Effects of supplementation with docosahexaenoic acid on reproduction of dairy cows

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Abstract

The objectives were to determine the effects of supplementing docosahexaenoic acid (DHA)-rich algae on reproduction of dairy cows. Holstein cows were assigned randomly to either a control ($n=373$) or the same diet supplemented daily with 100 g/cow of an algae product containing 10% DHA (algae, $n=366$) from 27 to 147 days postpartum. Measurements included yields of milk and milk components, fatty acids (FA) profiles in milk fat and plasma phospholipids, resumption of ovulation by 57 days postpartum, pregnancy per artificial insemination (AI) and expression of interferon-stimulated genes in leukocytes. Feeding algae increased resumption of estrous cyclicity (77.6 vs 65.9%) and pregnancy at first AI (47.6 vs 32.8%) in primiparous cows. Algae increased pregnancy per AI in all AI in both primiparous and multiparous cows (41.6 vs 30.7%), which reduced days to pregnancy by 22 days (102 vs 124 days) compared with control cows. Pregnant cows fed algae had greater expression of *RTP4* in blood leukocytes compared with those in pregnant control cows. Feeding algae increased the incorporation of DHA, eicosapentaenoic acid, conjugated linoleic acid isomers *cis*-9 *trans*-11, *trans*-10 *cis*-12 and total n-3 FA in phospholipids in plasma and milk fat. Yields of milk and true protein increased by 1.1 kg/day and 30 g/day respectively, whereas fat yield decreased 40 g/day in algae compared with that in control. Supplementing DHA-rich algae altered the FA composition of lipid fractions and improved reproduction in dairy cows. The benefits on reproduction might be mediated by enhanced embryo development based on changes in interferon-stimulated gene expression.

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Introduction

Fatty acids (FA) perform important roles as regulators of biological processes such as cell membrane stability, gene expression, cell adhesion and proliferation and both intracellular and intercellular transport (Wahle *et al.* 2003). They are important components of cellular membranes, and changes in tissue FA composition can influence cellular function and gene expression (Abayasekara & Wathes 1999, Bilby *et al.* 2006a). Fat supplementation has beneficial effects on reproductive performance of dairy cows (Rodney *et al.* 2015), and the improvements in fertility caused by fat feeding go beyond an increase in energy intake (Staples *et al.* 1998). In fact, some of the positive effects seem to be influenced by the type of FA fed (Santos *et al.* 2008). Also, it is known that fat supplementation can influence lactation performance, and a meta-analysis of the published literature reported an overall increase in milk yield of approximately 1.05 kg/day in cows that were fed

supplemental fat compared with those fed diets without supplemental fat (Rabiee *et al.* 2012).

One of the long-chain polyunsaturated FA that has been shown to have important biological effects is docosahexaenoic acid (DHA, C22:6 n-3). It is a member of the n-3 family of FA and is found in marine fish and algae oils. Algal biomass produced in bulk fermenters provides a consistent, high-quality source of DHA for dairy cattle (Stamey *et al.* 2012). Incorporation of supplemental n-3 FA into diets of lactating dairy cows can increase their uptake into membrane phospholipids and attenuate innate immunity and inflammatory responses and favor maintenance of pregnancy in dairy cattle (Santos *et al.* 2008, Silvestre *et al.* 2011a,b). Feeding DHA, however, may modify the ruminal environment and promote milk fat depression, typically accompanied by increased proportions of conjugated linoleic acid (CLA) and *trans*-C18:1 FA in milk fat (Hostens *et al.* 2011). This response can be exacerbated if diets already contain unsaturated FA or are high in starch.

Altering the dietary intake of FA may promote changes in circulating concentrations of metabolic hormones and ovarian steroids that, in turn, might affect the development and competence of ovarian follicles and/or the composition and secretions of reproductive tissues (Bilby *et al.* 2006b, Zachut *et al.* 2008). Changes in FA composition of reproductive tissues concurrent with the altered endocrine milieu might modulate prostaglandin synthesis and improve fertility (Staples *et al.* 1998). In fact, supplementation of n-3 FA promoted the downregulation of genes related to prostaglandin synthesis in the endometrium and influenced spontaneous release of PGF_{2α} in dairy cows (Bilby *et al.* 2006a, Greco *et al.* 2014). Several studies reported improvements in pregnancy per AI (P/AI) or embryo quality in lactating dairy cows supplemented with polyunsaturated FA (Santos *et al.* 2008, Rodney *et al.* 2015). Furthermore, lipid uptake and metabolism is likely a key feature regulating conceptus elongation (Ribeiro *et al.* 2016a,b). Therefore, it is proposed that supplementation with small amounts of n-3 FA such as DHA may improve the development of the conceptus, which in turn would facilitate embryo-maternal crosstalk and improve peripheral signaling through complementary actions with interferon-tau secretion that might benefit pregnancy.

Although supplementing diets with FA have been linked to beneficial effects on reproduction in lactating dairy cows, incorporation of large doses of n-3 FA can increase the risk of tissue peroxidation and increase oxidative stress (Gobert *et al.* 2009, Wullepitt *et al.* 2012). Such negative effects of polyunsaturated n-3 FA might be exacerbated if large quantities are fed during the transition period, when lipomobilization is already extensive (Wullepitt *et al.* 2012) or when diets have low content of antioxidant compounds such as vitamin E (Gobert *et al.* 2009). On the other hand, at low amounts, n-3 FA acts as anti- rather than prooxidant in endothelial vascular cells and placental tissues by reducing inflammation and reducing the formation of reactive oxygen species (Jones *et al.* 2013, Giordano & Visioli 2014). Furthermore, although moderate amounts of supplemental fat fed in early lactation does not seem to depress intake (Staples *et al.* 1998), feeding large quantities of unsaturated FA might suppress appetite (Harvatine & Allen 2006), which could exacerbate negative energy balance and defeat any benefit to reproduction. Because FA can be used as energy substrate for oocytes and embryos (Sturmey *et al.* 2009), and they can work as signaling molecules during conceptus elongation (Ribeiro *et al.* 2016a), it is anticipated that FA with regulatory cellular function might have greater benefit to reproduction. Therefore, strategies to improve reproduction should incorporate strategic feeding of moderate amounts of FA that are expected to have regulatory effects on tissue and cell metabolism.

The hypotheses of the experiment were that supplementation of a lactating dairy cow diet with an algae product rich in DHA to supply a small amount of FA will alter the FA composition of tissues and improve reproduction by increasing P/AI. Therefore, the objectives were to evaluate the effects of supplementing algae rich in DHA on reproductive performance, interferon-stimulated gene expression in blood leukocytes and FA composition of plasma and milk in dairy cows. Secondary objectives were to evaluate the effects of supplementing algae rich in DHA on productive performance and plasma concentration of hormones.

Materials and methods

Ethics approval

All procedures and protocols for animal handling and care were approved by the University of Florida Institutional Animal Care and Use Committee protocol number ARC 002-14ANS.

Cows and housing

The experiment was conducted at a commercial dairy farm located in central California comprised of 1800 Holstein cows. Lactating cows ($n=739$) were enrolled in the experiment between January and July of 2014. Primiparous and multiparous cows were housed separately in free-stall barns identical in design, size, location and orientation and equipped with sprinklers and fans. Cows were offered water and a totally mixed ration in ad libitum amounts to meet or exceed the requirements of lactating Holstein cows weighing 650 kg and producing 45 kg of milk per day with 3.5% fat and 3.2% true protein when consuming 26 kg of dry matter per day (National Research Council 2001). Cows were fed and milked twice daily.

Dietary treatments

Weekly cohorts of cows were blocked by parity and matched for milk yield recorded during the first 2 weeks of lactation and allocated randomly to receive the control diet ($n=373$: 115 primiparous cows and 258 multiparous cows) or the same diet supplemented with an algae product containing 10% docosahexaenoic acid or DHA (Algae, $n=366$: 105 primiparous cows and 261 multiparous cows). Cows were housed in 6 experimental pens per treatment and the experiment started at 27 ± 5 (mean $\pm$ s.d.) days postpartum and lasted 120 days. At enrollment, body condition was evaluated using a 1–5 scale (0=emaciated and 5=obese) according to the Elanco body condition score (BCS) chart (Elanco 2009). For purposes of statistical analyses, cows were categorized as having low (≤ 2.75) or moderate (≥ 3.00) BCS. All cows were identified with colored ear clips according to the assigned diet to ensure accurate pen location.

Diets were the same for both experimental groups, with the exception that cows fed the Algae received individually 100 g of a supplement (All-G-Rich, Alltech Inc., Nicholasville,

KY, USA) to supply approximately 10 g of DHA per cow per day. Upon return from the morning milking, cows were locked with self-lock up stanchions and treated cows were supplemented individually to assure intake of the daily dose of the algae

Table 1 Chemical composition of the diet and algae supplement fed to cows (mean $\pm$ S.D.).

