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Polyunsaturated fatty acids and fertility in female mammals - an update

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Abstract:	Both n-3 and n-6 polyunsaturated fatty acids (PUFAs) are derived from the diet, with concentrations in the reproductive tract reflecting dietary intake. PUFAs have multiple functions: as precursors to eicosanoids, regulators of steroid biosynthesis, inflammatory mediators and supplying energy (particularly in oocytes). The PUFA composition of cell membranes affects signalling pathways and susceptibility to oxidative damage. All of these roles may influence reproduction although results are often inconsistent between studies. Supplementation of cows with various PUFAs can increase the numbers of antral follicles although work on polyovular species (pigs, rodents) has usually failed to detect a change in ovulation rate. The anti-inflammatory actions of n-3 PUFAs may reduce follicular PGE production, delaying ovulation and allowing ovulatory follicles to grow larger and produce more steroid. Various PUFA supplements can reduce the interval from calving until first ovulation in cattle although the mechanism is uncertain. Both n-3 and n-6 PUFA supplements have been fed to various species before collecting oocytes for in vitro fertilization. Positive, negative and no effects on subsequent embryo development have all been reported. When PUFAs are added directly to oocyte maturation medium, high doses of linoleic acid (18:2 n-6) are consistently deleterious, while α-linolenic acid (18:3n-3) has been associated with positive outcomes. Uterine prostaglandin production regulates luteal regression and pregnancy recognition. Supplementary n-3 PUFAs have either increased or decreased PGF2α production in different studies. There is some evidence that cattle and pigs fed a PUFA supplement post insemination may have an increased pregnancy rate.

Responses to Reviewers' comments:

Reviewer #1: Overall I felt the review paper was comprehensive and well written; however, there are a few minor concerns that I would like to see addressed.

Q. My first concern is related to the section on eicosanoid synthesis (lines 64-88). I found this section of the paper hard to read, as it did not clearly explain the metabolic pathways. I believe the overall description is correct, it is just that some of the initial steps are not defined well and create minor confusion. Initially, when the authors describe eicosanoids being synthesized from 20 carbon PUFAs, they do not mention which PUFA's are the 20 carbon ones. I believe this causes some confusion throughout the section. I think AA and EPA need to be introduced a bit more thoroughly to help things flow better and give the reader a tighter grasp on the pathways. A lot of fatty acids are mentioned initially as required, and I think explaining the 20 carbon fatty acids as precursors more clearly will help.

A. We have now given more clear explanation of the PUFA metabolic pathways and stated the importance of AA and EPA in these pathways.

Q. On line 69 the authors mention the 'two' PUFA families. Lines 39/40 talk about the 3 PUFA families. Just clarify that it is the n-6 and n-3 pathways which are of concern, not the n-9's.

A. We have now clarified the importance of n-3 and n-6 pathways.

Q. Line 72 mentions the 'rate-limiting steps'. Elaborate on this slightly.

A. The explanation is now added.

Q. Line 73 discusses membrane phospholipids. Perhaps this is a good place to discuss what the most common PUFA's are in membrane lipids and tie the 20 carbon fatty acids to the pathway more clearly.

A. As to the most common PUFAs in the membrane, this depends on the cell types and stages of the development. For example, AA is one of the most common PUFAs in many “activated” immune cells and endometrial tissues and placenta can actively transfer the long chain PUFAs. This is very complicated and is somewhat out of the scope of this review.

Q. My final concern for the eicosanoid section is line 87. The authors state that AA is the preferred substrate for the enzymes being discussed and I would either like a reference included here or clarification. My understanding is that with the delta-5 and -6 desaturase enzymes, they prefer n-3 fatty acids over n-6 (see Palmquist, 2009). I am unaware of the substrate preferences for PTGS enzymes and would like clarification.

A. We understand that it has been reported that delta-5 and -6 desaturase enzymes prefer n-3 PUFAs. The substrates for PG production (DGLA, AA and EPA) may be provided by both the metabolisms by the desaturases and dietary intake. Therefore the key enzymes which determine PG production are PTGS1 and PTGS2. Although the Km values of both PTGS1 and PTGS2 are similar between AA and EPA, their Vmax values for the enzyme reaction are much different. The turnover of PGs for EPA is 10% and 35% of those of AA with PTGS1 and PTGS2,

respectively. This was discussed in details previously (Smith WL. Cyclooxygenases, peroxide tone and the allure of fish oil. Curr Opin Cell Biol 2005; 17:174-182). We have now added it to our references.

Q. I found the rest of the paper to be very clear and well written.

A. Many thanks.

Q. Line 208 - please clarify if the number of animals required to see differences vary between mono vs. poly ovulators since both groups of animals are covered in the review.

A. This is a good point. We have now added some actual power calculations to the text for polytocous and monotocous species.

Q. Again, the overall paper was very well written, and with revisions to that first section on eicosanoid synthesis, I see no reason why it should not be published. I think the authors touched on and attempted to seek valid explanations as to the high variability across studies in terms of results and touched on relevant issues such as the n-6 to n-3 ratios.

A. Many thanks for your positive comments. We tried our best to address these issues.

Reviewer #2:

Q. The paper is well written and informative. Not a lot is published in this area and the authors are correct in commenting that there are equivocal results published and difficulties in interpreting some papers due to different methodologies and different sources of PUFAs. There are some published papers from pig studies that could be included in the review (see below).

A. Again, many thanks for your positive comments. We have tried our best to address these issues.

Q. *P3, L36: suggest changing to "...roles within the mammalian body, including the supply of energy; as structural components of membranes; and by acting...".*

A. Done.

Q. P3, LN46: suggest changing to "LA and ALA cannot be synthesized by animals as they lack the desaturase enzymes capable of inserting a double bond between C9 and the terminal methyl group of the acyl chain".

A. Done

Q. P3, LN58: "...to supply energy and become incorporated into..."
A. Done

Q. P4, LN87: Some mention of rate of bioconversion from C18 PUFA to C20 and C22 (LCPUFA) would be worthwhile.

A. This is covered by the changes made in response to Reviewer 1.

Q. P4, LN92: "...production, again through a variety..."

A. Done

Q. P7, LN 166-167: suggested change "...reduces the production of pro-inflammatory eicosanoids (2-series) derived from AA. EPA instead give rise to eicosanoid mediators that are less inflammatory (3-series), while both ...."

