The effect of flushing with fatty acid supplementation in ewes ration on folliculogenesis

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Abstract This study aims to evaluate the effect of flushing with different fatty acids docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) from lemuru fish oil in the rations of local sheep on folliculogenesis. Sixteen ewes divided into 4 groups fed rations T0= basal ration; T1= flushing rations containing linoleic without enriched EPA and DHA; T2= flushing rations containing linoleic enriched with EPA and DHA, T3= flushing rations containing linoleic enriched with EPA and DHA with two times from maintenance requirement. Ratio forages and concentrates in the ration were 30%:70%. The parameter observed were body weight gain (BWG), body condition score (BCS), nutrient consumption, corpus luteum, diameter and amount of follicle, blood cholesterol, and blood glucose. The data obtained are analyzed by analysis of variance follow by Duncan Test. The results showed that the treatments did not significant effect to the dry matter consumption, protein, fiber, and total digestible nutrient, but significantly effect to fat consumptions (P<0.05). Flushing treatment for three weeks increased BCS from 0.75 to 0.87. There were significant effect to large follicles, the diameters of large follicle and corpus luteum. Plasma glucose and cholesterol concentrations during the flushing period have significantly different (P< 0.05) among all treatments.

1. Introduction

Local sheep have characteristics that are prolific or the ability to give birth to more than one, non-seasonal breeding, which means that they can mate without being limited to certain seasons and have adapted well to tropical environmental conditions such as in Indonesia. However, this prolific characteristic is often not expressed despite the high mortality in lambs with more than one litter size. Generally, these failure cases occur because of the low nutrient consumed by the parent. This is compounded by the low Body Condition Score (BCS) of the parent. Nutritional deficiencies in the parent can cause Body Condition score (BCS) at the time of mating to be not ideal and the performance of reproduction is reduced. Nutritional deficiencies in livestock can cause delays in puberty, anesthesia, ovulation, low secretion of reproductive hormones such as GnRH, estrogen, FSH, and LH, and inhibition of folliculogenesis [1].

A good feeding strategy is a way to overcome this problem. One strategy to support reproductive performance is flushing. Flushing is an effort to improve the condition of the animal's body by
providing high-quality rations so that the ewe is ready to carry out the process of reproduction (breeding, lambing, and lactating). Flushing can be given to ewes before mating so that when mating the body condition is more optimal. Feeding with content that exceeds the standard daily needs for 2-3 weeks before mating can increase the level of ovulation and fertility [1]. Improved nutrient quality can increase ovulation [2] increasing the percentage of estrus, pregnancy, calving rate, daily milk production of the mother, childbirth weight, early post-partum estrus, calving interval becomes shorter, and improves BCS [3].

Supplementation of fatty acids in the ration can increase fertility and the development of embryo [4]. Fat increases energy density in feed to reduce post-partum negative energy balance, it can also improve milk quality and animal reproductive performance [5]. Supplementation of fatty acids, especially polyunsaturated fatty acids (PUFA), is a strategy to stimulate follicular development and production of steroid hormones [6] also increases plasma progesterone concentration. Polyunsaturated fatty acids especially linolenic acid (omega 3) has a large impact on reproductive performance [7]. Linolenic acid has an inhibitory effect on cyclooxygenase activity and then stops the synthesis of PGF2α [8]. It is known that PGF2α plays a direct role in the corpus luteum (CL) regression and decreased blood progesterone, as a result of preventing pregnancy. However, with the inhibition of PGF2α synthesis, can prevent early embryonic death. [7] state that the benefits of high consumption of omega-3 infertility indicate that ovarian stimulation in a mild level produces only a few follicles, consequently only the best follicles and oocytes develop into embryos. Linoleic supplemented sheep also showed a higher pregnancy rate and twin birth rates compared to sheep supplemented with linoleate [8].