Item	Diet ^a	Algae supplement ^b
Dry matter (%)	53.1 $\pm$ 1.7	97.4 $\pm$ 0.1
	Dry matter basis	
Net energy of lactation ^c (mcals/kg)	1.59	–
Organic matter (%)	91.8 $\pm$ 0.1	96.2 $\pm$ 0.1
Crude protein (%)	17.1 $\pm$ 0.3	15.6 $\pm$ 0.2
Starch (%)	23.0 $\pm$ 1.2	–
Nonfibrous carbohydrates ^d (%)	42.0 $\pm$ 0.7	15.3 $\pm$ 2.1
Neutral detergent fiber (%)	30.9 $\pm$ 0.9	2.0 $\pm$ 1.8
Acid hydrolysis ether extract (%)	4.8 $\pm$ 0.4	63.2 $\pm$ 2.7
Fatty acids (%)	4.1 $\pm$ 0.3	33.2 $\pm$ 3.0
Ca (%)	0.99 $\pm$ 0.07	–
P (%)	0.42 $\pm$ 0.02	–
Mg (%)	0.33 $\pm$ 0.01	–
K (%)	1.40 $\pm$ 0.04	–
S (%)	0.28 $\pm$ 0.02	–
Na (%)	0.45 $\pm$ 0.02	–
Cl (%)	0.62 $\pm$ 0.04	–
Dietary cation–anion difference ^e (mEq/kg)	205 $\pm$ 20	–
Fatty acids, g/100 g of total FA		
<C16	0.63	5.74 $\pm$ 0.01
C16:0	22.34	52.58 $\pm$ 0.36
C18:0	2.55	1.41 $\pm$ 0.01
C18:1	27.86	0.13 $\pm$ 0.01
C18:2 <i>cis</i> -9, <i>cis</i> -12	40.66	Not detected
C18:3 <i>cis</i> -9, <i>cis</i> -12, <i>cis</i> -15	3.41	0.03 $\pm$ 0.01
C20:4 <i>cis</i> -5, <i>cis</i> -8, <i>cis</i> -11, <i>cis</i> -14	Not detected	0.08 $\pm$ 0.01
C20:5 <i>cis</i> -5, <i>cis</i> -8, <i>cis</i> -11, <i>cis</i> -14, <i>cis</i> -17	Not detected	0.41 $\pm$ 0.01
C22:5 <i>cis</i> -4, <i>cis</i> -7, <i>cis</i> -10, <i>cis</i> -13, <i>cis</i> -16	Not detected	6.31 $\pm$ 0.06
C22:6 <i>cis</i> -4, <i>cis</i> -7, <i>cis</i> -10, <i>cis</i> -13, <i>cis</i> -16, <i>cis</i> -19	Not detected	29.98 $\pm$ 0.28
n-6	40.66	6.56 $\pm$ 0.07
n-3	3.41	30.50 $\pm$ 0.29

^aDiet contained (% dry matter): 27.9% corn silage, 14.8% alfalfa hay, 0.3% wheat straw, 19.8% stem-flaked corn, 1.9% citrus pulp, 2.1% delactose whey, 5.9% whole cottonseed, 5.1% dried distiller's grains, 3.3% soybean hulls, 1.6% almond hulls, 12.1% canola meal, 0.7% molasses, 2.3% of animal protein blend, and 2.2% mineral and vitamin premix. Each kg of the mineral/vitamin premix contained 1800 mg of Zn, 450 mg of Cu, 1360 mg of Mn, 14 mg of Se, 23 mg of I, 14 mg of Co, 180,000 IU of vitamin A, 55,000 IU of vitamin D, and 1800 IU of vitamin E. ^bAll-G-Rich, Alltech. ^cCalculated according to National Research Council (2001) and adjusted for 26 kg of dry matter intake. ^dCalculated as: organic matter – (crude protein + (neutral detergent fiber – neutral detergent insoluble protein) + fat). ^eCalculated as: (mEq/kg K + mEq/kg Na) – (mEq/kg Cl + mEq/kg S).

supplement. Cows remained in the experiment for 120 days or until they were sold or died, whichever occurred first.

Samples of the diet and the supplement were collected weekly throughout the experiment and stored at -20°C . Samples were subsequently dried in air-forced oven at 55°C for 48 h and moisture loss was recorded. Dried samples were ground to pass through a 1.0-mm screen (Wiley Mill, Philadelphia, PA, USA) and later dried at 105°C for the determination of dry matter content. Monthly composite of diet samples were analyzed for organic matter (512°C for 8 h), neutral detergent fiber using a heat stable α -amylase and acid detergent fiber, N using an automated quantitative combustion digestion method, starch using an enzymatic digestion method and subsequent quantification of glucose and minerals using inductively coupled plasma mass spectrometry at a commercial laboratory (Dairyland Laboratories Inc., Arcadia, WI, USA). The crude fat content was analyzed by acid hydrolysis ether extraction at a commercial laboratory (Eurofins Scientific Inc., Des Moines, IA, USA). The algae biomass was produced by heterotrophic fermentation of *Schizochytrium limacinum* CCAP 4087/2 in closed controlled stainless steel fermenters at the Alltech Algae facility in Winchester, KY, USA. After fermentation, the material was centrifuged to separate the algae from water, and the concentrate algae cells were spray dried and packaged. Table 1 depicts the chemical composition of the diet and the supplemental algae product. The amounts of feed offered and refused per cow were weighed and recorded thrice a week to calculate dry matter intakes.

Milk yield and composition

Cows were milked twice-daily starting at 05:00 and 15:00 h, and milk weights were recorded electronically at each milking. Milk samples were collected from each cow every 3 weeks and submitted for analysis of fat, true protein, lactose, solids-not-fat and somatic cells at the local dairy herd improvement laboratory (Hanford, CA).

Fatty acid analyses of milk fat, phospholipid fraction of plasma and feeds

A subset of 25 cows from each dietary treatment underwent milk and blood collection on day 78 ± 3 of the experiment for the determination of FA composition of milk fat and plasma phospholipids.

Milk samples were collected during the first milking of the day and stored at -20°C without preservative. Samples were thawed and centrifuged at 17,800 g for 30 min at 8°C to collect the fat cake. Milk lipids were extracted, and FA methyl esters were prepared and quantified according to the methods described previously (Lock et al. 2013) and analyzed using gas liquid chromatography. Yield of individual FA (g/day) in milk fat was calculated by using milk fat yield and FA concentration to determine yield on a mass basis using the molecular weight of each FA while correcting for glycerol content and other milk lipid classes (Piantoni et al. 2013).

Blood was sampled by puncture of coccygeal vessels into evacuated tubes containing K_2EDTA (Vacutainer, Becton Dickinson, Franklin Lakes, NJ, USA) and placed immediately

in ice. Plasma was separated by centrifugation at 2000g for 15 min and stored at -20°C until assayed. Total lipids were extracted from plasma with chloroform and methanol using a modified method of Folch and coworkers (Folch *et al.* 1957), dried under nitrogen gas and reconstituted in 0.5 mL chloroform. The phospholipid fraction of plasma lipids was separated from other lipid fractions by solid-phase extraction using a modified method of Agren and coworkers (Agren *et al.* 1992) as described by Boerman and Lock (2014). Fatty acid methyl esters from the phospholipid fraction were prepared with a modified 2-step transmethylation procedure as described by Boerman and Lock (2014). Fatty acid methyl esters were quantified by gas liquid chromatography as described for milk fat (Lock *et al.* 2013). The FA content in phospholipids was calculated as g/100g of FA or based on the total plasma phospholipids as μg of FA/mL of plasma.

Two composite samples of the diet and two of the algae products had FA extracted and FA methyl esters prepared with a modified 2-step transmethylation procedure, followed by quantification by gas liquid chromatography as described previously.

Reproductive management

All cows had their ovaries evaluated by ultrasonography using a portable ultrasound equipped with a 7.5-MHz transrectal linear transducer (Easi-Scan, BCF Technology, Rochester, MN, USA) on days 43 ± 3 and 57 ± 3 postpartum to determine the presence or absence of a corpus luteum (CL). Cows without a detected CL in both examinations were considered anovular, whereas those with a CL in at least one of the two examinations were considered to have resumed estrous cyclicity. Cows were subjected to the Presynch-Ovsynch 56-h protocol for the first postpartum AI. Cows received an i.m. injection of $\text{PGF}_{2\alpha}$ (Lutalyse sterile solution, 25 mg of dinoprost as tromethamine salt, Zoetis, Florham Park, NJ, USA) administered on days 44 ± 3 and 58 ± 3 postpartum. Cows detected in estrus after 58 days postpartum were inseminated. Cows not observed in estrus were enrolled in the Ovsynch protocol on day 70 ± 3 postpartum. The protocol consisted of an i.m. injection of GnRH (Cystorelin, 100 μg of gonadorelin diacetate tetrahydrate, Merial Ltd., Duluth, GA, USA) followed 7 days later by an injection of $\text{PGF}_{2\alpha}$ and a final GnRH injection 56 h after the $\text{PGF}_{2\alpha}$. Cows were inseminated 16 h after the final GnRH injection, at 80 ± 3 days postpartum. Throughout the experiment, after 58 days postpartum, cows had their tailheads painted using paint sticks (All-Weather Paintstik, LA-CO Industries Inc., Chicago, IL, USA), and detection of estrus was evaluated each morning, based on the removal of the tail chalk. Cows identified in estrus were inseminated on the same morning. On day 32 ± 3 after an insemination, cows diagnosed as nonpregnant that had not been re-inseminated were resynchronized with the Ovsynch protocol.

Pregnancy diagnosis and calculation of reproductive responses

Pregnancy was diagnosed by transrectal ultrasonography on day 32 ± 3 after AI. The presence of an amniotic vesicle containing

an embryo with heartbeat was used as the determinant of pregnancy. Pregnant cows on day 32 were re-examined for pregnancy by transrectal ultrasonography 4 weeks later, on day 60 ± 3 after AI. Pregnancy per AI was calculated by dividing the number of cows diagnosed pregnant at 32 ± 3 or 60 ± 3 days after AI by the number of cows receiving AI. Pregnancy loss was calculated as the number of cows that lost pregnancy between days 32 ± 3 and 60 ± 3 after AI divided by the number of cows diagnosed pregnant on day 32 ± 3 after AI. Cows that were detected in estrus before pregnancy diagnosis were re-inseminated and considered as nonpregnant. For the time to pregnancy, the interval from calving to pregnancy or censoring was used. Cows that remained nonpregnant by 120 days in the experiment and those nonpregnant that were sold or died before 120 days in the experiment were censored. For the analysis of time to pregnancy, pregnancy was based on the diagnosis at 60 ± 3 days after insemination.

