This item is the archived peer-reviewed author-version of:

Non-esterified fatty acids in early luteal bovine oviduct fluid mirror plasma concentrations : an ex vivo approach

Reference:

Jordaens Lies, Van Hoeck Veerle, De Bie Jessie, Berth Mario, Marei Waleed, Desmet Karolien, Bols Peter, Leroy Jo.- Non-esterified fatty acids in early luteal bovine oviduct fluid mirror plasma concentrations : an ex vivo approach
Reproductive Biology - ISSN 1642-431X - 17:3(2017), p. 281-284
Full text (Publisher's DOI): <https://doi.org/10.1016/J.REPBIO.2017.05.009>
To cite this reference: <http://hdl.handle.net/10067/1461710151162165141>

1 Non-esterified fatty acids in early luteal bovine oviduct fluid mirror plasma concentrations:
2 an *ex vivo* approach

3 Lies Jordaens^a, Veerle Van Hoeck^a, Jessie De Bie^a, Mario Berth^b, Waleed F.A. Marei^a,
4 Karolien L.J. Desmet^a, Peter E.J. Bols^a, Jo L.M.R. Leroy^a

5 ^a *Laboratory for Veterinary Physiology and Biochemistry, Gamete Research Centre, University of*
6 *Antwerp, Universiteitsplein 1, B-2610 Wilrijk, Belgium*

7 ^b *Algemeen Medisch Laboratorium, AML, Emiel Vloorsstraat 9, B-2020 Antwerpen, Belgium*

8 Corresponding author: Lies Jordaens; Laboratory for Veterinary Physiology and Biochemistry,
9 Gamete Research Centre, University of Antwerp, Universiteitsplein 1, B-2610 Wilrijk, Belgium;
10 +32 3 265 23 98; lies.jordaens@uantwerpen.be

11 **Acknowledgements**

12 The authors acknowledge Dr. Mortelmans and co-workers of the Public Abattoir Heist-op-den-
13 Berg for the use of their facility, and Silke Andries for the outstanding technical assistance
14 throughout the experiments.

15 **Abstract**

16 We investigated whether and to which extent plasma non-esterified fatty acids (NEFAs) are
17 reflected in oviduct fluid (OF) using an improved *ex vivo* flushing method. OF and plasma NEFA
18 concentrations were respectively 0.29 ± 0.19 and 0.31 ± 0.14 mmol/L, they didn't differ
19 significantly ($P = 0.13$) and tended to be positively correlated (Pearson correlation coefficient =
20 0.56 ; $P = 0.07$). Results suggest that OF NEFAs mirror the concentrations seen in plasma of healthy
21 cattle.

22 **Key words:** oviduct micro-environment, cattle, maternal metabolism, free fatty acids

Introduction

The oviduct is a complex organ that facilitates oocyte fertilization and the earliest stages of embryo development. In cattle, the early embryo resides in the oviduct for 4 days during which the embryo is highly sensitive to environmental changes [1]. During this early luteal phase in the estrous cycle, the fate of the early embryo may, therefore, be influenced by the composition of the oviductal luminal fluid (OF). OF is essentially an ultrafiltrate from the blood, complemented with estradiol dependent intraluminal secretions of oviductal epithelial cells [2], and follicular fluid (FF) [3]. As maternal metabolic and nutritional state modulate blood and FF metabolites, they may also influence OF composition. Several studies already expanded on OF metabolite composition, but specific information on its fatty acid (esterified and non-esterified) content is still lacking in literature. As such, fatty acids in the embryo environment largely dictate *in vitro* embryo lipid profiles through intracellular fatty acid uptake and metabolism [4]. In this respect, non-esterified fatty acids (NEFAs) are highly relevant, as they are typically upregulated in females suffering from lipolytic metabolic disorders. These elevated NEFA concentrations are shown to directly impact early embryo development, as they affect embryo DNA methylation and transcript patterns [5].

