

1 **Sex- and bone-specific responses in bone structure to exogenous leptin and leptin**
2 **receptor antagonism in the ovine fetus**

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24

25 **Abstract**

26 Widespread expression of leptin and its receptor in developing cartilage and bone suggests
27 that leptin may regulate bone growth and development in the fetus. Using micro-computed
28 tomography, this study investigated the effects of exogenous leptin and leptin receptor
29 antagonism on aspects of bone structure in the sheep fetus during late gestation. From 125-
30 130 days of gestation (term ~145 days), chronically-catheterised singleton sheep fetuses were
31 infused intravenously for five days with either saline (0.9% saline, n=13), recombinant ovine
32 leptin at two doses (0.6 mg/kg/day LEP1, n=10 or 1.4 mg/kg/day LEP2, n=7) or recombinant
33 super-active ovine leptin receptor antagonist (4.6 mg/kg/day SOLA, n=6). No significant
34 differences in plasma insulin-like growth factor-I, osteocalcin, calcium, inorganic phosphate
35 or alkaline phosphatase were observed between treatment groups. Total femur midshaft
36 diameter and metatarsal lumen diameter were narrower in male fetuses treated with
37 exogenous leptin. In a fixed length of femur midshaft, total and bone volumes were reduced
38 by the higher dose of leptin; non-bone space volume was lower in both groups of leptin-
39 treated fetuses. Leptin infusion caused increments in femur porosity and connectivity density,
40 and vertebral trabecular thickness. Leptin receptor antagonism decreased trabecular spacing
41 and increased trabecular number, degree of anisotropy and connectivity density in the
42 lumbar vertebrae. The increase in vertebral porosity observed following leptin receptor
43 antagonism was greater in the male, compared to female, fetuses. Therefore, leptin may have
44 a role in the growth and development of the fetal skeleton, dependent on the concentration of
45 leptin, sex of the fetus and bone type examined.

46

47 **Introduction**

48 Leptin is a hormone primarily secreted by white adipose tissue which was first identified as
49 an important regulator of appetite and energy expenditure (50), and, in adult life, is now
50 known to have a wide range of biological actions, including modulation of immune,
51 neuroendocrine and reproductive function and bone metabolism (37, 47). Before birth, the
52 expression of leptin and its receptors is widespread in fetal and placental tissues, although, to
53 date, the role of leptin in the control of growth and development *in utero* is poorly understood
54 (14). In the mouse fetus, mRNA and protein for leptin and its long-form signalling receptor,
55 Ob-Rb, have been localised in particular to the skeleton, including vertebrae, ribs and the
56 bones of the fore- and hind-limbs (7, 23, 24). Leptin and its receptor were expressed in
57 different cell types in the rib of the murine fetus, indicating that leptin may exert paracrine as
58 well as endocrine actions in the developing cartilage-bone (23).

59

60 In human fetuses sampled by cordocentesis at 18-35 weeks of gestation, a negative correlation
61 has been observed between plasma leptin and a marker of bone resorption (cross-linked
62 carboxy-terminal telopeptide of type I collagen; 36). Leptin may, therefore, inhibit bone
63 resorption to promote growth of the fetal skeleton. Indeed, at birth, umbilical leptin
64 concentration has been shown to correlate positively with whole body bone mineral content
65 and estimated bone density in human neonates (27). However, in a study examining
66 umbilical samples from large, small and average-sized babies, plasma leptin did not relate to
67 whole body bone mineral density or content determined within the first 24 hours of life (1).
68 In addition, there are conflicting reports detailing changes in bone density in infants born to
69 diabetic mothers who are exposed to high concentrations of leptin *in utero* (18, 29, 42).

