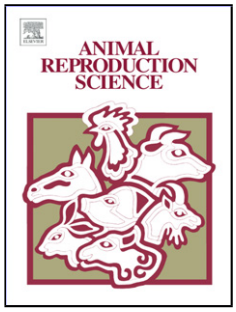

Title: Relationships among body composition, circulating concentrations of leptin and follistatin, and the onset of puberty and fertility in young female sheep

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Highlights

1. Accelerating the accumulation of muscle and adipose tissues can advance puberty.
2. Accelerating the accumulation of muscle and adipose tissues improved fertility rate.
3. Possible physiological link between muscle and the reproductive system of female sheep.
4. Muscle accumulation and leptin concentration were significant positive correlated.

Relationships among body composition, circulating concentrations of leptin and follistatin, and the onset of puberty and fertility in young female sheep

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Abstract

The onset of puberty depends on the attainment of critical body mass, so should also be affected by increases in the rate of accumulation of muscle and adipose tissue. Adipose tissue and reproduction are linked by leptin. For muscle, a link has not yet been identified, although one possibility is follistatin. We assessed the relationships among circulating concentrations of follistatin and leptin and the rates of growth and accumulation of muscle and fat during pubertal development in female sheep. We used 326 animals with known phenotypic values for live weight (LW), depths of eye muscle (EMD) and fat (FAT), and known breeding values at post-weaning age for body mass (PWT) and depths of eye muscle (PEMD) and fat (PFAT). Leptin concentration was positively correlated with values for EMD, PEMD, FAT, PFAT, LW and PWT ($P<0.001$), whereas follistatin concentration was negatively correlated with values for EMD and PWT ($P<0.001$), and PEMD ($P<0.01$) and FAT ($P<0.05$). Leptin concentration was negatively related to age and positively related to live weight at first oestrus and the proportion of females that attained puberty ($P\leq0.05$), and to fertility and reproductive rate ($P<0.01$). Follistatin concentration was negatively related to live weight at first oestrus and to fertility ($P<0.01$) and reproductive rate ($P<0.05$). There were positive correlations ($P<0.001$) between muscle accumulation and leptin concentration, and between muscle accumulation and reproductive performance. We conclude that leptin and follistatin are probably both involved in effects of accelerated accumulation of muscle and adipose tissues on the onset of puberty.

Keywords: Ewe lambs, follistatin, leptin, puberty, fertility

1. Introduction

The onset of puberty depends on an interaction between chronological age and accumulation of body mass so, for example, female sheep usually enter puberty when they attain 50-70% of their mature body mass (Hafez, 1952; Dýrmundsson, 1973). The 'body mass' concept has recently been refined by consideration of individual tissue types and we now appreciate that, in young ewes, genetic merit for accelerated accumulation of muscle and fat is associated with advanced puberty and improved fertility (Rosales Nieto et al., 2013a, 2013b).

The dependency of puberty on tissue mass reveals the importance of physiological signals from metabolic regulatory tissues to the reproductive axis (Martin et al., 2008). For adipose tissue, the primary signal is leptin (review: Foster and Nagatani, 1999) but, for muscle, endocrine factors associated with reproduction have not been clearly identified. One possibility is follistatin, a binding protein that inactivates several members of the transforming growth factor β family, including activin and myostatin, with diverse effects on growth, metabolism, immunity and reproduction (Rodino-Klapac et al., 2009; Hedger et al., 2011). Follistatin is secreted by the sheep ovary (Tisdall et al., 1992) but circulating concentrations vary little during the oestrous cycle (McFarlane et al., 2002), probably because it is produced in a variety of tissues, particularly muscle, where its importance for muscle growth and development has been demonstrated (Matzuk et al., 1995; Lee and McPherron, 2001; Gilson et al., 2009). With respect to reproduction, follistatin seems to have no effect on hypothalamic GnRH secretion in sheep (Padmanabhan et al., 2002), but it does act at pituitary level to inhibit FSH secretion in rodents (Ueno et al., 1987). The ultimate trigger for the first ovulation at puberty might be GnRH and LH pulses, but FSH is essential for the months-long process of ovarian development leading to that point – without it, there

would be no follicular development, no oestradiol production and therefore, no ovulation (Schwartz, 1974). Follistatin also appears to play a direct role in ovarian function – in mice, deletion of follistatin in adult granulosa cells leads to effects that range from reduced fertility and litter size to complete termination of ovarian activity and reproduction (Jorgez et al., 2004; Kimura et al., 2010). Overall, therefore, follistatin appears to play roles in oocyte maturation and in the inhibition of pituitary FSH synthesis (Shimasaki et al., 1989; review: Knight and Glister, 2001; Knight et al., 2012).

Thus, we hypothesized that accelerating the onset of puberty and improving reproductive performance by increasing the accumulation of muscle and fat will be associated with changes in the circulating concentrations of leptin and follistatin. We tested this hypothesis in young female sheep, using large field studies in which we analyzed the statistical relationships among leptin and follistatin concentrations, phenotypic and genotypic values for rates of growth and accumulation of muscle and adipose tissues, age and live weight at puberty, fertility and reproductive rate. Large correlation-based studies can detect potential physiological linkages so are valuable as a first step towards the development of hypotheses that guide intervention studies.

2. Material and Methods

These experiments were undertaken in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and was approved by the Animal Ethics Committee of the Department of Agriculture and Food, Western Australia.

2.1. Experiment 1

104 *2.1.1. Experimental location and animals*

105 We used Merino ewe lambs (n = 136) that had been born in August-September 2009 on a
106 commercial farm ('Moojepin'). The dams (mothers) of the experimental animals had been
107 sourced from two ram-breeding flocks in Western Australian and sires (fathers) were chosen
108 to supply a wide range in Australian Sheep Breeding Values (ASBV) for growth, muscle and
109 fat. Data were collected for birth date, birth weight, birth type (single or twin) and rear type
110 to weaning (single or twin). Ewes were transported to Medina Research Station (32.2° S,
111 115.8° E) where the experiment was conducted from February to June 2010. Merino sheep
112 present an extended breading season and the months with lowest ovarian activity are
113 November and December, although there is variation among years due to environmental
114 conditions (Watson, 1953; Radford, 1959). The ewes were weighed bi-weekly and the data
115 were used to calculate the average daily gain (ADG) and to estimate the live weight at
116 puberty and the date of conception. The depths of the *longissimus dorsi* muscle and
117 subcutaneous fat at a point 45 mm from the midline over the twelfth rib were measured using
118 ultrasound when the ewes were 164 (range 134 to 176) and 251 (range 221 to 263) days of
119 age. For both measurements, the range in eye muscle depth (EMD) was 20-33 mm and the
120 range in C-site fat depth (FAT) was 2-8 mm. Using MERINOSELECT (Brown et al., 2007),
121 the ultrasound data were used to generate estimates of Australian Sheep Breeding Values at
122 post-weaning age, which can be measured from 7 to 10 months of age, for weight (PWT;
123 range 0–9 kg), depth of eye muscle (PEMD; range 0.0–2.6 mm) and depth of fat (PFAT;
124 range 0.0–1.2 mm). In this year, the national average values in MERINOSELECT for
125 females were 0.9 for PWT, 0.0 mm for PFAT and 0.2 mm for PEMD.
126