Therefore, we investigated in this study whether and to which extent NEFAs are present in the oviduct micro-environment. Hereto, we aimed to characterize the OF composition for NEFAs in relation to plasma concentrations, during the early luteal phase of the estrous cycle. Other energy substrates, such as cholesterol and triglyceride, were also analyzed, while glucose, lactate, total protein and albumin concentrations were measured for validation purposes of an improved *ex vivo* OF collection protocol. Existing collection methods were adapted to inhibit ongoing glycolysis in OF samples, to maximize OF recovery, and to reduce common confounders. Furthermore, OF and plasma concentrations for these compounds were compared to investigate potential interrelations.

Materials and methods

In a local abattoir, a total of 220 Belgian Blue cows were sampled within a period of 2 weeks. **Blood samples** were collected during exsanguination in sodium fluoride/potassium oxalate Vacuette® blood collection tubes (Greiner Bio-One, Vilvoorde, Belgium), and stored on ice until further processing under laboratory conditions. Within 4h after collection, all samples were centrifuged (10 minutes, 1250 x g, 4°C) for plasma retrieval and individually frozen at -80°C until analysis (< 1 month of collection). Due to retrospective selection, only 49 samples were retained, as plasma samples were linked to specific OF samples selected using strict criteria: i) early luteal oviducts (= day 1-4 after ovulation), selected on ovarian morphology, typically showing a corpus hemorrhagicum, [6]; ii) normal tissue quality (no infectious or anatomical abnormalities), and iii) minimal oviduct length of 25 cm.

For **OF collection**, only oviducts ipsilateral to the ovulation site, were withheld and immediately clipped with a hemostat to avoid compromising of the oviduct lumen. Next, oviducts were surgically trimmed, and both infundibulum and uterotubal junction were removed for standardization purposes. OF-samples (n = 49) were isolated using flushing: hereto, 200µL of flushing medium (i.e. 2-4mg/mL sodium fluoride, 1-3mg/mL potassium oxalate (w/v) (Vacuette®, Greiner Bio-One) and 0.01mg/mL amaranth (w/v) (Sigma Aldrich) in sterile saline (Braun)) was infused in the oviduct via cannulation (Terumo, Surflo winged), after which the ampulla was clipped with a hemostat. The oviduct was gently massaged to mix flushing medium and OF, and subsequently coiled around the hemostat, while applying mild manual pressure on the serosa. All samples were kept on ice until centrifugation (10 min, 1250 x g, 4°C) and snap frozen in liquid nitrogen. Samples were stored at -80°C and submitted to biochemical analyses (<1 month of collection).

During **biochemical analyses**, cholesterol, triglyceride, glucose, lactate, total protein, and albumin concentrations were routinely measured using commercially available photometric assays on the Architect c16000 (Abbott Diagnostics) according to manufacturer's instructions. Total NEFA concentrations were measured using RX Daytona (Randox Laboratories). Inter- and intra-assay

coefficients of variation were < 5%, except for NEFA where the values were 1.8-3.1% and 5.3%, respectively.

The absorbance of amaranth was measured in each sample and performed in triplicate (inter- and intra-assay coefficient of variation < 5%) at 520 nm using 2 µL droplets in a NanoQuant plate and a Tecan microplate reader, Infinite® 200 Pro (Tecan Trading AG), in order to calculate original OF volumes. Plate blanking was performed for each measurement using distilled water, and by subtracting OF background using OF from oviducts flushed with flushing medium without amaranth. Individual amaranth concentrations were calculated by linear equation using a 7 point standard curve ranging from 0 to 0.01 mg/mL amaranth in saline ($R^2 = 0.998$), and used to calculate dilution factors. Sample dilution was used to retrospectively calculate original OF volumes.

Data were expressed as means $\pm$ SD and were analyzed using IBM SPSS Statistics version 23 for Windows, (Chicago, IL, USA). Outliers were identified using the interquartile range rule and omitted from further analyses. Mean differences per biochemical parameter were compared between OF and plasma with a *paired student's t-test* and submitted to *Pearson correlation* testing for all cases. Correlations between the concentrations of energy metabolites and NEFA (i.e. cholesterol and NEFA, triglycerides and NEFA, glucose and NEFA) within plasma or OF were determined via the same tests. Differences with $P < 0.05$ were considered statistically significant; $P < 0.1$ was considered a trend.