A. Done

Q. P7, LN190: Suggested change "Ruminant diets usually have a fodder component..."

A. Done

Q. P7, LN192: suggest an inserted sentence: "This can be overcome by the use of protected oils as calcium soaps which bypass the rumen and release LCPUFA into the small intestine" (see Staples, Burke, Thatcher 1998. J Dairy Science 81:856-871).

A. We have added something similar although this does not remove the problem that the diet for cows still has to contain fodder!

Q. P8, LN210: Other limitations to interpreting outcomes between studies occur due to synchronisation protocols using exogenous prostaglandins or progesterone? See Estienne et al 2006.
A. We agree this is another issue but decided it was outwith the scope of this review

Q. P9, LN230: see also paper by Perez-Rigau, A. et al 1995 J Animal Science 73:1372-1380 on ovulation response to PUFAs in pigs
A. This reference is now included (no. 66).

Q. P9, LN252: "... but evidence to support this is lacking."
A. Changed

Q. P9, LN259: Is this statement correct? "...almost all animals experience a uterine infection at this time...". I would disagree that it occurs in 'almost all animals' It doesn't often occur in pigs after uterine involution.

A.Yes it is correct for cows so changed the word

Q. P12, LN 323: In contrast to the references given, others have found some positive responses to embryo survival in pigs. See reference Perez-Rigau et al 1995 J Animal Science 73:1372-1380 and Smits RJ et al 2013, Animal Production Science 53: 57-66. It is also reported omega 3 LCPUFAs

have positive efects on litter size in pigs (see Palmer WM et al 1970 J Animal Science 31: 535-539; Smits RJ et al 2011 J Animal Science 89: 2731-2738) P14, LN389: See also paper by Brazle AE et al 2009 J Animal Science 87:994-1002 for PUFA uptake in uterine tissue in pigs P15, LN451: For reports in pigs on pregnancy rate/farrowing rate responses see Palmer et al 1970 and Smits et al 2011 as per above references)

A. We have added more on embryo survival in pigs to include these references.

Q. P20, LN35: formatting change in references

A. corrected

Q. P30, Figure 1: The use of the abbreviation COX enzymes. In the text on P4, LN57 the authors refer to PTGS1 and PTGS2 to superceed the terms COX 1 and COX 2 cycloxygenases. The terminology needs to be consistent.

A. Changed on figure and legend

Polyunsaturated fatty acids and fertility in female mammals – an update

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Abstract

Both n-3 and n-6 polyunsaturated fatty acids (PUFAs) are derived from the diet, with concentrations in the reproductive tract reflecting dietary intake. PUFAs have multiple functions: as precursors to eicosanoids, regulators of steroid biosynthesis, inflammatory mediators and supplying energy (particularly in oocytes). The PUFA composition of cell membranes affects signalling pathways and susceptibility to oxidative damage. All of these roles may influence reproduction although results are often inconsistent between studies. Supplementation of cows with various PUFAs can increase the numbers of antral follicles although work on polyovular species (pigs, rodents) has usually failed to detect a change in ovulation rate. The anti-inflammatory actions of n-3 PUFAs may reduce follicular PGE production, delaying ovulation and allowing ovulatory follicles to grow larger and produce more steroid. Various PUFA supplements can reduce the interval from calving until first ovulation in cattle although the mechanism is uncertain. Both n-3 and n-6 PUFA supplements have been fed to various species before collecting oocytes for *in vitro* fertilization. Positive, negative and no effects on subsequent embryo development have all been reported. When PUFAs are added directly to oocyte maturation medium, high doses of linoleic acid (18:2 n-6) are consistently deleterious, while α-linolenic acid (18:3n-3) has been associated with positive outcomes. Uterine prostaglandin production regulates luteal regression and pregnancy recognition. Supplementary n-3 PUFAs have either increased or decreased $PGF_{2\alpha}$ production in different studies. There is some evidence that cattle and pigs fed a PUFA supplement post insemination may have an increased pregnancy rate.

Keywords: fertility, prostaglandin, steroid, embryo, follicle, endometrium, ovulation

Review methodology

CAB Abstracts and PubMed were searched for papers combining the term polyunsaturated (or PUFA) with keywords relating to female fertility (fertility, ovary, oocyte, follicular fluid, granulosa, ovulation, fertilization, luteal/corpus luteum, endometrium). Reference lists in recent relevant review articles and recent articles citing earlier reviews were also scrutinised. The main focus was on papers published since 2007.

Introduction

Lipids have many important roles within the mammalian body, including the supply of energy, as structural components of membranes and by acting as signalling molecules. They are obtained from fats and oils in the diet which are broken down in the stomach and small intestine into fatty acids, cholesterol, triglycerides and phospholipids. Fatty acids are carboxylic acids with long-chain hydrocarbon side groups. There are three families of polyunsaturated fatty acids (PUFAs), omega-3 (n-3), omega-6 (n-6) and omega-9 (n-9). These all have more than one double bond present in the molecule and are classified into these families on the position of the first double bond relative to the methyl end of the molecule. Further members of the n-6 family are derived from linoleic acid (18:2 n-6, LA) by a process of desaturation and elongation, while n-3 family members are derived from α-linolenic acid (18:3n-3, ALA) (Figure 1). The enzymes involved, Δ6- and Δ5-desaturases and elongases, are most abundant in liver, where PUFA metabolism principally occurs [1]. LA and ALA themselves cannot be synthesised by animals, as they lack the desaturase enzymes capable of inserting a double bond between C9 and the terminal methyl group of the acyl chain [2]. They are, however, essential to life so must be obtained from the diet. The main sources of LA are vegetable oils, while ALA is present in green leafy vegetables, marine algae, seeds and nuts (e.g. linseed, walnuts) and the longer chain n-3 PUFAs eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are found at high concentrations in fish oils. During the process of digestion, a significant proportion of the dietary PUFAs become saturated, so the amounts reaching the circulation are less than those initially consumed. This is particularly true in ruminants, where ingested food is subjected to biohydrogenation by rumen microbes [3]. Nevertheless, the proportions of different PUFAs found in cell membranes throughout the body do generally reflect the amounts consumed in the diet [4].

Mechanisms of action

As lipids, PUFAs can be metabolized within the body to supply energy and become incorporated into cellular components. A tiny proportion of them are metabolised into signalling molecules with important biological functions.