127 *2.1.2. Animal management and feeding*

Animals were initially allocated on the basis of live weight to two 20 m x 60 m pens, where they had *ad libitum* access to water and to a pelleted diet (introduced over a 7-day period). The pellets were based on barley, wheat and lupin grains, cereal straw and hay, canola meal, minerals and vitamins, and had been formulated to provide 11.5 MJ of metabolizable energy per kilogram of dry matter, 15% protein, and sufficient minerals and vitamins for maximum growth.

On February 24 (Day –69), when the ewes were 179 days old (range 149 to 191) and weighed 36.8 ± 0.4 kg, four Merino wethers (male sheep castrated before puberty) with marking harnesses (MatingMark®; Hamilton, NZ) were introduced to detect the onset of oestrus (pre-mating period). The wethers had received a 2 mL subcutaneous injection of testosterone enanthate (75 mg/mL; Ropel®, Jurox, NSW) one week before they were placed with the young ewes. Every 2 weeks, the injections were repeated and the crayons on the harnesses were changed. The wethers were removed on May 4 (Day 0), when the ewes were 249 (range 219 to 261) days old and weighed 41 ± 0.5 kg. The ewes received an intramuscular injection (1 mL) of supplement of vitamins (Vitamin A 500,000 iu; Vitamin D3 75,000 iu; Vitamin E 50 iu/mL; Vet ADE®, Auckland, New Zealand). For the mating period, they were allocated, on the basis of live weight and sire, into 8 management groups of 15 and moved into 3 m x 7 m pens where they had *ad libitum* access to clean water and the sheep pellets. A single, experienced Merino ram was introduced into each group of ewes. The rams were removed on Day 47 and the ewes remained indoors.

Crayon marks on ewe rumps were recorded three times per week to estimate the date of first standing oestrus. Crayon marks were scored (1, 2 or 3), with Score 1 being one narrow mark on the middle or the edge of the rump and Score 3 as being a single large mark covering the rump. The date when the first Score 2-3 crayon mark was recorded was used to estimate age at first oestrus and the closest live weight recorded to that date was deemed to be

153 live weight at first oestrus. Pregnancy rate and the number of fetuses per ewe were confirmed
154 by ultrasound scanning 60 d after rams were removed. The data were used to generate values
155 for fertility (percentage of pregnant ewes per 100 ewes mated) and reproductive rate (number
156 of fetuses in utero per 100 ewes mated).
157
158 *2.1.3. Blood sampling and immunoassay*
159 Blood (5 ml) was sampled, without fasting, by jugular venipuncture on 4 occasions, at
160 beginning and middle of the teasing period and the mating periods, when the ewes were on
161 average 199, 227, 248, and 269 days old. The samples were placed immediately on ice,
162 centrifuged at 2000 g for 20 min and the plasma harvested and stored at –20º C until analysis.
163 The plasma concentration of total follistatin was measured in duplicate 100 μL aliquots by
164 radioimmunoassay using purified heterologous bovine follistatin as standard and iodinated
165 bovine follistatin as tracer, as previously described and validated for ovine samples (Klein et
166 al., 1991; O'Connor et al., 1999). The limit of detection was 1.16 ng/mL and the intra- and
167 inter-assay CVs were 7.9% and 7.8%. Plasma leptin concentrations were determined by
168 radioimmunoassay in duplicate 100 μL aliquots, as described by Blache et al. (2000). The
169 limit of detection was 0.06 ng/mL and the intra-assay CVs were 7.3% at 0.73 ng/mL, 4.4% at
170 0.84 ng/mL, and 2.4% at 1.61 ng/mL.
171
172 *2.2. Experiment 2*
173
174 *2.2.1. Experimental location and animals*
175 The Merino ewe lambs (n = 190) used in this experiment were born in June 2010 on the
176 research farm (‘Ridgefield’) of the University of Western Australia (32.2° S, 115.8° E). The
177 mothers of the experimental animals had been sourced from two ram-breeding flocks in

178 Western Australian and their fathers had a wide range in Australian Sheep Breeding Values
179 (ASBV) for growth, muscle and fat. In November 2010, ewes were transported to Medina
180 Research Station (32.2° S, 115.8° E) for the first stage of the experiment and, in late
181 December, they returned to 'Ridgefield' where they remained until the end of the experiment
182 (September 2011). Female sheep were moved from one experimental station to another due
183 to project's objectives and goals. Although, animals were moved from one location to
184 another, the data were always recorded similarly.

185 Data were collected using the same protocols as for Experiment 1, except for live
186 weight. The ewes were weighed every 2 weeks at the Medina site and every week at the
187 Ridgefield site. Data were combined and used to calculate the average daily gain (ADG) and
188 to estimate the live weight at puberty and the date of conception. The depths of the
189 *longissimus dorsi* muscle and subcutaneous fat at a point 45 mm from the midline over the
190 twelfth rib were measured using ultrasound when the ewes were 167 (range 146 to 186) and
191 218 (range 198 to 228) days of age. For both measurements, the range in eye muscle depth
192 (EMD) was 20-33 mm and the range in C-site fat (FAT) was 2-8 mm. Using
193 MERINOSELECT (Brown et al., 2007), the ultrasound data were used to generate estimates
194 of Australian Sheep Breeding Values at post-weaning age, which can be measured from 7 to
195 10 months of age, for weight (PWT; range 0–9 kg), depth of eye muscle (PEMD; range 0.0–
196 2.6 mm) and depth of fat (PFAT; range 0.0–1.2 mm). In this year, the national average values
197 in MERINOSELECT for females were 1.3 for PWT, 0.0 mm for PFAT and 0.2 mm for
198 PEMD.

