Effects of Lemuru Fish Oil (Sardinella Sp.) on Estrous Response, Hormonal Profile and Conception Rates in Garut Ewes

Aeni Nurlatifah1,3, Herdis Herdis1, Jean-Baptiste Menassol2, Lilis Khotijah3, Raden Iis Arifiantini4, Mashitah ShikhMaidin3, Dewi Apri Astuti3 and Pradita Iustitia Sitaresmi1

1Research Center for Animal Husbandry, National Research and Innovation Agency, Jalan Raya Jakarta-Bogor, 16915, Indonesia; 2SELMET, Institut Agro Montpellier, CIRAD, INRAE, Univ Montpellier, F-34000 Montpellier, France; 3Department of Nutrition and Feed Technology, Faculty of Animal Science, IPB University, Bogor, 16680, Indonesia; 4Department of Veterinary Clinics, Reproduction and Pathology, Faculty of Veterinary Medicine, IPB University, Bogor, 16680, Indonesia; 5Institute of Tropical Agriculture and Food Security (ITAFos), Universiti Putra Malaysia, 43400, Malaysia

ABSTRACT

The aim of this study was to determine the effects of short-term feed supplementation of Lemuru fish oil on the reproductive performance of 20 primiparous Garut ewes. For this purpose, the effects of four diets were compared: a control diet (C) and three diets with various oil supplements. These diets were: 6% palm oil (P1), 3% palm oil with 3% Lemuru fish oil (P2), and 6% Lemuru fish oil (P3). Experimental ewes were subjected to estrous synchronization using two injections of PGF2α 11 days apart. The hormonal profiles and blood metabolites were monitored for animals of each group. Estrous expression was observed at mating, and embryo counts were recorded individually using trans-retal ultrasonography 20 days after mating. Results showed that plasma concentrations of cholesterol were significantly (P<0.05) higher for P2 and P3 (44.90±10.51 and 49.81±14.37 mg/dL, respectively) than control and P1 groups. Similarly, blood estradiol concentrations were significantly (P<0.05) higher for P3 than P1 and P2 groups before estrus, while during estrus P3 had higher blood estradiol than control group (P<0.05). All treatment diets significantly enhanced the onset of estrus in comparison with control animals (P<0.05), while receptivity was higher for P3 (21.80±2.86 times) than for control group (P<0.05). The proportion of ewes pregnant after the first estrus was the highest for P3 (100%) compared to all other diets. However, there was no effect of diets on the number of embryos. In conclusion, feed supplementation of Lemuru fish oil improves reproductive performance of ewes following natural mating by stimulating onset of estrus and increasing pregnancy rates, making Lemuru oil an attractive feed supplement.

INTRODUCTION

The reproductive performance of our animals is still quite low in many developing countries with large human population such as Indonesia; which adversely affects the success of livestock farming. Meeting livestock nutritional requirements is important to improve their productive and reproductive performance, increase livestock population and improve food security (Makela et al., 2022). Nutrition acts on the reproductive axis through two components, the static and dynamic effects of nutrition, which are mediated through different neuro-hormonal pathways (Abecia et al., 2006). In sheep, short-term feed supplementation or flushing mobilizes the dynamic components of nutrition to act directly on the ovary and improve folliculogenesis (Scaramuzza et al., 2006). This effect is mediated through metabolic hormones and growth factors, including insulin, IGF-I, leptin and growth hormone (GH), acting within the follicular environment to modulate gonadotrophin-stimulated folliculogenesis (Scaramuzza et al., 2006; Alves et al., 2019). Thus, flushing practices aim at boosting the nutritional sensing...
pathways that act on the functioning of the ovaries. When performed at the timing of reproduction, this can be viewed as an effective on-farm technique to improve reproductive performances in small ruminants.

Among the various feed and nutritional supplements that can be used in a flushing program, Lemuru fish oil is of particular interest. According to Kosashik et al. (2021), Lemuru fish oil contains 20 to 26% of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Like other oils, Lemuru fish oil is an energy-dense supplement and considered as an economically competitive alternative source of omega-3 fatty acids. Thus, in goats, a diet containing 1.75% fish oil increased the number of pre-ovulatory follicles, an effect mediated by Omega-3 fatty acids (Hammiche et al., 2011). According to Kia and Safdar (2015), Omega-3 fatty acids rich diets inhibit cyclooxygenase activity and inhibit the synthesis of prostaglandins (PGF2α), thereby preventing early embryonic death. Accordingly, sheep diet supplemented with Omega-3 fatty acids showed higher pregnancy and twin birth rates than sheep diet supplemented with linoleate (Kia and Safdar, 2015). Moreover, feeding Lemuru fish oil as source of Omega-3 fatty acids in ewes can produce twin lambs with good growth performances (Nurlatifah et al., 2022). However, the specific effects of nutritional flushing with Omega-3 fatty acids rich supplements containing EPA and DHA on estrous behaviour and pregnancy rates in ewes are not clearly known. Therefore, this study was conducted to evaluate the effect of Lemuru fish oil flushing at the timing of reproduction on estrous response, hormone profiles and pregnancy rates in Garut ewes.

