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Suggested Reviewers:

Revision Note

Title: " The different effects of linseed and fish oil supplemented diets on insulin sensitivity of rabbit does during pregnancy"

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Authors: Menchetti L., Canali C., Castellini C., Boiti C., Brecchia G.

To the Editor-in-Chief
Research in Veterinary Science

We thank the editor and referees for providing constructive comments and help in improving the contents of our paper. The paper has been strongly revised by full professional revision and the bibliography has been updated.

The file “Manuscript PUFA-IR_rev3_highlighted” shows the revisions.

Specific comments

Reviewer #5:

Dear Authors,

The manuscript entitled "The different effects of linseed and fish oil supplemented diets on insulin sensitivity of rabbit does during pregnancy" is an interesting and improved paper, but English is too poor and difficult to read, therefore needs to be improved. I suggest to have it professionally revised.

A complete revision of the English language was carried out by two English mother tongue specialized translators (Eleanor Fabri and Dr Alda Quattrone).

Highlights

1. We evaluate the effects of n-3 PUFA supplementation during pregnancy in rabbit
2. Linseed supplementation negatively influences gestational insulin resistance
3. Fish oil supplementation improves insulin sensitivity
4. Rabbit's BW, before and during pregnancy, is related to insulin resistance indices

The different effects of linseed and fish oil supplemented diets on insulin sensitivity of rabbit does during pregnancy

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Abstract

This study investigates the effects of linseed (rich in α-linolenic acid (ALA)) and fish oil (rich in eicosapentaenoic (EPA) and docosahexaenoic acid (DHA)) supplementation on the insulin resistance of pregnant rabbits. Two months before insemination, the rabbits (15 animals/group) were fed different diets: commercial standard (group C), supplemented with 10% extruded linseed (group L), and 3% fish oil (group FO). The L group does showed both the highest feed intake before AI ($P<0.01$) and the highest body weight (BW) throughout pregnancy ($P<0.001$). The L does yielded less milk than the C does ($P<0.001$); however, no differences were observed in either weight or size of litter at weaning. Regardless of diet, insulin concentrations and HOMA-IR values were higher during the first half of pregnancy ($P<0.001$). Nevertheless, the L does showed higher mean insulin concentrations than FO rabbits ($P<0.01$) and the lowest glucose clearance ($P<0.01$) during pregnancy. On the other hand, pregnant FO rabbits showed the lowest glucose concentrations ($P<0.05$) and the lowest Homeostasis model assessment values for insulin resistance (HOMA-IR, $P<0.05$) as well as a faster restoration of baseline glucose levels following glucose load ($P<0.001$). Before and during pregnancy, the BW of the rabbits was positively related to fasting sample- and tolerance test-derived indices of insulin resistance ($P<0.05$) suggesting that a high pre-pregnancy BW predisposes to gestational insulin resistance. Linseed supplementation increased BW and predisposed to insulin resistance during pregnancy; whereas, fish oil improved insulin sensitivity without significant changes in BW.

Key words: insulin sensitivity; ALA, EPA and DHA; HOMA-IR; gestational diabetes; reproductive performance; rabbit

1. Introduction

Using n-3 polyunsaturated fatty acids (n-3 PUFAs) as functional foods became popular when the low incidence of cardiovascular disease in Eskimo population (Bang et al., 1980) was attributed to their fish - rich diet which is high in both EPA (20:5n-3) and DHA (22:6n-3). These long-chain PUFAs can be obtained indirectly from other sources, such as linseed which is rich in ALA (18:3n-3). In fact, humans and animals are able to convert ALA into EPA and DHA through a series of elongations and desaturations. Δ-desaturases are the rate-limiting enzymes in both n-3 and n-6 PUFA metabolisms (Das, 2005). n-3 PUFAs are generally associated with anti-inflammatory and cardioprotective effects (Djoussé et al., 2012; Oh et al., 2011; Soulimane-Mokhtari et al., 2008). Moreover, fatty acids provide energy, are structural and functional components of cell membranes, and affect signal transduction pathways as well as gene transcription.

Intakes of ALA or preformed EPA and DHA may have specific and potentially independent effects on physiological and pathological processes; indeed, dietary ALA has multiple metabolic fates, and the conversion efficiency to its longer chain counterparts is generally poor (Burdge, 2006; McCloy et al., 2004).

The effects of n-3 PUFA on reproduction have been evaluated mainly in ruminants. However, conflicting results were obtained for *in vivo* dietary supplementations, while the effects on pregnancy rates and embryo survival are still not fully known (for a general review, see Wathes et al., 2007). To our knowledge, only one study has been carried out regarding the effect of n-3 PUFA supplementation on the reproductive performance of rabbit does (Rebollar et al., 2014).

Conversely, the effects of EPA and DHA on metabolic diseases of humans and animals are well documented; for example, rodents and rabbits fed with EPA and DHA supplements showed reduced body weight and insulin resistance (Ivanova et al., 2014; Pérez-Matute et al., 2007). Several meta-analyses confirm an association between fish oil supplementation and reduced risk of type 2 diabetes in

humans (Djoussé et al., 2012; Zhang et al., 2013). However, these studies have some limitations. Firstly, differential effects of EPA/DHA and ALA on disease susceptibility have not been fully elucidated despite the potential differences in their metabolism. Secondly, the role of n-3 PUFAs in insulin sensitivity during pregnancy remains largely unknown. A Cochrane meta-analysis found no significant effects of fish oil supplements on pregnancy complications in women (Makrides et al., 2006). Finally, although the rabbit is a suitable model for studying reproductive disorders and the influence of nutrition during pregnancy (Brecchia et al., 2014, 2006, Menchetti et al., 2018, 2015a, 2015b), strategies for improving gestational insulin resistance in this species have not yet been evaluated.

In order to fill these gaps, we evaluated the effects of supplementation with two different sources of n-3 PUFAs, extruded linseed and fish oil, on insulin sensitivity of rabbit does during pregnancy. To this purpose, both fasting sample-derived indices of insulin resistance and intravenous glucose tolerance tests were used.

2. Materials and methods

2.1. Animals and diets

A total of 45 multiparous (third to fifth parity order) hybrid rabbit does were housed at the experimental farm of the Department of Agricultural, Food and Environmental Sciences of the University of Perugia. The does were kept individually in flat deck cages; the temperature ranged from +15 to +28 °C, and the light schedule was 16 L:8 D. Two months before artificial insemination (AI), the rabbits were randomly assigned to one of three groups (15 animals/group): C group was fed with commercial standard diet, L group with 10% extruded linseed supplementation (Nlest®, Valorex), and

FO group with 3% fish oil supplementation (Nordos ®).Vitamin E (200 mg/kg feed, synthetic alpha-tocopheryl acetate) was included in all diets. The feeding program of the rabbit does was continued until weaning (35th day post-partum). Lactation was controlled until day 20 post-partum by opening the nest once a day for 5-10 minutes. After the 20th day of lactation, the nest was opened and the kits were given access to solid food. The feed intake of the rabbit does was recorded daily until the end of controlled lactation (20th day). The diets (Table 1 and 2) were isoenergetic, isonitrogenous, and formulated according to the current dietary recommendations for rabbit does (De Blas and Mateos, 1998). The rabbit does received 130 g/d of food from the beginning of the experiment until the day of AI; subsequently, they had *ad libitum* access to food.

Ovulation was induced by injecting 0.8 μg of synthetic GnRH (Receptal, Hoechst-Roussel Vet, Milan, Italy) just before AI (Brecchia et al., 2006). AI was performed with 0.5 ml of diluted fresh semen. The day of AI was named day 0. Pregnancy was diagnosed by manual palpation 10 days after AI and non-pregnant does were submitted to successive AI after 21 days. Two cycles of AI were performed in order to achieve 10 pregnancies per group.

2.2 Body weight and reproductive performance

From the beginning of the experiment until weaning, the BW of overnight fasted rabbits was recorded weekly between 8:00 and 10:00 A.M.. The following productivity indices were calculated: fertility (number of parturitions/number of inseminations x 100), prolificacy (total number of born and stillborn kits per doe), litter weight at kindling as well as perinatal and pre-weaning mortality. In addition, litter size and weight were recorded at days 20 and 35 (weaning). The perinatal period

102 comprised the first 48 h after parturition. The pre-weaning mortality rate was calculated as the
103 percentage of weaned kits/litter size following the perinatal period. Daily milk production was
104 measured from parturition until day 20 of lactation by weighing the doe immediately before and after
105 suckling.
106
107 **2.3. Fasting sample-derived insulin resistance indices**
108 *2.3.1. Blood sampling and measurements of insulin and glucose*
109 Blood samples were taken from 10 randomly selected fasted pregnant does/group between 8:00 and
110 10:00 A.M at days 0, 7, 14, 21, and 28 after AI. Blood samples were collected from the marginal ear
111 vein and placed in EDTA containing tubes. The tubes were centrifuged at 3000xg for 15 min, and the
112 plasma was stored frozen until assayed for hormones and metabolites.
113 Plasma insulin concentrations were determined by the double antibody technique using an insulin
114 IRMA kit (Immunotech s.r.o., Prague, Czech Republic). The limit of sensitivity was 1.35 μU/ml and
115 intra- and inter-assay coefficients of variations were 4.0 and 4.8%, respectively. Glucose was analysed
116 with the hexokinase method using the Glucose GLUC-HK kit from RANDOX (Randox Laboratories
117 Limited, Country Antrim, UK). The limit of sensitivity was 0.71 mmol/l, and intra- and inter-assay
118 coefficients of variations were 3.6 and 5.1%, respectively.
119
120 *2.3.2 Determination of fasting-derived blood indices*
121 Fasting-derived blood indices were steady-state measures of insulin resistance that did not require
122 the administration of exogenous glucose, which included fasting *Glucose Concentrations* (in mmol/l),

123 *Insulin Concentrations* (in μU/ml) as well as mathematical models based on these measures. These
124 models included *Glucose-to-Insulin Ratio* and *Homeostasis model assessment for insulin resistance*
125 *(HOMA-IR)* calculated as follows: [insulin concentration x (glucose concentration/18)]/22.5 (Menchetti
126 et al., 2015a).
127
128 **2.4. Glucose Tolerance test-derived indices**
129 *2.4.1. Intravenous glucose tolerance test*
130 Intravenous glucose tolerance tests were performed on 5 randomly selected does/group between
131 8:00 and 10:00 A.M. on day 21 of pregnancy. The rabbits were fasted overnight (at least 16 h). A
132 single bolus of glucose (0.6 g/kg of body weight) was rapidly infused into the ear marginal vein
133 through an 18G catheter. A small drop of capillary blood was collected by puncturing the ear just
134 before glucose administration and subsequently after 5, 10, 30, 60, and 120 min. Blood glucose
135 (mmol/l) was measured with a calibrated glucometer (OneTouch ® UltraEasy, LifeScan Europe,
136 Johnson & Johnson, Zug, Switzerland), using test strips from the same supplier. The inter-assay
137 coefficient of variation for glucose was < 5%.
138
139 *2.4.2. Determination of kinetic parameters of glucose*
140 The pharmacokinetics of the glucose load was analysed using a one-compartment open model. The
141 *elimination rate constant* was calculated from the slope of the line during the elimination phase by
142 linear regression analysis of the semilogarithmic plot of glucose concentration versus time. The *half-*
143 *life* of the exogenous glucose load was obtained as follows: (ln 2 /elimination rate constant); the

