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Oxylipin profiling by LC-ESI-MS/MS in canine serum and plasma to investigate ovulation-specific changes

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ABSTRACT

New biomarkers that are directly associated with canine ovulation would be of value to ensure mating on optimal days of heat. In this study, canine plasma and serum were analyzed with liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) to quantify a broad range of oxylipins for the purpose of developing a method for biomarker discovery studies in canine reproduction. A majority of the 67 oxylipins probed for were detected at comparable levels in both sample types, but more oxylipins at higher concentrations were detected in serum than in plasma. Nine of the oxylipins were detected in a pilot study of serum at levels that significantly differed ($p \leq 0.1$) between time-points before ($n = 10$), during ($n = 10$) and after ($n = 10$) ovulation, and might serve as putative biomarkers for canine ovulation. One oxylipin (20-HETE) was significantly altered after adjusting for multiple comparisons. In conclusion, the results showed that the LC-ESI-MS/MS method was suitable for quantification of canine oxylipins, revealing important similarities and differences between plasma and serum profiles as well as preliminary ovulation-specific changes in a subset of the investigated oxylipins.

1. Introduction

A responsible breeding of dogs is a prerequisite to achieve sustainable dog breeds for the different tasks in society. Within each dog breed, effort is put on choosing breeding animals that fulfil certain requirements. Because of this, bitches are often several years old before deemed suitable as breeding animals, and the number of years for breeding is limited. Most bitches only come into heat twice a year, and there is consequently a strong desire to optimize each breeding occasion for a maximal chance of pregnancy.

The importance of defining the best breeding date increases with use of artificial insemination (AI) with chilled or frozen-thawed semen, because of the reduced viability of the sperm cells after these treatments [1,2]. The use of AI is important to facilitate a varying genetic background of dogs at a reduced risk of transmission of infectious diseases [3]. The heat in dogs is several weeks, and the length varies between bitches. There are no overt signs related to ovulation. There is a big individual variation also in length of time the bitch allows mating, and bitches may accept the male already several days before ovulation and until after ovulation. The preovulatory luteinization of the follicles, leading to increasing progesterone concentrations in the circulation provides an opportunity to determine the optimal days for breeding by analyzing serum progesterone concentrations [4]. However, several samples are often needed to establish the best days for mating. An alternative is the measurement of luteinizing hormone (LH) which also increases preceding ovulation, and usually is measured using a semi-quantitative test (presence of the LH peak or not). When measuring LH, daily blood samples are recommended. At ovulation, the concentrations of LH have decreased, and the progesterone concentrations vary within a range. Therefore, current techniques for the determination of ovulation are sub-optimal for both the clinic and the canine subject.

Challenges related to dog breeding and determination of ovulation highlight the need for novel tools to enhance the understanding of canine reproduction in general and ovulation in particular. The approach in this study focused on quantification of metabolites derived *via* catabolic pathways in the metabolism of fatty acids. Within these pathways, metabolites collectively known as bioactive lipids, or oxylipins (including eicosanoids), are produced [5]. A limited number of such metabolites have previously been studied in relation to canine reproduction [6–8].

Oxylipins belong to a family of bioactive lipid molecules derived from polyunsaturated fatty acids (PUFA), such as the omega-3 fatty

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acids α-linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), as well as the omega-6 fatty acids dihomo-γ-linolenic acid (DGLA), arachidonic acid (AA) and linoleic acid (LA) [5]. Oxylipins display a wide variety of biological functions and are derived *via* enzymatic pathways that involve enzymes such as lipoxygenases (LOX), cyclooxygenases (COX), and cytochrome P450s (CYP). They can act as both pro-inflammatory and anti-inflammatory mediators depending on the oxylipin species and the context in which it is produced. Oxylipins derived from both omega-3 and omega-6 PUFAs are attributed to pro- or anti-inflammatory effects depending on the metabolic pathway, but the DHA and EPA metabolites studied to date are generally anti-inflammatory in nature [9].