Results and discussion

The current protocol was designed to allow early postmortem OF sampling of estrous stage specific oviducts, without sacrifice of experimental animals. *In vivo* OF collection is often confounded through cellular contamination of the samples by erythrocytes, leucocytes and oviduct epithelial cells, low amount of OF recovery and unknown absolute OF volumes. Therefore, specific measures were taken to inhibit ongoing post-collection glycolysis with sodium fluoride, while amaranth was added as a tracer molecule to correct data for variations in OF dilution after

flushing. Amaranth was specifically selected to comply with abattoir regulations and for its wide range of concentration dependent linear absorbance at 520nm, high water solubility, and lacking photosensitivity, pH-dependency, or interference with routine laboratory analyses. Flushing was combined with application of gentle manual pressure to assure thorough sampling of typically viscous OF in extensive anatomically folded oviducts.

Samples from a total of 49 animals were selected for analysis. The average age was 5.4 ± 1.6 years, and carcass weights ranged between 459.2 and 591.2 kg. The interval between blood and OF collection was on average 32 ± 6 minutes. OF samples were on average 32 ± 11 % diluted by the flushing medium, resulting in an average OF volume of 70 ± 24 μ L per oviduct (after retrospective calculation).

Table 1: Concentrations of the biochemical parameters measured in plasma and oviduct fluid (OF) samples ($n = 49$). Values are expressed as means $\pm$ SD. Pearson correlation coefficients and mean comparison with paired t-tests were calculated between plasma and OF concentrations for each metabolite. All metabolite concentrations were significantly different in OF and plasma ($P < 0.01$), with the exception of NEFA concentrations ($P = 0.13$).

	NEFA (mmol/L)	Cholesterol (mg/dL)	Triglycerides (mg/dL)	Glucose (mg/dL)	Lactate (mg/dL)	Tot.protein (g/L)	Albumin (g/L)
OF	0.29 ± 0.19	33.7 ± 16.5	43.8 ± 20.6	43.2 ± 30.2	269.0 ± 103.1	37.84 ± 13.74	n.d.
Plasma	0.31 ± 0.14	124.9 ± 27.3	16.5 ± 6.4	110 ± 31.3	63.1 ± 22.4	79.8 ± 6.1	36.4 ± 2.4
Correlations (P)	0.56 (0.07)	0.29 (0.13)	0.14 (0.73)	0.16 (0.21)	0.28 (0.05)	0.46 (0.42)	n.d.
Paired t-test (P)	0.133	<0.001	<0.001	<0.001	<0.001	<0.001	n.d.

n.d. = below detection limit

In OF, **NEFAs** were on average 0.29 ± 0.19 mmol/L and were similar to the concentrations found in plasma. Plasma and OF NEFA concentrations showed a trend for positive correlation (figure 1, Pearson correlation coefficient = 0.56, $P = 0.07$). These data suggest that plasma NEFAs are reflected in OF, which is the earliest embryonic environment. This might explain why the conditions in the reproductive tract have been shown to be less supportive for early embryo development in metabolically stressed lactating dairy cattle compared to heifers [7] and to non-

lactating cows [8]. *In vitro* studies, reported on the effects of altered environmental conditions during bovine embryo culture, suggested a well-established association between elevated serum NEFAs and hampered blastocyst formation, altered DNA methylation and transcriptomic fingerprints [5], with potentially lasting effects for the offspring [9]. Increased OF NEFAs might add to this complex pathogenesis of in- and subfertility in females suffering from metabolic disorders, such as negative energy balance in high yielding dairy cattle or obesity and type II diabetes in women. Therefore, future research should focus on (changes of) OF NEFAs during metabolic disorders.