144 *apparent volume of distribution* was obtained as the ratio between dose and plasma concentrations after
145 bolus administration; whereas, the *clearance* was obtained as the volume of distribution x elimination
146 rate constant. The *area under the concentration-time curve* (AUC) was calculated by the linear
147 trapezoidal method using GraphPad Prism version 5.01 software (Inc., San Diego, CA, USA).
148
149 **2.5. Statistical analysis**
150 The data were analysed with the linear mixed model procedures. In these models, the animals were
151 treated as the random factor; whereas, the cycle of AI and time (day pre or post AI) were treated as
152 repeated factors. The models evaluated the effect of time (9 and 5 levels during pre- and post- AI,
153 respectively), group (3 levels: C, FO, and L), and interaction between time and group. In order to
154 evaluate the responses to the glucose tolerance test, the time factor (minutes after glucose
155 administration) had 6 levels: 0, 5, 10, 30, 60, 120 min after loading. In models including BW as
156 predictor and indices of insulin resistance as dependent variables, the b-parameters were estimated in
157 order to investigate the relationship between BW and insulin resistance. In order to limit the influence
158 of longitudinal changes in the BW of does caused by foetal growth, the stage of pregnancy (3 levels: AI
159 = day 0; Early-mid pregnancy = days 7 and 14; Late pregnancy = days 21 and 28) was included in the
160 model used for analysing the relationships between BW and fasting sample-derived indices. Only
161 group effect was considered to evaluate reproductive and kinetic parameters. The number of kits was
162 included as covariate whenever appropriate. Parity order was not included as a covariate as the
163 preliminary analyses did not show any significant effect. Diagnostic graphics were used to check
164 assumptions and outliers. Logarithmic transformations were used whenever appropriate (insulin

concentration, glucose-to-insulin ratio, HOMA-IR). The results were expressed as estimated marginal means ± standard error (SE). The logarithms were back transformed. Chi-square tests were used to analyse fertility and mortality rates. Statistical analyses were performed with SPSS Statistics version 20 (IBM, SPSS Inc., Chicago, IL, USA). We defined $P \leq 0.05$ as significant and $P < 0.1$ as a trend.

3. Results

3.1. Feed intake, BW, and productive performance

The BW of does at the beginning of the experiment was 4.07±0.05 kg in C, 4.04±0.05 kg in L, and 4.12±0.05 kg in FO groups. From the beginning of the experiment until AI, feed intake was higher in L group than in C and FO groups ($P < 0.01$; Table 3). The BW of all rabbits increased during the 2 months prior to AI (time effect $P < 0.05$); however, the weight gain was higher in L group (group effect $P < 0.001$). The rabbits of L group were heavier at AI than at the beginning of the experiment (+0.25±0.11 kg; $P < 0.05$). Their BW at AI was also higher when compared to the rabbits in the other groups (+0.25±0.11 kg; $P < 0.05$; Table 3).

Following AI, feed intake was unaffected by either dietary regimen or litter size; however, it was affected by the physiological phase ($P < 0.001$; Table 3). From AI until day 27 of pregnancy, the mean daily feed intake was 150±0, 144±2, and 144±2 g for C, FO and L groups, respectively. During the last three to four days before parturition, feed intake decreased by 13%. Feed intake increased progressively during the first 20 days of lactation from 128±8 to 318±8 g/d in all groups ($P < 0.001$) and was affected by litter size ($P < 0.001$; Table 3).

186 The BW during pregnancy differed between groups ($P < 0.001$; Fig. 1) with higher mean values
187 observed in L does ($P < 0.001$) than in C and FO groups.
188 During the trial, three does (one from group C and two from group FO) died or were excluded due
189 to severe health problems. However, no differences were found between groups regarding the mortality
190 rate of does (6.7%, 13.3% and 0.0% in C, FO and L groups, respectively; $P = 0.762$).
191 Fertility did not differ between groups (Table 3). The length of pregnancy did not differ among
192 groups (Table 3) although it was negatively related to litter size ($b = -0.21$, $P < 0.001$). Perinatal
193 mortality was higher ($P < 0.001$; Table 3) in C and FO groups than in L group due to the death of 2
194 litters in group FO and 1 litter in group C. No differences were observed for both weight and size of
195 litter between groups (Table 3). Milk yield was lower in L than C group (Table 3) while there were no
196 differences either in weight or size of litter at day 20 of lactation and at weaning (Table 3).
197
198 **3.2. Insulin sensitivity**
199 *3.2.1. Fasting-derived indices*
200 Plasma glucose concentrations were affected by time ($P < 0.01$), group ($P < 0.001$) and interaction
201 time x group ($P < 0.01$; Table 4). Plasma glucose concentrations ranged from 4.2 to 10.0 mmol/l, and
202 decreased from early (7.2±0.2 mmol/l at day 7 of pregnancy) to late pregnancy (6.2±0.2 mmol/l at day
203 28 of pregnancy; $P < 0.001$). Glycaemia during pregnancy was lower in FO group (6.2±0.1 mmol/l)
204 than in C (+9%, $P < 0.001$) and L (+5%, $P < 0.05$) groups. Pairwise comparisons for each day showed
205 significant differences on days 7 and 21 of pregnancy (Table 4).

206 Insulin concentrations ranged from 2.0 to 26.9 μU/ml and were affected by time ($P < 0.001$), group
207 ($P < 0.01$), and interaction time x group ($P < 0.05$; Table 4). Insulin concentrations were higher in mid
208 pregnancy (12.0±0.5 μU/ml at day 14) than in late pregnancy (3.9±0.2 μU/ml at day 28; $P < 0.01$).
209 Mean insulin concentrations during pregnancy were higher in L group (7.9±0.3 μU/ml; +32%, $P <$
210 0.01) than FO group (5.4±0.2 μU/ml). Pairwise comparisons showed significant differences at days 14
211 and 28 of pregnancy (Table 4).

212 The glucose-to-insulin ratio was affected by time ($P < 0.001$) as it was lower on day 14 (0.6±0.1)
213 and higher on day 28 (1.8±0.1) than at AI (1.1±0.1; $P < 0.01$). It was also affected by group x time
214 interaction ($P < 0.01$; Table 4). In early pregnancy (day 7), the glucose-to-insulin ratio was lower in L
215 group, while at the end of pregnancy (day 28) the ratio was higher in FO than in C group (Table 4).

216 During pregnancy, HOMA-IR values (Table 4) were affected by group ($P < 0.01$) and by time as
217 they were higher in early- and mid-pregnancy than in late-pregnancy ($P < 0.001$). A trend was observed
218 for time x group effect ($P < 0.1$). Does belonging to FO group had lower estimated marginal means of
219 HOMA-IR during pregnancy (0.08±0.01) than C (-38%, $P < 0.05$) and L groups (-62%, $P < 0.01$).
220 Pairwise comparisons on each day of this study showed lower HOMA-IR in FO at days 7 and 14
221 compared to L, and at day 28 compared to C group (Table 4).

222 Parameters of models including BW as predictor and fasting-derived indices as dependent variables
223 showed a significant positive relationship between the BW and plasma insulin concentrations of does
224 (log transformation: $b = 0.22$, $P < 0.05$) and HOMA-IR (log transformation: $b = 0.23$, $P < 0.01$; Fig. 2).

225

226 *3.2.2. Glucose Tolerance test-derived indices*

227 Regardless of nutritional regimen, the intravenous administration of glucose caused a rapid increase
228 in blood glucose concentrations. Glucose reached the highest value 5 min post-administration ($P <$
229 0.001) and returned to baseline values at 60 min.

230 The rabbits belonging to FO group showed lower glucose concentrations 60 min post-loading than C
231 (+14%, $P < 0.001$) and L (+14%, $P < 0.001$) groups (Table 5). The rabbits of the L group showed
232 higher glucose half-life than C does (+10%, $P < 0.05$) and the lowest clearance ($P < 0.01$; Table 5). The
233 elimination-rate constant was affected by group ($P < 0.05$) and tended to be lower in pregnant L does
234 than in C does (-11%; Sidak correction: $P < 0.1$). A trend of differences was found in AUC ($P < 0.1$
235 Table 5).

236 In order to investigate the relationship between BW and insulin resistance measured with the
237 glucose tolerance test, we calculated the parameters of models including BW at AI or at day 21 of
238 pregnancy as predictor and test-derived indices as dependent variables. Significant relationships were
239 observed between BW at day of test (day 21 of pregnancy) and some glucose tolerance test-derived
240 indices: the higher the value of BW, the higher the values of AUC ($b = 219.1$, $P < 0.05$; Fig. 3) and
241 maximum concentration of glucose ($b = 5.3$, $P < 0.05$), while clearance decreased ($b = - 0.2$, $P < 0.1$).
242 When BW at AI was included in the model, significant relationships were found with AUC ($b = 259.6$,
243 $P < 0.01$; Fig. 3) and maximum glucose concentration ($b = 5.6$, $P < 0.05$).

244

245 **4. Discussion**

246 For the first time in rabbits, we evaluated the effects of n-3 PUFA on gestational insulin resistance,
247 using diets supplemented with extruded linseed which is rich in ALA, and fish oil rich in preformed

248 EPA and DHA. The different n-3 PUFA diets affected body weight and milk production as well as
249 fasting- and glucose tolerance test-derived indices of insulin resistance.

250 According to Rebollar et al. (2014), n-3 PUFA supplementation did not affect significantly the
251 fertility of rabbits; however, in our study, the does who received extruded linseed showed low fertility
252 (46%). Linseed can, in fact, influence reproductive functions in several ways; for example, it is a rich
253 source of phytoestrogens (Tou et al., 1999) and contains ALA which inhibits PGF2α synthesis (Pérez-
254 Matute et al., 2007; Wathes et al., 2007). However, the differences that we observed in fertility were
255 not significant and suboptimal rates were recorded for all groups.

256 Moreover, Rebollar et al. (2014) showed that n-3 PUFA supplementation reduced the number of
257 stillborn and increased the weight and size of live born kits. Although the rabbits supplemented with
258 linseed showed lower perinatal mortality and milk yield, both the number and weight of the offspring at
259 weaning remained unaffected. The different experimental protocols and diets may explain the
260 inconsistencies between the results. The lower percentage of perinatal mortality of does receiving
261 extruded linseed was due to the death of litters belonging to the control and fish oil groups, while the
262 reduction in milk yield was unexpected. In cows supplemented with linseed, unchanged or increased
263 milk yields has been reported (Jahani-Moghadam et al., 2015; Zachut et al., 2010). In our study, the
264 low milk production of linseed supplemented does could be attributed to their excessive BW at
265 insemination (Rommers et al., 2004); however, further studies are required to confirm this hypothesis.

266 A diet rich in n-3 PUFAs was associated with an increase in gestational length in rats and humans
267 through alterations of prostaglandins or adrenal steroid synthesis (Wathes et al., 2007). Although a
268 similar involvement of prostaglandins and cortisol on pregnancy and parturition can be hypothesized in

rabbits (Boiti et al., 2006; Menchetti et al., 2015a), in agreement with Rebollar et al. (2014), we cannot confirm the influence of PUFAs on the duration of rabbit pregnancy.

Higher feed intake and BW increase occurred with the linseed supplemented diet during the pre-pregnancy period. Previous studies did not find significant effects of linseed supplementation on the performance of growing rabbits (Dal Bosco et al., 2004; Kouba et al., 2008), probably because different diet composition and animals at different ages were evaluated. The pregnant rabbits supplemented with linseed remained heavier throughout pregnancy. High BW values may have negatively influenced their fertility rate (Cardinali et al., 2008) as well as their insulin sensitivity (Pérez-Matute et al., 2007; Skvarca et al., 2013).