Oxylipins have been extensively studied in mammals, mainly in the context of inflammation [17], but also with regard to reproduction [10,11]. The few select oxylipins that have been studied in relation to canine reproduction have not been evaluated in relation to ovulation [6–8]. Interestingly, ovulation is not only an endocrine event; it is also an inflammatory reaction. The LH surge enhances granulosa cell expression of many key enzymes, leading to increased follicular fluid levels of several eicosanoids known for their association with inflammation and the immune response [12]. Ovulation is associated with increased concentrations of prostaglandins (PG) of the F and E series, and of hydroxyeicosatetraenoic acids (HETE) [13]. PGE_2 is a key ovulatory prostaglandin, involved in several processes related to female ovulation, including cumulus expansion and follicle rupture [14]. An expanded view of oxylipins associated with ovulation requires studies of the entire oxylipin profile in canine samples. In the cow for instance, a rich oxylipin production has been described from the corpus luteum [15]. The oxylipins under study may have diverse biological functions, but they are chemically similar to each other, which necessitates a robust, sensitive and selective analytical method for concentration measurements. In the current study, mass spectrometry (MS) coupled *via* an electrospray ionization (ESI) source to equipment for separation of metabolites based on liquid chromatography (LC) were used for a comprehensive detection of a broad panel of oxylipins.

The overall aim of the study was to investigate changes in oxylipin profiles in relation to canine ovulation and thereby pinpoint potential biomarkers. To reach this goal, normal oxylipin profiles were first studied in both canine serum and plasma collected from dogs that were not at or near ovulation (male dogs and bitches in anestrus). Then, serum was used to identify candidate ovulation biomarkers in bitches before, during and after ovulation as determined by their progesterone levels. Oxylipin levels were measured by an LC-ESI-MS/MS method previously validated for other types of biological samples (e.g. human plasma and serum). To our knowledge, this is the most comprehensive oxylipin profile detected in the circulation of dogs in relation to ovulation presented to date.

2. Materials and methods

2.1. Samples for comparing blood serum and plasma

Three female and three male dogs of the breeds Labrador retriever, Beagle, and Border collie, aged three to seven years were included for comparisons of oxylipin profiles in blood serum and plasma (Table 1). All bitches were in anestrus. Blood samples were collected from the cephalic vein into tubes with EDTA for plasma and without anticoagulants for serum. Samples were centrifuged within 2 h after collection and plasma or serum was separated. From each dog both serum and plasma was stored at −80 °C until analysis.

Table 1
Dogs included in the study. The breed and age of the dogs are provided together with the number of oxylipins detected in plasma and serum, respectively.

			Number of detected oxylipins	
	Breed	Age (years)	Plasma	Serum
Female 1	Border collie	7	46	49
Female 2	Beagle	4	45	49
Female 3	Beagle	3.5	45	49
Male 1	Labrador retriever	4.5	47	51
Male 2	Beagle	3	45	49
Male 3	Beagle	3	41	48

2.2. Samples for studies of peri-ovulatory events

Excess serum samples (n = 30) from clinically healthy bitches of 19 different breeds in heat that visited the University Animal Hospital in Uppsala, Sweden, for breeding management were used for the present study. All samples were collected at rest. Progesterone was analyzed using a chemiluminescence assay (Immulite 2000XPi®, Siemens Healthcare AB, Solna, Sweden). A selection of ten samples from the period preceding ovulation (progesterone concentrations 0.8–4.5 nmol/l), ten samples around ovulation (progesterone concentrations 15.1–21.1 nmol/l) and ten samples after ovulation (37.8–60.1 nmol/l) were analyzed for oxylipins. The samples were stored at − 20 °C for 1–7 months and then moved to − 80 °C until analysis.