199

200 *2.2.2. Animal management and feeding*

The ewes were allocated on the basis of live weight to eight groups of 23 or 24 animals and housed in separate pens (6 x 14 m) at Medina research station. They had *ad libitum* access to water and a pelleted diet, introduced over a 7-day period, formulated as described above.

The pre-mating period commenced on November 30 (Day –70), when the ewes were on average 157 days old (range 136 to 176) and weighed 36.2 ± 0.3 kg (range 24.8 to 50.8). A vasectomized Merino ram with a marking harness was introduced into each pen to detect the first oestrus. On December 29 (Day –41), the ewes and vasectomized rams were returned to 'Ridgefield', where each group was allocated to a separate 30 x 120 m plot, with access to clean water, *ad libitum* oaten hay (9 Mj/kg and 9% protein) plus lupin grain (13.5 Mj/kg and 32% protein). It was anticipated that the combination of supplement plus dry pasture would allow the ewes to gain approximately 100 g/day. Although, animals were moved from one location to another and changes in diet from pellets to oat hay and lupins cannot affect the circulating concentration of follistatin and leptin. The vasectomized rams were removed on February 8 (Day 0) and ewes were allocated on the basis of their live weight and sire into 8 groups. An experienced ram with a marking harness was introduced into each group to begin the mating period when the ewes were on average 226 days old (range 206 to 246) and weighed 42.4 ± 0.3 kg (range 24.3 to 56.4). The rams were removed after 45 days.

Crayon marks on ewe rumps were scored and recorded three times per week at Medina and once per week on 'Ridgefield' to estimate the date of first standing oestrus, as described for Experiment 1. Age and live weight at first oestrus, fertility (percentage of pregnant ewes per 100 ewes mated) and reproductive rate (number of fetuses in utero per 100 ewes mated) were also estimated using the same protocol as in Experiment 1.

2.2.3. Blood sampling and immunoassay

We used the same protocols for sampling and assay as in Experiment 1. Blood was sampled when the ewes were 144, 186, 227 and 254 days old. For follistatin in Experiment 2, the limit of detection was 1.16 ng/mL and the intra- and inter-assay CVs were 7.9% and 7.8%. For leptin in Experiment 2, the limit of detection was 0.05 ng/mL and the intra-assay CVs were 16% at 0.47 ng/mL, 3.3% at 1.10 ng/mL, and 3.6% at 1.79 ng/mL.

2.3. Data analysis

The data were analyzed using SAS version 9.3 (2010). Ewe live weight during the experiment was analyzed using the linear mixed model procedures allowing repetitive measures (PROC MIXED) and included dam source and age (mother) and birth type as fixed effects.

Average daily gain (ADG) during the experiments was determined for each young ewe using a random coefficient regression including a cubic smoothing spline for time (TRANSREG). ADG was analyzed using the linear mixed model procedures (PROC MIXED). Fixed effects in the model were source and age of dam (mother), birth type and age at start of the experiments. Follistatin and leptin concentrations were each independently tested as a covariate, and sire (father) of the ewes was used as a random effect. We fitted follistatin and leptin, due to their role in regulation of feed intake and energy balance and muscle growth and development, to test whether or not these two proteins are involved in the ADG of the ewe lambs.

The correlations among live weight, follistatin concentration, leptin concentration, PWT, PEMD, PFAT, EMD and FAT were computed using PROC GLM with MANOVA option which allows removal of major fixed effects. Fixed effects included in the model were source and age of dam (mother), birth type and age at the day of the muscle and fat scan.

Age and live weight at first oestrus were analyzed using mixed models (PROC Mixed), including dam source and age, birth type and age as fixed effects. Concentrations of follistatin and leptin were each independently tested as a covariate. The sire (father) of the ewes was used as a random effect.

Puberty and fertility data were analyzed using the generalized linear mixed model procedures with a binomial distribution and logit link function (PROC GLIMMIX). Fixed effects were dam source and age, birth type, age and live weight at the sampling date. Concentrations of follistatin and leptin were each independently tested as a covariate. Sire (father) of the ewe was used as random effect. Reproductive rate data were analyzed using the generalized linear mixed model procedures with a multinomial distribution and logit link function (PROC GLIMMIX). The same fixed effects, covariates and random effects were used as for the fertility analysis.

Average live weight, PWT, EMD, PEMD, FAT and PFAT were analyzed using mixed models (PROC MIXED), and included as fixed effects: dam source and age, and birth-rear type. Average hormone concentration for leptin and total follistatin were each independently tested as a covariate. Sire (father) of the ewe was used as random effect.

Hormone concentration (follistatin, leptin) was analyzed using mixed models (PROC MIXED) allowing for repeated-measures, and included as fixed effects: dam source and age, birth-reared type and age and live weight at start of teasing. FAT, EMD, PWT, PEMD or PFAT were each independently tested as a covariate. Sire (father) of the ewe was used as random effect. Mean hormone concentration was analyzed using analysis of variance model procedures, where Factor A was hormone concentration and Factor B was date at sampling (PROC ANOVA). Differences among groups for live weight, leptin and follistatin concentration within date of sampling were analyzed using PROC GLM.

All 2-way interactions among the fixed effects were included in each model and non-significant ($P > 0.05$) interactions were removed from the final model. The data for puberty, fertility and reproductive rate are presented as logit values and back-transformed percentages.

3. Results

3.1. Live weight, leptin and follistatin

In Experiment 1, mean live weight increased from around 37 kg on Day –75 to around 54 kg on Day +57 (Fig. 1A), with an ADG of 144 ± 2.4 g. There was a clear set-back in growth between Days –20 and +20. Mean leptin concentration increased from 1.3 ± 0.02 ng mL^{-1} on Day –50 to 1.7 ± 0.02 ng mL^{-1} on Day +20 ($P < 0.001$; Fig. 1B), and the progression in this rise was also interrupted around Day 0, at the time of the arrest in weight gain (upper panel). By contrast, follistatin concentration decreased gradually from 3.1 ± 0.1 ng mL^{-1} on Day –50 to 2.7 ± 0.1 ng mL^{-1} on Day 0 ($P < 0.001$), after which it did not change (Fig. 1B).