High insulin resistance was observed in all rabbits during the first half of pregnancy. Insulin resistance indices as well as glucose and insulin concentrations decreased in late pregnancy probably due to an increased transfer of maternal glucose to the foetuses. Our previous study (Menchetti et al., 2015a) showed increased HOMA-IR values in mid-pregnant rabbits although different categories of does (primiparous *vs.* pluriparous) and different experimental protocols were used. The reduced insulin sensitivity during pregnancy irrespective of dietary regimen confirms the data obtained for other animal species and women (Cardinali et al., 2017; Ciampelli et al., 1998; Corson et al., 2008; Skvarca et al., 2013). The pregnancy-dependent insulin resistance is probably due to the high concentrations of oestrogen, progesterone, and hormones from the placenta, but the exact mechanism is not fully understood. A certain reduction in insulin sensitivity during pregnancy has an adaptive role in saving glucose for foetal growth (Cardinali et al., 2017; Ciampelli et al., 1998; Menchetti et al., 2015a). However, when a pregnant woman is unable to produce an adequate amount of insulin to compensate

290 for this insulin resistance, this physiological phenomenon becomes a pathological condition called
291 gestational diabetes mellitus. Gestational diabetes is associated not only with various complications
292 during pregnancy and birth, but also with increased risk of type 2 diabetes mellitus and cardiovascular
293 diseases for both mothers and their offspring (Chu et al., 2007; Nasu-Kawaharada et al., 2013).
294 In this study, compared to the does of the control group, linseed supplementation increased the
295 indices of insulin resistance and the half-life of the exogenous glucose while it decreased its clearance,
296 thus suggesting a reduced insulin sensitivity and an impaired glucose tolerance. Studies on the effects
297 of ALA on insulin sensitivity are few and contradictory. Several authors (Correia-Santos et al., 2015;
298 Makni et al., 2011; Shomonov-Wagner et al., 2015) showed that in pregnant rats and mice, ALA
299 supplementation decreased glycaemia and stimulated secretion of insulin; on the contrary, no
300 improvement in insulin sensitivity was observed by Ibrahim et al. (2009). In agreement with our
301 findings, Andersen et al. (2008) found lower insulin sensitivity in rats fed with ALA compared to rats
302 receiving EPA or DHA. These discrepancies could be due to differences in experimental protocol or
303 physiological stage, animal model or diet composition. Moreover, several mechanisms may be
304 involved in the contradictory actions of linseed. Several authors claim that the beneficial properties of
305 ALA are primarily linked to their role as a substrate for the synthesis of EPA and DHA (Burdge, 2006;
306 Das, 2005; McCloy et al., 2004). The activity of the enzymes involved in the endogenous conversion of
307 ALA into EPA and DHA is modulated by both dietary and genetic factors such as n-3/n-6 PUFA ratio
308 and genotype (Burdge, 2006; Dal Bosco et al., 2012; Das, 2005). Dal Bosco et al. (2014) found a poor
309 desaturase activity in rabbits selected for productive performance that could also characterize the
310 hybrid used in our study. Das (2005) argued that a genetic predisposition to low activity of Δ-6 and Δ-5

desaturase increases the risk of type 2 diabetes. On the other hand, Fukumitsu et al. (2008) found that linseed lignans increased the expression of PPARγ and adiponectin mRNAs resulting in the improvement of insulin resistance. However, linseed contains phytates that can have anabolic effects, probably related to iron reduction and zinc increase as well as hyper-glycaemia (Figueiredo et al., 2009; Szkudelski, 2005; Taneja et al., 2012). Moreover, the preferential usage of ALA in humans and rodents is not EPA/DHA conversion but (i) β-oxidation and recycling of carbon for *de novo* fatty acid synthesis and (ii) direct storage into adipose tissue (Burdge, 2006; McCloy et al., 2004).

For the first time in rabbits, our findings showed an association between insulin resistance and excess weight during pregnancy. Interestingly, as demonstrated in women (Chu et al., 2007; Skvarca et al., 2013), this relationship was also found with the BW at the time of AI, thus confirming that the pre-pregnancy BW is a significant predictor of insulin sensitivity during pregnancy. Obesity is associated with a reduction in insulin sensitivity in rodents (Pérez-Matute et al., 2007; Samuelsson et al., 2008), and leads to a 3-fold increased risk of gestational diabetes mellitus in women (Chu et al., 2007). Gestational diabetes seems to be caused by post-receptor defects that reduce insulin receptor autophosphorylation and translocation of GLUT4 from intracellular vesicles to the cell surface (Shao et al., 2002). Several factors including adiponectin, leptin (Skvarca et al., 2013), tumour necrosis factor-α (TNF-α; Pérez-Matute et al., 2007; Uysal et al., 1997), and oestrogens (Barros et al., 2008) are involved in the mechanisms associating obesity, pregnancy, and insulin resistance. Since linseed contains lignans and phytates that can alter the adipogenesis, the bioavailability of minerals, and oestrogen levels, we can also speculate a role of these components on insulin resistance of L does.

Contrary to the results obtained with linseed supplement, the FO enriched diet improved insulin sensitivity as indicated by the response to glucose load and the fasting sample-derived indices during pregnancy. Positive effects of EPA and DHA supplementation were observed in pregnant sows (Corson et al., 2008) and diabetic pregnant rats (Nasu-Kawaharada et al., 2013; Soulimane-Mokhtari et al., 2008). Several mechanisms may explain the effects of EPA and DHA on insulin sensitivity such as the increase in the number and affinity of insulin receptors (Das, 2005), and the modulation of PPARs which both result in an increase in GLUT4 and adiponectin (Pérez-Matute et al., 2007). Improved insulin resistance can be also secondary to the reduction in the adipose mass via increased expression of enzymes involved in β-oxidation and via reduced expression of enzymes promoting lipogenesis (Ivanova et al., 2014; Pérez-Matute et al., 2007). Unlike the linseed group, the rabbits fed with fish oil supplements showed no differences in BW compared to the rabbits fed with a control diet, thus suggesting that the improvement of insulin sensitivity caused by EPA and DHA did not depend on the amount of adipose mass. However, several hypotheses can be made concerning the EPA/DHA-dependent effects on the function of adipose tissue. For example, since insulin resistance is associated with the chronic inflammation of insulin sensitive tissues, EPA and DHA could have reversed adipose inflammation through the down-regulation of nuclear factor-kappa B, Toll-like receptors and TNFα (Oh et al., 2011; Pérez-Matute et al., 2007; Uysal et al., 1997). The different effect on the expression of these genes between preformed dietary DHA and their precursor ALA could contribute to their differential effects on insulin resistance.

Their similarities with women confirm rabbits as valid experimental models for gestational diabetes. Other authors have successfully used the rabbit for studying metabolic syndromes induced by

352 castration (Ivanova et al., 2014) and high-fat diets (Maneschi et al., 2013). In fact, this nutritional
353 treatment induces all metabolic syndrome features, including hyperglycemia and glucose intolerance,
354 hypertension, dyslipidemia and increased visceral fat weight (Maneschi et al., 2013). Further studies
355 should be carried out to assess the effect of high-fat diets on the insulin resistance of pregnant rabbits as
356 well as the risk of metabolic syndromes following gestational diabetes.
357
358 **5. Conclusions**
359 Supplementation with linseed rich in ALA and fish oil rich in EPA and DHA, had differential
360 effects on insulin sensitivity during pregnancy. Pregnant rabbits fed with linseed showed elevated BW
361 and reduced milk production as well as high fasting plasma glucose concentration and poor response to
362 the glucose load. The deterioration of insulin sensitivity in the linseed group seems to be due to their
363 excessive weight gain since we demonstrated a positive correlation between BW, both before and
364 during pregnancy, and gestational insulin resistance. Conversely, preformed EPA and DHA provided
365 by fish oil did not affect BW but improved insulin sensitivity. Further studies using the rabbit as
366 experimental model are needed to clarify the activities of linseed components, the metabolic fates of
367 ALA, and the mechanisms involved in obesity-associated insulin resistance during pregnancy.
368
369
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536
537

538 Table 1. Formulation and chemical composition of control (C) and enriched diets supplemented with
539 fish oil (FO), and extruded linseed (L).
540

	Unit	Diet		
		C	FO	L
Ingredients				
Barley	%	17.0	17.0	17.0
Bran	%	7.0	7.0	5.0
Dehydrated alfalfa meal	%	50.0	50.0	50.00
Soybean meal 44%	%	18.0	18.00	13.00
Soybean oil	%	3.0	-	-
Extruded flaxseed	%	-	-	10.0
Nordos ®	%	-	3.0	-
Beet molasses	%	1	1	1
Calcium diphosphate	%	1.35	1.35	1.35
Vitamin-mineral premix*	%	1.2	1.2	1.2
Calcium carbonate	%	0.7	0.7	0.7
Salt	%	0.7	0.7	0.7
DL-methionine	%	0.05	0.05	0.05
Analytical data				
Crude Protein	%	17.80	17.81	17.95
Ether extract	%	5.55	5.63	6.02
Crude fiber	%	14.90	14.44	14.88
Ash	%	9.70	9.59	9.68
Starch*	%	11.2	11.1	10.8
NDF*	%	29.10	29.10	30.00
ADF*	%	19.80	19.80	20.45
ADL*	%	4.30	4.30	4.45
Digestible Energy**	Mj/kg	9.85	9.85	9.81

541 * Per kg diet: vitamin A 11,000 IU; vitamin D3 2000 IU; vitamin B1 2.5 mg; vitamin B2 4 mg; vitamin B6 1.25 mg;
542 vitamin B12 0.01 mg; alpha-tocopherol acetate 50 mg; biotine 0.06 mg; vitamin K 2.5 mg; niacin 15 mg; folic acid 0.30 mg;
543 D-pantothenic acid 10 mg; choline 600 mg; Mn 60 mg; Fe 50 mg; Zn 15 mg; I 0.5 mg; Co 0.5 mg.
544 * Calculated
545 ** Estimated by Maertens et al. (1988)
546

547 Table 2. Main fatty acid composition (expressed as percentage of total fatty acids) of diets.
548

Fatty acids	Diets		
	C	**FO**	**L**
SFAs	20.0	18.4	18.7
MUFAs	18.5	16.6	17.1
C18:2n-6 (LA)	39.0	32.2	33.3
C18:3n-3 (ALA)	22.2	29.2	30.6
C20:5n-3 (EPA)	0.2	1.0	0.2
C22:6n-3 (DHA)	0.1	2.2	0.1
PUFAs	61.5	65.0	64.2
n-6	39.0	32.2	33.3
n-3	22.5	32.8	30.9
LCP n-3	0.3	3.2	0.3
n-6/n-3	1.73	0.98	1.08

549

550

Table 3. Reproductive performance in C (control diet), FO (supplemented with fish oil), and L (supplemented with extruded linseed) rabbits (n = 15/groups).