2.3. Extraction and quantification of oxylipins in canine plasma and serum

All samples went through a solid phase extraction (SPE) protocol for isolation and concentration of the oxylipins according to Gouveia-Figueira et al. [16] using Waters Oasis HLB cartridges (60 mg of sorbent, 30 μm particle size). The internal standard (IS) mixture containing 500 pg of each IS (12,13-DiHOME-d4, 20-HETE-d6, 12(13)-EpOME-d4, 9-HODE-d4, PGE_2-d4, TXB_2-d4, PGD_2-d4, 5-HETE-d8, 11(12)-EpETrE-d11, and 11(12)-DiHET-d11) was spiked to each sample when loaded onto the SPE cartridge in line with Späth et al. [17]. Oxylipins were eluted with 3 mL acetonitrile followed by 2 mL of MeOH before evaporation and reconstitution in 110 μL methanol. Quantification of each oxylipin was obtained through separation of the analytes by LC followed by detection using a tandem mass spectrometry (MS/MS) instrument. 67 oxylipins were probed for using a validated protocol with an Agilent UHPLC system (Infinity 1290, Agilent Technologies, CA, USA) together with an Agilent 6495 Triple Quadrupole system equipped with an ESI source operating in negative ionization mode [17]. During the analysis, the autosampler was kept at 10 °C. Injection volume was 10 μL and a Waters BEH C18 column (2.1 mm × 150 mm, 1.7-μm particle size) was used for chromatographic separation, held at 40 °C. The mobile phase consisted of: (A) MilliQ water (0.1% acetic acid) and (B) acetonitrile/isopropanol (90/10). The following gradient was used: 10–35% B during 0.0–3.5 min, 40% B during 3.5–5.5 min, 42% B during 5.5–7.0 min, 50% B during 7.0–9.0 min, 65% B during 9.0–15.0 min, 75% B during 15.0–17.0 min, 85% B during 17.0–18.5 min, 95% B during 18.5–19.5 min, 10% B during 19.5–21 min, and 10% B during 21.0–25.0 min at a constant flow rate of 0.3 mL/min. For all analytes, the dynamic multiple reaction monitoring option was used. Two MS/MS transitions were monitored and the most intense was used for quantification purposes, whereas the second was used for qualification purposes. To each native analyte, a deuterated internal standard was assigned based on structural similarities and hence retention times. Transitions together with retention times, instrument settings and assigned IS can be found in Supplementary Table S1. Validation parameters have previously been established [23], and can be found in Supplementary Tables S2 – S4 together with a description of the validation protocol and IS recovery for the current study. The relative standard deviation of the injection standard (CUDA) used for quality control purposes was within 7%. Extraction blanks used as quality control samples did not display any peaks indicative of background levels of the analytes under study, except in a few cases noted in Supplementary Tables S5 and S6. Background levels were taken into

account when determining LOQ for each oxylipin. Separation of critical pairs of analytes, such as PGE_2 and PGD_2, Resolvin D1 and D2, as well as DiHETrEs was previously established by Gouveia-Figueira et al. [18].

2.4. Statistical analysis

Statistical analysis in the study of peri-ovulatory events was performed in Minitab®. Three groups of serum samples were defined based on progesterone levels and referred to as before (0.8–4.5 nmol/l), during