**MATERIALS AND METHODS**

**Experimental animals and diets:** The current research protocol was approved by the Animal Care and Use Committee (ACUC) at IPB University No. 119-2018 IPB. This study was conducted during the period from October 2022 to March 2023. The animals included in the study were 20 primiparous Garut ewes, aged 12-14 months, with a body condition score (BCS) of 2.69±0.10. These ewes were divided into 4 treatment groups (n=5 per group) on the basis of their live weight (LW), as follows: 28.84±2.52kg (control group), 29.26±2.65kg (P1 group), 28.50±1.91kg (P2 group) and 28.88±2.61kg (P3 group). Ewes were kept in individual pens equipped with feeding and water troughs.

The base daily diet for the ewes consisted of Napier grass (*Cenchrus purpureus*) and concentrate. Daily feed amount was adjusted to 3.5% of individual LW with a 30:70 ratio of Napier grass to concentrate, based on DM, as recommended by the NRC (2007). This diet remained unchanged for the control group (Group C) with protein content of 14.79% and TDN of 71.34%. Flushed groups received additional concentrate feed designed to be isoenergetic and isoproteic with total protein content of 17% and TDN of 75%. The different experimental groups were then subjected to different dietary treatments as follows: P1=Flushing concentrate with 6% palm oil, P2=Flushing concentrate with 3% palm oil and 3% Lemuru fish oil, and P3=Flushing concentrate with 6% Lemuru fish oil. Flushing period began 14 days before mating and continued till 14 days after mating (28 days in total). Drinking water was provided *ad libitum*.

**Estrous synchronization:** Synchronization of estrus was performed by an intramuscular injection of prostaglandin-F2α (Lutalyse®, Zoetis, US) at a dose of 2.0 mg dinoprost per animal. It was administered using the double injection method, with an interval of 11 days between each injection (Sözüblir et al., 2006).

**Estrous observation, mating and pregnancy diagnosis:** Experimental ewes were closely monitored daily for onset of estrus after the 2nd PGF2α injection. Estrous observations were conducted by placing ewes and ram in the same flock. The estrous response was calculated from the number of estrus ewes in one treatment group. The onset of estrus was taken as the time period (hours) when the ewe showed first estrus symptoms and accepted the male for mating after the 2nd PGF2α injection. The duration of estrus (hours) was calculated starting from the first expression of estrus symptoms until the ewe refused to accept the male for mating. Receptivity was determined as the number of mounts one ewe received from the start until the end of its estrous period.

Ewes in estrus were allowed to mate with fertile rams of good semen quality. At the timing of estrus, one fertile ram was presented with 4 ewes (one from each treatment group). Using trans-rectal ultrasonography on day 20 after mating, ewes that were diagnosed as non-pregnant were presented to the ram during their next estrus. In this way, mating of non-pregnant ewes was allowed for three successive estrous cycles.

Pregnancy diagnosis and the number of embryos per ewe were determined by trans-rectal ultrasonography 20 days after mating, using the ultrasound scanner ALOKA model SSD-500 (ALOKA Co. LTD, Japan) equipped with a 7.5 MHz linear-array probe.

**Blood collection and hormone assays:** Blood samples were collected at 3 days before estrus (assuming that estrus would occur 48 hours after the 2nd PGF2α injection), estrus day, and then 3, 6, 9, 12 and 15 days after estrus. Blood was collected from the jugular vein in the morning before feeding using sterile tubes that contained EDTA as an anticoagulant. Blood samples were immediately centrifuged at 3000 rpm for 10 minutes (4°C) and plasma was stored at 4°C until used for hormones assay. The estradiol and progesterone assays were carried out following the procedure provided by the company providing the commercial ELISA kits (DRG International Inc., Germany). Plasma estradiol concentrations were determined using the estradiol ELISA Kit No. 1907 with recovery standard of 99.84%. Plasma progesterone concentrations were determined using the progesterone ELISA Kit No. 60126 with recovery standard of 97.97% and R² of 0.99%. Minimum detection limit (sensitivity) for progesterone was 0.045 ng/ml and for estradiol it was 9.714 pg/ml. Inter-assay and intra-assay CV for estradiol was 2.14 to 6.02% and for progesterone it was 6.98 to 8.35%.