Parameter	Unit	C	FO	L	P-value
Baseline[1] BW	**kg**	4.1±0.1	4.0±0.1	4.1±0.1	*0.752*
Feed intake during pre-pregnancy period[1]	**g/d**	124_{a}±1	122_{a}±1	128_{b}±1	***0.001***
BW at AI	**kg**	4.1_{A}±0.1	4.1_{A}±0.1	4.4_{B}±0.1	***0.046***
Fertility	**%**	59.1	66.7	45.8	*0.357*
Feed intake during pregnancy	**g/d**	147±2	142±2	143±2	*0.316*
Duration of gestation	**d**	31.9±0.3	31.7±0.3	32.2±0.3	*0.498*
Litter size at kindling[2]	**n**	6.3±0.9	7.0±0.8	7.3±0.7	*0.660*
Litter weight at kindling	**g**	368±97	445±189	504±74	*0.426[6]*
Perinatal mortality[3]	**%**	31.2_{a}	40.2_{a}	9.6_{b}	***0.0001***
Litter size at 20 d	**n**	6.3±0.6	6.9±0.6	7.0±0.5	*0.631*
Litter weight at 20 d	**kg**	2.1±0.1	2.0±0.1	1.8±0.1	*0.264[6]*
Feed intake during lactation	**g/d**	236±4	241±3	245±4	*0.217[6]*
Milk production[4]	**g/d**	$153±5_{b}$	$142±4_{ab}$	$137±5_{a}$	***0.0001[6]***
Litter size at weaning[5]	**n**	6.1±2.8	6.3±2.2	6.8±2.2	*0.216*
Litter weight at weaning[5]	**kg**	4.9±0.2	4.6±0.2	4.7±0.2	*0.740[6]*
Pre-weaning mortality	**%**	2.3	10.2	7.6	*0.310*

Values in the same row not sharing the same subscript are significantly different (a, b: P<0.05; A, B: P < 0.1; Sidak correction). Bold P-values are significant at the 0.05 level.

[1] Two month before AI

[2] Kits dead during perinatal period not included

[3] Death of entire litter included

[4] Estimated marginal means from 0 to 20th day of lactation

[5] 35 days post-partum

[6] Corrected for the number of kits

Table 4. Fasting derived indices of insulin resistance during pregnancy. C = control diet; FO = maternal diet supplemented with fish oil; L = maternal diet supplemented with extruded linseed (n = 10/group). Values are estimated marginal means ± SE.

Index	Days of pregnancy	Group			P-value		
		C	FO	L	Time	Group	Time x Group
Fasting glucose (mmol/l)	**0**	$7.08\pm0.26_{a}$	$6.55\pm0.30_{a}$	$6.32\pm0.25_{a}$	***0.003***	***0.0001***	***0.001***
	7	$8.46\pm0.34_{b}$	$6.12\pm0.30_{a}$	$7.00\pm0.39_{a}$			
	14	$6.60\pm0.25_{a}$	$6.62\pm0.30_{a}$	$6.64\pm0.25_{a}$			
	21	$6.42\pm0.25_{ab}$	$5.55\pm0.30_{a}$	$6.95\pm0.25_{b}$			
	28	$6.31\pm0.25_{a}$	$6.01\pm0.30_{a}$	$6.37\pm0.25_{a}$			
Fasting insulin* (µU/ml)	**0**	$4.91\pm0.33_{a}$	$6.15\pm0.45_{a}$	$8.14\pm0.60_{a}$	***0.0001***	***0.006***	***0.010***
	7	$6.05\pm0.63_{a}$	$6.53\pm0.68_{a}$	$12.86\pm1.33_{a}$			
	14	$11.25\pm0.90_{ab}$	$9.18\pm0.74_{a}$	$16.95\pm1.24_{b}$			
	21	$5.88\pm0.40_{a}$	$4.76\pm0.38_{a}$	$4.39\pm0.30_{a}$			
	28	$5.84\pm0.40_{b}$	$2.54\pm0.20_{a}$	$4.06\pm0.28_{ab}$			
Glucose-to-Insulin Ratio*	**0**	$1.51\pm0.26_{a}$	$1.04\pm0.26_{a}$	$0.87\pm0.26_{a}$	***0.0001***	*0.087*	***0.004***
	7	$1.48\pm0.33_{b}$	$1.15\pm0.33_{ab}$	$0.58\pm0.33_{a}$			
	14	$0.64\pm0.26_{a}$	$0.74\pm0.26_{a}$	$0.55\pm0.26_{a}$			
	21	$1.12\pm0.22_{a}$	$1.28\pm0.26_{a}$	$1.94\pm0.22_{a}$			
	28	$1.15\pm0.22_{a}$	$2.42\pm0.26_{b}$	$1.76\pm0.23_{ab}$			
HOMA-IR*	**0**	$0.09\pm0.01_{a}$	$0.11\pm0.01_{a}$	$0.13\pm0.01_{a}$	***0.0001***	***0.004***	*0.059*
	7	$0.13\pm0.01_{ab}$	$0.10\pm0.01_{a}$	$0.22\pm0.02_{b}$			
	14	$0.19\pm0.02_{ab}$	$0.15\pm0.01_{a}$	$0.26\pm0.02_{b}$			
	21	$0.09\pm0.01_{a}$	$0.07\pm0.01_{a}$	$0.07\pm0.01_{a}$			
	28	$0.09\pm0.01_{b}$	$0.04\pm0.00_{a}$	$0.06\pm0.00_{ab}$			

Values in the same row not sharing the same subscript are significantly different at P< 0.05 (Sidak correction).
Bold P-values are significant at the 0.05 level.
HOMA-IR = Homeostasis model assessment for insulin resistance
* Back-transformed values.

572 Table 5. Intravenous Glucose tolerance test -derived indices obtained in does at day 21 of pregnancy. C
573 = control diet; FO = maternal diet supplemented with fish oil; L = maternal diet supplemented with
574 extruded linseed (n = 5/group). Values are estimated marginal means ± SE.
575

Parameter	C	FO	L	*P-value*
Glucose 60 min (mmol/l)	6.39_{b}±0.02	5.60_{a} ±0.01	6.36_{b}±0.01	***0.0001***
AUC ((mmol/l) x min)	1104±20	1102±13	1154±15	*0.085*
K_{el} (%/min)	1.33_{B}±0.03	1.25_{AB} ±0.06	1.19_{A} ±0.04	***0.038***
$t_{1/2}$ (min)	52.00_{a} ±1.12	55.43_{ab} ±2.70	58.32_{b} ±1.48	***0.031***
V_{d} (dl/kg)	1.36±0.06	1.38±0.05	1.27±0.06	*0.388*
C_{max} (mmol/l)	24.53±1.16	24.30±1.00	26.24±1.08	*0.391*
CL (ml//kg/min)	1.67_{b}±0.05	1.75_{b}±0.04	1.43_{a}±0.04	***0.002***

576 Values in the same row not sharing the same subscript are different (a, b: P<0.05; A, B: P < 0.1). Adjustment for
577 multiple comparisons: Sidak. Bold P-values are significant at the 0.05 level.
578 Glucose 60 min = glucose concentrations 60 min post-loading; AUC = area under the concentration time curve;
579 K_{el} = elimination rate constant; $t_{½}$ = half-life; V_{d} = apparent volume of distribution; C_{max} = maximum
580 concentration; CL = clearance.
581
582

583 Figure 1. Body weight of rabbit does (n=10/group) during pregnancy. C = control diet; FO = maternal
584 diet supplemented with fish oil; L = maternal diet supplemented with extruded linseed. Values are
585 means ± SE. $^{\#}P < 0.1$, $*P < 0.05$ supplemented groups *versus* control group for each gestational day.
586
587 Figure 2. Relationship between Homeostasis model assessment for insulin resistance (HOMA-IR) and
588 body weight of does (BW). The points of scatter plot show the BW of the rabbits and the corresponding
589 values of HOMA-IR recorded on day 0 (AI, empty circle), in early-mid-pregnancy (days 7 and 14 post
590 AI, gray triangle), and in late pregnancy (days 21 and 28 post AI, black square). In the model including
591 BW as predictor and HOMA-IR as dependent variable, the parameters showed that insulin resistance
592 measured by HOMA-IR increases with the increase in BW (b after log transformation = 0.23, $P <$
593 0.01). This relationship was most evident in early-mid pregnancy.
594
595 Figure 3. Relationship between area under the concentration-time curve (AUC) and body weight of
596 does (BW). The points of scatter plot show the BW of the rabbits recorded on day 0 (AI, empty circle)
597 and day 21 of pregnancy (black square), and the value of AUC measured on day 21. In the model
598 including BW as predictor and AUC as dependent variable, parameters showed that insulin resistance
599 on day 21 of pregnancy measured by AUC increases with the increase in BW at AI ($b = 259.6$, $P <$
600 0.01) and on day 21 of pregnancy ($b = 219.1$, $P < 0.05$).

The different effects of linseed and fish oil supplemented diets on insulin sensitivity of rabbit does during pregnancy

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Abstract

This study investigates the effects of linseed (rich in α-linolenic acid (ALA)) and fish oil (rich in eicosapentaenoic (EPA) and docosahexaenoic acid (DHA)) supplementation on the insulin resistance of pregnant rabbits. Two months before insemination, the rabbits (15 animals/group) were fed different diets: commercial standard (group C), supplemented with 10% extruded linseed (group L), and 3% fish oil (group FO). The L group does showed both the highest feed intake before AI ($P<0.01$) and the highest body weight (BW) throughout pregnancy ($P<0.001$). The L does yielded less milk than the C does ($P<0.001$); however, no differences were observed in either weight or size of litter at weaning. Regardless of diet, insulin concentrations and HOMA-IR values were higher during the first half of pregnancy ($P<0.001$). Nevertheless, the L does showed higher mean insulin concentrations than FO rabbits ($P<0.01$) and the lowest glucose clearance ($P<0.01$) during pregnancy. On the other hand, pregnant FO rabbits showed the lowest glucose concentrations ($P<0.05$) and the lowest Homeostasis model assessment values for insulin resistance (HOMA-IR, $P<0.05$) as well as a faster restoration of baseline glucose levels following glucose load ($P<0.001$). Before and during pregnancy, the BW of the rabbits was positively related to fasting sample- and tolerance test-derived indices of insulin resistance ($P<0.05$) suggesting that a high pre-pregnancy BW predisposes to gestational insulin resistance. Linseed supplementation increased BW and predisposed to insulin resistance during pregnancy; whereas, fish oil improved insulin sensitivity without significant changes in BW.

Key words: insulin sensitivity; ALA, EPA and DHA; HOMA-IR; gestational diabetes; reproductive performance; rabbit

1. Introduction

Using n-3 polyunsaturated fatty acids (n-3 PUFAs) as functional foods became popular when the low incidence of cardiovascular disease in Eskimo population (Bang et al., 1980) was attributed to their fish - rich diet which is high in both EPA (20:5n-3) and DHA (22:6n-3). These long-chain PUFAs can be obtained indirectly from other sources, such as linseed which is rich in ALA (18:3n-3). In fact, humans and animals are able to convert ALA into EPA and DHA through a series of elongations and desaturations. Δ-desaturases are the rate-limiting enzymes in both n-3 and n-6 PUFA metabolisms (Das, 2005). n-3 PUFAs are generally associated with anti-inflammatory and cardioprotective effects (Djoussé et al., 2012; Oh et al., 2011; Soulimane-Mokhtari et al., 2008). Moreover, fatty acids provide energy, are structural and functional components of cell membranes, and affect signal transduction pathways as well as gene transcription.

Intakes of ALA or preformed EPA and DHA may have specific and potentially independent effects on physiological and pathological processes; indeed, dietary ALA has multiple metabolic fates, and the conversion efficiency to its longer chain counterparts is generally poor (Burdge, 2006; McCloy et al., 2004).