70

71 A variety of experimental studies *in vivo* and *in vitro* have demonstrated that the actions of
72 leptin on bone growth and development in postnatal animals are complex and depend on
73 factors including i) the leptin dose, ii) route of administration, iii) age of the animal and iv)
74 the skeletal region and type of bone tissue examined (30). In prepubertal mice, the epiphyseal
75 growth plate has been shown to express Ob-Rb and leptin treatment increases the size of the
76 tibial growth plate in association with proliferation and differentiation of chondrocytes (16).
77 Leptin receptors are also present in isolated fetal rat osteoblasts and in primary cultures of
78 adult osteoblasts and chondrocytes (9, 43). Studies *in vitro* have shown that leptin directly
79 stimulates proliferation and differentiation of osteoblasts, while inhibiting differentiation of
80 bone adipocytes (9, 45). In contrast, it has also been reported in rodents and sheep that leptin
81 can suppress bone formation indirectly by hypothalamic control of sympathetic and cocaine
82 amphetamine regulated transcript (CART) pathways (12, 13, 40, 49). Both hypothalamic and
83 peripheral administration of leptin have been shown to correct the skeletal abnormalities seen
84 in leptin-deficient ob/ob mice, in association with elevated serum insulin-like growth factor-I
85 (IGF-I) and osteocalcin levels, a marker of osteoblast activity (2, 26, 46). The overall effect
86 of leptin on bone development, therefore, may depend upon the balance between peripheral
87 and central leptin signalling pathways, although the relative importance of these mechanisms
88 in bone remodelling remains controversial (30).

89

90 The role of leptin in the control of bone growth and development before birth is unclear.
91 Previous studies have shown that plasma leptin concentration is elevated in hypothyroid fetal
92 sheep that show abnormalities in bone growth and development (22, 28), although the extent
93 to which leptin contributes to the bone phenotype in this model remains unknown. The
94 present study investigated the effects of leptin treatment and leptin receptor antagonism on
95 plasma IGF-I and osteocalcin concentrations, and aspects of bone structure determined by

96 micro-computed tomography, in the sheep fetus during late gestation. The study hypothesised
97 that exogenous leptin treatment would promote, while antagonism of the leptin receptor
98 would inhibit, the normal development of bone, and plasma IGF-I and osteocalcin
99 concentrations, in the sheep fetus.

100

101 **Methods**

102 *Animals*

103 All surgical and experimental procedures were approved by the local animal ethics committee
104 and were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986
105 under Home Office project licence PPL70/7645. Thirty-six Welsh Mountain sheep with
106 singleton pregnancies of known gestational age were used in this study. The pregnant ewes
107 were housed in individual pens and maintained on 200g/kg concentrates with free access to
108 hay, water and a salt-lick block.

109

110 *Surgical procedures*

111 The pregnant ewes were fasted for 18-24 h before surgery with free access to water. At
112 between 118 and 120 days of pregnancy (term 145 ± 2 days) and under general anaesthesia
113 (1.5% halothane in O₂-N₂O), catheters were inserted into the femoral artery and vein of the
114 fetus and the femoral artery of the ewe using techniques previously described (8). All
115 catheters were exteriorised through the flank of the ewe and secured in a bag sutured to the
116 skin. The vascular catheters were flushed daily with heparinised saline solution (100 IU
117 heparin in 0.9% saline) from the day after surgery. At surgery, all fetuses were administered
118 i.v. with 100 mg ampicillin (Penbritin, Beecham Animal Health, Brentford, UK) and 2 mg
119 gentamycin (Frangen-100, Biovet, Mullingar, Ireland). Ewes were administered with

120 antibiotics i.m. (Depocillin, Mycofarm, Cambridge, UK) on the day of surgery and for 3 days
121 thereafter.