In Experiment 2, live weight increased from 37 kg on Day –75 to 48 kg on Day +59 (Fig. 1C). There were brief periods when growth was negative (eg, Days –50,–12, –8, +24) so the overall ADG (69 ± 4.7 g) was about half that observed in Experiment 1. The concentrations of both leptin and follistatin were higher in Experiment 2 (Fig. 1D) than in Experiment 1(Fig. 1C), but the dynamics were similar. In both experiments, leptin concentrations were initially high and increasing, then fell on Day 0 ($P < 0.001$), in association with declines in growth. In Experiment 2, follistatin concentrations began at high levels then decreased markedly on Day 0 ($P < 0.001$; Fig. 1D), before rising again.

In both experiments, neither follistatin concentration nor leptin concentration were correlated with age or source of dam, or birth or rear type of the young ewes. However, leptin concentration was strongly positively correlated with live weight in both experiments ($P < 0.001$; Fig. 2A, B). By contrast, for follistatin concentration, the relationship with live weight

was weak, if still significant, in Experiment 1 ($P < 0.01$; Fig. 2A) but not significant in Experiment 2 (Fig. 2B).

3.2. Growth, muscle and fat

The correlations among live weight, PWT, EMD, PEMD, FAT and PFAT are shown in Table 1. In both experiments, there were strong positive relationships between EMD and live weight and PWT, FAT and live weight, EMD and FAT and PEMD and PFAT, but all other relationships were relatively weak. Leptin concentration was strongly related to PWT, EMD, FAT and PFAT in both experiments (Table 2). The correlation with PEMD was also significant in both experiments, but strong in Experiment 2 and relatively weak in Experiment 1. By contrast, where the relationships with follistatin concentration were significant, they were all negative. In Experiment 1, the relationships were strong for PWT, EMD, PEMD and FAT but, in Experiment 2, there were only two significant correlations, with EMD and PEMD, and both were relatively weak (Table 2).

The potential effects of accumulation of muscle on leptin and follistatin concentrations are of particular interest, so the relationships with EMD are explored in Figure 3. For leptin, the correlations are relatively robust in both experiments whereas, for follistatin, the correlations are significant but explain only 2-10% of the variation.

3.3. Puberty

As shown in Table 3, leptin concentration was positively associated with the proportion of ewe lambs that attained puberty for Experiment 2 ($P \leq 0.05$), but the relationship was not significant for Experiment 1 ($P = 0.08$). Age at first oestrus was weakly negatively correlated with leptin concentration in Experiment 1 ($P < 0.01$), but the association was not significant in Experiment 2 ($P > 0.05$). Live weight at first oestrus was weakly positively correlated with

leptin concentration for Experiment 2 ($P < 0.05$), but not Experiment 1. After adjustment for effects of live weight, these effects of leptin concentration on age at first oestrus and puberty were no longer evident (Table 3). Follistatin concentration was not related to the proportion of ewe lambs entering puberty, or to age at first oestrus. There was a weak and significant negative relationship with live weight at first oestrus, but only in Experiment 1 (Table 3).

3.4. Fertility and reproductive rate after puberty

As shown in Table 3, for Experiment 1, leptin concentration was positively correlated with the proportion of ewe lambs that became pregnant ($P < 0.01$) and with reproductive rate ($P < 0.01$). By contrast, follistatin concentration was negatively correlated with the proportion of young female sheep that became pregnant ($P < 0.01$) and with reproductive rate ($P < 0.05$). The relationships between leptin and fertility and between leptin and reproductive rate were significant after adjustment for effects of live weight, but the relationships between follistatin and fertility and between follistatin and reproductive rate were not significant.

The strong but contrasting relationships observed in Experiment 1 between fertility and leptin, and between fertility and follistatin, are illustrated in Figure 4. For Experiment 2, neither leptin nor follistatin concentration were related to the proportion of ewe lambs that became pregnant, or with reproductive rate (Table 3).

4. Discussion

These correlation-based field studies with young Merino ewes offer several robust novel observations: the positive relationships between muscle development and puberty and fertility and, and the positive relationships between muscle development and leptin concentrations. Moreover, these studies provide the first observations of relationships between follistatin concentrations, puberty and fertility in sheep, although they are somewhat

problematical because of differences between the two experiments (as we will discuss below). Importantly, the expected relationships between leptin and reproduction, evident in both studies, verifies the validity of large, correlation-based field studies for detecting potential physiological linkages, as well as the validity of our sampling regime and statistical methodology. We can therefore interpret the correlations with some confidence and use them as a solid basis for development of hypotheses for guiding direct intervention studies.

Circulating concentrations of leptin increased progressively as the animals grew and puberty approached, consistent with previous reports (review: Foster and Nagatani, 1999). However, the progressive increase was interrupted by brief periods in both experiments when the growth of the ewe lambs was negative, such that the animals lost weight rather than continue to grow. The losses in body mass were small in absolute terms, although arguably larger in the context of the growth trajectory, and the associated changes in the leptin profile suggest a greater impact on metabolic homeostasis than is indicated by a relatively insensitive measure such as body mass. Indeed, even the more mechanistic relationship between mass of adipose tissue and leptin concentration (Maffei et al., 1995; Blache et al., 2000) is probably incapable of reflecting dynamic changes in metabolic homeostasis. With respect to the control of ovarian function, the 'acute' response to short-term changes in nutrition seems to be best explained changes in circulating concentrations of metabolic hormones that precede detectable changes in live weight (reviewed by Scaramuzzi et al. 2006). Moreover, across both experiments, there was a consistent relationship between circulating leptin concentrations and the rate of tissue accumulation, whether it was measured by the rate of growth or by the rates of accumulation of muscle or fat. As a consequence, leptin concentration was positively correlated to age and live weight at first oestrus, the success of puberty and fertility, and final reproductive rate, observations that are consistent with previous studies (reviewed: Smith et al., 2002). Overall, these observations support the

concept that increases in the rates of accumulation of adipose tissue, perhaps acting through the leptin that it produces, inform to the brain centres that control reproduction about the body composition of the animal and thus affect the onset of puberty.