The effects of n-3 PUFA on reproduction have been evaluated mainly in ruminants. However, conflicting results were obtained for *in vivo* dietary supplementations, while the effects on pregnancy rates and embryo survival are still not fully known (for a general review, see Wathes et al., 2007). To our knowledge, only one study has been carried out regarding the effect of n-3 PUFA supplementation on the reproductive performance of rabbit does (Rebollar et al., 2014).

Conversely, the effects of EPA and DHA on metabolic diseases of humans and animals are well documented; for example, rodents and rabbits fed with EPA and DHA supplements showed reduced body weight and insulin resistance (Ivanova et al., 2014; Pérez-Matute et al., 2007). Several meta-analyses confirm an association between fish oil supplementation and reduced risk of type 2 diabetes in

humans (Djoussé et al., 2012; Zhang et al., 2013). However, these studies have some limitations. Firstly, differential effects of EPA/DHA and ALA on disease susceptibility have not been fully elucidated despite the potential differences in their metabolism. Secondly, the role of n-3 PUFAs in insulin sensitivity during pregnancy remains largely unknown. A Cochrane meta-analysis found no significant effects of fish oil supplements on pregnancy complications in women (Makrides et al., 2006). Finally, although the rabbit is a suitable model for studying reproductive disorders and the influence of nutrition during pregnancy (Brecchia et al., 2014, 2006, Menchetti et al., 2018, 2015a, 2015b), strategies for improving gestational insulin resistance in this species have not yet been evaluated.

In order to fill these gaps, we evaluated the effects of supplementation with two different sources of n-3 PUFAs, extruded linseed and fish oil, on insulin sensitivity of rabbit does during pregnancy. To this purpose, both fasting sample-derived indices of insulin resistance and intravenous glucose tolerance tests were used.

2. Materials and methods

2.1. Animals and diets

A total of 45 multiparous (third to fifth parity order) hybrid rabbit does were housed at the experimental farm of the Department of Agricultural, Food and Environmental Sciences of the University of Perugia. The does were kept individually in flat deck cages; the temperature ranged from +15 to +28 °C, and the light schedule was 16 L:8 D. Two months before artificial insemination (AI), the rabbits were randomly assigned to one of three groups (15 animals/group): C group was fed with commercial standard diet, L group with 10% extruded linseed supplementation (Nlest®, Valorex), and

FO group with 3% fish oil supplementation (Nordos ®).Vitamin E (200 mg/kg feed, synthetic alpha-tocopheryl acetate) was included in all diets. The feeding program of the rabbit does was continued until weaning (35th day post-partum). Lactation was controlled until day 20 post-partum by opening the nest once a day for 5-10 minutes. After the 20th day of lactation, the nest was opened and the kits were given access to solid food. The feed intake of the rabbit does was recorded daily until the end of controlled lactation (20th day). The diets (Table 1 and 2) were isoenergetic, isonitrogenous, and formulated according to the current dietary recommendations for rabbit does (De Blas and Mateos, 1998). The rabbit does received 130 g/d of food from the beginning of the experiment until the day of AI; subsequently, they had *ad libitum* access to food.

Ovulation was induced by injecting 0.8 µg of synthetic GnRH (Receptal, Hoechst-Roussel Vet, Milan, Italy) just before AI (Brecchia et al., 2006). AI was performed with 0.5 ml of diluted fresh semen. The day of AI was named day 0. Pregnancy was diagnosed by manual palpation 10 days after AI and non-pregnant does were submitted to successive AI after 21 days. Two cycles of AI were performed in order to achieve 10 pregnancies per group.

2.2 Body weight and reproductive performance

From the beginning of the experiment until weaning, the BW of overnight fasted rabbits was recorded weekly between 8:00 and 10:00 A.M.. The following productivity indices were calculated: fertility (number of parturitions/number of inseminations x 100), prolificacy (total number of born and stillborn kits per doe), litter weight at kindling as well as perinatal and pre-weaning mortality. In addition, litter size and weight were recorded at days 20 and 35 (weaning). The perinatal period

comprised the first 48 h after parturition. The pre-weaning mortality rate was calculated as the percentage of weaned kits/litter size following the perinatal period. Daily milk production was measured from parturition until day 20 of lactation by weighing the doe immediately before and after suckling.

2.3. Fasting sample-derived insulin resistance indices

2.3.1. Blood sampling and measurements of insulin and glucose

Blood samples were taken from 10 randomly selected fasted pregnant does/group between 8:00 and 10:00 A.M at days 0, 7, 14, 21, and 28 after AI. Blood samples were collected from the marginal ear vein and placed in EDTA containing tubes. The tubes were centrifuged at 3000xg for 15 min, and the plasma was stored frozen until assayed for hormones and metabolites.

Plasma insulin concentrations were determined by the double antibody technique using an insulin IRMA kit (Immunotech s.r.o., Prague, Czech Republic). The limit of sensitivity was 1.35 μU/ml and intra- and inter-assay coefficients of variations were 4.0 and 4.8%, respectively. Glucose was analysed with the hexokinase method using the Glucose GLUC-HK kit from RANDOX (Randox Laboratories Limited, Country Antrim, UK). The limit of sensitivity was 0.71 mmol/l, and intra- and inter-assay coefficients of variations were 3.6 and 5.1%, respectively.

2.3.2 Determination of fasting-derived blood indices

Fasting-derived blood indices were steady-state measures of insulin resistance that did not require the administration of exogenous glucose, which included fasting G*lucose Concentrations* (in mmol/l),

Insulin Concentrations (in µU/ml) as well as mathematical models based on these measures. These models included *Glucose-to-Insulin Ratio* and *Homeostasis model assessment for insulin resistance (HOMA-IR)* calculated as follows: [insulin concentration x (glucose concentration/18)]/22.5 (Menchetti et al., 2015a).

2.4. Glucose Tolerance test-derived indices

2.4.1. Intravenous glucose tolerance test

Intravenous glucose tolerance tests were performed on 5 randomly selected does/group between 8:00 and 10:00 A.M. on day 21 of pregnancy. The rabbits were fasted overnight (at least 16 h). A single bolus of glucose (0.6 g/kg of body weight) was rapidly infused into the ear marginal vein through an 18G catheter. A small drop of capillary blood was collected by puncturing the ear just before glucose administration and subsequently after 5, 10, 30, 60, and 120 min. Blood glucose (mmol/l) was measured with a calibrated glucometer (OneTouch ® UltraEasy, LifeScan Europe, Johnson & Johnson, Zug, Switzerland), using test strips from the same supplier. The inter-assay coefficient of variation for glucose was < 5%.

2.4.2. Determination of kinetic parameters of glucose

The pharmacokinetics of the glucose load was analysed using a one-compartment open model. The *elimination rate constant* was calculated from the slope of the line during the elimination phase by linear regression analysis of the semilogarithmic plot of glucose concentration versus time. The *half-life* of the exogenous glucose load was obtained as follows: (ln 2 /elimination rate constant); the

144 *apparent volume of distribution* was obtained as the ratio between dose and plasma concentrations after
145 bolus administration; whereas, the *clearance* was obtained as the volume of distribution x elimination
146 rate constant. The *area under the concentration-time curve* (AUC) was calculated by the linear
147 trapezoidal method using GraphPad Prism version 5.01 software (Inc., San Diego, CA, USA).
148
149 **2.5. Statistical analysis**
150 The data were analysed with the linear mixed model procedures. In these models, the animals were
151 treated as the random factor; whereas, the cycle of AI and time (day pre or post AI) were treated as
152 repeated factors. The models evaluated the effect of time (9 and 5 levels during pre- and post- AI,
153 respectively), group (3 levels: C, FO, and L), and interaction between time and group. In order to
154 evaluate the responses to the glucose tolerance test, the time factor (minutes after glucose
155 administration) had 6 levels: 0, 5, 10, 30, 60, 120 min after loading. In models including BW as
156 predictor and indices of insulin resistance as dependent variables, the b-parameters were estimated in
157 order to investigate the relationship between BW and insulin resistance. In order to limit the influence
158 of longitudinal changes in the BW of does caused by foetal growth, the stage of pregnancy (3 levels: AI
159 = day 0; Early-mid pregnancy = days 7 and 14; Late pregnancy = days 21 and 28) was included in the
160 model used for analysing the relationships between BW and fasting sample-derived indices. Only
161 group effect was considered to evaluate reproductive and kinetic parameters. The number of kits was
162 included as covariate whenever appropriate. Parity order was not included as a covariate as the
163 preliminary analyses did not show any significant effect. Diagnostic graphics were used to check
164 assumptions and outliers. Logarithmic transformations were used whenever appropriate (insulin

concentration, glucose-to-insulin ratio, HOMA-IR). The results were expressed as estimated marginal means ± standard error (SE). The logarithms were back transformed. Chi-square tests were used to analyse fertility and mortality rates. Statistical analyses were performed with SPSS Statistics version 20 (IBM, SPSS Inc., Chicago, IL, USA). We defined $P \leq 0.05$ as significant and $P < 0.1$ as a trend.

3. Results

3.1. Feed intake, BW, and productive performance

The BW of does at the beginning of the experiment was 4.07±0.05 kg in C, 4.04±0.05 kg in L, and 4.12±0.05 kg in FO groups. From the beginning of the experiment until AI, feed intake was higher in L group than in C and FO groups ($P < 0.01$; Table 3). The BW of all rabbits increased during the 2 months prior to AI (time effect $P < 0.05$); however, the weight gain was higher in L group (group effect $P < 0.001$). The rabbits of L group were heavier at AI than at the beginning of the experiment (+0.25±0.11 kg; $P < 0.05$). Their BW at AI was also higher when compared to the rabbits in the other groups (+0.25±0.11 kg; $P < 0.05$; Table 3).

Following AI, feed intake was unaffected by either dietary regimen or litter size; however, it was affected by the physiological phase ($P < 0.001$; Table 3). From AI until day 27 of pregnancy, the mean daily feed intake was 150±0, 144±2, and 144±2 g for C, FO and L groups, respectively. During the last three to four days before parturition, feed intake decreased by 13%. Feed intake increased progressively during the first 20 days of lactation from 128±8 to 318±8 g/d in all groups ($P < 0.001$) and was affected by litter size ($P < 0.001$; Table 3).

186 The BW during pregnancy differed between groups ($P < 0.001$; Fig. 1) with higher mean values
187 observed in L does ($P < 0.001$) than in C and FO groups.
188 During the trial, three does (one from group C and two from group FO) died or were excluded due
189 to severe health problems. However, no differences were found between groups regarding the mortality
190 rate of does (6.7%, 13.3% and 0.0% in C, FO and L groups, respectively; $P = 0.762$).
191 Fertility did not differ between groups (Table 3). The length of pregnancy did not differ among
192 groups (Table 3) although it was negatively related to litter size ($b = -0.21$, $P < 0.001$). Perinatal
193 mortality was higher ($P < 0.001$; Table 3) in C and FO groups than in L group due to the death of 2
194 litters in group FO and 1 litter in group C. No differences were observed for both weight and size of
195 litter between groups (Table 3). Milk yield was lower in L than C group (Table 3) while there were no
196 differences either in weight or size of litter at day 20 of lactation and at weaning (Table 3).
197
198 **3.2. Insulin sensitivity**
199 *3.2.1. Fasting-derived indices*
200 Plasma glucose concentrations were affected by time ($P < 0.01$), group ($P < 0.001$) and interaction
201 time x group ($P < 0.01$; Table 4). Plasma glucose concentrations ranged from 4.2 to 10.0 mmol/l, and
202 decreased from early (7.2±0.2 mmol/l at day 7 of pregnancy) to late pregnancy (6.2±0.2 mmol/l at day
203 28 of pregnancy; $P < 0.001$). Glycaemia during pregnancy was lower in FO group (6.2±0.1 mmol/l)
204 than in C (+9%, $P < 0.001$) and L (+5%, $P < 0.05$) groups. Pairwise comparisons for each day showed
205 significant differences on days 7 and 21 of pregnancy (Table 4).