122

123 *Experimental procedures*

124 Starting at 125 days of gestation and for a period of 5 days, one group of fetuses was infused
125 i.v. with saline (0.9% sodium chloride, n=13) while a further three groups received either
126 recombinant ovine leptin at two doses (0.56 ± 0.02 mg/kg/day LEP1, n =10 or 1.35 ± 0.11
127 mg/kg/day LEP2, n=7) or recombinant super-active ovine leptin antagonist (4.56 ± 0.24
128 mg/kg/day SOLA, n=6; Protein Laboratories Rehovot, Israel; 17, 34). The doses of leptin
129 administered increased circulating leptin to supra-physiological concentrations in the sheep
130 fetus (10) and by a similar magnitude as that seen in the umbilical blood of babies born to
131 women with obesity and/or diabetes during pregnancy (6, 18). The leptin antagonist was
132 produced by D23L/L39A/D40A/F41A mutation of recombinant ovine leptin (34). The leptin
133 mutant competes with endogenous leptin for binding sites on all forms of the leptin receptor
134 but lacks biological activity (34). In fetal sheep, a less potent form of the recombinant ovine
135 leptin receptor antagonist (mutant L39A/D40A/FA1A/I42A, OLA) at a dose of 1.5 mg/kg/day
136 i.v. has previously been shown to reduce STAT-3 phosphorylation by approximately 50% in
137 the adrenal cortex (11). The treatments were administered via the fetal venous catheter at a
138 rate of 3 ml/day using a Graseby portable infusion pump. Arterial blood from the fetus and
139 ewe (3 ml) was collected daily from 2 days before and during the 5-day infusion period.

140

141 On the fifth day of infusion at 130 days of gestation, the fetuses were delivered by Caesarean
142 section under maternal general anaesthesia (20 mg/kg sodium pentobarbitone i.v.). After
143 administration of a lethal dose of barbiturate (200 mg/kg sodium pentobarbitone i.v.) to the
144 ewe and fetus, the fetus was weighed and a variety of tissues were collected. In all fetuses,

145 bodyweight, crown-rump length and fore-limb (humerus, radius and metacarpus) and hind-
146 limb (femur, tibia and metatarsal) lengths were measured. Three selected bones from the
147 axial and appendicular skeleton (femur, metatarsal and lumbar vertebra L2-L4) were dissected
148 and frozen at -80°C.

149

150 *Biochemical analyses*

151 All blood samples were collected into EDTA-containing tubes and centrifuged at 1000g for 5
152 minutes at 4°C; the plasma was stored at -20°C until analysis. Plasma concentrations of
153 leptin and IGF-I were determined by RIA as previously described (4, 15). The intra-assay
154 coefficients of variation were 4-5%, and the minimum levels of detection were 0.09 and 0.08
155 ng/ml, respectively. Plasma osteocalcin concentrations were determined using an ELISA kit
156 (Immunodiagnosics Systems Ltd, Boldon, UK); the intra-assay coefficient of variation was
157 4% and the lower limit of assay detection was 0.5 ng/ml. Total plasma calcium, inorganic
158 phosphate and alkaline phosphatase concentrations were measured using a Siemens
159 Dimension RXL-2 autoanalyser (Siemens Healthcare, Camberley, UK). The minimum levels
160 of detection were 1.25 mM, 0.1 mM and 11 U/l, respectively.

161

162 *Micro-computed tomography*

163 The femur, metatarsal and lumbar vertebrae were scanned using a Skyscan 1176 *in vivo*
164 micro-CT scanner (Bruker micro-CT, Kontich, Belgium). All scans were taken at 50 kV, 50
165 µA with 0.5 mm aluminium filter and 0.4° rotation step. Individual 2D cross-sectional images
166 were reconstructed using Bruker NRecon software version 1.6.5.8. Voxel resolution was 18
167 µm. Reconstructed images were analysed using Bruker CTAn software version 1.13.5.1 to
168 calculate bone volume, bone volume to total volume ratio, bone surface to bone volume ratio,
169 and trabecular thickness, number and spacing. In addition, measurements were made of

170 trabecular pattern factor (relative convex or concave nature of the total bone surface),
171 porosity, connectivity density, structural model index (SMI, surface convexity) and degree of
172 anisotropy (DOA, orientation of trabeculae). In the femur and metatarsal, a 3.56 mm length
173 of midshaft bone was assessed for volumes of lumen, bone tissue and space between the bone
174 tissue.

175

176 *Statistical analysis*

177 All data were tested for normality, and parametric and non-parametric tests were used as
178 appropriate (SPSS Statistics 20 statistical analysis software, Richmond, USA). Values
179 obtained from the four groups were compared separately to assess the effects of leptin
180 infusion (saline, LEP1, LEP2) and the effects of leptin receptor antagonism (saline, SOLA).
181 Initially, all data were analysed by two-way ANOVA, with treatment and sex of the fetus as
182 factors, followed by Tukey's *post hoc* test. Where data were not influenced by the sex of the
183 fetus, one-way ANOVA followed by Tukey's *post hoc* test, or paired or Student's unpaired t-
184 test as appropriate, was used to assess the effects of treatment. Differences where $p < 0.05$
185 were regarded as significant. All data are presented as mean \pm SEM values.