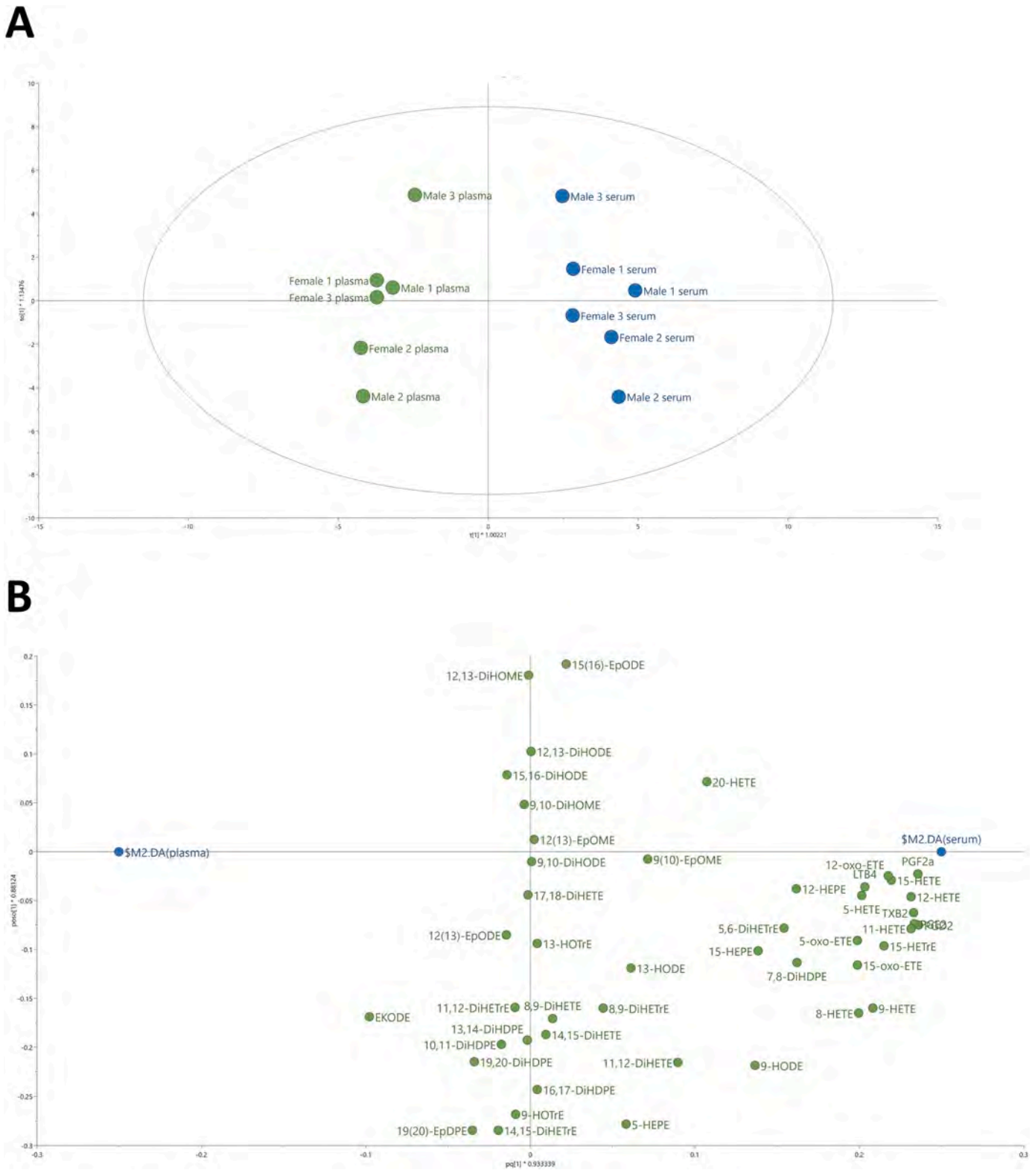

Fig. 1. Score plot (A) and loading plot (B) of orthogonal partial least squares discriminant analysis (OPLS-DA) of oxylipin profiles. The model contained one predictive and one orthogonal component (R2X(cum) = 0.499, R2Y(cum) = 0.955, and Q2(cum) = 0.829). The separation of samples in the score plot (A) are due to the oxylipins shown in the loading plot (B) with emphasis on the oxylipins to the far right being indicative of serum samples.

(15.1–21.1 nmol/l) and after (37.8–60.1 nmol/l) ovulation. The Mann-Whitney U test was applied to compare the oxylipin levels between groups. In this exploratory study, p-values ≤0.1 were considered significant. Kruskal-Wallis and Dunn's post hoc tests were used to adjust for multiple testing. Solely oxylipins with at least 50% of measurements above the limit of detection (LOD) were taken into consideration, and measurements below LOD were substituted with the LOD value (where applicable). Multivariate statistical modelling was performed using SIMCA v. 17.0.2.34594. Data evaluation to compare oxylipins in canine plasma and serum samples was performed in Microsoft Excel 2016.

2.5. Ethical considerations

The study has an ethical permission from the Uppsala Animal Experiment Ethics Board, 5.8.18–17395/2018. All owners gave their informed consent.