**Blood metabolites:** For blood metabolites (glucose and cholesterol) determination, samples were collected from
the jugular vein of each ewe using EDTA tubes one day before mating in the morning 2 hours after morning feeding. Blood samples were immediately centrifuged at 3000 rpm for 10 minutes to collect plasma. Analysis of total blood glucose was conducted using glucose Kit (Cat. No. 112191, Greiner, AU), while total cholesterol Kit (Cat. No. 101592, Greiner, AU) was used for blood cholesterol analysis. Enzymatic Kit techniques and a spectrophotometer at 546 nm (Genesys 10S UV-Vis, USA) were used to measure blood metabolites concentrations.

**Experiment design and data analysis:** The experiment followed a randomized complete block design (RCBD), taking body weight as block due to its CV of >10%, which might affect nutrition consumption and influence reproductive performance. The percentages of pregnant ewes per group were analyzed using ANOVA and the differences between treatments were tested using the Duncan’s multiple range test. All analyses were performed using the SAS software and differences were considered as significant whenever P<0.05. Data are presented as mean±SEM.

### RESULTS

**Feed intake:** There was non-significant differences between experimental groups in terms of total dry matter intake, crude fiber, nitrogen free extract and total digestible nutrients (Table 1). However, ewes of groups P1 and P2 showed significantly higher (P<0.05) crude protein intake compared to control and P3 groups, the difference between the latter two groups was non-significant. Crude fat intake was significantly lower in control group than the other treatments, while it was the highest in ewes of groups P1 and P2 (P<0.05).

**Body condition score:** Compared to control, all three short term nutritional supplementations using palm oil or Lemuru fish oil for four weeks did not affect BCS (Table 2). However, BCS after supplementation increased by 7.5-13.5% compared to BCS before supplementation.

**Blood metabolites:** While there was non-significant difference between groups in terms of plasma glucose concentrations, plasma cholesterol concentrations were higher (P<0.05) in P2 and P3 groups compared to control and P1 groups (Table 3). However, differences in plasma cholesterol levels between P2 & P3, as well as control & P1 groups, were non-significant.

**Steroid hormones profiles:** Short-term nutritional supplementation significantly affected (P<0.05) plasma estradiol levels (Fig. 1). The highest (P<0.05) plasma estradiol levels were found for the animals in the P3 group. However, 3 days before estrus, this difference was only significant when compared with the animals of the P1 and P2 groups and, at the timing of estrus, it was only significantly different when compared with the animals from the control group. When plasma progesterone was considered, there was non-significant difference among all four groups on different days before and after estrus (Fig. 2).

**Estrous expression and mating behaviour:** The characteristics of estrous expression are presented in Table 4. There were non-significant differences between groups in the proportion of estrus response and duration of estrus. However, the onset of estrus was significantly earlier for the animals in P1, P2 and P3 groups when compared with the animals in the control group (P<0.05). The flushing treatments increased the receptivity to the males in comparison with animals in the control group, but this difference was only significant (P<0.05) for the animals in the P3 group.

**Pregnancy rate and number of embryos:** The pregnancy rate cumulated over three consecutive estrus cycles (Fig. 3) was high in this study, with variable results between the experimental groups. While the animals in the P1 and P2 groups had a pregnancy rate of 80% (at the second and first estrus cycles, respectively), the animals in the control and P3 groups showed pregnancy rate of 100%. This pregnancy rate was reached at the first estrus cycle for the animals in the P3 group and at the third estrus cycle for the animales in the control group. The effects of our experimental treatments on the number of embryos observed by ultrasonography at 20 days after mating were non-significant (Fig. 4). However, number of embryos tended to be slightly higher for the animals in the groups supplemented with fish oil (P2 and P3) compared to animals of control and P1 groups.

**Table 1: Nutrient intake in ewes of each experimental treatment**

<table>
<thead>
<tr>
<th>Consumption (g/head/d)</th>
<th>Control</th>
<th>P1</th>
<th>P2</th>
<th>P3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry matter</td>
<td>857.7±25</td>
<td>922.6±75.38</td>
<td>886.8±44.32</td>
<td>804.9±39.87</td>
</tr>
<tr>
<td>Crude protein</td>
<td>110.0±8.00</td>
<td>139.7±11.5</td>
<td>136.1±6.49</td>
<td>119.2±5.40</td>
</tr>
<tr>
<td>Crude fat</td>
<td>12.6±0.93</td>
<td>57.6±8.1</td>
<td>53.6±2.49</td>
<td>46.2±1.90</td>
</tr>
<tr>
<td>Crude fiber</td>
<td>141.5±10.65</td>
<td>146.4±11.68</td>
<td>139.9±7.88</td>
<td>133.5±8.25</td>
</tr>
<tr>
<td>Nitrogen free extract</td>
<td>557.3±40.57</td>
<td>614.4±5.30</td>
<td>589.6±28.63</td>
<td>527.5±24.71</td>
</tr>
<tr>
<td>Total digestible nutrient</td>
<td>497.5±3.22</td>
<td>476.3±9.06</td>
<td>457.6±22.55</td>
<td>414.3±19.94</td>
</tr>
</tbody>
</table>