Concentrations of progesterone in plasma and ovarian ultrasound

Seventy-eight cows, 39 control (14 primiparous cows and 25 multiparous cows) and 39 Algae cows (8 primiparous cows and 31 multiparous cows) that underwent the Presynch-Ovsynch protocol had blood sampled during the synchronization protocol for the assessment of concentrations of progesterone in plasma. All cows had a synchronized estrous cycle based on ovulation to the initial GnRH, regression of the CL after the $\text{PGF}_{2\alpha}$ and ovulation within 48 h after the final GnRH of the Ovsynch protocol. Ovulation was based on ultrasonographic evaluation of the ovaries, and CL regression based on a decline in progesterone concentration in plasma to less than 1.0 ng/mL 72 h after $\text{PGF}_{2\alpha}$ treatment, when AI was performed. Ovaries were scanned by ultrasound (Aloka SSD-500 equipped with a 7.5 MHz linear transducer, Aloka Co., Tokyo, Japan) during the Ovsynch protocol concurrent with the injections of the first GnRH, $\text{PGF}_{2\alpha}$, second GnRH and 48 h after AI. Follicle and CL location and diameter were recorded. Ovulation in response to the first GnRH of the Ovsynch was based on the presence of a follicle ≥ 10 mm in diameter and appearance of a new CL in the same ovary on the day of the $\text{PGF}_{2\alpha}$ treatment.

Additional blood was sampled from 149 cows, 77 control (22 primiparous cows and 55 multiparous cows) and 72 Algae (22 primiparous cows and 50 multiparous cows) on days 2, 4, 6, 8, 10, 12, 14, 16, 17, 18 and 19 after AI. Only cows that had a synchronized ovulation based on low concentration of progesterone (<1.0 ng/mL) on day 2 after AI and high concentrations of progesterone (>1.0 ng/mL) on days 6 and 8 after AI remained for statistical analyses. Of the initial sampled cows, 9 control (4 primiparous cows and 5 multiparous cows) and 12 Algae (6 primiparous cows and 6 multiparous cows) were removed because of asynchronous estrous cycle. The remaining sampled cows (68 control and 60 Algae) were evaluated for pregnancy on day 32 after AI and characterized as nonpregnant ($n=64$, 40 control and 24 Algae) and pregnant cows ($n=64$, 28 control and 36 Algae).

Blood was handled as described previously. Progesterone concentrations in plasma were evaluated by radioimmunoassay using a commercial kit (Coat-a-Count, Siemens Healthcare

Diagnostics). Plasma harvested from cows on days 4 (~1.5 ng/mL) and 10 (~5.5 ng/mL) of the estrous cycle were incorporated into each assay and used to calculate the CV. The intra- and inter-assay CVs were 8.9 and 11.8% respectively.

Concentrations of insulin and IGF-1 in plasma before and after treatment with bovine somatotropin

Forty-eight cows, 24 control (7 primiparous cows and 17 multiparous cows) and 24 Algae cows (7 primiparous cows and 17 multiparous cows), had blood sampled from coccygeal vessels to evaluate the concentrations of insulin and IGF-1 in plasma. Within each treatment, half of the cows received treatment with 500 mg of bovine somatotropin (bST, Posilac, sometribove zinc suspension for injection, Elanco Animal Health, Greenfield, IN, USA) and half received an injection of saline. Samples were collected in all cows starting at 62 ± 3 days postpartum and, in cows receiving bST, treatment was initiated 6 days later, at 68 ± 3 days postpartum and repeated 11 days later. Blood was sampled in all 48 cows on days -6, -2, 2, 6, 10, 14, 18 and 22 relative to bST treatment.

Blood was handled as described previously. Concentrations of total IGF-1 were determined by a commercial ELISA kit (Quantikine ELISA Human IGF1 Immunoassay, R&D Systems) as described previously (Ribeiro *et al.* 2014). Plasma concentrations of insulin were determined by a commercial bovine ELISA kit (Mercodia Bovine Insulin ELISA, Mercodia AB, Uppsala, Sweden). The intra- and inter-assay CVs were 6.5 and 10.5% for IGF-1 and 2.3% and 4.0% for insulin.

Peripheral blood leukocyte isolation and mRNA extraction

Blood was sampled from coccygeal vessels on day 19 after AI from 116 pregnant cows, 49 control and 67 Algae, for isolation of leukocytes as described by Ribeiro and coworkers (Ribeiro *et al.* 2014). Samples of blood also were collected from 9 nonpregnant cows (5 control and 4 Algae) that served as reference in the analyses. The pellets of isolated leukocytes were suspended with 0.8 mL of TRIzol (Molecular Research Center Inc., Cincinnati, OH, USA), transferred to microcentrifuge tubes and stored at -80°C until RNA extraction. On the day of RNA extraction, samples were removed from the freezer and 200 μL of chloroform were added for each 1 mL of solution. The microcentrifuge tubes were homogenized vigorously by hand for 15 s and incubated at room temperature for 3 min. Tubes were centrifuged at 12,000 g for 15 min at 4°C for the removal of the upper aqueous solution containing RNA. Subsequent RNA extraction was performed using a commercial kit (Purelink RNA Mini Kit, Cat. No. 12183018A; Life Technologies) according to the manufacturer's instructions.

Real-time qPCR

Isolated RNA was evaluated for concentration using a NanoDrop 2000 spectrophotometer (Thermo Scientific), and integrity was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies) before assays. A total of 250 ng of RNA were reverse transcribed to cDNA using a commercial kit

Table 2 Gene, primer orientation, primer sequence (5'–3') and National Center for Biotechnology Information (NCBI) accession number and sequence for primers used in RT-qPCR assays.

Gene	Primer	Sequence (5'–3')	NCBI sequence
ACTB	Forward	CTGGACTTCGAGCAGGAGAT	AY141970
	Reverse	GATGTCGACGTCACACTTC	
ISG15	Forward	GGTATCCGAGCTGAAGCAGTT	NM_174366
	Reverse	ACCTCCCTGCTGTCAAGGT	
RPL19	Forward	ATTGACCGCCACATGTATCA	NM_001040516
	Reverse	GCGTGCTTCCTTGGTCTTAG	
RTP4	Forward	TTCTCCCCAGAAAGCAGCAA	BC105539
	Reverse	TTCACAGTTGGCCTTGTCATG	

(High-capacity cDNA Reverse Transcription Kit, Cat. No. 4368813, Applied Biosystems) following manufacturer's instructions. Real-time qPCR was performed using SYBR Green PCR Master Mix (Cat. No. 4385614, Applied Biosystems) and the ABI 7300 Real-Time PCR System (Applied Biosystems). After an initial activation at 60°C for 2 min followed by denaturation at 95°C for 10 min, the amplification protocol followed 40 cycles of 95°C for 15 s and 60°C for 1 min. Each sample was evaluated in duplicate, and the specificity for amplification was verified by melting curve analysis. Primer efficiency averaged 102%. Four genes were investigated (Table 2) including two reference genes β -actin (*ACTB*) and ribosomal protein L19 (*RPL19*), and two target genes, ubiquitin-like IFN-stimulated gene 15-kDa protein (*ISG15*) and chemosensory receptor transporter protein 4 (*RTP4*).

Statistical analyses

The experiment followed a randomized block design. Weekly cohort of cows were blocked by parity and randomly assigned to control or Algae. Continuous variables were analyzed by ANOVA using the MIXED procedure of SAS, version 9.4 (SAS Institute Inc., Carry, NC, USA). Every response for continuous variables was evaluated for distribution of residuals and homogeneity of variance after model fitting. Data with violation of the assumptions of normality after model fitting were subjected to Box-Cox power transformation using the TRANSREG procedure of SAS before the final statistical analysis. The least-squares means and respective standard errors of transformed data were back transformed for presentation according to Jørgensen and Pedersen (1998). The statistical models included the fixed effects of treatment (control vs Algae), parity (primiparous cows vs multiparous cows) and the interaction between treatment and parity. Individual milk yield average during the first 14 days postpartum was used as covariate for analyses of production performance. For repeated measurements within experimental unit, the effect of time and the interaction between treatment and time were included as fixed effects, and cow nested within treatment was the random effect for testing the effect of treatment. For the subset of cows included in the analyses of responses to bST, the models also included the fixed effects of bST (yes or no) and the interactions between treatment and bST, bST and day and treatment and bST and day. For analysis of concentrations of progesterone, the fixed effects of pregnancy on day 32, treatment and the interactions between treatment and pregnancy, pregnancy and

day and treatment and pregnancy and day were also considered in the model. The Kenward–Roger method was used to obtain the approximate degrees of freedom. The covariance structure that resulted in the best fitted model based on the smallest Akaike's criterion was selected for the analysis of data with repeated measurements.

Binary data were analyzed by multivariable logistic regression using the GLIMMIX procedure of SAS. The models included the fixed effects of treatment, parity, interaction between treatment and parity and the BCS at experimental enrollment. Time to pregnancy was analyzed by the Cox's proportional hazard model with the PHREG procedure of SAS. The time variable considered was the number of days between calving and the day of AI that resulted in pregnancy or the day in which cows were censored from the analyses (i.e. cows sold, dead or remained nonpregnant at the end of the experiment). Models included the effects of treatment, parity, interaction between treatment and parity and BCS of cows at enrollment. The adjusted hazard ratio (AHR) and 95% confidence interval (CI) were computed. The median and mean days to pregnancy were obtained from the LIFETEST procedure of SAS.

Quantitative PCR data are presented using the comparative method developed by Livak and Schmittgen (2001) using nonpregnant cows as reference for relative expression of mRNA abundance, which was set to the relative value of 1. The delta cycle threshold (CT) values for each target gene were obtained after normalization of CT value of the gene with the geometric mean of CT values from the 2 reference genes. Data were analyzed using the delta CT with the MIXED procedure of SAS fitting a model with the fixed effects of treatment, parity and the interaction between treatment and parity. The delta–delta CT was obtained from delta CT least-squares means differences of pairwise comparisons among treatments and the reference nonpregnant cows (Yuan *et al.* 2006). Pairwise comparisons were adjusted by the Tukey's honest significant difference. The relative expression values were obtained by raising the PCR amplification efficiency ($E=2$) to the power delta–delta CT (Yuan *et al.* 2006). Confidence limits for

graphical representation of relative expression were generated from the lower and upper 95% CI obtained for delta CT least-squares means differences as described by Yuan and coworkers (Yuan *et al.* 2006).

Treatment differences with $P \leq 0.05$ were considered significant and those with $0.05 < P \leq 0.10$ were considered as tendencies.