3. Results and discussion

3.1. Oxylipin levels in canine serum and plasma

Serum and plasma samples (300–450 μL) from six dogs, three female and three male dogs, were analyzed using a validated LC-ESI-MS/MS protocol to establish normal oxylipin profiles in canine serum and plasma for dogs that were not at our near ovulation. Out of the 67 oxylipins under study, 51 were detected in at least one of the samples and 41 of the oxylipins were detected in both plasma and serum from all six dogs. Individual oxylipin values together with LOD and limit of quantification (LOQ) for each oxylipin are found in Supplementary Table S5. The number of detected oxylipins per dog are summarized in Table 1 and the result from multivariate modelling of the oxylipin plasma *vs* serum profiles is provided in Fig. 1. No obvious differences between female and male dogs were observed, neither with respect to the number of detected oxylipins nor with respect to the identity of the quantified oxylipins. However, in a principal component analysis, the oxylipin profiles found in the Beagle samples were more similar to each other than in the samples collected from other breeds (Supplementary Figure S1).

Comparisons of oxylipin levels showed that most of the oxylipins were detected at comparable levels in serum and plasma, as illustrated in Fig. 2 where the dotted straight line indicates a 1:1 concentration ratio. The ratio, serum *vs* plasma, is also presented in Supplementary Table S5 for all oxylipins. More oxylipins of higher concentrations were detected in serum than in plasma. This was mainly due to AA COX- and AA LOX-derived species. It was observed that LTB_4, PGD_2 and PGE_2 were solely detected in serum, and the levels of TXB_2, $PGF_{2\alpha}$, 11-HETE and 12-HETE were over 100 times higher in serum than in plasma. This was in line with the OPLS-DA findings (Fig. 1B), where e.g. PGD_2, $PGF_{2\alpha}$, PGE_2, TXB_2, 12-HETE, 11-HETE, 15-HETE, 12-oxo-ETE, 15-HETrE, 9-HETE, LTB_4, and 5-HETE were found to the far right in the loading plot.

To the best of our knowledge, the results presented in this study are the first to compare oxylipin profiles in canine serum and plasma. The findings are in accordance with those presented for human serum and plasma [19,20]. COX-derived oxylipins, including TXB_2 and PGE_2 have been reported to increase by 2–3 orders of magnitude in human serum as compared to plasma. LOX-derived 12-HETE and 12-HEPE have also been reported as significantly increased in human serum *vs* plasma [20]. The enzymes responsible for catalyzing the production of these oxylipins are known to be involved in coagulation and thrombosis, and are likely to be activated during serum formation, which may impact the oxylipin profiles. Clotting was prevented using EDTA as anti-coagulant for generation of plasma. In the process of generating serum, higher levels of a subset of the oxylipins were expected due to activation of white blood cells and platelets during blood clotting. Therefore, serum oxylipin levels may reflect *ex vivo* formation of certain metabolites, or the *ex vivo* ability to generate oxylipins.

For studies on canine ovulation, serum (but not plasma) samples were available and therefore analyzed to investigate the method's usefulness for biomarker discovery in canine reproduction in general, and for ovulation in particular. Analysis of oxylipin profiles in serum or plasma has been successfully conducted, even though only plasma oxylipin profiles can be considered to reflect endogenous circulating