Eicosanoid synthesis

Eicosanoids are physiologically active compounds derived from 20 carbon PUFAs which include prostaglandins (PGs), leukotrienes, thromboxanes, lipoxins, neuroprotectins and resolvins [5-8, Figure 1]. ALA and LA are essential PUFAs which cannot be synthesized in the mammalian body and must be provided from the diet. After intake, they are incorporated into membrane phospholipid pools and released by the action of phospholipase A2 or the co-ordinate actions of phospholipase C and diglyceride lipase [7]. Following sequential desaturation and elongation, ALA is converted to stearidonic acid (SDA, 18:4n-3), eicosatetraenoic acid (20:4n-3) and EPA while LA is catalysed to γ-linolenic acid (GLA, 18:3n-6), dihomo-γ-linolenic acid (DGLA, 20:3n-6) and AA. The above metabolites can incorporate back into cellular membrane phospholipids or are subject to further metabolism. The enzymes PTGS1 and PTGS2 (previously known as cyclooxygenase (COX)-1 and COX-2) catalyse DGLA, AA and EPA into 1-, 2- and -3 series PGs respectively. The 5-lipoxygenase (LOX) pathway generates 4-series leukotrienes (LTs) from AA and 5-series LTs from EPA. The 15-LOX and 5-LOX pathways catalyse AA sequentially to produce 4-series lipoxins (LXs), EPA to 5-series LXs or DHA into another family of lipid mediators, the neuroprotectins (Figure 1). Both aspirin-dependent and -independent pathways generate E series resolvins (RvEs) from EPA and D series resolvins (RvDs) from DHA [8]. These are produced in a tissue-specific manner which depends on the combination of precursor lipids and enzymes present in the cells.

Much work on the actions of different PUFAs has examined their influence on PG synthesis. The system is extremely complex. Each PUFA family produces its own specific metabolites and cross-metabolism between the families cannot happen [5]. The n-3 and n-6 PUFA families compete with each other for both cellular membrane lipid incorporation and metabolic enzymes [6]. This competition can influence the amounts of different longer chain PUFAs (LCPUFAs) produced from ALA and LA, although dietary supplementation with longer chain n-3 (SDA, EPA) or n-6 (GLA, AA) PUFAs can by-pass the rate-limiting step. This is the slowest step which determines the speed and efficiency of the reaction chain. The studies on supplementation and interactions between n-3 and n-6 PUFA families have attracted considerable interest as their metabolism leads to different families of PGs and resolvins as outlined above. Particular attention has been paid to EPA and AA because they are direct precursors for the production of many eicosanoids and their intracellular concentration can be influenced by both dietary and in vitro manipulation. The pattern of PUFA-derived mediators produced in any situation is tightly regulated and can be altered by a variety of mechanisms. In addition to varying amounts of precursor present in the cell, we and others have shown that PUFAs influence endometrial PG production by: (i) regulating PTGS expression [9-11]; (ii) altering the proportions of 1-, 2- and 3-series PGs produced [9] and (iii) changing the PGE:PGF ratios through altered expression of PG synthases [12]. PTGS1 and PTGS2 have similar actions but are encoded by different genes which are regulated differentially in a

cell-specific manner [13]. AA is the preferred substrate for both enzymes, so EPA metabolism to 3-series PGs is poor, and EPA also inhibits PTGS1 activity [14].

Steroid synthesis

PUFAs also have the ability to regulate steroid hormone production, again through a variety of both direct and indirect mechanisms. Steroids are derived from cholesterol as precursor and PUFAs can influence the function of transcription factors which regulate cholesterol metabolism [15]. For example, PUFAs can induce or suppress expression of the Liver X receptor, LXRα, which is a cholesterol-sensing transcription factor which plays a key role in lipid metabolism [16]. Steroid production is also influenced by PGs. For example, PGI_2 is stimulatory to progesterone synthesis by the early stage corpus luteum [17], whereas $PGF_{2\alpha}$ is the main luteolytic factor causing demise of the corpus luteum at the end of the oestrous cycle [18,19].

In order for cholesterol to become available for steroid hormone synthesis, it must first traverse the outer mitochondrial membrane to gain access to the enzyme cytochrome P450cscc, which resides on the inner membrane. This is considered the rate limiting step in steroid biosynthesis and is controlled by steroidogenic acute regulatory protein (StAR) [20]. Both PUFAs and PUFA metabolites can stimulate/inhibit StAR expression and these actions on StAR are inevitably associated with either an increase or decrease in steroid output [21]. For example, inhibition of endogenous release of AA inhibited dibutyryl cyclic AMP (dbcAMP)-induced steroid synthesis as well as StAR promoter activity, StAR mRNA and StAR protein, whereas addition of exogenous AA reversed all these effects [22].

With respect to reproductive physiology, a fish oil supplemented diet increased circulating oestrogen concentrations in pre- but not post- menopausal women [23]. Another recent observation was that fish oil altered oestradiol signalling pathways in human breast cancer cells to promote apoptosis at the expense of growth [24]. This change was probably mediated via the G protein coupled membrane receptor GPER1 rather than the classic oestradiol receptor, ERA.

There is also evidence for signalling in the opposite direction, with steroid hormones affecting PUFA metabolism. Female mammals have a greater ability to synthesize the LCPUFAs EPA and DHA from ALA than males [25]. During oestrous/menstrual cycles and pregnancy the reproductive tract is exposed to alternating periods of oestrogen and progesterone dominance. Oestrogen up-regulates the desaturases, so increasing the conversion of ALA and LA to LCPUFAs [26] to produce inflammatory/anti-inflammatory mediators. In contrast, progesterone inhibits uterine eicosanoid synthesis and stimulates and maintains production of PG dehydrogenase (PGDH) which inactivates PGs [27,28].

Transcription factor regulation

PUFAs can alter the function of a number of other transcription factors. Amongst the most studied are PPARs, a subfamily of nuclear receptors with three known subtypes α, β, and γ, which are expressed in a tissue specific manner [29]. A variety of long-chain 18C and 20C unsaturated-, polyunsaturated-, and branched chain fatty acids and prostaglandins act as endogenous ligands for PPARs [30]. PPARs influence steroidogenesis as they can induce the expression of a variety of genes whose encoded proteins are involved in the biosynthesis and metabolism of cholesterol and fatty acids [31]. PPAR activation also inhibits NF-kB signalling to decrease cytokine production and these pathways may play important roles in regulating inflammation [32,33]. AA acts through PPARα to increase PTGS2 expression in bovine endometrium [11]. There is also evidence that fish oil supplementation can influence gene expression in bovine endometrium [33]. Nuclear receptor subfamily 1, group H, member 3 (NR1H3) is another transcription factor known to respond to PUFAs [34].