Feed ingredients that are rich in omega 3 (linolenic acid) one of which is fish oil lemuru. Lemuru oil is a local product from fish processing waste so the price is affordable. Lemuru fish oil contains n-3 polyunsaturated fatty acids (PUFA) namely eicosapentaenoic acid 13.70% (EPA) and docohexasonoic acid (DHA) 8.91% [9]. Lemuru oil contains EPA and DHA as much as 22.47%. The use of 1.75% fish oil in goats can increase preovulatory follicles and ovulation rates which are related to the number of kidding rates. furthermore, decreasing E2 and PGFM during the critical period of pregnancy [7]. Supplementation of unsaturated oil of 6% in the lactated sheep diet without protection does not interfere with rumen fermentation, improving the availability of important bioactive components in milk [10]. However, the percent use of EPA and DHA which provides the most optimal reproductive performance results is not yet clearly known. Therefore, it is necessary to further study the effect of EPA and DHA on follicle formation. This study aims to evaluate the effect of flushing with different fatty acid profiles (DHA and EPA) from lemuru fish oil in local sheep feeds on the formation of follicles.

2. Materials and Methods

2.1 Animals and feeding treatment

This study was conducted in March 2019 to August 2019 at Field Laboratory, while sample analysis for blood metabolites was conducted in the Laboratory of Meat and Work Livestock Nutrition, Faculty of Animal Science, IPB University. Sixteen ewes with an average body weight of 21.45±3.41 kg were divided into four treatments (n= 4 per treatment) for the period from the start of estrus synchronization to the end of next estrus cycle (3 weeks). The flushing concentrate was formulated isocaloric (TDN= 75%) and isonitrogenous (CP= 17%). The experimental ewe was fed total mixed ration at the level of 3.5% body weight with forage: concentrate ratio (based on the dry matter) was 30:70, the animal gave feed two times daily and had free access to water. Composition of concentrate 100% dry matter basis has shown on table 1 and nutrient content of concentrate and grass dry matter basis has shown on table 2.
Table 1. Composition of concentrate 100% dry matter basis

| Feed Ingredients   | Treatment |
|--------------------|-----------|
|                    | T0        | T1        | T2        | T3        |
| Soybean meal       | 17.14     | 28.57     | 28.57     | 28.57     |
| Pollard            | 43.57     | 27.86     | 29.26     | 30.26     |
| Dried cassava      | 30.00     | 30.29     | 25.86     | 22.0      |
| Lemuru oil         | -         | -         | 3         | 6         |
| Sunflower oil      | -         | 4         | 4         | 4         |
| Molasses           | 7.14      | 7.14      | 7.14      | 7.14      |
| CaCO3              | 0.71      | 0.71      | 0.71      | 0.71      |
| Premix*            | 0.71      | 0.71      | 0.71      | 0.71      |
| Salt               | 0.71      | 0.71      | 0.71      | 0.71      |

T0 = basal ration, T1 = flushing ration without lemuru oil, T2 = flushing ration contain 3% lemuru oil, T3 = flushing ration contain 6% of lemuru oil

Table 2. Nutrient content of concentrate and grass dry matter basis.

| Nutrient         | Concentrate T0 | Concentrate T1 | Concentrate T2 | Concentrate T3 | Penisetum purpureum |
|------------------|----------------|----------------|----------------|----------------|---------------------|
| Dry matter a     | 85.61          | 85.88          | 86.42          | 87.07          | 24.64               |
| Crude protein a  | 14.45          | 17.05          | 17.06          | 17.06          | 8.93                |
| Crude fat         | 2.03           | 5.60           | 8.64           | 11.66          | 2.52                |
| Crude fiber a     | 4.83           | 3.63           | 6.07           | 5.83           | 26.6                |
| NFE a             | 65.62          | 60.28          | 57.32          | 54.60          | 53.51               |
| TDN a             | 73.51          | 75.76          | 76.04          | 76.49          | 61.89               |
| EPA and DHA b     | -              | -              | 0.67           | 1.34           | -                   |
| Linoleic acid b   | -              | 2.38           | 2.38           | 2.38           | -                   |
| Ca a              | 0.73           | 0.82           | 0.81           | 0.81           | 0.46                |
| P a               | 0.65           | 0.55           | 0.56           | 0.56           | 1.1                 |