Results

Reproductive responses

An interaction ($P=0.04$) between treatment and parity was detected for resumption of estrous cycles by 58 days postpartum because feeding Algae increased the proportion of primiparous cows that resumed estrous cyclicity, whereas no difference was detected for multiparous cows (Table 3). Regardless of dietary treatment, estrous cyclicity at 58 days postpartum increased ($P<0.01$) in cows that had moderate BCS at experiment enrollment compared with those with low BCS (77.9 vs 68.8%). A greater proportion of cows fed Algae tended ($P=0.09$) to be detected in estrus after treatment with PGF_{2α} than control cows (49.3 vs 42.2%). Also, detection of estrus increased ($P=0.02$) in primiparous cows compared with multiparous cows (50.9 vs 39.9%), but BCS at experiment enrollment or the interaction between treatment and BCS did not affect the detection of estrus. The day postpartum at first AI did not differ with treatment, parity or BCS (Table 3).

Pregnancy at first AI on day 32 was affected ($P=0.04$) by the interaction between treatment and parity. Feeding Algae improved P/AI at first AI in primiparous cows, but had no effect on multiparous cows when the diagnosis was performed 32 days after the first AI (Table 3). Likewise, this interaction tended ($P=0.07$) to be significant at 60 days after the first AI. The BCS at

Table 3 Effects of feeding an algae product rich in docosahexaenoic acid on reproductive performance of Holstein cows.

Item ^c	Treatment ^a				S.E.M.	P value ^b		
	Control		Algae			TRT	Parity	TRT × parity
	Primiparous	Multiparous	Primiparous	Multiparous				
Cows (n)	115	258	105	261	—	—	—	—
Estrous cyclic (%)	65.9	76.6	77.6	73.4	—	0.27	0.44	0.04
Estrus (%)	44.7	39.7	56.9	41.7	—	0.09	0.02	0.23
Day at first AI	71.8	71.5	69.3	71.6	1.2	0.15	0.35	0.12
P/AI at first AI (%)								
Day 32	33.7	38.7	49.3	36.6	—	0.11	0.39	0.04
Day 60	32.8	35.3	47.6	34.7	—	0.09	0.25	0.07
PL at first AI (%)	2.7	8.2	4.2	5.0	—	0.97	0.34	0.47
P/AI at all AI (%)								
Day 32	27.3	37.9	46.9	40.3	—	<0.01	0.48	<0.01
Day 60	26.8	35.0	45.1	38.1	—	<0.01	0.73	0.01
PL at all AI (%)	1.9	7.2	3.8	5.2	—	0.77	0.18	0.40

^aCows in control and Algae were fed the same diet but Algae was supplemented with 100 g/cow per day of an algae product rich in docosahexaenoic acid. ^bTRT=effect of treatment (control vs Algae); Parity=effect of parity (primiparous vs multiparous); TRT × parity=interaction between TRT and parity. ^cEstrous cyclic at 58 days postpartum; estrus=detection of estrus between 58 and 70 days postpartum; P/AI=pregnancy per AI; PL=pregnancy loss.

Figure 1 Survival curves for interval from calving to pregnancy in cows fed control (dotted line) or Algae (solid line). Control and Algae cows were fed the same diet, but Algae was supplemented with 100g/cow per day of an algae product rich in docosahexaenoic acid. Cows fed Algae became pregnant faster ($P < 0.01$) than those fed control (adjusted hazard ratio = 1.39; 95% CI = 1.14–1.70). The median and mean ($\pm$ S.E.M.) days to pregnancy were respectively 124 (95% confidence interval (CI) = 108–141) and 117.9 ± 2.2 for control and 102 (95% CI = 87–112) and 106.9 ± 1.9 for Algae.

experiment enrollment tended ($P = 0.07$) to influence P/AI because cows with moderate BCS had better P/AI than those with low BCS (43.2 vs 35.9%), and this response was detected regardless of the treatment. Pregnancy loss between gestation days 32 and 60 was not affected by treatment or by the interaction between treatment and parity and averaged 4.7%.

Pregnancy per AI for all inseminations increased ($P < 0.01$) in Algae compared with control cows on days 32 (43.6 vs 32.3%) and 60 (41.6 vs 30.7%) after AI (Table 3). The benefit of feeding Algae was greater in primiparous cows than that in multiparous cows based on the interaction ($P < 0.01$) between treatment and parity. Cows with BCS at experiment enrollment classified as moderate tended ($P = 0.09$) to have greater P/AI at all inseminations than those with low BCS (40.6 vs 34.4%), and this response was irrespective of dietary treatment. No difference in pregnancy loss was detected due to treatment, parity, BCS and interactions between treatment and parity or treatment and BCS.

Feeding Algae increased ($P < 0.01$) the rate of pregnancy compared with control by 39% (Fig. 1, AHR = 1.39, 95% CI = 1.14–1.70), which reduced the median days to pregnancy by approximately 22 days. The increase in pregnancy rate in cows fed Algae was detected in both parity groups, but the benefit was greater in primiparous cows than that in multiparous cows based on the interaction ($P = 0.02$) between treatment and parity (Table 4). Within primiparous cows, Algae almost doubled ($P < 0.01$) the rate of pregnancy (AHR = 1.99, 95% CI = 1.38–2.86), whereas in multiparous cows, feeding Algae tended ($P = 0.10$) to increase the rate of pregnancy by 22% (AHR = 1.22, 95% CI = 0.96–1.55).

Diameter of the pre-ovulatory follicle and concentrations of hormones in plasma

The diameter of the ovulatory follicle on day 9 of the Ovsynch protocol did not differ ($P = 0.83$) between treatments and averaged 15.4 ± 0.60 mm. Multiparous cows had a greater ($P = 0.05$) mean pre-ovulatory follicle diameter than those in primiparous cows (16.2 vs 14.6 ± 0.70 mm), regardless of treatment. The presence of a CL in the beginning of the Ovsynch protocol did not affect ($P = 0.14$) the diameter of the pre-ovulatory follicle.

Concentrations of progesterone in plasma on day 7 of the Ovsynch protocol did not differ ($P = 0.13$) between treatments (control = 6.36 and Algae = 5.24 ± 0.55 ng/mL). Cows that had a CL at the beginning of the Ovsynch protocol had greater ($P = 0.02$) concentrations of progesterone on day 7 of the protocol compared with those that did not have a CL (CL = 6.59 vs no CL = 5.00 ± 0.53 ng/mL), and this effect was not influenced by treatment. Parity, treatment or interaction between treatment and parity did not influence plasma concentrations of progesterone during the Ovsynch protocol. Mean concentrations of progesterone in plasma during the first 19 days after AI did not differ between treatments and averaged 5.02 ± 0.18 and 4.83 ± 0.19 for control and Algae respectively (Fig. 2). Cows that were pregnant on day 32 after AI had greater ($P < 0.001$) concentrations of progesterone than nonpregnant cows, but differences were only detected

Table 4 Cox's proportional hazard model for time to pregnancy in Holstein according to treatment and parity.

Treatment ¹	Pregnant (%)	Days to pregnancy		Adjusted HR ³ (95% CI)	P-value
		Median (95% CI) ²	Mean $\pm$ S.E.M.		
Control primiparous (n = 115)	42.6	142 ^{c,4}	113.1 $\pm$ 3.2	Reference	–
Control multiparous (n = 258)	51.6	121 ^{bc} (104–138)	116.5 $\pm$ 2.7	1.24 (0.89–1.73)	0.20
Algae primiparous (n = 105)	66.7	83 ^a (80–90)	94.0 $\pm$ 2.9	1.99 (1.38–2.86)	<0.01
Algae multiparous (n = 261)	57.5	111 ^b (96–121)	110.2 $\pm$ 2.2	1.48 (1.06–2.06)	0.02

^{a,b,c}Superscripts within the same column differ ($P < 0.05$). ¹Cows in control and Algae were fed the same diet but Algae was supplemented with 100g/cow per day of an algae product rich in docosahexaenoic acid. Effects of treatment ($P < 0.01$), parity ($P = 0.20$) and interaction between treatment and parity ($P = 0.02$). Within primiparous, effect of treatment ($P < 0.01$; adjusted HR = 1.99, 95% CI = 1.38–2.86). Within multiparous, effect of treatment ($P = 0.10$; adjusted HR = 1.22, 95% CI = 0.96–1.55). ²CI, confidence interval. ³HR, hazard ratio. ⁴Because the median days to pregnancy were close to the end of the experiment (147 ± 5 days postpartum), only the lower limit for the 95% CI was available for control primiparous (119 days). The upper limit was not calculated.

Figure 2 Progesterone concentrations in plasma after AI. Cows were fed the same diet as control (square symbol dashed line, $n=68$, 40 nonpregnant and 28 pregnant) or Algae (circle symbol solid line, $n=60$, 24 nonpregnant and 36 pregnant), but Algae cows were supplemented with 100 g/cow per day of an algae product rich in docosahexaenoic acid. Effects of treatment ($P=0.47$), day ($P<0.001$), pregnancy ($P<0.001$) and interactions between treatment and day ($P=0.19$), treatment and pregnancy ($P=0.81$), pregnancy and day ($P<0.001$) and treatment and pregnancy and day ($P=0.79$).

after day 14 after insemination. Parity did not affect the concentrations of progesterone. No interactions were detected between treatment and day, treatment and pregnancy and treatment and parity.

An interaction between dietary treatment and bST was detected for plasma concentrations of insulin (Fig. 3A and B). Control cows not treated with bST had greater ($P=0.04$) mean concentrations of insulin compared with Algae cows not treated with bST

(control = 0.79 ± 0.08 vs Algae = 0.57 ± 0.06), whereas dietary treatment did not affect insulin concentrations in plasma of cows injected with bST (control = 0.51 ± 0.05 vs Algae = 0.57 ± 0.06). Concentrations of IGF-1 increased (effect of week, $P<0.001$) after bST treatment and a tendency for interaction ($P=0.08$) between treatment and bST on concentrations of IGF-1 (Fig. 3C and D) was detected. Concentrations of IGF-1 did not differ between dietary treatments in cows not receiving bST and averaged 97.7 ng/mL (Fig. 3C). However, when cows were treated with bST, those receiving Algae tended ($P=0.08$) to have greater concentrations of IGF-1 than control cows (control-bST = 102.9 ± 6.7 ng/mL vs Algae-bST = 118.7 ± 7.7 ng/mL, Fig. 3D).