Follistatin concentrations, by contrast, decreased as the animals grew and as the amount of muscle increased. Interestingly, the actual concentration of follistatin was similar at the onset of puberty and conception in both experiments and, whenever follistatin concentration was significantly correlated with measures of reproductive function, the relationships were negative. These observations suggest that if follistatin plays a role in reproduction in young female sheep, it is inhibitory – delaying puberty and reducing fertility and reproductive rate. If circulating follistatin acts as a physiological signal between muscle tissue and the reproductive axis, it appears to be an inhibitory factor that needs to be reduced before reproduction can proceed. Clearly, this hypothesis needs to be tested with more detailed intervention studies.

There were inconsistencies among the observations from these two field studies. For example, the dependency of leptin concentration on the rate of growth, and on the rates of muscle and fat accumulation, were significant in both experiments, but much stronger in Experiment 2 than in Experiment 1, particularly for phenotypic and genotypic measures of muscle accumulation. For follistatin concentration, on the other hand, relationships with the rates of growth and of muscle and fat accumulation were weaker for Experiment 1 than for Experiment 2. Ewe lambs from Experiment 2 were heavier and had higher circulating concentrations of both hormones than the young females from Experiment 1, but the average growth rate was lower for Experiment 2 than for Experiment 1. Our interpretation is that the differences in fertility rate between the experiments is largely explained by differences in growth rate during the mating period, a variable that is difficult to control with field studies in an extensive management system. It is clear that there were uncontrolled external factors

affecting the secretion of leptin and follistatin, as well the reproductive performance of the animals. The significant relationships that we did observe were revealed because we used large numbers of animals.

Interestingly, there were significant relationships between muscle accumulation and leptin concentration and between muscle accumulation and reproductive performance. Leptin is thought to be produced primarily by adipose tissue, but the large variation in circulating leptin concentrations at similar levels of adiposity implies control by factors other than simple fat mass (Flier, 1997). The relationships with measures of muscle accumulation suggest that intramuscular adipose tissue might also be a biologically significant source of leptin, a concept supported by other findings: the leptin gene is expressed in muscle (Wang et al., 1998); leptin induces muscular hypertrophy and regulates energy expenditure and fat oxidation in muscle (Gong et al., 1997; Muoio et al., 1997); and, as muscle mass increases, the concentration of intramuscular fat increases (Zeidan et al., 2005; Zhong et al., 2011). It is therefore possible that the leptin produced in muscle, particularly in intramuscular fat, works in parallel with leptin from adipose tissue to inform the brain about metabolic reserves.

We hypothesized that follistatin concentration would be high during muscle growth and development, and therefore higher in ewe lambs selected for rapid muscle accumulation. Our data lead us to reject these hypotheses, although it is important to note that our observations were made under field conditions so uncontrolled, day-to-day variations in food intake and weight gain might have affected follistatin secretion (Silanikove, 2000). Indeed, Phillips et al. (1998) reported that reductions in food intake affected circulating concentrations of follistatin in Romney ewe lambs. On the other hand, we also need to explore the relationship between myostatin and follistatin. Myostatin is an important inhibitory regulator of muscle development (McPherron et al., 1997; Thomas et al., 2000; Lee and McPherron, 2001) and, in sheep, muscle development is enhanced by a mutation in

the myostatin gene and by increases in the production of follistatin, which blocks myostatin action (Clop et al., 2006; Rodino-Klapac et al., 2009). However, in animals selected for accelerated muscle accumulation, but less specialized for meat (eg, Merino), the ratio of follistatin to myostatin is probably more important in determining muscle mass (McPherron et al., 2009). To date, there have been no studies of the processes that underpin muscle accumulation in Merino sheep that have been selected for rapid muscle accumulation.

With respect to reproduction, the decline in total follistatin concentration at the approach of puberty and conception reflects previous observations (Foster et al., 2000; McFarlane et al., 2002). Among the variety of effects that follistatin has been reported to have on the reproductive axis, our observations are consistent with its role in blocking the activins – at pituitary level, reducing FSH synthesis and at ovarian level reducing the actions of FSH on granulosa cells. Under these circumstances, withdrawal of follistatin as mature live weight is achieved would aid the progress of puberty and the maximization of fecundity.

In conclusion, in sheep with higher breeding values for accumulation of muscle or fat, puberty will be advanced and reproductive performance improved, perhaps because of the effects of the changes in tissue accumulation on the circulating concentrations of leptin and follistatin. These hypotheses have been generated from correlations with large numbers of animals in field studies, and now need to be tested in intervention experiments.

Conflict of interest

Please disclose any potential conflict of interest pertaining to your contribution or the Journal; or write 'NONE' to indicate you declare no such conflict of interest exists. A conflict of interest might exist if you have a competing interest (real or apparent) that could be considered or viewed as exerting an undue influence on you or your contribution. Examples could include financial, institutional or collaborative relationships. The Journal's editor(s) shall contact you if any disclosed conflict of interest may affect publication of your contribution in the Journal.

Potential conflict of interest: NONE

On behalf of all the authors, I declare that there are no potential conflicts of interest.

Cesar Rosales Nieto

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585
586

586 **Fig. 1.** Changes in live weight in Experiment 1 (A) and Experiment 2 (C) and circulating
587 concentrations of follistatin (○) and leptin (●) in the young Merino ewes in Experiment 1 (B)
588 and Experiment 2 (D). Day 0 is the day fertile Merino rams were introduced. Values are
589 mean ± sem.
590
591 **Fig. 2.** Correlation between live weight and mean circulating concentrations of follistatin (○
592 grey lines) and leptin (● black lines) in the young Merino ewes in Experiment 1 (A; $P < 0.01$
593 for follistatin and $P < 0.001$ for leptin) and Experiment 2 (B; $P > 0.05$ for follistatin and $P <$
594 0.001 for leptin).
595
596 **Fig. 3.** Correlation analysis for the effect of depth of eye muscle (EMD) on the
597 concentrations of mean total follistatin (○ grey lines) and mean leptin (● black lines) in the
598 young Merino ewes from Experiment 1 (A; $P < 0.05$ for leptin and $P < 0.01$ for follistatin)
599 and Experiment 2 (B; $P < 0.001$ for leptin and $P < 0.05$ for follistatin).
600
601 **Fig. 4.** Effect of mean concentration of leptin (black line) and follistatin (grey line) on
602 fertility in the young Merino ewes in Experiment 1. The closest sample to the date of
603 conception was used to plot these regressions. The broken lines represent upper and lower
604 95% confidence limits (both relationships: $P < 0.01$).
605

Tables

Table 1: Correlations (*r*) among post-weaning phenotypic and genotypic values for live weight (LW, PWT), depth of muscle (EMD, PEMD) and depth of fat (FAT, PFAT) in young Merino ewes from Experiments 1 and 2.