T0 = basal ration, T1 = flushing ration without lemuru oil, T2 = flushing ration contain 3% lemuru oil, T3 = flushing ration contain 6% of lemuru oil

aResult of Nutrition and Feed Technology Laboratory, IPB University (2019)

bResult of Integrated Laboratory, IPB University (2018)

2.2 Oestrus synchronization and ultrasonographic

The estrus cycles of the experimental do were synchronized by injecting Dinoprost prostaglandin (PGF2α) hormone preparations at a dose of 5 mg per doe intramuscularly two times with 11 days interval. The experimental goats were observed for the next estrus cycle (day 18 after the first estrus cycle) using a teaser buck. The developments of follicles and corpus luteum were observed using ultrasound (USG) ALOKA model SSD-500 (ALOKA Co.LTD, Japan) equipped with a 7.5 MHz linear probe (ALOKA Co.LTD, Japan). Ultrasonographic scanning of the ovaries was done on the next estrus cycle (day 18 after the first estrus cycle) to ascertain the population of different classes of follicles. The follicles were counted, measured, and classed into 3 categories: (i) small (SF = 2-3 mm); medium (MF = 3.1-5 mm), and large follicles (LF > 5 mm) (Crozet et al. 1995). Further, ultrasonographic scanning was carried out every day until ovulation to assess the number and diameter of POF at estrus. The ovulation rate was determined based on CL number.
2.3 Blood sampling
Blood samples were carried out before and after flushing periods (the day of the next estrus onset), collected at the jugular vein using 5 mL disposable sterile syringe into a glass tube containing anticoagulant (EDTA) to obtain plasma. The blood samples plasma was separated immediately after blood sampling using a centrifuge (3000 rpm for 15 min) and stored in microtubes inside a freezer at −20°C until the time of analysis. Glucose and cholesterol concentrations were analyzed from blood plasma using Glucose kit (Manufactured in Germany for PT. Rajawali Nursindo) Cat No 112191 and Reg No AKL 20101803460.

2.4 Experimental design
The experimental diets included 4 treatments and 4 replications, as follows: P1= basal ration, P2= flushing ration without lemuru oil, P3= flushing ration contain 3% lemuru oil, and P4= flushing ration contains 6% of lemuru oil.

2.5 Data analysis
The experimental was assigned to a completely randomized blocked design. Data of consumption, BCS, the number of large POF, the diameters of large POF and corpus luteum, the plasma glucose and cholesterol concentrations were analyzed statistically with variance analysis and were continued with Duncan Multiple Range Test

2.6 Research flow
Flushing period, time for blood sampling, USG and hormone describe in figure 1.

![Figure 1](image_url)

**Figure 1.** Flushing period, time for blood sampling, USG and hormone

3. Results and Discussion
3.1 Nutrient Consumption
The following average consumption of ewe during flushing presented in Table 1. The value of dry matter consumption, crude protein, crude fiber, and TDN during pregnancy is not influenced by the treatment in the ration, but for fat consumption, it is significantly affected by the treatment. Dry matter consumption during pregnancy ranged from 3.16% to 3.32% of body weight (Table 3). dry matter consumption not significantly different shows that the addition of lemuru oil as a source of EPA and DHA up to 6% in the concentrate did not reduce the palatability of ration.
Table 3. Nutrient Consumption