206 Insulin concentrations ranged from 2.0 to 26.9 µU/ml and were affected by time ($P < 0.001$), group
207 ($P < 0.01$), and interaction time x group ($P < 0.05$; Table 4). Insulin concentrations were higher in mid
208 pregnancy (12.0±0.5 µU/ml at day 14) than in late pregnancy (3.9±0.2 µU/ml at day 28; $P < 0.01$).
209 Mean insulin concentrations during pregnancy were higher in L group (7.9±0.3 µU/ml; +32%, $P <$
210 0.01) than FO group (5.4±0.2 µU/ml). Pairwise comparisons showed significant differences at days 14
211 and 28 of pregnancy (Table 4).
212 The glucose-to-insulin ratio was affected by time ($P < 0.001$) as it was lower on day 14 (0.6±0.1)
213 and higher on day 28 (1.8±0.1) than at AI (1.1±0.1; $P < 0.01$). It was also affected by group x time
214 interaction ($P < 0.01$; Table 4). In early pregnancy (day 7), the glucose-to-insulin ratio was lower in L
215 group, while at the end of pregnancy (day 28) the ratio was higher in FO than in C group (Table 4).
216 During pregnancy, HOMA-IR values (Table 4) were affected by group ($P < 0.01$) and by time as
217 they were higher in early- and mid-pregnancy than in late-pregnancy ($P < 0.001$). A trend was observed
218 for time x group effect ($P < 0.1$). Does belonging to FO group had lower estimated marginal means of
219 HOMA-IR during pregnancy (0.08±0.01) than C (-38%, $P < 0.05$) and L groups (-62%, $P < 0.01$).
220 Pairwise comparisons on each day of this study showed lower HOMA-IR in FO at days 7 and 14
221 compared to L, and at day 28 compared to C group (Table 4).
222 Parameters of models including BW as predictor and fasting-derived indices as dependent variables
223 showed a significant positive relationship between the BW and plasma insulin concentrations of does
224 (log transformation: $b = 0.22$, $P < 0.05$) and HOMA-IR (log transformation: $b = 0.23$, $P < 0.01$; Fig. 2).
225
226 *3.2.2. Glucose Tolerance test-derived indices*

227 Regardless of nutritional regimen, the intravenous administration of glucose caused a rapid increase
228 in blood glucose concentrations. Glucose reached the highest value 5 min post-administration ($P < 0.001$) and returned to baseline values at 60 min.

230 The rabbits belonging to FO group showed lower glucose concentrations 60 min post-loading than C
231 (+14%, $P < 0.001$) and L (+14%, $P < 0.001$) groups (Table 5). The rabbits of the L group showed
232 higher glucose half-life than C does (+10%, $P < 0.05$) and the lowest clearance ($P < 0.01$; Table 5). The
233 elimination-rate constant was affected by group ($P < 0.05$) and tended to be lower in pregnant L does
234 than in C does (-11%; Sidak correction: $P < 0.1$). A trend of differences was found in AUC ($P < 0.1$
235 Table 5).

236 In order to investigate the relationship between BW and insulin resistance measured with the
237 glucose tolerance test, we calculated the parameters of models including BW at AI or at day 21 of
238 pregnancy as predictor and test-derived indices as dependent variables. Significant relationships were
239 observed between BW at day of test (day 21 of pregnancy) and some glucose tolerance test-derived
240 indices: the higher the value of BW, the higher the values of AUC ($b = 219.1$, $P < 0.05$; Fig. 3) and
241 maximum concentration of glucose ($b = 5.3$, $P < 0.05$), while clearance decreased ($b = - 0.2$, $P < 0.1$).
242 When BW at AI was included in the model, significant relationships were found with AUC ($b = 259.6$,
243 $P < 0.01$; Fig. 3) and maximum glucose concentration ($b = 5.6$, $P < 0.05$).

244

245 **4. Discussion**

246 For the first time in rabbits, we evaluated the effects of n-3 PUFA on gestational insulin resistance,
247 using diets supplemented with extruded linseed which is rich in ALA, and fish oil rich in preformed

248 EPA and DHA. The different n-3 PUFA diets affected body weight and milk production as well as
249 fasting- and glucose tolerance test-derived indices of insulin resistance.
250 According to Rebollar et al. (2014), n-3 PUFA supplementation did not affect significantly the
251 fertility of rabbits; however, in our study, the does who received extruded linseed showed low fertility
252 (46%). Linseed can, in fact, influence reproductive functions in several ways; for example, it is a rich
253 source of phytoestrogens (Tou et al., 1999) and contains ALA which inhibits PGF2α synthesis (Pérez-
254 Matute et al., 2007; Wathes et al., 2007). However, the differences that we observed in fertility were
255 not significant and suboptimal rates were recorded for all groups.
256 Moreover, Rebollar et al. (2014) showed that n-3 PUFA supplementation reduced the number of
257 stillborn and increased the weight and size of live born kits. Although the rabbits supplemented with
258 linseed showed lower perinatal mortality and milk yield, both the number and weight of the offspring at
259 weaning remained unaffected. The different experimental protocols and diets may explain the
260 inconsistencies between the results. The lower percentage of perinatal mortality of does receiving
261 extruded linseed was due to the death of litters belonging to the control and fish oil groups, while the
262 reduction in milk yield was unexpected. In cows supplemented with linseed, unchanged or increased
263 milk yields has been reported (Jahani-Moghadam et al., 2015; Zachut et al., 2010). In our study, the
264 low milk production of linseed supplemented does could be attributed to their excessive BW at
265 insemination (Rommers et al., 2004); however, further studies are required to confirm this hypothesis.
266 A diet rich in n-3 PUFAs was associated with an increase in gestational length in rats and humans
267 through alterations of prostaglandins or adrenal steroid synthesis (Wathes et al., 2007). Although a
268 similar involvement of prostaglandins and cortisol on pregnancy and parturition can be hypothesized in

rabbits (Boiti et al., 2006; Menchetti et al., 2015a), in agreement with Rebollar et al. (2014), we cannot confirm the influence of PUFAs on the duration of rabbit pregnancy.

Higher feed intake and BW increase occurred with the linseed supplemented diet during the pre-pregnancy period. Previous studies did not find significant effects of linseed supplementation on the performance of growing rabbits (Dal Bosco et al., 2004; Kouba et al., 2008), probably because different diet composition and animals at different ages were evaluated. The pregnant rabbits supplemented with linseed remained heavier throughout pregnancy. High BW values may have negatively influenced their fertility rate (Cardinali et al., 2008) as well as their insulin sensitivity (Pérez-Matute et al., 2007; Skvarca et al., 2013).

High insulin resistance was observed in all rabbits during the first half of pregnancy. Insulin resistance indices as well as glucose and insulin concentrations decreased in late pregnancy probably due to an increased transfer of maternal glucose to the foetuses. Our previous study (Menchetti et al., 2015a) showed increased HOMA-IR values in mid-pregnant rabbits although different categories of does (primiparous *vs.* pluriparous) and different experimental protocols were used. The reduced insulin sensitivity during pregnancy irrespective of dietary regimen confirms the data obtained for other animal species and women (Cardinali et al., 2017; Ciampelli et al., 1998; Corson et al., 2008; Skvarca et al., 2013). The pregnancy-dependent insulin resistance is probably due to the high concentrations of oestrogen, progesterone, and hormones from the placenta, but the exact mechanism is not fully understood. A certain reduction in insulin sensitivity during pregnancy has an adaptive role in saving glucose for foetal growth (Cardinali et al., 2017; Ciampelli et al., 1998; Menchetti et al., 2015a). However, when a pregnant woman is unable to produce an adequate amount of insulin to compensate

for this insulin resistance, this physiological phenomenon becomes a pathological condition called gestational diabetes mellitus. Gestational diabetes is associated not only with various complications during pregnancy and birth, but also with increased risk of type 2 diabetes mellitus and cardiovascular diseases for both mothers and their offspring (Chu et al., 2007; Nasu-Kawaharada et al., 2013).

In this study, compared to the does of the control group, linseed supplementation increased the indices of insulin resistance and the half-life of the exogenous glucose while it decreased its clearance, thus suggesting a reduced insulin sensitivity and an impaired glucose tolerance. Studies on the effects of ALA on insulin sensitivity are few and contradictory. Several authors (Correia-Santos et al., 2015; Makni et al., 2011; Shomonov-Wagner et al., 2015) showed that in pregnant rats and mice, ALA supplementation decreased glycaemia and stimulated secretion of insulin; on the contrary, no improvement in insulin sensitivity was observed by Ibrahim et al. (2009). In agreement with our findings, Andersen et al. (2008) found lower insulin sensitivity in rats fed with ALA compared to rats receiving EPA or DHA. These discrepancies could be due to differences in experimental protocol or physiological stage, animal model or diet composition. Moreover, several mechanisms may be involved in the contradictory actions of linseed. Several authors claim that the beneficial properties of ALA are primarily linked to their role as a substrate for the synthesis of EPA and DHA (Burdge, 2006; Das, 2005; McCloy et al., 2004). The activity of the enzymes involved in the endogenous conversion of ALA into EPA and DHA is modulated by both dietary and genetic factors such as n-3/n-6 PUFA ratio and genotype (Burdge, 2006; Dal Bosco et al., 2012; Das, 2005). Dal Bosco et al. (2014) found a poor desaturase activity in rabbits selected for productive performance that could also characterize the hybrid used in our study. Das (2005) argued that a genetic predisposition to low activity of Δ-6 and Δ-5

desaturase increases the risk of type 2 diabetes. On the other hand, Fukumitsu et al. (2008) found that linseed lignans increased the expression of PPARγ and adiponectin mRNAs resulting in the improvement of insulin resistance. However, linseed contains phytates that can have anabolic effects, probably related to iron reduction and zinc increase as well as hyper-glycaemia (Figueiredo et al., 2009; Szkudelski, 2005; Taneja et al., 2012). Moreover, the preferential usage of ALA in humans and rodents is not EPA/DHA conversion but (i) β-oxidation and recycling of carbon for *de novo* fatty acid synthesis and (ii) direct storage into adipose tissue (Burdge, 2006; McCloy et al., 2004).

For the first time in rabbits, our findings showed an association between insulin resistance and excess weight during pregnancy. Interestingly, as demonstrated in women (Chu et al., 2007; Skvarca et al., 2013), this relationship was also found with the BW at the time of AI, thus confirming that the pre-pregnancy BW is a significant predictor of insulin sensitivity during pregnancy. Obesity is associated with a reduction in insulin sensitivity in rodents (Pérez-Matute et al., 2007; Samuelsson et al., 2008), and leads to a 3-fold increased risk of gestational diabetes mellitus in women (Chu et al., 2007). Gestational diabetes seems to be caused by post-receptor defects that reduce insulin receptor autophosphorylation and translocation of GLUT4 from intracellular vesicles to the cell surface (Shao et al., 2002). Several factors including adiponectin, leptin (Skvarca et al., 2013), tumour necrosis factor-α (TNF-α; Pérez-Matute et al., 2007; Uysal et al., 1997), and oestrogens (Barros et al., 2008) are involved in the mechanisms associating obesity, pregnancy, and insulin resistance. Since linseed contains lignans and phytates that can alter the adipogenesis, the bioavailability of minerals, and oestrogen levels, we can also speculate a role of these components on insulin resistance of L does.