186

187 **Results**

188 *Plasma hormone and metabolite concentrations*

189 Plasma leptin concentrations in the fetuses treated with recombinant ovine leptin increased
190 significantly over the period of the infusion ($p < 0.05$, Table 1). The RIA method used to
191 measure plasma leptin detected the recombinant ovine leptin receptor antagonist as leptin and,
192 therefore, the apparent plasma leptin concentrations in the fetuses infused with the antagonist
193 were also increased from pre-treatment levels ($p < 0.05$, Table 1). On the fifth day of
194 treatment, plasma leptin concentrations in the fetuses infused with either leptin or leptin

195 receptor antagonist were significantly higher than those observed in the control fetuses
196 infused with saline; values were increased by leptin infusion in a dose-dependent manner
197 ($p < 0.05$, Table 1).

198
199 Plasma concentrations of IGF-I, osteocalcin, calcium and inorganic phosphate did not differ
200 between the treatment groups before or after infusion, and were unaffected by administration
201 of leptin or leptin receptor antagonist over five days (Table 1). Plasma alkaline phosphatase
202 concentrations were increased by gestational age over the five days of treatment in all the
203 groups of fetuses ($p < 0.05$, Table 1). There was no difference in the change in plasma alkaline
204 phosphatase observed over the period of study between the treatment groups (Table 1).

205

206 *Body morphometry*

207 No significant differences in fetal bodyweight, crown-rump length or limb lengths were
208 observed between the treatment groups at the end of the 5-day infusion period, when
209 measurements were made before dissection (Table 2). When data from the fetuses treated
210 with saline or the leptin receptor antagonist were assessed, a significant effect of sex was
211 identified for the metatarsal, radius and metacarpal bone lengths ($p < 0.05$ in all cases);
212 however, although the data indicated that values were greater in the male compared to female
213 fetuses, the results of the Tukey *post-hoc* tests failed to reach significance for each pair-wise
214 comparison ($p > 0.05$). There were no interactions between sex and treatment for any of the
215 measurements of body weight or limb length.

216

217 *Bone structure*

218 *Exogenous leptin infusion*

219 Femur midshaft diameter was significantly narrower in the fetuses of the LEP2 group
220 compared to those infused with saline ($p<0.05$, Table 3); midshaft diameter in the LEP1
221 fetuses was intermediate to the values observed in the saline and LEP2 fetuses (Table 3).
222 When analysed by sex, femur midshaft diameter was significantly greater in the male
223 compared to female fetuses of the saline group alone; midshaft diameter was reduced by
224 leptin infusion in the male, but not female, fetuses of the LEP1 and LEP2 groups ($p<0.05$,
225 Table 3).

226
227 In a fixed length of femur midshaft bone, total volume was significantly lower in the LEP2-
228 treated fetuses, compared with the saline control group, while the values in the LEP1-treated
229 fetuses were intermediate ($p<0.05$, Figure 1). The midshaft volume composed of non-bone
230 space was significantly decreased by leptin treatment in both LEP1 and LEP2 groups ($p<0.05$,
231 Figure 1A). In LEP1-treated fetuses, the non-bone space expressed as a proportion of the
232 total volume was significantly lower than that observed in the saline-treated fetuses ($p<0.05$,
233 Figure 1B). A significant reduction in bone tissue volume was seen in the fetuses treated with
234 the higher dose of leptin compared to those treated with the lower dose ($p<0.05$, Figure 1A).
235 The bone surface to volume ratio in the femur tended to increase with leptin treatment, but
236 this change failed to reach statistical significance ($p=0.08$, Table 3).