Expression of interferon-stimulated genes in leukocytes on day 19 after AI

As anticipated, the normalized expression of interferon-stimulated genes in peripheral blood leukocytes on day 19 after AI increased ($P<0.0001$) with pregnancy (Fig. 4). The mRNA abundance for *ISG15* did not differ between treatments (Fig. 4A and C), but it was greater ($P=0.03$) in pregnant primiparous cows than in pregnant multiparous cows (Fig. 4C). Treatment affected ($P=0.02$) the expression of *RTP4*, which was greater in leukocytes of pregnant cows fed Algae than control (Fig. 4B). Expression of *RTP4* was greater ($P \leq 0.01$) in primiparous compared with multiparous cows (Fig. 4D). The increase in mRNA normalized the expression for *RTP4* when feeding Algae was observed in both primiparous and multiparous cows (Fig. 4D).

Figure 3 Concentrations of insulin (panels A and B) and insulin-like growth factor (IGF) 1 (panels C and D) in plasma. Cows were fed the same diet as control (square dashed line, $n=24$) or Algae (circle solid line, $n=24$), but Algae cows were supplemented with 100 g/cow per day of an algae product rich in docosahexaenoic acid. Within each treatment, half of the cows ($n=12$ /treatment) received bST on days 0 and 11. Panels A and C represent results from cows injected with saline, whereas panels B and D represent results of cows injected with bST. Insulin, effects of treatment ($P=0.33$), day ($P=0.19$), bST ($P=0.03$) and interactions between treatment and day ($P=0.36$), treatment and bST ($P=0.04$) and treatment and bST and day ($P=0.22$). Insulin-like growth factor-1, effects of treatment ($P=0.69$), day ($P<0.001$), bST ($P=0.06$) and interactions between treatment and day ($P=0.29$), treatment and bST ($P=0.08$) and treatment and bST and day ($P=0.23$).

Figure 4 Relative abundance of mRNA for interferon-stimulated genes (ISG) in leukocytes isolated from pregnant cows on day 19 after AI. Cows were fed the same diet as control (gray bars) or Algae (white bars), but Algae-fed cows were supplemented with 100g/cow per day of an algae product rich in docosahexaenoic acid. Nonpregnant cows were the reference group (black bars) for depicting relative mRNA expression. Panel A, *ISG15* expression on day 19 after AI according to treatment. Panel B, receptor transporter protein-4 (*RTP4*) expression on day 19 after AI according to treatment. Panel C, *ISG15* expression on day 19 after AI according to treatment and parity. Panel D, *RTP4* expression on day 19 after AI according to treatment and parity. For *ISG15*, effects of pregnancy ($P < 0.0001$), treatment ($P = 0.30$), parity ($P = 0.03$) and interaction between treatment and parity ($P = 0.92$). For *RTP4*, effects of pregnancy ($P < 0.0001$), treatment ($P = 0.02$), parity ($P < 0.001$) and interaction between treatment and parity ($P = 0.50$). The error bars represent the confidence interval (CI) of normalized expression generated from the lower and upper 95% CI obtained for delta CT least-squares means differences.

Lactation performance

Dry matter intake did not differ ($P = 0.51$) between control and Algae treatments averaging 25.5 and 24.8 ± 0.65 kg/day respectively. Considering the

Figure 5 Effect of dietary treatment on milk yield (kg/day) during the experimental period. Cows in control (dotted line, open square) and Algae (solid line, dark circle) were fed the same diet but Algae-fed cows were supplemented with 100g/cow per day of an algae product rich in docosahexaenoic acid. Weekly milk yield (kg/day) averages were calculated using data from daily individual measurements. Effects of treatment ($P = 0.01$), parity ($P < 0.01$), week ($P < 0.01$) and interactions between treatment and week ($P < 0.01$) and treatment and parity ($P = 0.35$). Within a week, differences are represented as follows: * $P \leq 0.05$; † $0.05 < P \leq 0.08$.

additional 33 g of supplemental FA provided by 100 g of Algae, the intake of FA by control and Algae cows did not differ ($P = 0.90$) and averaged 1.046 ± 0.027 and 1.051 ± 0.027 kg/day respectively. Multiparous cows had greater ($P = 0.05$) dry matter intake than primiparous cows (26.3 vs 23.0 ± 0.63 kg/day), regardless of treatment. Feeding Algae increased ($P \leq 0.01$) milk yield by 0.9 kg/day (Fig. 5) as well as yields of true protein by 20 g/day, lactose by 50 g/day and solids-not-fat by 100 g/day compared with control (Table 5). In contrast, concentration (3.08 vs $3.23 \pm 0.03\%$) and yield of milk fat (1.30 vs 1.34 ± 0.01 kg/day) decreased ($P < 0.02$) in Algae compared with control cows. No treatment differences were observed for concentrations of true protein, lactose and solids-not-fat in milk. Furthermore, no interaction between treatment and parity were detected for the production variables evaluated. Somatic cell score did not differ with treatments and averaged 1.49 ± 0.10 . The efficiency of feed conversion into milk yield (1.80 vs 1.70 ± 0.04), 3.5% fat-corrected milk (1.66 vs 1.61 ± 0.05) and energy-corrected milk (1.65 vs 1.59 ± 0.05) did not differ ($P > 0.10$) between the Algae and the control group.

Fatty acid composition of plasma phospholipids and milk fat

The FA composition in plasma phospholipids was affected markedly by dietary treatment (Table 6).

Table 5 Effects of feeding an algae product rich in docosahexaenoic acid on lactation performance in Holstein cows.

Item	Treatment ^a				S.E.M.	P-value ^b		
	Control		Algae			TRT	Parity	TRT × parity
	Primiparous	Multiparous	Primiparous	Multiparous				
Milk yield (kg/day)	36.1	47.2	37.3	47.7	0.42	0.01	<0.01	0.35
3.5% FCM ^c (kg/day)	35.1	44.4	35.5	43.6	0.42	0.54	<0.01	0.11
ECM ^d (kg/day)	34.6	43.7	35.1	43.2	0.39	0.97	<0.01	0.13
Fat								
%	3.32	3.13	3.17	2.98	0.05	<0.01	<0.01	0.85
Yield (kg/day)	1.20	1.47	1.19	1.41	0.02	0.03	<0.01	0.15
True protein								
%	2.91	2.83	2.93	2.84	0.02	0.76	<0.01	0.78
Yield (kg/day)	1.06	1.33	1.09	1.35	0.01	<0.01	<0.01	0.39
Lactose								
%	4.95	4.75	4.94	4.76	0.01	0.73	<0.01	0.84
Yield (kg/day)	1.79	2.24	1.86	2.28	0.02	0.01	<0.01	0.36
Solids not fat								
%	8.75	8.43	8.76	8.47	0.03	0.78	<0.01	0.47
Yield (kg/day)	3.17	3.97	3.29	4.06	0.03	<0.01	<0.01	0.46
SCS ^e	1.38	1.60	1.35	1.63	0.17	0.99	0.07	0.85

^aCows in control and Algae were fed the same diet but Algae was supplemented with 100 g/cow per day of an algae product rich in docosahexaenoic acid. ^bTRT=effect of treatment (control vs Algae); parity=effect of parity (primiparous vs multiparous); TRT × parity=interaction between TRT and parity. ^c3.5% fat-corrected milk=((0.4324 × milk kg) + (16.218 × fat yield kg)). ^dEnergy corrected milk=((0.3246 × milk kg) + (12.86 × fat yield kg) + (7.04 × protein yield kg)). ^eSomatic cell score=Log₁₀ (SCC/12.5)/Log₁₀ 2.

Feeding Algae increased ($P \leq 0.04$) concentrations of total n-3 and polyunsaturated FA, and the individual FA palmitic acid, vaccenic acid, linoleic acid, CLA *cis*-9 *trans*-11 and *trans*-10 *cis*-12 and DHA, whereas eicosapentaenoic acid tended ($P = 0.06$) to increase with feeding Algae compared with control. Moreover, cows fed Algae had reduced ($P < 0.01$) concentrations of oleic acid, total monounsaturated FA, gamma-linolenic acid and arachidonic acid in the phospholipid fraction of

plasma. When FA in phospholipids were calculated per mL of plasma, a similar pattern to that of the FA profile in phospholipids was also observed (Table 7). Feeding Algae resulted in enriched ($P < 0.01$) concentrations in plasma of total n-3 FA, and the individual FA vaccenic acid, CLA *trans*-10 *cis*-12 and DHA, whereas CLA *cis*-9 *trans*-11 tended ($P = 0.08$) to increase with feeding Algae compared with control. In addition, cows fed Algae had reduced ($P \leq 0.04$) concentrations in plasma

Table 6 Effects of feeding an algae product rich in docosahexaenoic acid on fatty acid composition of phospholipid fraction of plasma from Holstein cows ($n = 50$).