| Parameters             | Treatment         |
|------------------------|-------------------|
|                        | T0                | T1                | T2                | T3                |
| Dry matter consumption | 929,17±258,04     | 948,50±185,00     | 901±134,19        | 949,54±185,62     |
| (g·e⁻¹·h⁻¹)            |                   |                   |                   |                   |
| Crude protein          | 120,12±31,98      | 141,21±29,88      | 135,27±19,77      | 141,18±27,42      |
| consumption            | (g·e⁻¹·h⁻¹)       |                   |                   |                   |
| Fat consumption        | 36,54±11,97c      | 61,52±11,06a      | 78,55±11,71b      | 103,45±20,16a     |
| (g·e⁻¹·h⁻¹)            |                   |                   |                   |                   |
| Crude fiber            | 62,58±18,15       | 63,61±11,55       | 61,26±9,44        | 63,29±12,45       |
| consumption            | (g·e⁻¹·h⁻¹)       |                   |                   |                   |
| TDN consumption        | 517,52±128,18     | 549,80±126,47     | 532,20±79,22      | 553,39±106,88     |
| (g·e⁻¹·h⁻¹)            |                   |                   |                   |                   |
| DM consumption (%)     | 3,22±0,25         | 3,29±0,69         | 3,16±0,21         | 3,32±0,75         |

Different letters in the same line are significantly different (P<0.05)

The addition of lemuru oil as a source of fat in rations indicated increasing energy density ration in this study did not result in a decrease in the consumption of dry matter. Dry matter, protein and TDN intake has already fulfilled the needs of ewe recommended by [11]. According to [11] the nutritional needs of sheep weighing 20 kg in the initial period of pregnancy with the number of twin embryos namely total digestible nutrient (TDN) 670 g/day, crude protein 103 g/day, calcium 5.4 g/day, and phosphorus 3.3 g/day, at the end of the pregnancy period is TDN 970 g/day, crude protein 150 g/day, calcium 7.7 g/day, and phosphorus 4.1 g/day. The results of this study are in line with [12] which provided canola oil, sunflower oil, and astor oil to sheep did not affect the consumption of dry matter and protein consumption, but markedly increased consumption of fat, crude fiber, and Beta-N.

In line with the addition of oil in the ration can increase fat consumption followed by an increase in consumption of EPA and DHA (Table 1). An increase in fat consumption will indirectly increase the intake of fatty acids that play a role in the biosynthesis of prostaglandins that are important in fertilization and birth processes [12], EPA is a precursor for eicosanoids including prostaglandins. The removal of two double bonds from EPA (20: 5n-3) by prostaglandin H synthase leads to the formation of 3-series eicosanoids [13].

3.2 Body Condition Score

Body Condition Score before treatment was measured and recorded then BCS measured again after being given flushing ration treatment for 21 days. Changes in BCS are calculated from the initial BCS difference before treatment minus the final BCS after treatment. The treatment had no significant effect (P> 0.05) on BCS. BCS data and BCS changes are shown in table 4.

Table 4. Effects of flushing with fatty acid supplementation (DHA and EPA) on BCS.

| Parameters         | Treatment         |
|--------------------|-------------------|
|                    | T0                | T1                | T2                | T3                |
| BCS before flushing| 2,31±0,37         | 2,37±0,25         | 2,37±0,25         | 2,37±0,25         |
| BCS after flushing | 3,06±0,12         | 3,18±0,24         | 3,25±0,28         | 3,12±0,25         |
| ∆ BCS              | 0,75±0,35         | 0,81±0,24         | 0,87±0,25         | 0,75±0,28         |

T0 = basal ration, T1 = flushing ration without lemuru oil, T2 = flushing ration contain 3% lemuru oil, T3 = flushing ration contain 6% of lemuru oil
Although the BCS conditions were not significantly different between treatments, there was a tendency for BCS conditions in animals supplemented with oil. Giving flushing treatment for 3 weeks can improve the condition of BCS ranging from 0.75 to 0.87. With the increase in nutrient intake, especially energy, ewe can reach the ideal BCS so that ewe are ready for reproduction. Animals with high BCS have better ovulation rates than animals with low BCS. Animals that have good BCS are not inhibited in the production of reproductive hormones such as GnRH, estrogen, FSH and LH [1].