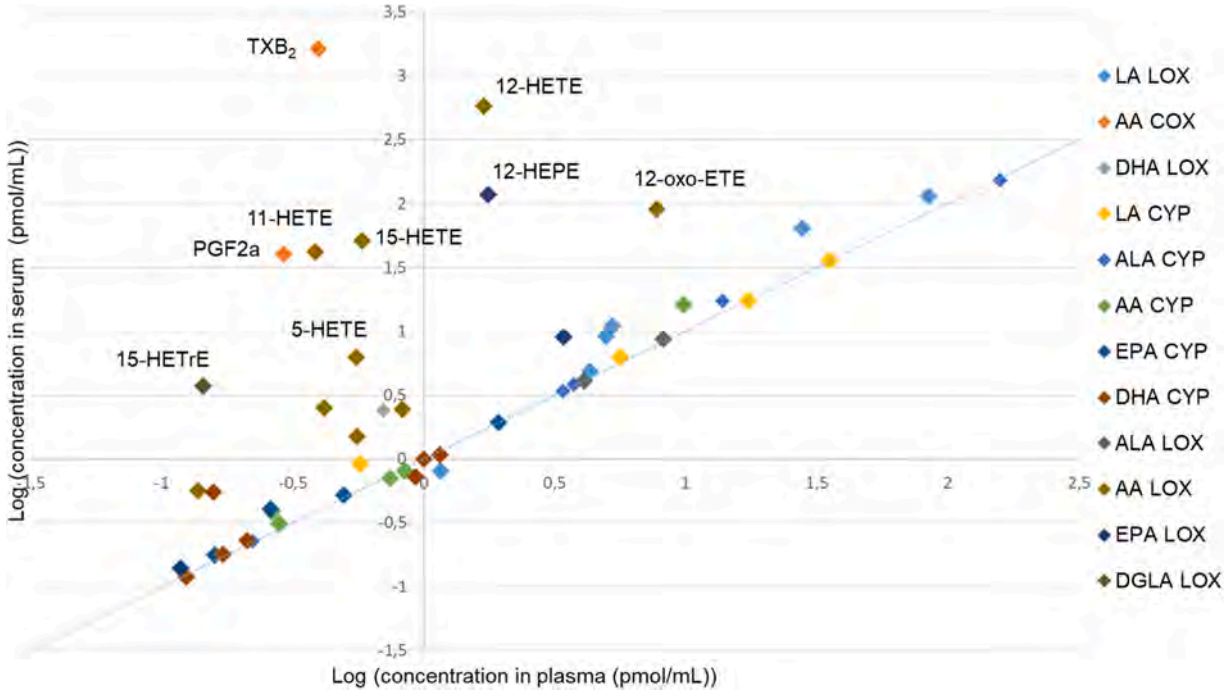

Fig. 2. Oxylipin levels in plasma and serum. Data are plotted on a logarithmic scale using average value of six dogs. The dotted straight line indicates a 1:1 concentration ratio. Most oxylipins were detected at similar levels in plasma and serum. The identities of the oxylipins for which the concentrations differed by more than 10 times are shown in the figure. LTB_4, PGD_2 and PGE_2 were solely detected in serum and are therefore not included.

oxylipins *in vivo*. In studies of serum oxylipin profiles, the coagulation procedure needs to follow a standardized protocol for collection and storage of samples to enable comparisons between groups. In such cases, absolute values will not reflect circulating levels, but group comparisons may be valid as long as the impact of coagulation is addressed by using standardized protocols for sample collection and storage. When choosing sample for analysis, additional factors such as sample availability and the possibility to perform several types of analyses on the same sample set should be taken into account, following the 3Rs in animal research, i.e. Replace, Reduce and Refine.

3.2. Ovulation-specific changes

The ovulation-specific changes in serum oxylipin profiles were determined to pinpoint potential biomarkers. The number of detected oxylipins in 250 – 400 μL serum varied from 50 to 58 between bitches, and 43 of the oxylipins were detected in all samples. In total, concentrations of 53 oxylipins, with values above LOD in at least 50% of the samples, were included in statistical analysis. Levels for individual oxylipins are reported in Supplementary Table S6, including measurements below LOD.

Nine of the oxylipins were detected at levels that differed significantly ($p \leq 0.1$) in comparison to values pre-ovulation, at ovulation and post-ovulation (Fig. 3). The findings provide evidence for the following oxylipins serving as potentially useful serum biomarkers for canine ovulation: 20-HETE, 11(12)-EpETrE, 14(15)-EpETrE, 9-HETE, EKODE, 5-HEPE, 12,13-DiHODE, TXB_2, and PGE_2. Three of these oxylipins were AA CYP-derived metabolites, i.e. 20-HETE, 11(12)-EpETrE and 14(15)-EpETrE. The level of 20-HETE decreased after ovulation to a significant extent ($p \leq 0.05$), see Supplementary Figure S2 for chromatograms, while a significant elevation at ovulation was observed for 11(12)-EpETrE and 14(15)-EpETrE. The significant decrease of 20-HETE was confirmed using a Kruskal-Wallis test ($p = 0.011$) followed by Dunn's post hoc test to adjust for multiple comparisons (before *vs* after, $p = 0.016$; during *vs* after $p = 0.056$).