Membrane properties

PUFAs become incorporated into the plasma membranes of all cells and can influence several aspects of membrane physiology which are important in reproductive biology. The fluidity of the membrane is strongly influenced by the lipid component. This property in turn affects the ability of sperm to fuse with the egg at fertilization and it also affects the sensitivity of sperm and oocytes to chilling and freezing, important in assisted reproduction [35]. For example, in oocytes collected from ewes fed a diet supplemented with Ca-soap of fish oil for 13 weeks the proportion of LCPUFAs in cumulus cell phospholipids increased by 12.7% and this was associated with improved integrity and physical properties of oocyte membranes and better resistance to chilling [36].

Unsaturated fatty acids are also vulnerable to attack by reactive oxygen species (ROS) which can initiate a damaging lipid peroxidation cascade [6,38]. Membrane potentials and intracellular ionic concentrations are controlled by different types of ion channels. Free PUFAs can modulate the voltage-dependence of voltage-gated channels. This has mainly been studied with respect to neuronal and cardiac cell activity [39] but is also important at fertilization [40].

Lipid rafts are localized regions in plasma membranes which are rich in cholesterol, sphingolipids and phospholipids. Specific proteins localize to these regions, including those involved in signal transduction pathways and T cell activation [41,42]. This partitioning promotes efficient signalling by

clustering of relevant proteins. When n-3 PUFAs, in particular DHA, become incorporated into membrane phospholipids they cause lipid raft regions to merge, with an associated depletion of cholesterol and sphingolipids and partitioning of some proteins away from the raft. This can interfere with some cell-signalling pathways, for example T-cell activation and epidermal growth factor (EGF) receptor signalling [42]. This is one mechanism by which the n-3 LCPUFAs act as anti-inflammatory agents. We have found that *in vitro* treatment of uterine epithelial cells with GLA or AA reduces responsiveness to oxytocin (OT) challenge [43,44]. This may possibly involve altered response through the oxytocin receptor (OTR), although the underlying mechanism has not been investigated.

Immune Function

PUFAs derived from fish oil (EPA and DHA) are known to have anti-inflammatory properties [45]. As outlined above, their incorporation into cells of the immune system decreases the AA content and so reduces the production of pro-inflammatory (2-series) eicosanoids derived from AA. EPA instead gives rise to 3-series eicosanoid mediators that are less inflammatory, while both EPA and DHA are precursors for resolvins that are actively anti-inflammatory and inflammation resolving. n-3 PUFAs can also alter immune function via effects on phagocytosis, T-cell signalling and antigen presentation mediated through changes in both cell membrane composition and eicosanoid signalling as outlined above. An extensive review of studies in which humans received n-3 supplementation concluded that there was good evidence for inhibition of lymphocyte proliferation, although changes in cytokine production were inconsistent [46]. Positive local effects were detected in patients with on-going inflammatory conditions, often in the absence of changes in immune markers of inflammation in the peripheral circulation. Healthy controls did not, however, show the same responses. With respect to reproduction, susceptibility of the genital tract to infection is greater in the luteal than the follicular phase of the cycle [27,47]. Progesterone inhibits NFkB activity which regulates cytokine and chemokine production, reducing the influx of neutrophils and monocytes to the uterus [48]. Supplementation with n-3 PUFAs may synergize with this effect since they also down-regulate NF-kB activity [33].

Problems of interpretation

This brief overview of possible mechanisms of action for PUFAs on reproduction illustrates just how complex the system is. Before reviewing studies which have investigated the effects of PUFAs on female reproduction, it is pertinent to consider briefly possible reasons for the frequent inconsistencies in the

results reported. For *in vivo* work, it is initially hard to formulate a diet in which only one PUFA is increased or decreased, as available food sources contain mixtures of different PUFAs. Other aspects of the diet such as protein and energy content also need to be equalised between treatment groups. This is particularly hard to achieve in human populations, but is also challenging for farm livestock such as ruminants. Ruminant diets require a fodder component such as grass or silage, whose PUFA levels may differ considerably, and in which the absorption level is further influenced by rumen dehydrogenation. The extent of the biohydrogenation can, however, be reduced in supplementary feeds by the use of protected oils such as calcium soaps which bypass the rumen and release LCPUFA into the small intestine [49]. Pure oils can be used for *in vitro* experiments but these often fail to reflect the complex biology of the whole body. PUFAs obtained from the diet will be metabolised to various extents and taken up in a tissue-specific manner. The way any one cell will react depends on both the balance of different PUFAs and other lipids stored within it and the precise signals it receives from the periphery and surrounding cells. In particular the n-3 to n-6 ratio is likely to be important and there are clear dose responses for individual PUFAs which can change effects from stimulation to inhibition. Although dietary input is clearly able to alter cellular PUFA concentrations, these will also depend to some extent on the levels present in the body before the experiment started, which in turn will vary between different experiments and species used.

Another consideration is the physiological state of the animal, as this will affect lipid metabolism in general and thus the balance of storage and release. For example, animals in early lactation undergo lipolysis to support milk production, leading to elevated concentrations of circulating non esterified fatty acids [50]. Such animals are likely to show different response to those which are gaining weight. Finally there is another key problem relating to all work where the measured outcome is conception and this is the need for adequate numbers of animals in each group to provide sufficient statistical power. The effects of dietary PUFAs on fertility are unlikely to be major. For a monotocous species such as the cow, power calculations show that about 800 animals are needed to show a 5% difference in conception rate at $P<0.05$. For polytocous species such as the pig around 40 sows should be sufficient to detect a 5% change in litter size. Much of the published work on fertility effects has therefore been underpowered.