Fatty acid, g/100 g of fatty acids	Treatment ^a		S.E.M.	P value ^b		
	Control	Algae		TRT	Parity	TRT × parity
C16:0	14.10	15.87	0.28	<0.01	0.11	0.82
C18:0	24.03	23.56	0.35	0.35	0.26	0.44
C18:1 <i>cis</i> -9	9.615	8.008	0.227	<0.01	0.83	0.89
C18:1 <i>trans</i> -11	0.481	0.774	0.044	<0.01	0.08	0.17
C18:2 <i>cis</i> -9, <i>cis</i> -12	31.57	33.20	0.35	<0.01	0.60	0.81
C18:3 <i>cis</i> -9, <i>cis</i> -12, <i>cis</i> -15	1.024	1.034	0.043	0.87	0.77	0.51
C18:3 <i>cis</i> -6, <i>cis</i> -9, <i>cis</i> -12	0.241	0.110	0.011	<0.01	0.95	0.38
CLA ^c <i>cis</i> -9, <i>trans</i> -11	0.098	0.113	0.005	0.04	0.75	0.85
CLA <i>trans</i> -10, <i>cis</i> -12	0.010	0.030	0.004	<0.01	0.47	0.80
C20:4 <i>cis</i> -5, <i>cis</i> -8, <i>cis</i> -11, <i>cis</i> -14	2.584	1.610	0.119	<0.01	0.79	0.15
C20:5 <i>cis</i> -5, <i>cis</i> -8, <i>cis</i> -11, <i>cis</i> -14, <i>cis</i> -17	0.308	0.373	0.025	0.06	0.74	0.20
C22:6 <i>cis</i> -4, <i>cis</i> -7, <i>cis</i> -10, <i>cis</i> -13, <i>cis</i> -16, <i>cis</i> -19	0.065	2.160	0.136	<0.01	0.42	0.34
Sum of fatty acids						
Saturated	43.99	43.17	0.35	0.11	0.86	0.36
Monounsaturated	14.44	12.65	0.26	<0.01	0.66	0.63
Polyunsaturated	37.57	39.54	0.42	<0.01	0.72	0.19
n-6	35.24	35.54	0.32	0.51	0.68	0.35
n-3	2.243	3.981	0.205	<0.01	0.86	0.21

^aCows in control and Algae were fed the same diet but Algae was supplemented with 100 g/cow per day of an algae product rich in docosahexaenoic acid. ^bTRT=effect of treatment (control vs Algae); parity=effect of parity (primiparous vs multiparous); TRT × parity=interaction between treatment and parity. ^cCLA, conjugated linoleic acid.

Table 7 Effects of feeding an algae product rich in docosahexaenoic acid on fatty acid content present in the phospholipid fraction of plasma from Holstein cows ($n=50$).

Fatty acid, μg of FA/mL of plasma	Treatment ^a			P value ^b		
	Control	Algae	S.E.M.	TRT	Parity	TRT $\times$ parity
C16:0	128.2	143.8	6.53	0.10	0.20	0.67
C18:0	221.8	213.9	11.4	0.63	0.64	0.82
C18:1 <i>cis</i> -9	87.74	72.29	4.01	<0.01	0.68	0.69
C18:1 <i>trans</i> -11	4.443	7.070	0.50	<0.01	0.30	0.34
C18:2 <i>cis</i> -9, <i>cis</i> -12	291.1	301.1	15.0	0.64	0.36	0.90
C18:3 <i>cis</i> -9, <i>cis</i> -12, <i>cis</i> -15	9.446	9.300	0.59	0.86	0.38	0.55
C18:3 <i>cis</i> -6, <i>cis</i> -9, <i>cis</i> -12	2.225	0.997	0.136	<0.01	0.90	0.51
CLA ³ <i>cis</i> -9, <i>trans</i> -11	0.882	1.026	0.058	0.08	0.55	0.91
CLA <i>trans</i> -10, <i>cis</i> -12	0.099	0.227	0.043	<0.01	0.34	0.90
C20:4 <i>cis</i> -5, <i>cis</i> -8, <i>cis</i> -11, <i>cis</i> -14	23.48	14.45	1.385	<0.01	0.90	0.16
C20:5 <i>cis</i> -5, <i>cis</i> -8, <i>cis</i> -11, <i>cis</i> -14, <i>cis</i> -17	2.831	3.313	0.261	0.20	0.81	0.13
C22:6 <i>cis</i> -4, <i>cis</i> -7, <i>cis</i> -10, <i>cis</i> -13, <i>cis</i> -16, <i>cis</i> -19	0.576	19.34	1.382	<0.01	0.23	0.18
Sum of fatty acids						
Saturated	403.8	391.8	19.9	0.67	0.43	0.95
Monounsaturated	132.2	114.0	6.05	0.04	0.65	0.82
Polyunsaturated	345.9	358.0	17.6	0.63	0.38	0.66
n-6	324.6	322.1	16.3	0.91	0.38	0.79
n-3	20.57	35.68	2.27	<0.01	0.46	0.15

^aCows in control and Algae were fed the same diet but Algae was supplemented with 100 g/cow per day of an algae product rich in docosahexaenoic acid. ^bTRT=effect of treatment (control vs Algae); parity=effect of parity (primiparous vs multiparous); TRT $\times$ parity=interaction between treatment and parity. ^cCLA, conjugated linoleic acid.

of oleic acid, total monounsaturated FA, gamma-linolenic acid and arachidonic acid present in the phospholipid fraction.

As observed for the FA profile in plasma phospholipids, the FA composition of milk fat also was affected

drastically by treatment (Table 8). Cows fed Algae had milk fat with reduced ($P<0.01$) concentrations of total *de novo* synthesized, preformed and saturated FA. However, feeding Algae increased ($P\leq 0.05$) concentrations of total n-6, n-3 and polyunsaturated FA,

Table 8 Effects of feeding an algae product rich in docosahexaenoic acid on the fatty acid composition of milk fat in Holstein cows ($n=50$).

Fatty acid, g/100 g fatty acids	Treatment ^a			P value ^b		
	Control	Algae	S.E.M.	TRT	Parity	TRT $\times$ parity
Summation by source ^c						
<i>De novo</i>	21.77	19.78	0.45	<0.01	0.02	0.25
Mixed	45.38	49.43	0.72	<0.01	0.03	0.14
Preformed	32.83	30.78	0.50	<0.01	0.35	0.28
Selected individual fatty acids						
C16:0	31.39	29.29	0.47	<0.01	0.45	0.25
C18:0	11.04	11.60	0.38	0.30	0.15	0.22
C18:1 <i>cis</i> -9	20.44	21.54	0.40	0.06	0.18	0.18
C18:1 <i>trans</i> -11	0.843	1.218	0.060	<0.01	0.04	0.62
C18:2 <i>cis</i> -9, <i>cis</i> -12	2.450	2.716	0.060	<0.01	0.89	0.62
C18:3 <i>cis</i> -9, <i>cis</i> -12, <i>cis</i> -15	0.273	0.318	0.007	<0.01	0.15	0.66
CLA ^d <i>cis</i> -9, <i>trans</i> -11	0.389	0.549	0.023	<0.01	0.05	0.37
CLA <i>trans</i> -9, <i>cis</i> -11	0.007	0.016	0.002	<0.01	0.67	0.79
CLA <i>trans</i> -10, <i>cis</i> -12	0.007	0.015	0.003	0.05	0.97	0.55
C20:5 <i>cis</i> -5, <i>cis</i> -8, <i>cis</i> -11, <i>cis</i> -14, <i>cis</i> -17	0.022	0.028	0.001	<0.01	0.48	0.07
C22:6 <i>cis</i> -4, <i>cis</i> -7, <i>cis</i> -10, <i>cis</i> -13, <i>cis</i> -16, <i>cis</i> -19	0.002	0.242	0.010	<0.01	0.17	0.12
Sum of fatty acids						
Saturated	65.90	62.06	0.67	<0.01	0.19	0.32
Monounsaturated	25.17	26.39	0.43	0.05	0.30	0.15
Polyunsaturated	3.167	3.630	0.069	<0.01	0.39	0.99
n-6	2.655	2.880	0.062	0.01	0.74	0.75
n-3	0.512	0.751	0.015	<0.01	0.01	0.17

^aCows in control and Algae were fed the same diet but Algae was supplemented with 100 g/cow per day of an algae product rich in docosahexaenoic acid. ^bTRT=effect of treatment (control vs Algae); parity=effect of parity (primiparous vs multiparous); TRT $\times$ parity=interaction between treatment and parity. ^c*De novo* FA originate from mammary *de novo* synthesis (<16 carbons); preformed FA originate from the diet or are mobilized from plasma pool or adipose depots (>16 carbons), and mixed FA originate from both sources. ^dCLA, conjugated linoleic acid.

and individual FA linoleic acid, α -linolenic acid, CLA *trans*-10 *cis*-12 and eicosapentaenoic acid compared with control cows. In addition, the milk fat of cows fed Algae had increased ($P<0.01$) incorporation of DHA compared with control-fed cows. Furthermore, an interaction ($P=0.07$) between treatment and parity was observed for eicosapentaenoic acid in milk fat, because incorporation of this FA milk fat of primiparous cows fed Algae was greater ($P<0.01$) than their counterparts fed control (control = 0.021 vs Algae = 0.030 ± 0.002 g/100 g FA), whereas for multiparous cows, feeding Algae only tended ($P=0.07$) to increase in eicosapentaenoic acid in milk fat (control = 0.023 vs Algae = 0.026 ± 0.001 g/100 g of FA).

Discussion

Daily dietary supplementation with algae rich in DHA resulted in increased proportion of estrous cyclicity and pregnancy at the first AI in primiparous cows and increased the overall P/AI and reduced days to pregnancy in all cows. In addition, the normalized expression of the interferon-stimulated gene *RTP4*, which is associated with the amount of interferon-tau secreted by the conceptus, was upregulated in peripheral blood leukocytes of pregnant cows fed Algae compared with pregnant control cows. Feeding Algae altered the profile of FA in plasma phospholipids and milk fat, resulting in increased incorporation of n-3 FA in those lipid fractions and reduced concentrations of arachidonic acid and gamma-linolenic acid in phospholipids from plasma. Furthermore, cows fed Algae produced 0.9 kg/day more milk, 30 g/day more milk true protein and 100 g more solids-not-fat than control cows. However, yield of milk fat decreased 30 g/day and energy-corrected milk remained unchanged with feeding of Algae.