237
238 In the saline control group alone, the midshaft lumen diameter of the metatarsal bone was
239 significantly greater in the male than the female fetuses; midshaft lumen diameter was
240 decreased by leptin infusion in male, but not female, fetuses of the LEP1 and LEP2 groups
241 ($p<0.05$, Table 3). In the fixed length of midshaft bone, the bone tissue volume was
242 significantly lower in the fetuses treated with the higher dose of leptin compared to those
243 treated with the lower dose ($p<0.05$, Figure 2A).

244

245 Significant increments in femur trabecular porosity and connectivity density, and vertebral
246 trabecular thickness, were observed in the LEP1-infused fetuses compared to the control
247 saline group ($p < 0.05$, Figure 3); these parameters were also elevated in the LEP2 fetuses but
248 failed to differ significantly from the values in the saline control group (Figure 3).

249

250 For all other parameters measured in the femur, metatarsal and lumbar vertebrae, no
251 significant differences were observed between the fetuses infused with saline or leptin (Table
252 3). Leptin treatment influenced trabecular thickness ($p = 0.07$) and DOA ($p = 0.08$) in the
253 metatarsal, and body length ($p = 0.09$), bone surface to volume ratio ($p = 0.08$), trabecular
254 pattern factor ($p = 0.07$) and structural model index ($p = 0.08$) in the lumbar vertebrae, but these
255 effects failed to reach statistical significance (Table 3).

256

257 *Leptin receptor antagonism*

258 In the lumbar vertebra, leptin receptor antagonism caused a significant decrease in trabecular
259 spacing and increases in trabecular number, DOA and connectivity density ($p < 0.05$, Figure 4).
260 Lumbar vertebral porosity was also increased following treatment with the leptin receptor
261 antagonist in a sex-dependent manner, with the increment in porosity greater in the male,
262 compared to the female, fetuses ($p < 0.05$, Figure 5).

263

264 In the other bones, there were no significant differences in any of the other measured
265 parameters between the fetuses infused with saline or the leptin antagonist (Table 4).

266 Measurements of femur midshaft total diameter, metatarsal midshaft total and lumen

267 diameter, and vertebral bone surface to volume ratio and structural model index were greater

268 in the male compared to female fetuses ($p < 0.05$), but these were not affected by leptin
269 receptor antagonism (Table 4).

270

271 **Discussion**

272 The findings of the present study demonstrate that exogenous leptin treatment and leptin
273 receptor antagonism have differential effects on bone structure in the sheep fetus during late
274 gestation, dependent on the bone type examined and, in some aspects, the sex of the fetus. In
275 the femur, exogenous leptin treatment caused significant decrements in total, bone and non-
276 bone space volumes and increments in trabecular porosity and connectivity density. In
277 addition, compared to the saline control group, a reduction in femur midshaft diameter was
278 observed in the male, but not female, fetuses treated with exogenous leptin. These findings
279 show that supra-physiological concentrations of leptin impair femoral bone growth, although
280 the trabecular bone may become a more organised and potentially stronger structure. In
281 contrast, leptin receptor antagonism predominantly affected the developing lumbar vertebra.
282 Leptin receptor antagonism resulted in an increase in trabecular number, DOA and
283 connectivity density, with less space between the structures and no change to trabecular
284 thickness. Therefore, while exogenous leptin promoted growth of vertebral trabeculae, the
285 leptin receptor antagonist caused generation and organisation of the vertebral trabecular bone
286 structure. These findings indicate that leptin normally suppresses these aspects of bone
287 development in the axial skeleton. The responses to exogenous leptin and leptin receptor
288 antagonism occurred without any change in circulating IGF-I, osteocalcin or other markers of
289 bone turnover. In newborn mice, primary ossification centres in the limb bones were enlarged
290 in size following maternal treatment with leptin during mid-gestation (3). The present study
291 is the first to investigate the consequences of direct leptin administration to the fetus for its

292 bone structure, with potentially fewer confounding effects of leptin on maternal and placental
293 physiology.