The AA LOX-derived 9-HETE were elevated at ovulation, and the same trend was observed for EKODE and EPA LOX-derived 5-HEPE. The level of ALA CYP-derived 12,13-DiHODE was increased after ovulation.

Concentrations of the AA COX-derived TXB_2 and PGE_2 were lower at ovulation. However, as previously discussed, the TXB_2 and PGE_2 levels detected in serum differ significantly from those detected in plasma and might reflect the capacity of *ex vivo* formation of these metabolites. This illustrates that the choice of biofluid (serum or plasma) influences the study outcome with regard to oxylipin markers for canine ovulation, in line with an investigation on obesity-related biomarkers that has been performed in plasma and serum [21]. Further studies using methods optimized for detection of TXB_2 and PGE_2 in not only serum, but also plasma (e.g.[19]) are required before conclusions can be drawn on endogenous levels in the peripheral circulation of the dog and thereby the utility of these oxylipins as potential biomarkers in plasma. Since PGE_2 is a key ovulatory prostaglandin and involved in several processes related to female ovulation [14], such methodology are of notable clinical interest.

In the ovary, especially in the preovulatory follicle, synthesis of prostaglandins increases after LH stimulation due to induction of the COX-2 isoform [22]. For example, changes in PGE_2, 9,10-DiHOME, 12, 13-DiHOME, 11,12-DiHETrE and 14,15- DiHETrE have been described in relation to ovulation in porcine follicular fluid, possibly related to follicular maturation [23]. The ratio of 14,15-DiHETrE to 11, 12-DiHETrE reached a maximum at estrus, which correlated with the estradiol concentration. The post-LH concentration of 5,6-DiHETrE showed a similar trend. In the present study, variations were found in the peripheral circulation, which is interesting and opens up for further studies and investigations e.g. of cases of anovulation in dogs.

Strengths of the study include analyses of a broad range of oxylipins in an already collected set of well-defined serum samples. However, limitations with regard to the exploratory nature of the study allows only for indications of changes in oxylipin levels correlating with