Supplementation with Algae was initiated past the period when cows have extensive lipomobilization and feed intake is low, which makes it more challenging to alter tissue lipid profiles. Also, previous research showed that supplementing n-3 FA starting at 30 days postpartum was beneficial to embryo survival (Silvestre *et al.* 2011a). It is important to note that cows were supplemented with a small amount of n-3 FA, 10.2 g/day, because feeding large quantities of polyunsaturated FA to early lactation cows can increase tissue peroxidation and elicit more oxidative stress (Gobert *et al.* 2009, Wullepitt *et al.* 2012). Such negative effects of n-3 FA might be exacerbated if large quantities are fed during the transition period, when lipomobilization is already extensive (Wullepitt *et al.* 2012). On the other hand, at low amounts, n-3 FA acts as anti- rather than pro-oxidant in endothelial vascular cells and placental tissues by reducing inflammation and reducing the formation of reactive oxygen species (Jones *et al.* 2013, Giordano & Visioli 2014). Although the Algae product contained several FAs, other non-FA lipids, carbohydrates

and protein, the only identified compound fed in appreciable amounts sufficient to influence dairy cow fertility was DHA. The provision of 16 g/day of palmitic acid is unlikely to have any impact on reproduction, other than supplying a small quantity of calories. The other polyunsaturated FA, docosapentaenoic acid (C22:5 n-6), was supplemented in small quantity and only a marginal increase was observed in plasma phospholipids with no increase in milk fat, whereas DHA increased 33-fold in plasma phospholipids and in milk fat in cows fed Algae. The amounts of crude protein and carbohydrates were insufficient to alter the intake of those compounds by cows. Therefore, although we did not have pure source of a single FA, we suggest that the animal responses observed in the current experiment are attributed primarily to DHA given the expressive tissue incorporation and the known biological effects of this FA.

It is well established that fat supplementation in diets of lactating dairy cows often benefits reproduction. A recent meta-analysis of the literature included 17 experiments with 26 comparisons reported a 27% increase in the relative risk of pregnancy, and the results showed little heterogeneity suggesting that responses were consistent across different fat sources and conditions evaluated (Rodney *et al.* 2015). Nevertheless, it is also known that different FAs have distinct effects on cellular functions and gene expression (Wahle *et al.* 2003), including differential impacts on tissues of the reproductive tract (Bilby *et al.* 2006a,b, Zachut *et al.* 2008). Therefore, it is not surprising that different FAs might have distinct effects on fertility responses of dairy cows (Santos *et al.* 2008, Silvestre *et al.* 2011a). Feeding n-3 FA promotes changes in the FA composition of reproductive tissues that can alter cell membrane fluidity and gene expression, which might favor the establishment and maintenance of pregnancy in dairy cattle (Santos *et al.* 2008). Indeed, feeding Algae rich in DHA increased the incorporation of polyunsaturated FA into milk fat and plasma phospholipids and improved both P/AI and pregnancy rate. In agreement, Silvestre and coworkers (Silvestre *et al.* 2011a,b) showed that feeding Ca salts containing fish oil FA, which were rich in DHA and eicosapentaenoic acid, increased the incorporation of those FA into maternal tissues and improved P/AI by reducing pregnancy loss. Others have also shown that feeding n-3 FA improved P/AI (Petit *et al.* 2001) and reduced pregnancy loss in dairy cows (Ambrose *et al.* 2006).

Feeding Algae increased the concentrations of DHA and other n-3 FA and reduced arachidonic acid and gamma-linolenic acid in plasma phospholipids, thereby confirming that those FA were absorbed and transferred to the plasma pool becoming available for use by all body tissues. It is important to mention that feeding Algae not only altered the relative concentrations of FA in plasma phospholipids but similar changes were also

observed when the absolute concentrations of those FA were analyzed. Thus, the availability of specific FA was altered by the dietary treatment imposed, which likely not only influenced plasma phospholipid and milk FA composition but also the composition of FA in other tissues. The transfer efficiency of n-3 FA into bovine tissues is low, and only 5–7% of eicosapentaenoic acid and DHA fed appear in milk fat (Mattos *et al.* 2004). However, feeding fish oil FA consistently increased the incorporation of eicosapentaenoic acid and DHA in placentomes, immune cells, endometrial cells, liver, follicular fluid and in conceptuses (Bilby *et al.* 2006a, Moussavi *et al.* 2007a, Childs *et al.* 2008, Silvestre *et al.* 2011b, Greco *et al.* 2014). Strong positive relationships between plasma and uterine endometrial concentrations of eicosapentaenoic acid ($r^2=0.86$) and total n-3 FA ($r^2=0.77$) have been reported in beef heifers (Childs *et al.* 2008). Based on the changes in phospholipid FA in the plasma pool, it is likely probable that cows fed Algae had reduced concentrations of arachidonic acid and gamma-linolenic acid in phospholipid pools of the endometrium, which limits the amount of these precursors for production of the series 2 prostaglandins in the uterus. Feeding fish oil FA increased the incorporation of n-3 FA in the endometrial tissue, altered the expression of genes involved in the luteolytic cascade and attenuated the spontaneous release of $\text{PGF}_{2\alpha}$ in lactating dairy cows (Greco *et al.* 2014). Also, feeding Algae likely increased the incorporation of DHA and other n-3 FA in the endometrium of dairy cows, which might favor pregnancy by either influencing embryonic development or altering the synthesis of $\text{PGF}_{2\alpha}$ (Mattos *et al.* 2004, Greco *et al.* 2014).

Feeding n-3 FA to dairy cows increased tissue content of those FA and attenuated the proinflammatory response *in vivo* and *in vitro* (Silvestre *et al.* 2011b, Greco *et al.* 2015). Eicosapentaenoic and DHA influence the expression of genes involved in the luteolytic cascade in dairy cows (Bilby *et al.* 2006a, Greco *et al.* 2014), although pulses are attenuated (Mattos *et al.* 2003), the secretion of $\text{PGF}_{2\alpha}$ is not abolished, and prostaglandins are essential for proper embryo development and maintenance of pregnancy in ruminants (Dorniak *et al.* 2011). It is possible that improvements in reproduction observed with feeding n-3 FA might be mediated by changes in immune and inflammatory responses. Inflammatory diseases in the early lactation reduces P/AI and increases the risk of pregnancy loss, and conceptuses derived from cows that had disease have increased the expression of proinflammatory genes and genes responsible for antigen presentation, suggesting a potential for increased fetal antigen presentation to the surveilling maternal immune system (Ribeiro *et al.* 2016c). Because n-3 FA such as DHA can attenuate inflammation and innate immune responses *in vivo* and *in vitro* (Silvestre *et al.* 2011b, Greco *et al.* 2015), it is plausible to postulate that improvements in P/AI

or maintenance of pregnancy in dairy cattle fed n-3 FA might have been mediated by attenuation of the maternal immune system to favor the establishment and maintenance of pregnancy in dairy cows (Santos *et al.* 2008, Silvestre *et al.* 2011a, Ribeiro *et al.* 2016a). Also, FA and lipid derivatives are essential for conceptus development in the bovine. During early gestation, remarkable changes in the expression of genes related to FA and lipid metabolism occur as the conceptus develops from the ovoid to the elongated stage at around day 16 of gestation (Ribeiro *et al.* 2016b). The bovine conceptus takes up FA from the surrounding environment and incorporates those lipids into tissues for synthesis of phospholipids or for energy generation (Ribeiro *et al.* 2016a). In fact, expression of genes related to lipid uptake, FA biosynthesis and modification of FA increase substantially during the onset of conceptus elongation in dairy cattle (Ribeiro *et al.* 2016b). Some polyunsaturated FA such as DHA and other n-3 FA are natural ligands of transcription factors essential for conceptus elongation. One such transcription factor is peroxisome-proliferator activator receptor gamma (PPARG), which is a highly expressed gene in bovine conceptus during elongation (Ribeiro *et al.* 2016b), and loss of function of *PPARG* gene in utero resulted in growth retardation in ovine embryos (Brooks *et al.* 2015).

The mRNA expression of one of the two interferon-stimulated genes investigated in maternal peripheral blood leukocytes increased in pregnant cows fed Algae compared with pregnant cows fed the control diet. During early pregnancy, trophoblast cells produce interferon-tau, which is responsible for the servomechanism for the maintenance of pregnancy during early gestation by blocking the luteolytic cascade and endometrial pulses of $\text{PGF}_{2\alpha}$. Interferon-tau produced by the conceptus reaches the maternal circulation exposing maternal peripheral cells to this protein (Oliveira *et al.* 2008), which might have implications for the maintenance of pregnancy in cattle. Interferon-tau in the maternal circulation influences immune cells, and expression of interferon-stimulated genes in peripheral blood cells was correlated positively to the amount of interferon-tau produced by the trophoblast (Matsuyama *et al.* 2012), which suggests that greater expression of these genes in leukocytes indicate a more advanced conceptus (Ribeiro *et al.* 2014). The increase in mRNA for *RTP4* in leukocytes of Algae cows suggests that day 19 conceptuses were more advanced and produced greater amounts of interferon-tau, a possible mechanism for improved P/AI observed with feeding n-3 FA in the current experiment.

The gene *RTP4* and its protein are expressed in a multitude of reproductive tissues (Gifford *et al.* 2008), in addition to leukocytes, and massive increments in mRNA expression are detected during early pregnancy in response to interferon-tau. *RTP4* is a gene originally identified in olfactory neurons, but pregnant ewes express

RTP4 mRNA localized in different cell layers of the glandular endometrium, and expression was upregulated in response to pregnancy (Gifford *et al.* 2008). Indeed, both pregnant ewes and heifers express *RTP4* mRNA and protein in endometrial luminal epithelium, glandular epithelium, stroma, corpus luteum and in granulosa cells (Gifford *et al.* 2008, Horsley *et al.* 2016, Wilson *et al.* 2016). Because *RTP4* is a class of G-protein-coupled receptor transporters, it is plausible to speculate that expression of these proteins in the endometrium might influence chemokine receptors involved in the attachment of the trophoblast and subsequent invasion during the peri-implantation period and/or lymphocyte recruitment such as T regulatory cells important for promoting the tolerance to paternal alloantigens expressed in the conceptus. The differences in expression of *RTP4* in pregnant cows fed Algae compared with control cows were observed in spite of similar concentrations of progesterone after AI. Progesterone is known to stimulate histotroph secretion that increases trophoblast elongation and proliferation. The lack of difference in progesterone concentrations between treatments suggests that benefits of supplementing DHA on interferon-stimulated gene expression were likely a direct effect of FA on embryo development and not an indirect action of distinct concentrations of progesterone. However, the possibility that DHA might have sensitized blood leukocytes to respond to conceptus-derived interferon-tau cannot be ignored.