Evidence for actions

Follicle development

A number of studies provide evidence that various LCPUFAs (both n-3 and n-6) can influence the growth and development of ovarian follicles, ovulation rate and the timing of ovulation. One consistent, although not universal, finding across a number of studies on both dairy and beef cattle was an increase in the numbers of antral follicles present on the ovaries [51-57]. In some dairy cow studies, there was also an increase in the size which the dominant follicle reached before ovulation [51,55,58-60]. Effects on follicular steroidogenesis have also been noted. High n-3 PUFAs increased the level of progesterone in the follicular fluid [61] which was mainly produced in the theca cells and was associated with an increase in StAR expression [62]. Higher circulating concentrations of oestradiol were present in the follicular phase in cows supplemented with ALA [57] and granulosa cells collected from follicles of n-6 PUFA supplemented cows showed increased steroid secretion *in vitro* [53]. In humans, higher baseline concentrations of oestradiol were found in women consuming more ALA in their diet [63], concentrations of n-3 LCPUFAs were positively associated with circulating oestradiol and progesterone [25] and fish oil supplementation increased both oestradiol and oestrone levels in pre- (but not post) menopausal women [23].

The ovulation rate was not altered following dietary supplementation with saturated fatty acids or PUFAs (LA, AA, ALA, EPA and/or DHA) in superovulated cows [64,65], pigs [66-68], or rats [69]. In other papers, however, EPA or fish oil was reported to decrease the number of ova released by rats [70] and mice [71], in which more oocytes became trapped in luteinized follicles, whereas both ALA and EPA+DHA increased the ovulation rate in rats [72]. Some experiments have examined possible effects of PUFAs on ovarian PG synthesis as this could alter the ability of follicles to ovulate, a process which is dependent on increased PGE production [73]. One difficulty with interpretation is that the PG assays used (which are mainly antibody binding assays) generally fail to differentiate 2-series from 3-series PGs as this can only be done reliably following separation by high performance liquid chromatography or gas/liquid chromatography-mass spectrometry systems. Feeding dairy cows with a high n-3 PUFA diet resulted in a lower level of PGE in the follicular fluid from large follicles [74]. Similarly feeding fish oil to mice deceased ovarian production of both PGF2 and PGE via reduced PTGS2 [71]. The work of Broughton and colleagues showed that DHA alone increased production of 3-series prostaglandin E and F in rat ovaries [69], EPA increased PGE and PGF [70] and ALA increased PGF but reduced PGE [72]. While there are inconsistencies, there is thus a trend across several species to suggest that the anti-inflammatory properties of n-3 PUFAs can reduce follicular production of PGE_2, so causing dominant follicles to grow larger and produce more steroid before ovulating.

Uterine activity

Evening primrose and borage oil, which contain high concentrations of GLA, are promoted to the human population for their anti-inflammatory properties. This may be because they increase the synthesis of 1-series rather than 2-series PGs [9,75]. It has been suggested that GLA containing oils increase uterine contractions [76], thus leading to induction of labour [77,78] but evidence to support this is lacking.

Postpartum period

Uterine involution after calving in cows is associated with an up-regulation of PGF production, commonly measured as a rise in the metabolite 15-keto-dihydro-PGF2 alpha (PGFM) in the circulation for about 3-4 weeks after calving [79]. This is part of a normal physiological response, although almost all dairy cows experience a uterine infection at this time [80] and such infections may prolong the period when PGFM is elevated [79]. Dietary PUFAs could potentially influence several aspects of reproductive function during the postpartum period: PG synthesis, the immune response to uterine infection and the timing of the first ovulation. In practice all these are inter-related.

Several studies in cattle have investigated the effects of PUFA supplementation pre-partum on plasma PGFM concentrations after calving. In general this increased PGFM levels in both dairy [3,81] and beef [82,83] cows, whereas Mattos et al. [84] found that a fish oil supplement reduced PGFM. Supplementing with C18:2 n-6 decreased the incidence of uterine disease after calving and feeding a calcium salt rich in LA and *trans*-octadecenoic acid (LTFA) from 25 days prepartum to 80 days postpartum tended to decrease the incidence of puerperal metritis (15.1 vs. 8.8%) but had no effect on retained placenta or other aspects of uterine disease [81].

Various fat supplements have led to a shorter interval to first ovulation postpartum in some studies. This was observed in cows fed calcium salts of long chain fatty acids (Ca-LCFA) [55,85]. Similarly cows fed either LA (linola) or ALA (flaxseed) supplementation exhibited shorter calving to first ovulation interval than those fed oleic acid (canola) (23.7 ± 3.2 d and 21.0 ± 3.1 d, vs. 34.7 ± 3.1 d respectively) [86]. Two studies found that transition cows supplemented with a rumen inert fatty acid mixture (Megalac®, composition: 47% palmitic acid (C16:0), 5% stearic acid (C18:0), 38% oleic acid (C18:1), 9% LA, 1% ALA) ovulated sooner after calving than cows on a control or soybean supplemented diet [87,88]. Similarly, dairy cows on pasture which received a soybean oil by-product had an earlier first ovulation (26.7 vs. 42.4 day postpartum) [89]. A sunflower seed supplement increased the likelihood that the first dominant follicle to develop after calving would ovulate, but only in primiparous and not

multiparous cows [90]. Earlier ovulation after calving therefore seems to be a fairly consistent finding, but it is not currently clear if this is due to a specific PUFA effect or just the supply of extra energy.

Fatty acid profiles of follicular fluid and oocytes

Removal of the oocyte from the follicular environment *in vitro* initiates a spontaneous resumption of meiosis. In contrast, follicular fluid of the preovulatory follicles supports oocyte maturation *in vivo* [91]. Follicular fluid thus plays has a key regulatory role in oocyte development. PUFAs accumulate in follicular fluid via a concentration gradient reflecting serum levels [92] and constitute the major portion of the fatty acid content of bovine follicular fluid. The most predominant fatty acid, contributing about a third of the total, is LA with important contributions from oleic, palmitic, stearic acids and ALA [93]. The relative contributions are influenced by follicle size, with more LA (18:2) in small follicles and ALA (18:3) in large follicles [93].

The oocytes in turn take up PUFAs from the follicular fluid. In cattle, cumulus oocyte complexes (COCs) contain a greater proportion of saturated FA (45-87% of total FAs) compared to MUFAs (11-34%) and PUFAs (2-21%) [94]. Palmitic, stearic and oleic acids were again the most prominent together with some LA and AA [95,96]. One study in cattle [97] found that dietary changes can alter the PUFA content of oocytes. In contrast, supplementation of dairy cows with Ca salt of FA increased the PUFA content of plasma and follicular fluid but not the COCs [94]. The fatty acid composition of lipids in the oocytes was also found to vary according to many factors including species [96], quality of the oocyte [98] and season [99].