3.3 Average daily gain and feed efficiency

Average daily gain and feed efficiency during flushing were not significantly different between treatments. Bodyweight gain before mating is related to the readiness to achieve the ideal BCS before mating. There is no difference in body weight gain during pregnancy in line with the level of food consumption which is also not significantly different. Feed efficiency shows a tendency to increase in P2 and P3, although not significantly different (table 5).

Table 5 Flushing with fatty acid supplementation on average daily gain and feed efficiency

| Parameters                   | T0            | T1            | T2            | T3            |
|------------------------------|---------------|---------------|---------------|---------------|
| BWG (g tail⁻¹day⁻¹)          | 147.73±73.93  | 242.28±151.32 | 218.05±74.49  | 168.63±43.60  |
| Feed Efficiency (%)          | 15.22±5.03    | 24.15±10.43   | 24.76±9.45    | 17.79±2.79    |

T0 = basal ration, T1 = flushing ration without lemuru oil, T2 = flushing ration contain 3% lemuru oil, T3 = flushing ration contain 6% of lemuru oil

The increase is in line with the addition of sunflower oil and lemuru oil in the ration. Ewe that consumed linoleic-rich ration and DHA EPA sourced from sunflower oil and lemuru oil have better feed efficiency compared to controls. This shows that the fat or fatty acids contained in sunflower oil can increase energy intake and improve body metabolism so that good body weight is produced during mating preparation.

Average daily gain (ADG) in this study ranged from 147.73-242.28 with feed efficiency 15.22-24.76, ADG was greater than the research conducted on sheep fed flushing rations containing sunflower seed oil 99-121 with feed efficiency 13-28% [14]. Average daily gain in this study was also higher than sheep ADGs reported in sheep fed with sunflower seeds and flaxseed 26-49 g / e / h with feed efficiency of 5-11.5 [15]. The high daily body weight gain can help prospective parents more quickly get the ideal BCS in preparation for pregnancy and lambing. It also shows that the fat or fatty acids contained in lemuru oil can increase energy intake and improve body metabolism so that good body weight is produced.

3.4 Blood Metabolite

Blood metabolites can describe the nutrients that are absorbed and distributed throughout the body through blood vessel tissue. The treatment flushing ration significantly increased the concentration of glucose and cholesterol and blood glucose in ewe (P<0.05). The total protein and cholesterol in the blood of the goats are presented in table 6.

Glucose is the main energy source that will affect energy sufficiency during the pregnancy phase. In this study, the plasma glucose levels before treatment were not significantly different. Then after being treated for three weeks glucose levels were significantly different (P<0.05) influenced by flushing treatment. Both before and after treatment, glucose levels contained in sheep blood plasma are still in the normal range. The normal range for healthy sheep is 44-81.18 mg/dl [16]. The highest plasma blood glucose P4 treatment was 70.97 ± 3.73 mg/dL, then P3 and P2 69.84-70.45 mg/dL, and 51.93 mg/dL (control).

Glucose levels are significantly different in line with the consumption of crude fat which is also significantly different for each treatment. Glucose levels are relatively increased and normal as an increase in the amount of lemuru oil in the ratio indicates the contribution of feed fat which is hydrolyzed into fatty acids and glycerol, where glycerol is one of the ingredients to produce propionate and is useful as a source of glucose in the process of gluconeogenesis. Glucose level is related to the level of release of the hormone FSH and LH which also plays a role in the process of ovulation,
according to the statement of [17], that glucose derived from propionate is the main energy source for the work of the hypothalamus in the process of egg maturation, through the regulation of FSH and LH until ovulation and corpus luteum formation.