In a functional analysis of the transcriptome of day 16 bovine conceptuses, FA and *PPARG* were placed as upstream regulators of changes in gene expression during conceptus elongation (Ribeiro *et al.* 2016b). Peroxisome-proliferator-activated receptor gamma is known to regulate immune cell function and plasticity, particularly the differentiation of T helper lymphocytes to regulatory T cells, which have been described as the main cell type-controlling immune responses in the endometrium, avoiding rejection of the conceptus by the maternal immune system (Samstein *et al.* 2012). Expression of *PPARG* in conceptus cells was highly correlated with length of the conceptus and concentration of interferon-tau in the uterine flushing collected from pregnant cows (Ribeiro *et al.* 2016b). This biological association infers that conceptus elongation is mediated, in part, by the activation of *PPARG*, which stimulates trophoblast elongation and proliferation in dairy cattle. Activation of *PPARG* signaling is important for placental development, immunomodulation and stimulation of conceptus elongation. It is possible that feeding DHA stimulated such pathways to improve P/AI in dairy cows observed in the current experiment. In cell culture, DHA stimulates angiogenesis in placental trophoblast cell lines by increasing mRNA expression of angiogenic genes such as angiopoietin-like 4 (*ANGPTL4*) and vascular endothelial growth factor (*VEGF*; Johnsen *et al.* 2011), which is important for normal placentation.

The benefits of improving fertility when cows were fed Algae, however, were more pronounced in primiparous than that in multiparous cows. A greater proportion of primiparous cows fed Algae resumed ovulation by 58 days postpartum compared with primiparous control cows. In agreement, Jahani-Moghadam and coworkers (Jahani-Moghadam *et al.* 2015) reported that dairy cows supplemented with linseed, which is rich in n-3 FA, had earlier resumption of estrous cyclicity compared with cows fed a diet supplemented with Ca salts of palm oil FA distillate, which contains mostly saturated and monounsaturated FA. It is well accepted that cows that have resumed estrous cyclicity by the end of the voluntary waiting period at approximately 60 days postpartum have improved reproductive performance compared with cows that remain anovular by the same time postpartum (Ribeiro *et al.* 2016c). This may explain partially the improved P/AI and reduced days to pregnancy in primiparous cows fed Algae. The exact reason why a greater proportion of primiparous cows fed Algae were estrous cyclic is not fully understood. Estrous cyclicity by 58 days postpartum was not altered by treatment in multiparous cows. It is possible that the amount of supplemental FA supplied to multiparous cows was not sufficient to promote these benefits in the beginning of the breeding period, and longer feeding was needed in cows that have larger body size and greater milk yield. Also, it is possible that a longer period of feeding might be needed for multiparous cows to elicit the benefits on fertility. A tendency for increased rate of pregnancy was observed in multiparous cows when fed Algae, but the response was observed after approximately 85 days postpartum, when they had received the treatments for at least 60 days. Because multiparous cows are larger animals and have greater milk secretion of FA, it is plausible to suggest that a longer feeding period or a larger quantity of DHA might be needed for tissue accumulation of specific FA to elicit the same response in fertility as those observed in primiparous cows. Although the benefit of Algae was more pronounced in primiparous cows, after 120 days of treatments, feeding Algae increased P/AI and reduced days to pregnancy in both multiparous and primiparous cows.

Feeding fat often influences pre-ovulatory follicle diameter and concentrations of progesterone in blood of dairy cows (Santos *et al.* 2008); however, the type of FA fed usually has minor effects on those responses, including n-3 FA (Moussavi *et al.* 2007a). Because the intake of total FA by cows in the current experiment did not differ between treatments, it is not surprising that pre-ovulatory follicle diameter and progesterone concentrations before and after insemination remained unaltered by dietary treatments.

Pregnant primiparous cows had greater mRNA expression of interferon-stimulated genes in maternal leukocytes, regardless of the diet assigned. Green and coworkers (Green *et al.* 2010) also reported

greater expression of interferon-stimulated genes in primiparous cows than that in multiparous cows. Likely, the concentration of interferon-tau in the maternal circulation was greater in primiparous cows than that in multiparous cows either because primiparous cows had larger conceptuses or because of their smaller body size and less body weight or a combination of both.

Concentrations of insulin and IGF-1 in plasma were modestly affected by dietary treatments. Algae cows not receiving bST had the lowest concentrations of insulin in the current study. Others have shown that feeding fish oil FA, which contains DHA, reduced concentrations of insulin in blood of dairy cows (Bilby *et al.* 2006b), although this response is not consistent (Moussavi *et al.* 2007b). In cases when dry matter intake increased with feeding n-3 FA as Ca salts of fish oil, then concentrations of insulin in blood also increased in early lactation dairy cows (Moussavi *et al.* 2007b). It is well accepted that concentrations of insulin reflect the availability of glucose that stimulates pancreatic beta cells to produce and release insulin. Fatty acids of the n-3 family usually have insulinotropic activity in monogastrics stimulating insulin release and tissue sensitivity to insulin (Bhaswanta *et al.* 2015). Nevertheless, in dairy cattle, supplementing FA to the diet usually reduces concentrations of insulin, except when total caloric intake increased with fat feeding (Staples *et al.* 1998). When cows received bST, those fed Algae had greater concentrations of IGF-1 than control cows. Somatotropin stimulates hepatic IGF-1 production and the greater concentrations in cows fed Algae suggest that response to bST might have been enhanced by feeding n-3 FA. In monogastrics, the insulinotropic and anti-inflammatory effects of n-3 FA usually enhance hepatic IGF-1 secretion and tissue sensitivity to IGF-1 (Bhaswanta *et al.* 2015).

Elevated basal plasma concentrations of IGF-1 were observed in cyclic cows fed with n-3 from fish oil compared with cows fed a control isolipidic diet lacking DHA and eicosapentaenoic acid. However, in response to bST, n-3-fed cows had increased GH and reduced IGF-1 plasma concentrations compared with control-fed cows (Bilby *et al.* 2006b). Thus, it appears that n-3 may be modulating the GH-IGF-1 system. Likewise, feeding n-3 FA as Ca salts of fish oil tended to increase the concentrations of IGF-1 in early lactation dairy cows compared with cows fed Ca salts of palm oil distillate FA (Moussavi *et al.* 2007b). Because cows fed n-3 FA also had greater dry matter intake and insulin concentrations in Moussavi and coworkers (Moussavi *et al.* 2007b), it is possible that the IGF-1 response reflected the improved energy status and earlier recoupling of the somatotropic axis. Additional experiments need to confirm if supplementing cows with DHA influence the IGF-1 response to bST.

Supplementation with Algae had no impact on dry matter intake, but increased yields of milk, true protein

and lactose compared with cows fed the control diet. The increases in yields of milk components were caused by the greater milk yield. Nevertheless, the concentration and yield of milk fat decreased in cows fed Algae compared with control cows. Collectively, these responses resulted in the same yields of 3.5% fat-corrected milk and energy-corrected milk. Lactation responses to feeding n-3 FA depend upon the amounts offered and the interactions with other dietary components. Large quantities supplemented to diets that are low in forage can depress dry matter intake and negatively influence lactation performance. Hostens and coworkers (Hostens *et al.* 2011) offered dairy cows 2 kg of a supplement containing 44 g of DHA and reported an increase in milk yield, but an abrupt decrease in concentration and yield of milk fat. Although cows in the current experiment experienced a depression in milk fat yield when fed Algae, the total energy output as milk remained similar to that of control cows. Feeding polyunsaturated FA may favor the accumulation of numerous mono and dienoic trans-isomers of C18 FA in the rumen. Some of these intermediates (e.g., CLA *trans*-10 *cis*-12 and *trans*-9 *cis*-11) are known to promote milk fat depression by the downregulation of key lipogenic genes in the mammary gland (Bauman *et al.* 2011). Indeed, feeding Algae increased the aforementioned CLA in milk fat, which likely caused the observed depression in milk fat yield. Cows fed Algae had reduced concentrations of *de novo*-synthesized FA, which were replaced by preformed FA originating from the diet or from storage pools.

Feeding the Algae supplement to supply 10 g of DHA per cow per day improved reproductive performance in dairy cows. A greater proportion of primiparous cows fed Algae were estrous cyclic by 58 days postpartum compared with primiparous control cows, which resulted in an increased proportion of Algae cows detected in estrus before the first postpartum AI. Feeding algae improved P/AI at first AI in primiparous cows and at all AIs performed in the 120-days treatment period in both primiparous and multiparous cows. The increased P/AI resulted in an increased hazard of pregnancy by 38%, which reduced the median days to pregnancy by 21 days. Pregnant cows fed algae had increased expression of one of the two interferon-stimulated genes investigated, *RTP4*, in peripheral blood leukocytes, perhaps suggesting advanced embryo development, despite similar concentrations of progesterone in control and Algae pregnant cows during the first 19 days after insemination. Algae altered the profile of FA in milk fat and in the phospholipid fraction of plasma, particularly increasing the concentration of DHA and total n-3 FA, thereby indicating the incorporation of the supplemented FA into tissues. Feeding Algae reduced milk fat yield, but yields of milk and true protein increased, resulting in similar yields of energy-corrected milk between Algae and control cows. Collectively, results presented herein suggest that fat supplementation

based on algae product to provide 10g of DHA per cow per day improves the reproductive performance and alters tissue fatty acid composition. The benefits in reproduction might be associated with improvements in embryo development, potentially through improvements in embryo–maternal crosstalk.

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.

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Author contribution statement

Letícia D P Sinedino, Paula M Honda and Letícia R L Souza conducted the animal and laboratory portions of the experiment. Adam L Lock assisted with laboratory analyses of fatty acids and manuscript preparation. Maurice P Boland, Charles R Staples, William W Thatcher and José E P Santos designed the experiment and assisted with preparation of the manuscript. Letícia D P Sinedino and José E P Santos conducted the statistical analyses and wrote the manuscript. All authors reviewed and approved the final version of the manuscript.

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