Oocyte quality and embryo development

Many studies, mainly conducted in cattle, have investigated the effects of PUFAs on oocyte and embryo development. This is achieved either by feeding the dam differing diets and then flushing oocytes from antral follicles, or using abattoir derived oocytes followed by maturation in media of differing PUFA composition. Dietary supplementation with sunflower or other vegetable oils, linseed oil, fish oil or oleic, palmitic or stearic acids did not affect oocyte quality, fertilisation rate or embryo quality in dairy cows [58] or beef heifers [65], although another study reported a lower blastomere number in embryos derived from cows fed saturated fats [64]. In contrast, high fat supplementation of lactating dairy cows with Megalac® significantly improved blastocyst production and also improved the quality of the blastocysts produced in terms of increased total, inner cell mass and trophectoderm cell numbers [100]. Moreover,

feeding more unsaturated fatty acids in the form of Ca-LTFA (rich in LA) tended to increase the fertilisation rate, significantly increased the proportion of excellent and good quality embryos, decreased degenerated embryos and resulted in greater numbers of blastomeres compared to embryos from cows fed Ca salt of palm oil (rich in palmitic and oleic acids) [101]. For COCs collected from cows fed encapsulated flaxseed or sunflower oil, diet had no effect on the maturation rate; flaxseed however resulted in a higher cleavage rate compared to the control cows receiving no supplemental fat [97]. In complete contrast, feeding flaxseed to dairy cows decreased the fertilisation rate, percentage of grade 1 and 2 embryos, and increased the percentage of degenerated embryos compared with Ca salts of palm oil [102].

Turning to other mammalian species, fish oil supplementation to sheep improved oocyte yield and quality [103]. Feeding ewes fish oil or n-6 PUFA enriched diets did not affect embryo size or number following superovulation but the n-6 diet reduced development to blastocysts [61]. In contrast, a later study from the same group found that both the n-3 and n-6 diets increased blastocyst yield but these were of lower quality [62]. In pigs, n-3 supplementation did not alter ovulation rate or number and size of embryos [67,68]. Experiments on mice have also produced conflicting reports. Fish oil had little effect on oocyte quality and blastocyst development in one study [71], but altered mitochondrial distribution in oocytes, increased production of ROS and decreased embryo development to blastocysts in another [104]. Also in mice, conjugated linoleic acid (CLA) reduced the fertilization and blastocyst development rate but had no effect on litter size or ovulation rate [105]. In women undergoing a fertility treatment of IVF/ICSI, a high n-3 intake based on assessment of preconception diet improved embryo morphology, but the n-6 content of the diet had no effect [63]. Another large scale study of over 18,000 married, subfertile women trying to establish a pregnancy reported that diets with high contents of trans unsaturated fats were at increased risk of ovulatory infertility but no associations were found with intakes of total, n-6 or n-3 PUFAs [106].

Using the *in vitro* approach, we have shown that n-3 ALA supplementation (50 μM) during maturation induced molecular and biochemical changes leading to improved bovine oocyte maturation and subsequent early embryo development [107], whereas supplementation with n-6 LA (100 μM) was detrimental [108]. Similarly, Hochi et al. [109] reported that embryos cultured in the presence of LA had reduced development to the morula and blastocyst stages compared with embryos cultured in LA free media. Carro et al. [110] found that low doses of LA (9 and 43 μM) were beneficial whereas they agreed that 100 μM LA was harmful. Al Darwich et al. [111] concluded that supplementation with CLA or DHA during bovine IVF both reduced embryo development but ALA had a minor benefit. This was supported by work on IVF in goats where 50 μM ALA increased maturation and cleavage rates and blastocyst

formation [112]. Van Hoeck et al. [113] changed the order of diets fed to heifers then used serum collected from these animals to supplement *in vitro* bovine zygote development. The serum from the animals fed a diet high in saturated fat (C16:0) reduced blastocyst yield compared to controls, whereas the unsaturated fat diet (ALA) either reduced or improved embryo production depending on whether it was fed after or before the saturated fat diet.

Based on the evidence to date it is therefore hard to make a convincing case that any particular PUFA supplemented to the diet before breeding will consistently improve embryo yield or quality. It is however possible that, where oocytes have been collected for IVF, the use of the same culture medium for all oocytes irrespective of the original diet may have masked any treatment effects. The *in vitro* supplementation effects are more consistent in showing a generally harmful effect of LA and beneficial effect of ALA, although the results are dose-dependent.

A number of mechanisms have been suggested whereby PUFAs may influence oocyte quality. Firstly, triglycerides are the major component of the lipid content of the oocyte and act as an important energy reservoir [114,115]. Inhibition of fatty acid metabolism and β-oxidation during bovine oocyte maturation resulted in reduced development to blastocysts [116]. Dietary PUFAs could potentially alter the exogenous fatty acids available as an energy supply. Carro et al. [110] examined the uptake and nuclear status of bovine embryos matured *in vitro* with LA. All doses increased triacylglycerol accumulation in the cytoplasm. Low doses (9 and 43 μM) had no effect on the nucleus but the highest dose (100 μM) inhibited germinal vesicle breakdown (GVB), so a much higher proportion of oocytes arrested at the germinal state. Homa and Brown [93] also found that 50 μM LA inhibited GVB. This may be because LA influenced mitochondrial activity and increased ROS concentrations in the oocytes [117]. ALA supplementation to oocyte maturation media increased intracellular cAMP concentration and increased phosphorylation of MAPK1 and MAPK3 and AKT during oocyte maturation in cattle [103], while LA resulted in the opposite effects [108]. Both cAMP and phosphorylation of MAPK in cumulus cells are downstream to G-protein coupled receptors, mainly gonadotrophin, EGF and prostaglandin receptors, suggesting that PUFAs can differentially alter membrane receptor functions in the COCs.

Another possible action is via eicosanoid signalling. The bi-directional cross talk between the oocyte and surrounding cumulus cells is crucial for oocyte development. PGE_2 is an important mediator of both oocyte maturation and cumulus expansion [118-121]. LA and ALA supplementation increased PGE_2 production by bovine COCs during maturation. LA, but not ALA, also increased $PGF_{2\alpha}$ levels to a lesser extent resulting in an overall increase in the PGE:PGF ratio in both treatments. The LA treatment resulted in production of extreme levels of PGs ($\geq$ 20 times) compared to ALA, which may have contributed to the reduced maturation rate in LA-treated COCs [107,108].