Contrary to the results obtained with linseed supplement, the FO enriched diet improved insulin sensitivity as indicated by the response to glucose load and the fasting sample-derived indices during pregnancy. Positive effects of EPA and DHA supplementation were observed in pregnant sows (Corson et al., 2008) and diabetic pregnant rats (Nasu-Kawaharada et al., 2013; Soulimane-Mokhtari et al., 2008). Several mechanisms may explain the effects of EPA and DHA on insulin sensitivity such as the increase in the number and affinity of insulin receptors (Das, 2005), and the modulation of PPARs which both result in an increase in GLUT4 and adiponectin (Pérez-Matute et al., 2007). Improved insulin resistance can be also secondary to the reduction in the adipose mass via increased expression of enzymes involved in β-oxidation and via reduced expression of enzymes promoting lipogenesis (Ivanova et al., 2014; Pérez-Matute et al., 2007). Unlike the linseed group, the rabbits fed with fish oil supplements showed no differences in BW compared to the rabbits fed with a control diet, thus suggesting that the improvement of insulin sensitivity caused by EPA and DHA did not depend on the amount of adipose mass. However, several hypotheses can be made concerning the EPA/DHA-dependent effects on the function of adipose tissue. For example, since insulin resistance is associated with the chronic inflammation of insulin sensitive tissues, EPA and DHA could have reversed adipose inflammation through the down-regulation of nuclear factor-kappa B, Toll-like receptors and TNFα (Oh et al., 2011; Pérez-Matute et al., 2007; Uysal et al., 1997). The different effect on the expression of these genes between preformed dietary DHA and their precursor ALA could contribute to their differential effects on insulin resistance.

Their similarities with women confirm rabbits as valid experimental models for gestational diabetes. Other authors have successfully used the rabbit for studying metabolic syndromes induced by

352 castration (Ivanova et al., 2014) and high-fat diets (Maneschi et al., 2013). In fact, this nutritional
353 treatment induces all metabolic syndrome features, including hyperglycemia and glucose intolerance,
354 hypertension, dyslipidemia and increased visceral fat weight (Maneschi et al., 2013). Further studies
355 should be carried out to assess the effect of high-fat diets on the insulin resistance of pregnant rabbits as
356 well as the risk of metabolic syndromes following gestational diabetes.
357
358 **5. Conclusions**
359 Supplementation with linseed rich in ALA and fish oil rich in EPA and DHA, had differential
360 effects on insulin sensitivity during pregnancy. Pregnant rabbits fed with linseed showed elevated BW
361 and reduced milk production as well as high fasting plasma glucose concentration and poor response to
362 the glucose load. The deterioration of insulin sensitivity in the linseed group seems to be due to their
363 excessive weight gain since we demonstrated a positive correlation between BW, both before and
364 during pregnancy, and gestational insulin resistance. Conversely, preformed EPA and DHA provided
365 by fish oil did not affect BW but improved insulin sensitivity. Further studies using the rabbit as
366 experimental model are needed to clarify the activities of linseed components, the metabolic fates of
367 ALA, and the mechanisms involved in obesity-associated insulin resistance during pregnancy.
368
369
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537

538 Table 1. Formulation and chemical composition of control (C) and enriched diets supplemented with

539 fish oil (FO), and extruded linseed (L).

540

	Unit	Diet		
		C	FO	L
Ingredients				
Barley	%	17.0	17.0	17.0
Bran	%	7.0	7.0	5.0
Dehydrated alfalfa meal	%	50.0	50.0	50.00
Soybean meal 44%	%	18.0	18.00	13.00
Soybean oil	%	3.0	-	-
Extruded flaxseed	%	-	-	10.0
Nordos ®	%	-	3.0	-
Beet molasses	%	1	1	1
Calcium diphosphate	%	1.35	1.35	1.35
Vitamin-mineral premix*	%	1.2	1.2	1.2
Calcium carbonate	%	0.7	0.7	0.7
Salt	%	0.7	0.7	0.7
DL-methionine	%	0.05	0.05	0.05
Analytical data				
Crude Protein	%	17.80	17.81	17.95
Ether extract	%	5.55	5.63	6.02
Crude fiber	%	14.90	14.44	14.88
Ash	%	9.70	9.59	9.68
Starch*	%	11.2	11.1	10.8
NDF*	%	29.10	29.10	30.00
ADF*	%	19.80	19.80	20.45
ADL*	%	4.30	4.30	4.45
Digestible Energy**	Mj/kg	9.85	9.85	9.81

541 * Per kg diet: vitamin A 11,000 IU; vitamin D3 2000 IU; vitamin B1 2.5 mg; vitamin B2 4 mg; vitamin B6 1.25 mg;
542 vitamin B12 0.01 mg; alpha-tocopherol acetate 50 mg; biotine 0.06 mg; vitamin K 2.5 mg; niacin 15 mg; folic acid 0.30 mg;
543 D-pantothenic acid 10 mg; choline 600 mg; Mn 60 mg; Fe 50 mg; Zn 15 mg; I 0.5 mg; Co 0.5 mg.
544 * Calculated
545 ** Estimated by Maertens et al. (1988)
546

547 Table 2. Main fatty acid composition (expressed as percentage of total fatty acids) of diets.
548

Fatty acids	Diets		
	C	**FO**	**L**
SFAs	20.0	18.4	18.7
MUFAs	18.5	16.6	17.1
C18:2n-6 (LA)	39.0	32.2	33.3
C18:3n-3 (ALA)	22.2	29.2	30.6
C20:5n-3 (EPA)	0.2	1.0	0.2
C22:6n-3 (DHA)	0.1	2.2	0.1
PUFAs	61.5	65.0	64.2
n-6	39.0	32.2	33.3
n-3	22.5	32.8	30.9
LCP n-3	0.3	3.2	0.3
n-6/n-3	1.73	0.98	1.08

549

550

551 Table 3. Reproductive performance in C (control diet), FO (supplemented with fish oil), and L

552 (supplemented with extruded linseed) rabbits (n = 15/groups).

553

Parameter	Unit	C	FO	L	P-value
Baseline[1] BW	**kg**	4.1±0.1	4.0±0.1	4.1±0.1	*0.752*
Feed intake during pre-pregnancy period[1]	**g/d**	124_{a}±1	122_{a}±1	128_{b}±1	***0.001***
BW at AI	**kg**	4.1_{A}±0.1	4.1_{A}±0.1	4.4_{B}±0.1	***0.046***
Fertility	**%**	59.1	66.7	45.8	*0.357*
Feed intake during pregnancy	**g/d**	147±2	142±2	143±2	*0.316*
Duration of gestation	**d**	31.9±0.3	31.7±0.3	32.2±0.3	*0.498*
Litter size at kindling[2]	**n**	6.3±0.9	7.0±0.8	7.3±0.7	*0.660*
Litter weight at kindling	**g**	368±97	445±189	504±74	*0.426*[6]
Perinatal mortality[3]	**%**	31.2_{a}	40.2_{a}	9.6_{b}	***0.0001***
Litter size at 20 d	**n**	6.3±0.6	6.9±0.6	7.0±0.5	*0.631*
Litter weight at 20 d	**kg**	2.1±0.1	2.0±0.1	1.8±0.1	*0.264*[6]
Feed intake during lactation	**g/d**	236±4	241±3	245±4	*0.217*[6]
Milk production[4]	**g/d**	$153±5_{b}$	$142±4_{ab}$	$137±5_{a}$	***0.0001***[6]
Litter size at weaning[5]	**n**	6.1±2.8	6.3±2.2	6.8±2.2	*0.216*
Litter weight at weaning[5]	**kg**	4.9±0.2	4.6±0.2	4.7±0.2	*0.740*[6]
Pre-weaning mortality	**%**	2.3	10.2	7.6	*0.310*

554 Values in the same row not sharing the same subscript are significantly different (a, b: P<0.05; A, B: P < 0.1;
555 Sidak correction). Bold P-values are significant at the 0.05 level.
556 [1] Two month before AI
557 [2] Kits dead during perinatal period not included
558 [3] Death of entire litter included
559 [4] Estimated marginal means from 0 to 20th day of lactation
560 [5] 35 days post-partum
561 [6] Corrected for the number of kits
562

Table 4. Fasting derived indices of insulin resistance during pregnancy. C = control diet; FO = maternal diet supplemented with fish oil; L = maternal diet supplemented with extruded linseed (n = 10/group). Values are estimated marginal means ± SE.

Index	Days of pregnancy	Group			P-value		
		C	FO	L	Time	Group	Time x Group
Fasting glucose (mmol/l)	**0**	$7.08\pm0.26_{a}$	$6.55\pm0.30_{a}$	$6.32\pm0.25_{a}$	***0.003***	***0.0001***	***0.001***
	7	$8.46\pm0.34_{b}$	$6.12\pm0.30_{a}$	$7.00\pm0.39_{a}$			
	14	$6.60\pm0.25_{a}$	$6.62\pm0.30_{a}$	$6.64\pm0.25_{a}$			
	21	$6.42\pm0.25_{ab}$	$5.55\pm0.30_{a}$	$6.95\pm0.25_{b}$			
	28	$6.31\pm0.25_{a}$	$6.01\pm0.30_{a}$	$6.37\pm0.25_{a}$			
Fasting insulin* (µU/ml)	**0**	$4.91\pm0.33_{a}$	$6.15\pm0.45_{a}$	$8.14\pm0.60_{a}$	***0.0001***	***0.006***	***0.010***
	7	$6.05\pm0.63_{a}$	$6.53\pm0.68_{a}$	$12.86\pm1.33_{a}$			
	14	$11.25\pm0.90_{ab}$	$9.18\pm0.74_{a}$	$16.95\pm1.24_{b}$			
	21	$5.88\pm0.40_{a}$	$4.76\pm0.38_{a}$	$4.39\pm0.30_{a}$			
	28	$5.84\pm0.40_{b}$	$2.54\pm0.20_{a}$	$4.06\pm0.28_{ab}$			
Glucose-to-Insulin Ratio*	**0**	$1.51\pm0.26_{a}$	$1.04\pm0.26_{a}$	$0.87\pm0.26_{a}$	***0.0001***	*0.087*	***0.004***
	7	$1.48\pm0.33_{b}$	$1.15\pm0.33_{ab}$	$0.58\pm0.33_{a}$			
	14	$0.64\pm0.26_{a}$	$0.74\pm0.26_{a}$	$0.55\pm0.26_{a}$			
	21	$1.12\pm0.22_{a}$	$1.28\pm0.26_{a}$	$1.94\pm0.22_{a}$			
	28	$1.15\pm0.22_{a}$	$2.42\pm0.26_{b}$	$1.76\pm0.23_{ab}$			
HOMA-IR*	**0**	$0.09\pm0.01_{a}$	$0.11\pm0.01_{a}$	$0.13\pm0.01_{a}$	***0.0001***	***0.004***	*0.059*
	7	$0.13\pm0.01_{ab}$	$0.10\pm0.01_{a}$	$0.22\pm0.02_{b}$			
	14	$0.19\pm0.02_{ab}$	$0.15\pm0.01_{a}$	$0.26\pm0.02_{b}$			
	21	$0.09\pm0.01_{a}$	$0.07\pm0.01_{a}$	$0.07\pm0.01_{a}$			
	28	$0.09\pm0.01_{b}$	$0.04\pm0.00_{a}$	$0.06\pm0.00_{ab}$			

Values in the same row not sharing the same subscript are significantly different at P< 0.05 (Sidak correction).
Bold P-values are significant at the 0.05 level.
HOMA-IR = Homeostasis model assessment for insulin resistance
* Back-transformed values.