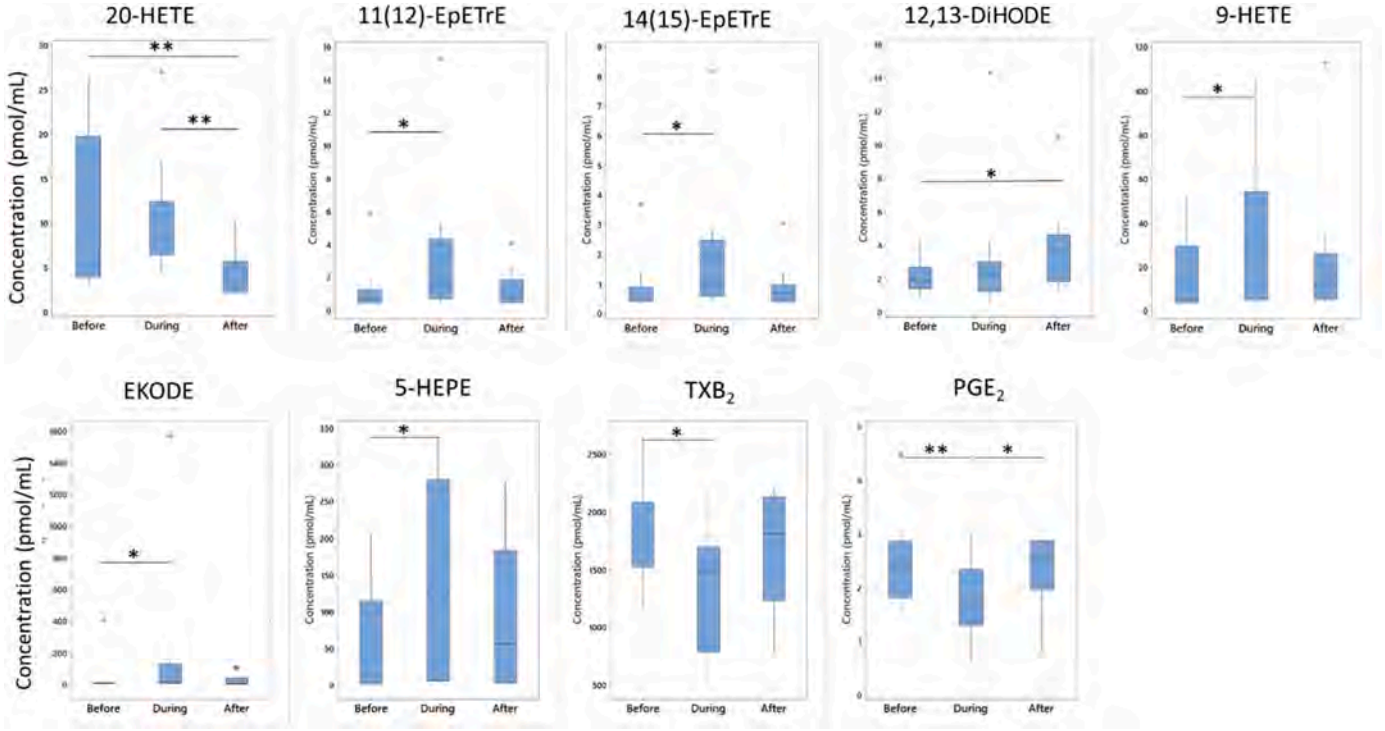

Fig. 3. Comparison of serum oxylipin levels pre-ovulation, at ovulation and post-ovulation. The three groups are referred to as before, during and after ovulation and significant differences based on the Mann-Whitney U test between groups are indicated (*$p \leq 0.1$, **$p \leq 0.05$), n = 10 for each group. Of the oxylipin measurements, only one (for PGE_2) was below LOD. Progesterone concentrations were 0.8–4.5 nmol/l (pre-ovulation), 15.1–21.1 nmol/l (at ovulation), and 37.8–60.1 nmol/l (post-ovulation).

ovulation. Furthermore, endogenous levels cannot be determined for all of the oxylipins by the use of serum. Previous studies of canine oxylipin profiles have shown that urinary eicosanoids may change in response to exercise [24], and that refrigerated storage of canine plasma has an impact on eicosanoid levels [25]. Conditions such as infection [26] and breed-specific vascular dysfunction [27] may also contribute to alterations in oxylipin profiles. These studies highlight that specifically PGE_2, TXB_2, HETEs, DiHETrEs and EpETrEs, several of which were prominent in the current study, are prone to changes in response to a number of naturally occurring circumstances that need to be taken into consideration when designing studies of canine oxylipin profiles. Hence, physical exercise, storage conditions, underlying disease, and breed are among the confounding factors that need to be addressed when the associations between oxylipin biomarkers and ovulation are investigated. In the current study all included dogs were clinically healthy and samples were collected at rest. Dogs of many different breeds were included, to avoid bias from any specific breed. Taken together, LC-ESI-MS/MS profiling of oxylipins in canine serum and plasma may serve as a useful method in carefully designed biomarker discovery studies of canine reproduction, and more research is needed to identify biomarkers for ovulation in dogs.

4. Conclusion

The LC-ESI-MS/MS method in this study has previously been validated for other types of biological samples (e.g. human blood), but never for the purpose of comprehensive oxylipin profiling of plasma and serum collected from dogs. The oxylipin panel included fatty acid metabolites beyond the usual eicosanoids that are derived from AA, and comprised also ALA, EPA, DHA, LA, and DGLA metabolites. The majority of the oxylipins under study were found at comparable levels in both plasma and serum, while a subset was typical for serum. In relation to ovulation, the findings indicated that 20-HETE and eight more oxylipins might serve as candidate biomarkers. Impacted pathways included LOX-, COX-, and CYP-derived oxylipins originating from both omega-3 and omega-6 PUFA precursors, which emphasized the value of analyzing a comprehensive oxylipin profile in investigations of putative biomarkers in future research. Several confounding factors should be taken into account, such as physical exercise, storage conditions, underlying disease, and breed for an optimal study design of future studies.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

Data availability

Data will be made available on request.

Acknowledgements

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.prostaglandins.2023.106790.

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