The average OF **cholesterol** concentration was 33.7 ± 16.5 mg/dL and cholesterol in plasma versus OF did not indicate any interrelations. Within OF samples, however, a significant positive correlation between cholesterol and NEFA concentrations was observed (table 2). This finding can be suggestive for upregulated NEFA metabolism in oviduct cells and increased conversion to cholesterol as seen in myocardial cells [10]. As such, oviduct cells may facilitate elimination of detrimental NEFAs in the embryo micro-environment in order to safeguard successful early embryo development.

The mean OF **triglyceride** (TG) concentration was 43.8 ± 20.6 mg/dL which was 3 times higher ($P < 0.001$) compared to plasma concentrations. Interestingly, our previous research, using a polarized cell culture system, showed that intracellular lipid accumulation in bovine oviduct epithelial cells was associated with a significant drop in NEFA concentrations in the overlaying medium [11]. As embryos from oocytes exposed to high NEFA levels, shunt fatty acids towards increased triglyceride storage as a protective reflex [12], we propose that the observed correlation between increased OF NEFAs and cholesterol levels might be the result of oviduct cell NEFA uptake, and TG and cholesterol release. This mechanism may also elucidate the marked increase in the OF TGs compared to the concentrations found in plasma samples. Further research, however, is required to confirm this theory.

OF **glucose** concentration was on average 43.2 ± 30.2 mg/dL, which was significantly lower compared to plasma but similar to earlier *in vivo* sampling reports [e.g. 13]. However, no correlation between OF and plasma glucose concentrations could be detected. Leese and Gray (1985) [14] reported that OF glucose is derived from the blood, but is highly metabolized by the oviduct epithelial cells. This may result in significantly decreased glucose and increased lactate concentrations in the OF compared to plasma concentrations, as observed in our samples. Glucose can be toxic for early embryo development, therefore, the low OF glucose concentrations detected in our samples probably meet the natural requirements of the early embryo [15].

Table 2: Comparison of specific energy metabolite versus NEFA concentrations in OF or plasma using Pearson correlation coefficients (*P*).

	Cholesterol	Triglycerides	Glucose
OF	0.83 (<0.01)	0.57 (<0.01)	0.58 (<0.01)
Plasma	-0.22 (0.13)	-0.2 (0.16)	-0.36 (0.01)

Only in OF, a positive correlation could be detected between cholesterol, triglycerides and glucose versus NEFA (table 2). Data suggest that OF is a more tightly controlled environment compared to blood.

Lactate concentrations in OF were 269.0 ± 103.1 mg/dL, furthermore, OF and plasma lactate tended to be positively correlated (Pearson correlation coefficient = 0.28; *P* = 0.05). The current findings exceed the concentrations reported in *in vivo* experiments on average 4 times [13], which is most likely due to ongoing cellular metabolism during the 30 minute interval between exsanguination and sampling.

OF **total protein** concentration was on average 37.8 ± 13.7 g/L. The lacking correlation between plasma and OF protein concentrations may be explained by OF protein originating from both the blood and oviduct epithelial cell secretions. **Albumin** concentrations could not be detected in the OF samples, due to high laboratory detection limits for this specific compound.

Glucose, lactate, total protein and albumin were analysed for validation purposes of the OF collection protocol, by comparing the current concentrations to earlier *in vivo* reports. With the exception of lactate, all parameters were very similar. The current protocol may, therefore, provide a valid alternative for extensive OF sampling.

In conclusion, NEFAs occur in OF and mirror the concentrations seen in plasma of healthy cattle. They may, therefore, affect the oviduct cells and may also alter the micro-environment for pre-implantation embryo development. Future research should focus on OF NEFA concentrations during metabolic disorders, as they may add to the complex pathogenesis of sub- and infertility in high yielding dairy cattle. Other OF metabolites approximated earlier *in vivo* reports which may propose the current protocol as a valid alternative to *in vivo* OF sampling, except when assessment of ions and lactate is intended.