Luteal development

After ovulation, the timing of the progesterone rise in the early luteal phase of dairy cows influences the likelihood of successful conception, with less evidence to support a major role for the mid cycle progesterone concentration [122]. Most studies have not found a change in luteal progesterone levels following various types of PUFA supplementation [56,59,123,124]. In one study, however, flaxseed supplementation resulted in higher progesterone levels in the late luteal phase [125] whereas another found a reduction in plasma progesterone associated with feeding either LA or ALA supplements to dairy cows [57].

Luteolysis

As in follicular fluid, dietary changes which alter circulating PUFA concentrations are also reflected in the endometrium [65,126-128]. It has been suggested that n-3 PUFA supplementation may suppress endometrial luteolytic $PGF_{2\alpha}$ production and that this in turn may be beneficial for embryo survival, particularly in cattle [3]. One potential problem is that uterine $PGF_{2\alpha}$ production is up-regulated in normal early pregnancy [129] with IFNτ treatment of bovine endometrial explants increasing both PGE_2 and $PGF_{2\alpha}$ output [130]. The topic was recently revisited by Ullbrich et al. [131], who concluded that up-regulation of PG synthesis in early pregnancy (PGI_2, PGE_2 and $PGF_{2\alpha}$) was a key component of the bi-directional signalling between the endometrium and the embryo. When ALA was fed to non-pregnant sheep the length of the luteal phase was indeed slightly prolonged, but only by about 1 day [132]. Similarly, Zachut et al. [97] found that dairy cows fed a high n-3 PUFA diet exhibited longer intervals from being given a $PGF_{2\alpha}$ injection to manifestation of oestrus behaviour, which delayed the beginning of the subsequent luteal phase. This group of animals also showed a longer duration of their pre-ovulatory oestradiol surge [74].

With respect to the effects on luteolysis, investigators have not measured the release pattern of the PGF metabolite PGFM in the blood during normal luteal regression as this requires taking serial blood samples over a number of days to determine the pattern of pulsatile release. An easier option is therefore to measure OT stimulated PGFM release. The problem with this approach is that the response is crucially dependent on how many OTR are present in the endometrium at the time of treatment. The OTR normally up-regulate on about day 17 of the bovine oestrous cycle [19], but in practice multiparous cows have quite variable cycle lengths (around 19-24 days). Some workers have primed cows with oestrogen, which

424 artificially increases the OTR population. To gain an accurate picture, the test should therefore be
425 repeated on more than one day of a natural cycle and progesterone profiles and baseline PGFM values are
426 required to confirm exactly when in the cycle the tests are performed. Using this approach, Robinson et
427 al. [57] found that n-6 PUFA supplementation increased the PGFM response to OT on day 17 but not on
428 days 15 or 16. In a study of beef heifers, increasing n-3 PUFA concentrations through higher fish oil
429 supplementation produced a clear dose response rise in PGFM on day 15 but this was no longer seen on
430 day 16 of the cycle [65].
431 Gulliver et al. [133] recently reviewed 10 studies where cows received PUFA supplements
432 followed by OT challenge during the oestrous cycle to measure PGFM response. Of these, three studies
433 reported no effect and in three the results differed according to either the progesterone profile or the day
434 of the cycle. Petit et al. [134] found that n-3 PUFAs increased the baseline for PGFM but reduced the
435 response to OT. There were two reports that PGFM release was lower with n-3 supplementation (fishmeal
436 or linseed) [124,135] and two that it was higher after sunflower seeds or Megalac (both high in n-6)
437 [136,137]. This clearly indicates the extreme inconsistency of the responses to n-3 in the diet although
438 there is more agreement for a rise in PGFM after n-6. Gulliver et al. [133] commented on difficulties of
439 interpretation given the variety of supplements used and the lack of FA analysis in the diet and/or plasma
440 in several studies.
441 One study fed beef heifers a fish oil supplemented diet for 45 days then collected tissues on day 17
442 of a synchronised oestrous cycle. The high n-3 diet altered gene endometrial expression of a number of
443 transcription factors, PG and steroid synthetic enzymes and immune regulators [34,127]. Similarly, a fish
444 oil diet altered expression of steroid receptors and PTGS2 in bovine endometrium, also on day 17 [137].
445 The significance of these changes to fertility remains uncertain.
446
447 ***Embryo development and pregnancy rate***
448
449 As PUFA supplemented diets are fed with the intention of improving pregnancy rate, this is a better
450 measure of success than PGFM responses to OT. Much larger numbers of animals are, however, required
451 to achieve statistical significance, so many studies have been under powered. Staples et al. [49]
452 summarized work investigating the effect of fat supplementation on reproductive performance of lactating
453 dairy cows, and stated that eleven out of twenty studies have shown an average of 17% improvement in
454 conception or pregnancy rates. This was achieved using different types of fats: rumen inert fat, fish meal,
455 tallow or prilled fat. In general, pregnancy rates were higher and/or pregnancy losses lower with a high n-
456 3 (ALA or fish oil) supplement compared with high n-6 or saturated fat [3,49,59,65,123,125,138]. On the

other hand, n-6 supplements may reduce pregnancy rates in cattle [139]. Yet other studies have not found any effect of either LA or ALA supplements on pregnancy rates [57,83,125,140-142]. More recently, Lopes et al. [139,143] fed rumen inert PUFA (Megalac-E, 31% LA, 2.7% ALA) to large numbers of *Bos indicus* cattle in two series of experiments to test the effect at different time periods in the 4 weeks after AI or embryo transfer in comparison with saturated fat or kaolin (control) supplements. The PUFA supplement had a consistent positive effect of around 10% on pregnancy rate which was better when fed over a longer time period, particularly after day 14 of gestation. Thus it can generally be concluded that fat supplemented diets can positively improve pregnancy rates in dairy cows when compared to cows receiving isoenergetic diets with no fat supplement and that ALA-rich diets tend to be more efficient when compared to diets rich in LA. The results are, however, inconsistent.