**Table 2: Effects of the experimental treatments on body condition score before and after supplementation in ewes**

<table>
<thead>
<tr>
<th>Body condition score</th>
<th>Control</th>
<th>P1</th>
<th>P2</th>
<th>P3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before supplementation</td>
<td>2.9±0.18</td>
<td>2.8±0.20</td>
<td>2.8±0.12</td>
<td>2.6±0.10</td>
</tr>
<tr>
<td>After supplementation</td>
<td>2.9±0.18</td>
<td>3.0±0.17</td>
<td>3.0±0.12</td>
<td>2.8±0.10</td>
</tr>
</tbody>
</table>

C=Control; P1=Supplementation concentrate with 6% palm oil; P2=Supplementation concentrate with 3% palm oil and 3% Lemuru oil; P3=Supplementation concentrate with 6% Lemuru oil. Values with different superscripts in the same row indicate significant difference due to different flushing treatments (P<0.05).
DISCUSSION

Total DMI did not differ in ewes of different group in this experiment, which indicates that oil supplementation of diet did not affect its palatability. The total DMI in this study met the recommendations of NRC (2007). Since the protein content of the flushing diets was higher when compared with the control diet (17% vs 14%), the higher protein intake in these groups was expected. However, this effect was only evident for the P1 and P2 groups, probably because the animals in the P3 group tended to have relatively lower dry matter intake. The total protein intake in this study also met the recommendations of NRC (2007). In terms of crude fat intake, animals in the control group showed significantly lower crude fat intake than animals in the flushed groups. Since the flushing treatment was a mix of concentrate feed and oil supplement, this result was expected. As previously stated, significantly higher (P<0.05) levels of crude fat intake for animals in P1 and P2 groups compared with the animals in the P3 group could be explained by the tendency of the animals in this latter group to have lower dry matter intake.

The mean BCS before supplementation was lower than the recommended target BCS of 3.00 in all groups at the timing of reproduction in sheep (Maurya et al., 2009). Other studies in sheep suggest that animals with BCS ranging from 3.00 to 3.50 show high reproductive performance (Sejian et al., 2010). However, higher BCS may show adverse effects on ovulation, embryo loss and...
conception rates, which in turn affect pregnancy success (Kenyon et al., 2014). According to Scaramuzzi et al. (2006), a low BCS at the timing of nutritional supplementation is expected to increase the positive effects of flushing on ovulation rate. There was a trend for the animals within each experimental group to gain BCS during the study that could be explained by our experimental conditions despite the fact that flushing protocols were not designed to increase the BCS or body weight of the animals.

The higher plasma cholesterol concentrations in ewes of P2 and P3 groups than those of control and P1 groups is likely due to the nature of the oil used for supplementation. Indeed, contrary to vegetable-based oils, animal-based oils like Lemuru fish oil, contain cholesterol-type sterols (Clifton, 2002). High levels of plasma cholesterol are important to support the biosynthesis of steroid hormones involved in the growth of follicles, estrous behaviour and ovulation (Schade et al., 2020). Cholesterol is known to be a precursor to the biosynthesis of steroid hormones such as progesterone, cortisol, corticosterone, and estradiol (Strauss and FitzGerald, 2019).

The high plasma concentrations of estradiol for the animals in the P3 group is in line with the high plasma cholesterol levels recorded for the animals in this same group, and could be due to the high-fat content of EPA and DHA in the feed (Mirzaei-Alamouti et al., 2018). Similarly, higher plasma concentrations of estradiol were reported when a diet supplemented with 3% fish oil was compared with a diet supplemented with sunflower seed oil and calcium soap from palm oil in Afshari sheep (Mirzaei-Alamouti et al., 2018). However, a decrease in plasma estradiol levels following the administration of 0.6 mL/kg BW of fish oil was found in goats (Mahla et al., 2017). According to Verma et al. (2018), administration of fish oil in low (<0.3 mL/kg feed) doses increases the blood estrogen response but medium and high (0.6-1.2 mL/kg feed) doses induce a decrease in blood estrogen levels in goats. In our study, the doses of fish oil could be considered as high, since these reached 2.1 and 4.2 mL/kg feed in the diets of P2 and P3 groups, respectively. Altogether, these differences might be attributable to differences in oil concentrations and species-specific responses. Our data emphasizes that the addition of fish oil at the appropriate dose in the diet promotes a favorable estrogen response compared to vegetable oil and a control situation.