Experiment	Variable	PWT	EMD	PEMD	FAT	PFAT
1	LW	0.68	0.64	0.28	0.56	0.29
2		0.79	0.70	0.24	0.52	0.26
1	PWT		0.53	0.40	0.39	0.34
2			0.63	0.29	0.33	0.11
1	EMD			0.41	0.59	0.34
2				0.76	0.54	0.52
1	PEMD				0.22	0.76
2					0.40	0.67
1	FAT					0.26
2						0.71

Table 2. Correlations (*r*) among post-weaning phenotypic and genotypic values for live weight (PWT), muscle accumulation **(**EMD, PEMD) and fat accumulation (FAT, PFAT) and the mean circulating concentrations of leptin and follistatin in young Merino ewes. Information has been provided for analyses with or without live weight (LW, PWT) included in the statistical model[1].

	Experiment 1		Experiment 2	
Variable	Leptin	Follistatin	Leptin	Follistatin
PWT (*r*)	*** (0.46)	*** (-0.28)	*** (0.27)	NS
EMD (*r*)	*** (0.47)	*** (-0.32)	*** (0.55)	* (-0.15)
EMD + LW	*	**	***	*
PEMD (*r*)	* (0.19)	** (-0.27)	*** (0.53)	** (-0.18)
PEMD + PWT	NS	*	***	**
FAT (*r*)	*** (0.41)	** (-0.23)	*** (0.47)	NS
FAT + LW	***	NS	***	NS
PFAT (*r*)	*** (0.26)	NS	*** (0.45)	NS
PFAT + LW	NS	NS	***	NS

P-values: * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; NS $P > 0.05$

[1]Note: values for *r* cannot be supplied for correlations when a third factor (LW or PWT) is included in the analyses.

620

621 **Table 3.** The correlations (*r*) between the hormone concentrations (follistatin and leptin) and
622 the advent of puberty, the age and live weight at first oestrus, and reproductive performance
623 (fertility, reproductive rate) in young Merino ewes mated at 8-9 months of age. Information
624 has been provided for analyses with live weight (LW) included or excluded in the statistical
625 model[2].

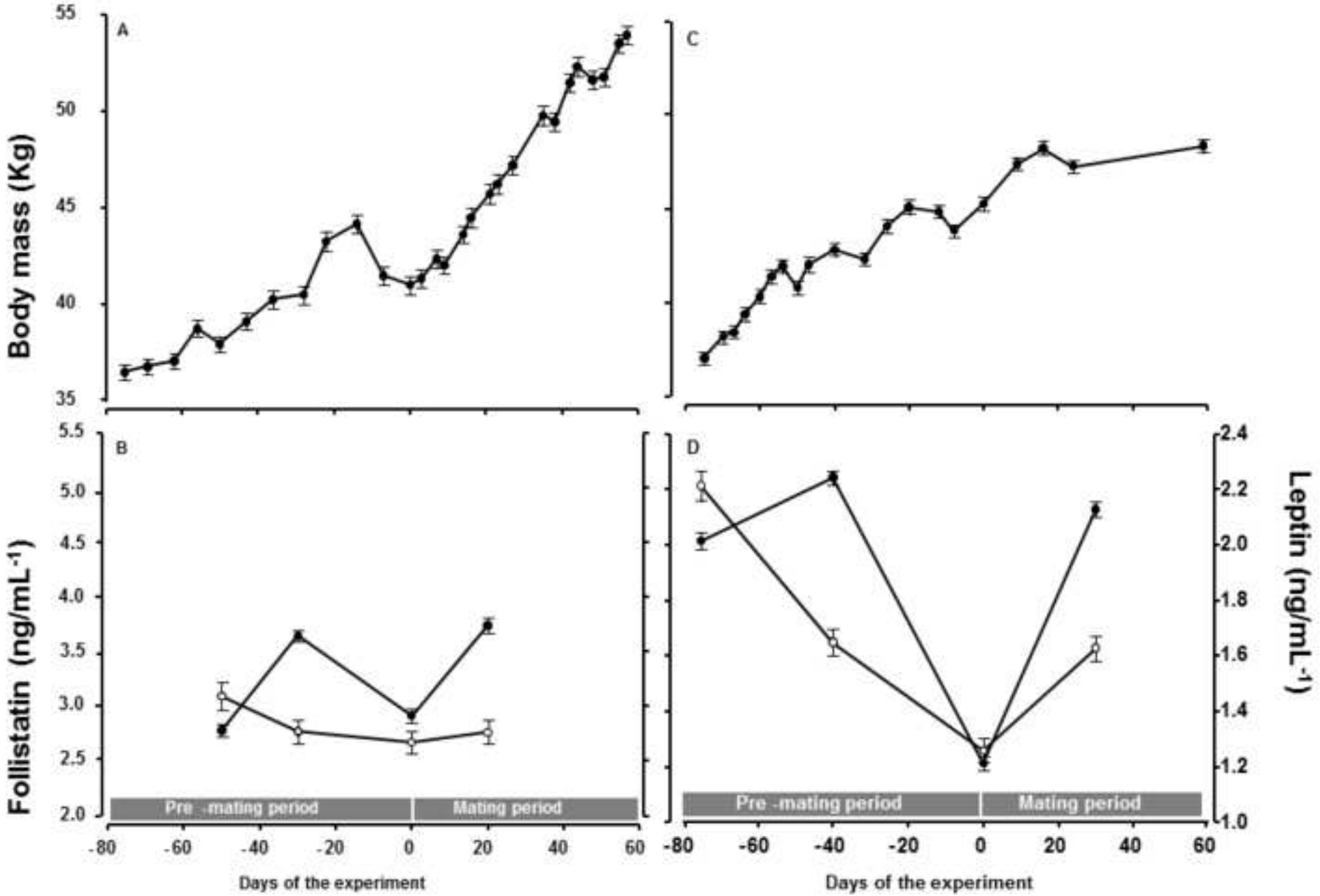
	Experiment 1		Experiment 2	
Variable	Leptin	Follistatin	Leptin	Follistatin
Puberty (%)	NS	NS	*	NS
Age at first oestrus (days) (r) – LW	** (-0.21)	NS	NS	NS
Age at first oestrus + LW	NS	NS	NS	NS
LW at first oestrus (kg) (r)	NS	* (-0.17)	* (0.19)	NS
Fertility (%) – LW	**	**	NS	NS
Fertility + LW	*	NS	NS	NS
Reproductive rate (%) – LW	**	*	NS	NS
Reproductive rate + LW	*	NS	NS	NS