Table 5. Intravenous Glucose tolerance test -derived indices obtained in does at day 21 of pregnancy. C = control diet; FO = maternal diet supplemented with fish oil; L = maternal diet supplemented with extruded linseed (n = 5/group). Values are estimated marginal means ± SE.

Parameter	C	FO	L	*P-value*
Glucose 60 min (mmol/l)	6.39_{b}±0.02	5.60_{a} ±0.01	6.36_{b}±0.01	***0.0001***
AUC ((mmol/l) x min)	1104±20	1102±13	1154±15	*0.085*
K_{el} (%/min)	1.33_{B}±0.03	1.25_{AB} ±0.06	1.19_{A} ±0.04	***0.038***
$t_{1/2}$ (min)	52.00_{a} ±1.12	55.43_{ab} ±2.70	58.32_{b} ±1.48	***0.031***
V_d (dl/kg)	1.36±0.06	1.38±0.05	1.27±0.06	*0.388*
C_{max} (mmol/l)	24.53±1.16	24.30±1.00	26.24±1.08	*0.391*
CL (ml//kg/min)	1.67_{b}±0.05	1.75_{b}±0.04	1.43_{a}±0.04	***0.002***

Values in the same row not sharing the same subscript are different (a, b: P<0.05; A, B: P < 0.1). Adjustment for multiple comparisons: Sidak. Bold P-values are significant at the 0.05 level.
Glucose 60 min = glucose concentrations 60 min post-loading; AUC = area under the concentration time curve; K_{el} = elimination rate constant; $t_{½}$ = half-life; V_d = apparent volume of distribution; C_{max} = maximum concentration; CL = clearance.

583 Figure 1. Body weight of rabbit does (n=10/group) during pregnancy. C = control diet; FO = maternal
584 diet supplemented with fish oil; L = maternal diet supplemented with extruded linseed. Values are
585 means ± SE. $^{\#}P < 0.1$, $*P < 0.05$ supplemented groups *versus* control group for each gestational day.
586
587 Figure 2. Relationship between Homeostasis model assessment for insulin resistance (HOMA-IR) and
588 body weight of does (BW). The points of scatter plot show the BW of the rabbits and the corresponding
589 values of HOMA-IR recorded on day 0 (AI, empty circle), in early-mid-pregnancy (days 7 and 14 post
590 AI, gray triangle), and in late pregnancy (days 21 and 28 post AI, black square). In the model including
591 BW as predictor and HOMA-IR as dependent variable, the parameters showed that insulin resistance
592 measured by HOMA-IR increases with the increase in BW (b after log transformation = 0.23, $P <$
593 0.01). This relationship was most evident in early-mid pregnancy.
594
595 Figure 3. Relationship between area under the concentration-time curve (AUC) and body weight of
596 does (BW). The points of scatter plot show the BW of the rabbits recorded on day 0 (AI, empty circle)
597 and day 21 of pregnancy (black square), and the value of AUC measured on day 21. In the model
598 including BW as predictor and AUC as dependent variable, parameters showed that insulin resistance
599 on day 21 of pregnancy measured by AUC increases with the increase in BW at AI (b = 259.6, $P <$
600 0.01) and on day 21 of pregnancy (b = 219.1, $P < 0.05$).

Figure 1

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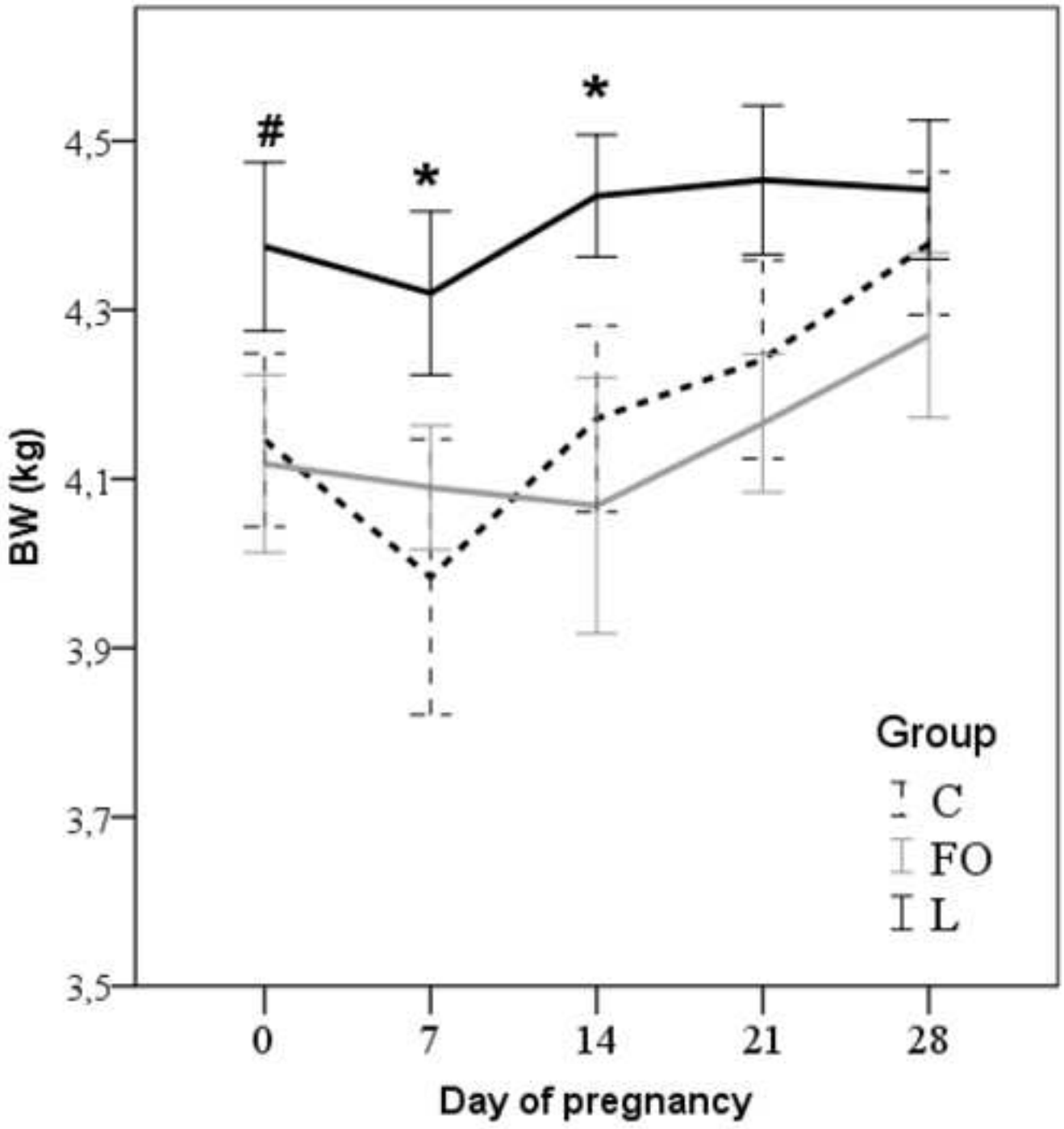

Figure 2

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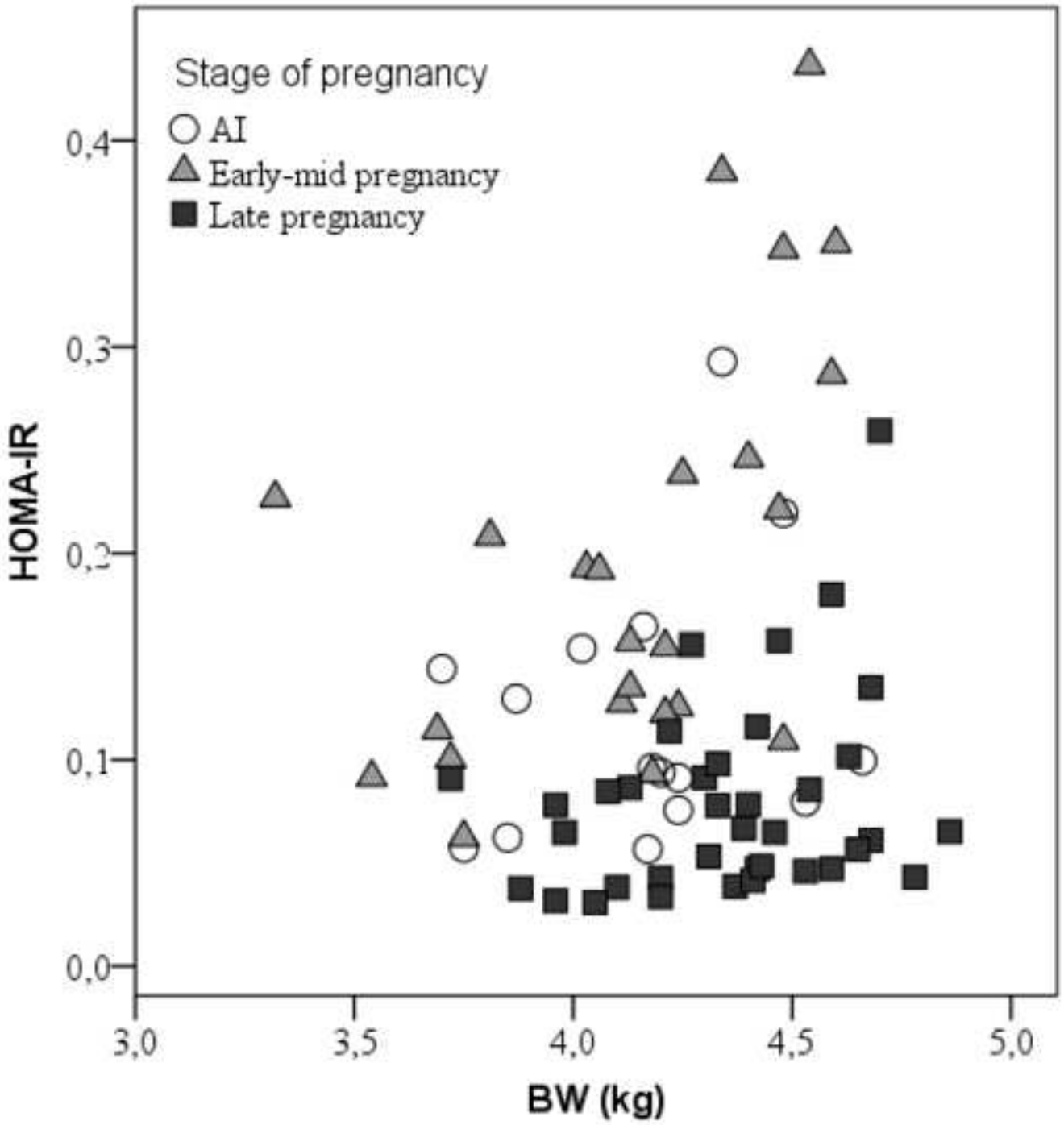

Figure 3

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