Plasma estradiol concentrations exhibited a drastic increase between the two blood sample collections in animals of flushed groups. A similar increase in plasma estradiol concentrations in ewes supplemented with fish oil has been previously reported (Mirzaei-Alamouti et al., 2018). The fact that this increase was observed in all flushed groups, including the animals in the P1 group that had a vegetal-based oil supplementation, would argue in favor of an interaction between oil supplementation and the lipid metabolism, thereby boosting the secretion of ovarian steroids (Meza-Villalvazo et al., 2018). This argument is favored by the higher levels of crude fat intake by animals in these groups compared to animals of control group, as fatty acids are crucial in steroidogenesis (Khajeh et al., 2017). Indeed, fatty acids are involved in the control of estrogen levels in livestock (Zeng et al., 2023).

Non-significant difference in plasma progesterone concentration was reported in sheep given 3% fish oil (Mirzaei-Alamouti et al., 2018) or supplemented with flaxseed oil four days before mating (Soydan et al., 2020). Altogether, this suggests that the nutrient-sensing pathways involved in mediating the effects of flushing on the ovarian functions, in this case concentrate as well as vegetal and animal-based oils, did not interfere with the pathways controlling progesterone secretion.

In this experiment, onset of estrus was earlier for the animals in the supplemented groups when compared with the animals in the control group. This highlights the fact that nutritional supplementation can influence the dynamics of follicular development. Habibzad et al. (2015) found that nutritionally supplemented sheep showed earlier symptoms of estrus in comparison with the animals given the control diet. Furthermore, providing high energy diets for a short period of time can stimulate follicular growth and ovulation (Zabuli et al., 2010; Alves et al., 2019; Domingues et al., 2020).

The present study showed that flushing treatments did not affect the duration of estrus, which is in alignment with previous reports in sheep fed moderate and high nutrition (Farrag, 2019). The flushing treatments with 6% Lemuru oil (P3 group) increased the receptivity to the males compared to control group. This supports the fact that animals in this group had higher levels of plasma estrogen concentrations during estrus when compared with the animal in the control group. Indeed, estrogen contributes to the establishment of mating behavior (Juengel et al., 2020). It has been established that this effect is mediated through central pathways, where estrogen stimulates catecholaminergic neurons involved in the central control of the mating behavior (Wersinger and Rissman, 2000). Another plausible pathway involves the influence of estrogen on the production of pheromones (Bakker et al., 2010). Therefore, the increased plasma estradiol concentrations for the ewes in the Lemuru oil flushed group could have increased their attractiveness (Rekwot et al., 2001).

The pregnancy rate at first estrus was highest in P3 group, which coincides with our results for estradiol concentrations at estrus and receptivity to males. Similar effect was previously observed in fat-tailed Iranian Afshari ewes supplemented with fish oil (Mirzaei-Alamouti et al., 2018). The number of embryos did not differ among flushed and non-flushed groups in this experiment, although it tended to be higher for the animals in the groups supplemented with fish oil (P2 and P3 groups). Similarly, Mahla et al. (2017) reported that goats given fish oil tended to have a higher number of embryos than those given palm oil but this difference was not statistically significant.

Conclusions: A short-term nutritional flushing including supplementation with vegetal (palm oil) or animal-based (Lemuru fish) oils improves reproductive performance of ewes following natural mating after estrus synchronization. However, this response was further improved when using animal-based oil supplementation, especially the Lemuru fish oil. These results highlight the
positive effect of specific components of animal-based oils, like DHA and EPA, and suggest that Lemuru fish oil constitutes an interesting feed supplement for ewes. It could be an interesting suggestion to increase the reproductive performance of sheep, specifically in Indonesia where this supplement is readily available and can be locally-produced.

**Conflict of interests:** The authors declare that they have no conflicts of interest.

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**Authors’ contributions:** AN, LK, RIA, MS and DAA conceived the idea and designed the study. AN, RIA, HH and DAA executed the experiment and analyzed samples. HH, AN, PIS and JBM analyzed the data. HH, AN, JBM and PIS prepared the manuscript. JBM, HH, RIA, AN, PIS, LK, MS and DAA reviewed the manuscript.

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