Whilst the majority of studies have focussed on cattle, Chavarro et al. [106] set a food questionnaire to over 18,000 subfertile women trying to establish a pregnancy. They concluded that intakes of total, n-3 or n-6 PUFAs were not associated with the chances of conception in women with ovulatory infertility. Another study fed an evening primrose oil supplement (high in GLA) to blue foxes. Both the conception rate and the abortion rate increased, so there was no overall effect on the number of females producing litters [144]. Other workers have examined the effects of dietary PUFAs on the subsequent pregnancy in pigs. The most common response has been a small increase in litter size. Palmer et al. [145] fed mated gilts fish meal and increased litter size by 0.5 to 1.2 piglets (although other components of the fish meal may also have been beneficial). Two other studies similarly increased litter size by 0.8 and 1.0 piglets respectively when sows received a protected fish oil before mating [146,147]. A subsequent experiment found a similar trend in gilts for survival to Day 25 of gestation but only when the supplementary feeding was continued during early pregnancy [148]. As ovulation rate was not increased, the most likely influence was on embryo survival, a similar situation to the cow.

Transgenic experiments

Some experiments in mice which have used a transgenic approach to manipulating endogenous PUFA production have also examined fertility. Pohlmeier et al. [149] increased the expression of Fat-1 (omega3 fatty acid desaturase), leading to higher synthesis of n-3 PUFA. This led to a decreased litter size from 7.2 to only 2.7 pups. The ovulation and fertilization rates were normal but there were fewer pre-implantation embryos and a higher rate of post implantation absorption. By transferring embryos between transgenic and wild type mice, the authors were able to show that the fault was in the oocyte regardless of the genotype of the female reproductive tract. A study by Stoffel et al. [150] knocked out the enzyme

FADS2 (Δ6 desaturase) thus stopping the onwards conversion of LA and ALA, so mice could not produce their own LCPUFAs. Both male and female mice were infertile. The structure of the testes and ovaries were very abnormal, with breakdown of the blood-testis barrier and disrupted folliculogenesis. Prostaglandin levels in these animals were very low, although they did make some as they acquired small amounts of LCPUFAs directly from the diet. It was possible to restore fertility by supplementing the diet with either AA or DHA/EPA. Both these studies therefore support the need for "normal" PUFA levels to achieve development of fertile eggs within the ovary.

Conclusions

PUFAs have multiple actions within the body which can impact on fertility. Most evidence for specific functions is based on *in vitro* work and this often fails to translate into consistent *in vivo* effects. There are many potential reasons for this but a pervading difficulty is to change concentrations of individual PUFAs and the ratios between them in particular tissues in a predictable manner. While tissue contents do reflect dietary intake, suitable diets which alter the levels to a sufficient extent are hard to devise and variations in metabolic status and health between individual animals will always be important. There is some evidence that n-3 PUFAs in particular can benefit some aspects of fertility, but this requires validation in larger studies to ensure that the supposed benefits are of sufficient size and consistency to be cost effective in practice.

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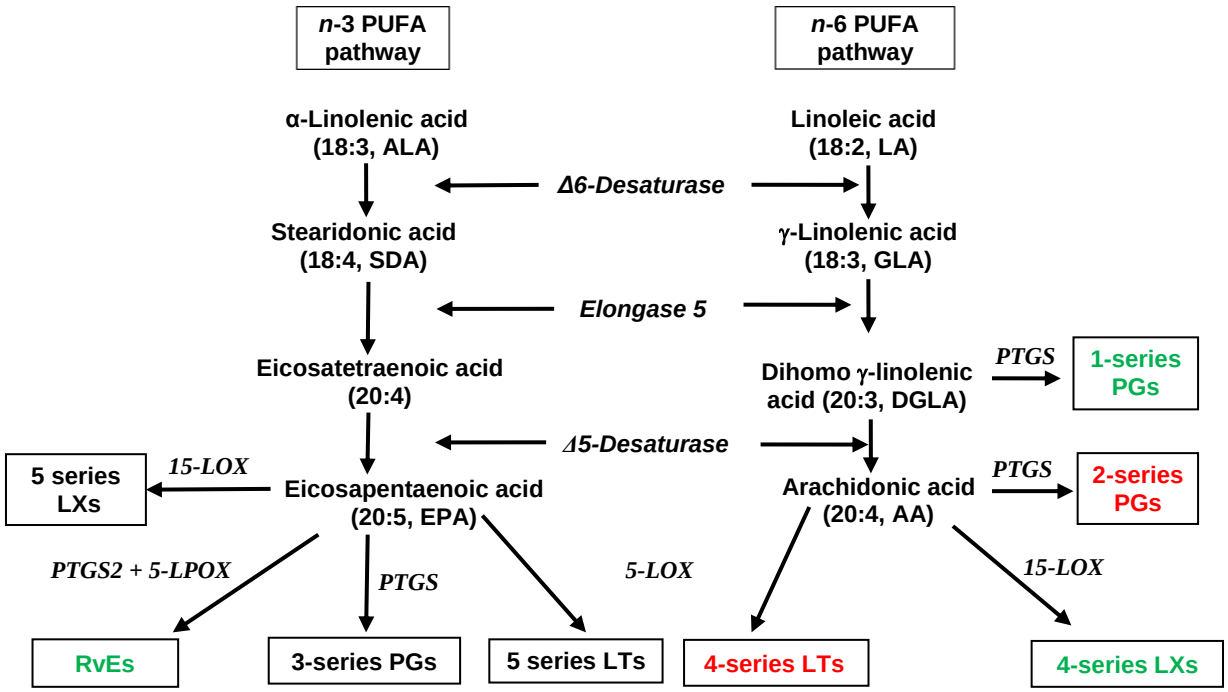
Figure Legend

Figure 1. Diagram showing the metabolic pathways for n-3 and n-6 PUFA metabolism. These lead to the production of lipid mediators with pro-inflammatory, anti-inflammatory and pro-resolution effects: LX, lipoxin; PG, prostaglandin; RvE, resolvin. Enzymes are shown in italics: LOX, lipoxygenase; PTGS, prostaglandin endoperoxide synthase.

Figure


n-3 PUFA pathway
n-6 PUFA pathway
α-Linolenic acid (18:3, ALA)
Linoleic acid (18:2, LA)
Δ6-Desaturase
Stearidonic acid (18:4, SDA)
γ-Linolenic acid (18:3, GLA)
Elongase 5
Eicosatetraenoic acid (20:4)
Dihomo γ-linolenic acid (20:3, DGLA)
PTGS
1-series PGs
Δ5-Desaturase
5 series LXs
15-LOX
Eicosapentaenoic acid (20:5, EPA)
Arachidonic acid (20:4, AA)
PTGS
2-series PGs
PTGS2 + 5-LPOX
PTGS
5-LOX
15-LOX
RvEs
3-series PGs
5 series LTs
4-series LTs
4-series LXs