Table 6. Flushing with fatty acid supplementation on glucose and blood cholesterol in sheep

| Parameters      | T0             | Treatment | T1           | T2           | T3           |
|-----------------|----------------|-----------|--------------|--------------|--------------|
| Glucose Before flushing (mg/dL) | 66.04 ± 2.06   | 68.32 ± 1.03 | 66.62 ± 4.38 | 66.69 ± 3.96 |
| Glucose flushing (mg/dL)          | 51.91±2.63 b   | 63.13±3.41 ab | 69.06±2.74 ab | 70.97±3.73 *  |
| Cholesterol Before flushing (mg/dL) | 69.73 ± 1.88   | 69.84 ± 2.28 | 70.43 ± 1.85 | 68.67 ± 6.05 |
| Cholesterol flushing (mg/dL)     | 66.25±5.13 b   | 71.05±8.17 ab | 74.61±10.57 ab | 78.02±4.89 *  |

Different letters in the same line are significantly different (P<0.05)

Cholesterol is a precursor to the formation of steroid hormones which has an important role in animal reproduction. Cholesterol levels of sheep obtained in the study are presented in Table 2. The treatment has a significant effect on blood cholesterol levels (P<0.05). Cholesterol levels at P4 higher than other treatments. Cholesterol levels were significantly different in line with the consumption of crude fat that is also significantly different. Fatty acids consumed will be used as a form of cholesterol in the blood. The availability of cholesterol which tends to increase is closely related to the status of hormones formed because cholesterol is a precursor of the synthesis of steroid hormones such as progesterone, cortisol, corticosterone, and estradiol [18]. before mating, cholesterol is needed for the synthesis of steroid hormones which play a role in the preparation of estrus. [19] states that fat supplementation in the ration can increase cholesterol concentrations which can further lead to an increase in the synthesis of progesterone which is important during pregnancy.

3.5 Follicles
The main component of diet that stimulates folliculogenesis is energy, in particular glucose, although energy derived from fatty acid oxidation also appears to be important. Total follicles, diameter follicles, and diameter CL are presented in table 7.

Table 7. Flushing with fatty acid on large follicles and diameter of follicular and corpus luteum

| Parameters                  | T0     | Treatment | T1     | T2     | T3     |
|-----------------------------|--------|-----------|--------|--------|--------|
| Total follicles             | 1.25±0.50 | 1.50±0.57 | 1.5±1.00 | 1.75±0.50 |
| Diameter follicles (mm)     | 5.37±1.11 | 6.00±0.41 | 6.29±1.15 | 5.68±0.50 |
| Diameter CL (mm)            | 5.90±0.67 | 6.07±0.32 | 6.32±0.62 | 6.17±0.35 |

T0 = basal ration, T1 = flushing ration without lemuru oil, T2 = flushing ration contain 3% lemuru oil, T3 = flushing ration contain 6% of lemuru oil

The total number of small, medium and large follicles is measured every week through observations with ultrasonography. The results show the treatment had no significant effect (P>0.05) on total follicles, follicular diameter, and CL diameter. However, there is a tendency for the greater diameter of the follicles resulting from the addition of flushing treatment. In the ewe, short-term nutritional supplementation stimulates folliculogenesis [20] and increases ovulation rate [21]. These results are in line with the research in sheep where short-term energy supplements stimulate folliculogenesis, only 30–50% of treated ewes will convert increases in follicle number to increased ovulation rate. In cattle, energy supplements also stimulate folliculogenesis but this is rarely converted into increased ovulation rate [1]. The diameter of the follicle in the treatment given flushing ration ranged from 5.68 to 6.00 mm, this result is higher than the average yield of follicles ovulated in arrowroot sheep is 5.5 - 7 mm [22]. The CL diameter in this study ranged from 5.90 to 6.32 mm, this figure is by the average CL normal of 5.8-6.5 mm [22]. Growth and regression of the corpus luteum are related to the profile of the hormone progesterone during one cycle in sheep [23],[24].
4. Conclusion
Flushing diet with high EPA and DHA (6% lemuru oil) can increase BCS, cholesterol and glucose concentration, but still not improved the number and diameter of follicle. The cholesterol in flushing treatment is important as a precursor for improving the reproductive system. Plasma glucose also important as an energy source during pregnancy.

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