeur D, Michel G, Disenhaus C. Hierarchy of factors affecting behavioural signs used for oestrus detection of Holstein and Normande dairy cows in a seasonal calving system. Anim Reprod Sci 2009;113:22–37. https://doi.org/10.1016/j.anireprosci.2008.07.001.
[47] Leroy J, Opsomer G, Van Soom A, Goovaerts I, Bols P. Reduced fertility in high-yielding dairy cows: are the oocyte and embryo in danger? Part I: the importance of negative energy balance and altered corpus luteum function to the reduction of oocyte and embryo quality in high-yielding dairy cows. Reprod Domest Anim 2008;43:612–22. https://doi.org/10.1111/j.1439-0531.2007.00960.x.
[48] Giordano E, Visioli F. Long-chain omega 3 fatty acids: molecular bases of potential antioxidant actions. Prostagl Leukot Essent Fat Acids 2014;90:1–4. https://doi.org/10.1016/j.plefa.2013.11.002.
[49] Shim J-S, Oh K, Kim HC. Dietary assessment methods in epidemiologic studies. Epidemiol Health 2014;7. https://doi.org/10.4178/epih/e2014009. e2014009.
[50] Naska A, Lagiou A, Lagiou P. Dietary assessment methods in epidemiological research: current state of the art and future prospects. F1000Research 2017;6:926. https://doi.org/10.12688/f1000research.10703.1.
[51] Glasser F, Doreau M, Maxin G, Baumont R. Fat and fatty acid content and composition of forages: a meta-analysis. Anim Feed Sci Technol 2013;185:19–34. https://doi.org/10.1016/j.anifeedsci.2013.06.010.
[52] Soyeurt H, Dehareng F, Gengler N, McParland S, Wall E, Berry DP, et al. Mid-infrared prediction of bovine milk fatty acids across multiple breeds, production systems, and countries. J Dairy Sci 2011;94:1657–67. https://doi.org/10.3168/jds.2010-3408.
[53] Ferrand-Calmels M, Palhière I, Brochard M, Leray O, Astruc JM, Aurel MR, et al. Prediction of fatty acid profiles in cow, Ewe, and goat milk by mid-infrared spectrometry. J Dairy Sci 2014;97:17–35. https://doi.org/10.3168/jds.2013-6648.