Declaration of interest

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

Funding

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

References

- [1] Latham KE, Schultz R M. Embryonic genome activation. *Front Biosci*, 2001; 6: 748-759.
- [2] Bauersachs S, Rehfeld S, Ulbrich SE, Mallok S, Prella K, Wenigerkind H, Einspanier R, Blum H, WOF E. Monitoring gene expression changes in bovine oviduct epithelial cells during the oestrous cycle. *J Mol Endocrinol* 2004; 32: 449-466.
- [3] Leese HJ. The formation and function of oviduct fluid. *J Reprod Fertil*, 1988; 82, 843-856.
- [4] McKeegan PJ, Sturmey RG. The role of fatty acids in oocyte and early embryo development. *Reprod Fertil Dev* 2012; 24: 59–67.
- [5] Desmet KLJ, Van Hoeck V, Gagné D, Fournier E, Thakur A, O'Doherty AM, Walsh CP, Sirard MA, Bols PEJ, Leroy JLMR. Exposure of bovine oocytes and embryos to elevated non-esterified fatty acid

198 concentrations: integration of epigenetic and transcriptomic signatures in resultant blastocysts. BMC
199 Genomics, 2016; 17:1004.

200 [6] Ireland JJ, Murphee RL, Coulson PB. Accuracy of predicting stages of bovine estrous cycle by gross
201 appearance of the corpus luteum; J Dairy Sci, 1980; 63: 155-160.

202 [7] Rizos D, Carter F, Besenfelder U, Havlicek V, Lonergan P. Contribution of the female reproductive
203 tract to low fertility in postpartum lactating dairy cows. J Dairy Sci 2010; 93 1022-1029.

204 [8] Maillo V, Rizos D, Besenfelder U, Havlicek V, Kelly AK, Garrett M, Lonergan P. Influence of
205 lactation on metabolic characteristics and embryo development in postpartum Holstein dairy cows. J
206 Dairy Sci, 2012; 95 3865-3876.

207 [9] Jungheim ES, Loudon ED, Chi MMY, Frolova AI, Riley JK, Moley KH. Preimplantation exposure of
208 mouse embryos to palmitic acids results in fetal growth restriction followed by catch-up growth in
209 the offspring. Biol Reprod, 2011; 85: 678-683.

210 [10] Lopaschuk GD, Ussher JR, Folmes CDL, Jaswal JS, Stanley WC. Myocardial fatty acid metabolism
211 in health and disease. Physiol Rev, 2010; 90: 207-258.

212 [11] Jordaens L, Van Hoeck V, Maillo V, Gutierrez-Adan A, Marei WFA, Vlaeminck B, Thys S, Sturmey
213 RG, Bols PEJ, Leroy JLMR. Maternal metabolic stress may affect oviduct gatekeeper function.
214 Reproduction, 2016; doi:10.1530/REP-16-0569?

215 [12] Aardema H, Vos PLAM, Lolicato F, Roelen BA, Knijn HM, Vaandrager AB, Helms JB, Gadella BM.
216 Oleic acid prevents detrimental effects of saturated fatty acids on bovine oocyte developmental
217 competence. Biol. Reprod. 2011; 85: 62-69.

218 [13] Hugentobler SA, Humpherson PG, Leese HJ, Sreenan JM, Morris DG. Energy substrates in bovine
219 oviduct and uterine fluid and blood plasma during the oestrous cycle; Mol Reprod Dev, 2008 75: 496-
220 503.

221 [14] Leese HJ, Gray SM. Vascular perfusion: a novel means of studying oviduct function. Am J Physiol,
222 1985; 245: E624-632.

223 [15] Kim JH, Funahashi H, Niwa K, Okuda K. Glucose requirement at different developmental stages
224 of in vitro fertilized bovine embryos cultured in semi-defined medium. Theriogenology, 1993; 39(4):
225 875-886.

226

227

228

229 **Figure 1:** Scatterplot showing the relationship between plasma and OF NEFA concentrations in 44
230 early luteal cows ($R^2 = 0.31$)

Figure 1