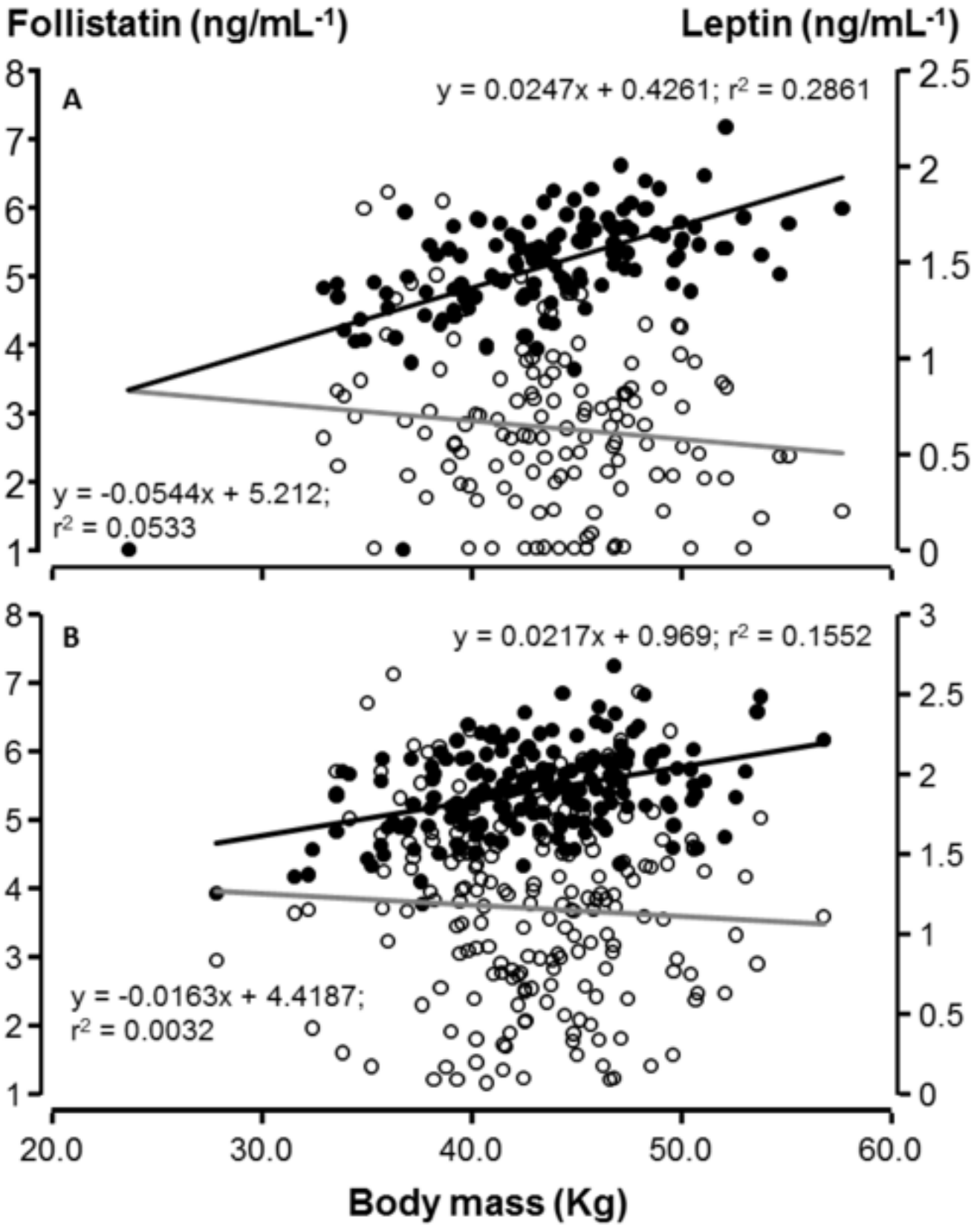
626 P-values: * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; NS $P > 0.05$

627 [2]Note: values for *r* cannot be supplied for correlations when a third factor (LW) is included in the analyses, or
628 when the distribution is binomial or multinomial (puberty, fertility, reproductive rate).

629

Figure1


A
B
C
D
Body mass (Kg)
Follistatin (ng/mL-1)
Leptin (ng/mL-1)
Pre -mating period
Mating period
Days of the experiment
55
50
45
40
35
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
-80
-60
-40
-20
0
20
40
60



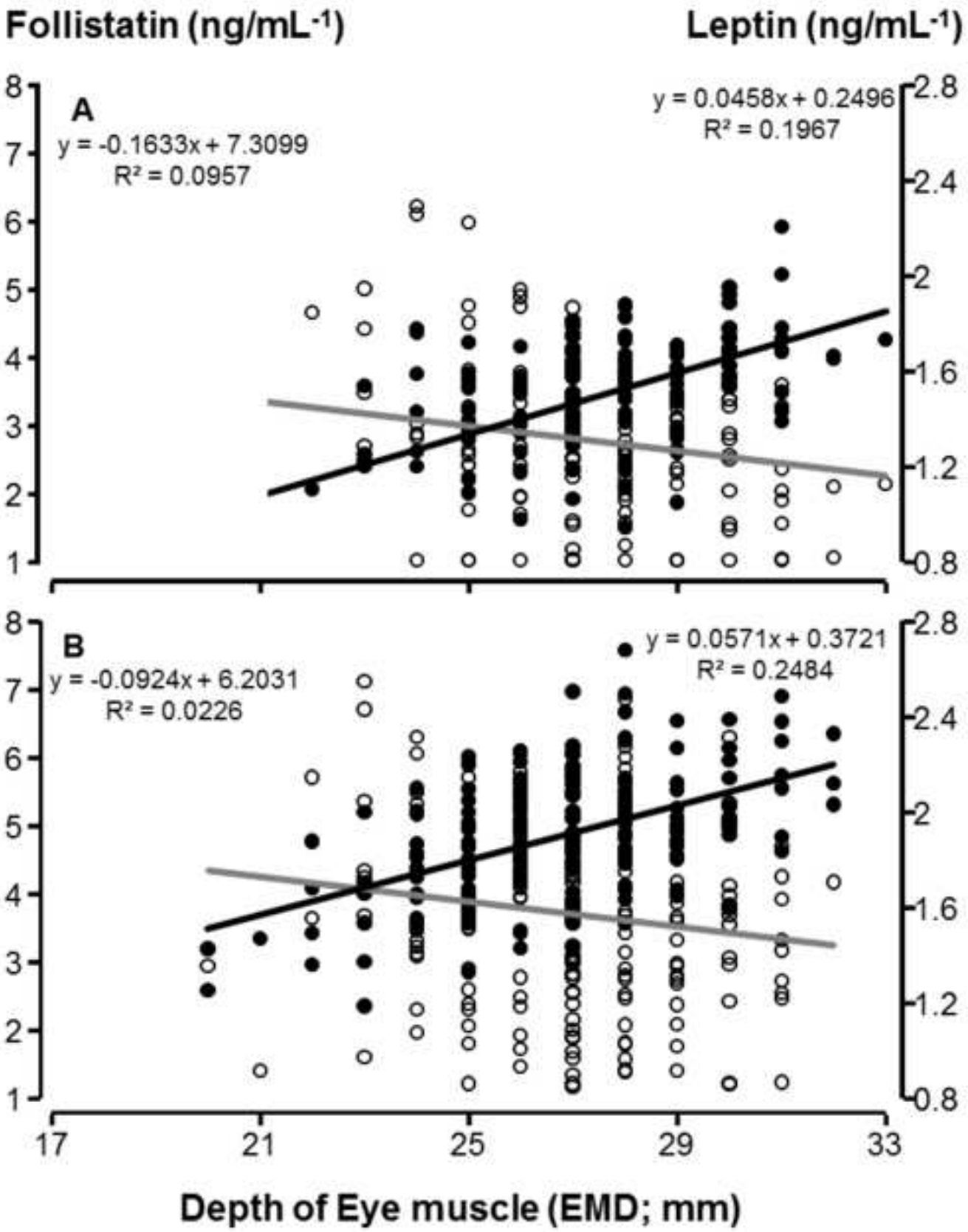
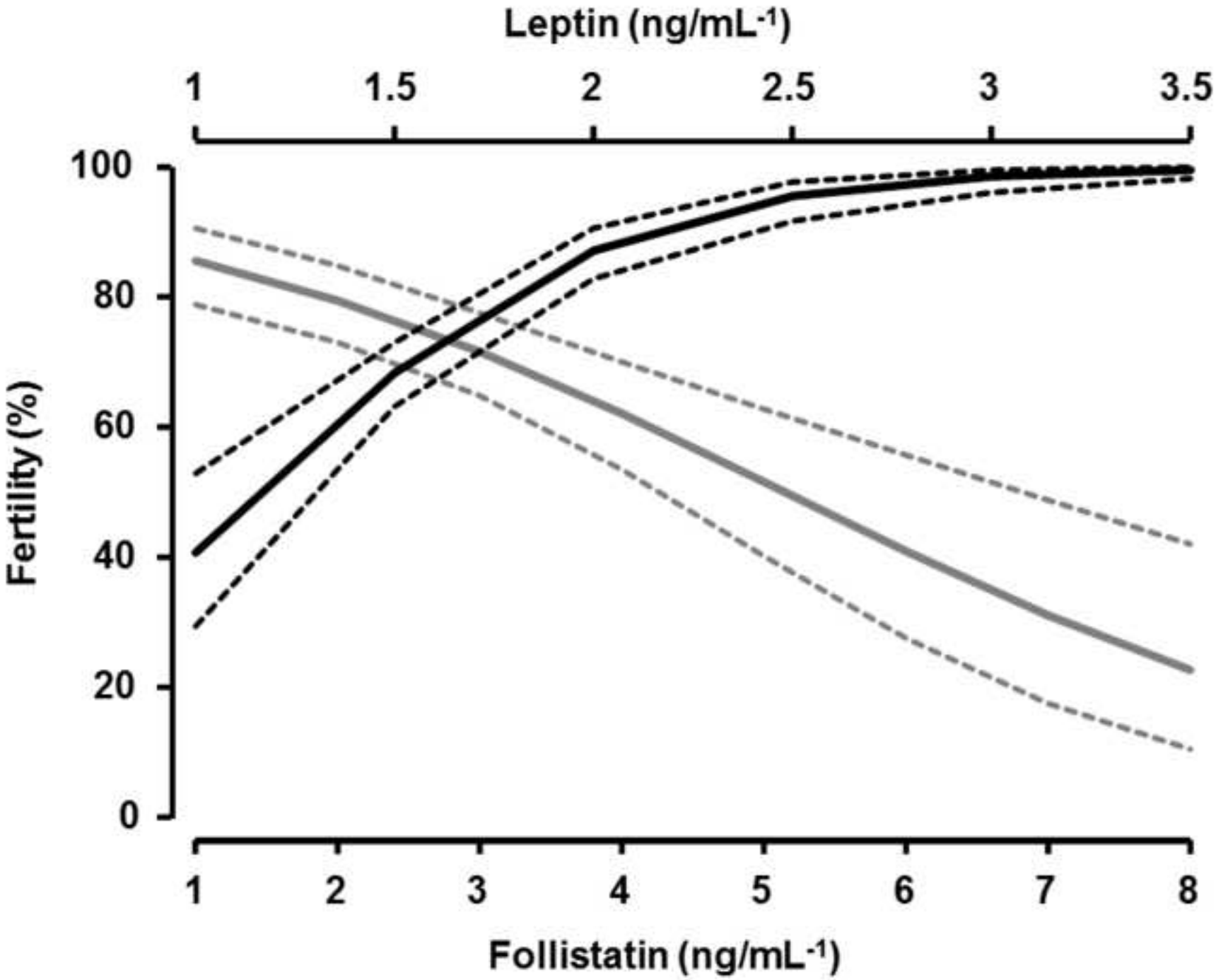


Figure4


Leptin (ng/mL-1)
1
1.5
2
2.5
3
3.5
100
80
60
40
20
0
Fertility (%)
1
2
3
4
5
6
7
8
Follistatin (ng/mL-1)