294

295 Regional differences have been observed in the effects of leptin excess and deficiency on the
296 appendicular and axial bones of the postnatal skeleton (19, 21). Intracerebroventricular
297 infusion of leptin in rats caused reductions in bone mineral content and density in the femur,
298 but not the lumbar vertebra (19). In *ob/ob* mice, the femur was reduced in length with lower
299 mineralization and trabecular bone volume, while trabecular volume and bone mineral content
300 and density were increased in the lumbar vertebrae (21). The bone phenotype of the leptin-
301 deficient rodent, however, is complex as previous studies have shown greater bone mass in
302 both the femur and vertebrae of *ob/ob* and leptin receptor-deficient *db/db* mice (12).

303 Measurements of bone volume and trabecular number, thickness and mineral density were
304 also elevated in the femur of the leptin-deficient rat, suggesting that leptin suppresses bone
305 formation in this species (48). The overall effects of leptin manipulation on bone structure
306 may depend on the balance between the peripheral stimulatory and central inhibitory control
307 of bone turnover by leptin, although the relative importance of these mechanisms, especially
308 within specific regions of the skeleton, remains poorly understood (30).

309

310 In the current study, the effects of exogenous leptin and leptin receptor antagonism on bone
311 structure in the ovine fetus may be mediated by direct and/or indirect mechanisms, in
312 particular via the hypothalamic relay. Leptin receptors are expressed on developing bone
313 cells in fetal rodents (7, 9, 23) and leptin stimulates proliferation of osteoblasts isolated from
314 fetal rats in late gestation (9). The hypothalamic control of bone development by sympathetic
315 and CART neurones, and the role of leptin in modulating these pathways, are unknown in
316 fetal life. In the sheep fetus during late gestation, Ob-Rb mRNA has been localised to several

317 hypothalamic nuclei, including the arcuate nucleus and dorsomedial, ventromedial and
318 paraventricular regions (31) and previous studies have shown that intracerebroventricular
319 infusion of leptin has effects on swallowing movements and hypothalamic-pituitary-adrenal
320 activity (25, 41). The permeability of the blood-brain barrier to supra-physiological systemic
321 concentrations of leptin and the leptin antagonist, however, remains to be established. The
322 leptin mutant antagonist can bind to all forms of the leptin receptor, including the soluble Ob-
323 Re which enables leptin to transfer across the blood-brain barrier. The blood-brain barrier is
324 functional in the ovine fetus from at least two-thirds of gestation although, in many regions of
325 the brain, it is more permeable to small hydrophilic molecules in fetal compared to neonatal
326 and adult life (44). It is possible that the effects of the leptin receptor antagonist on vertebral
327 bone structure *in utero* are largely due to prevention of the normal inhibitory effects of leptin
328 on bone growth via the hypothalamic relay.

329

330 Most studies using human and murine leptin receptors to examine receptor kinetics have
331 shown that the equilibrium dissociation constant (KD) is in the sub-nanomolar range; KD
332 values are reported to range from 0.1-15nM for leptin receptors in solution and 0.2-2.6nM for
333 those attached to the cell surface, with variation between studies possibly dependent on the
334 techniques and cell types used (38). The mean plasma concentration of leptin in the saline-
335 infused control fetuses at 130 days of gestation was 0.04 nM in the present study, and rises to
336 0.06 nM in sheep fetuses near term (35). In the fetuses infused with recombinant leptin, the
337 mean plasma leptin concentrations were 0.29 and 0.51 nM on the fifth day of infusion of the
338 two leptin doses, LEP1 and LEP2, respectively. Therefore, although plasma leptin
339 concentrations achieved in the infused fetuses were significantly above the normal
340 endogenous levels, they were still within the range of the leptin receptor KD.

341

342 It is also possible that exposure to supra-physiological concentrations of leptin may modify
343 tissue expression of the leptin receptor and the activity of downstream signalling pathways.
344 In a previous study examining the effect of leptin treatment on lung structure and function in
345 fetal sheep, the five-day infusion of the lower LEP1 dose caused a significant increase in
346 pulmonary leptin receptor mRNA abundance (10). The expression and activity of leptin
347 receptors in the bone and hypothalamus were not investigated in the present study, although it
348 has been shown that long-term exposure to leptin in obese adult animals and human subjects
349 leads to leptin insensitivity in the appetite networks of the hypothalamus (32).

350

351 In the present study, sexual dimorphism was evident in a variety of bone measurements, and
352 male fetuses appeared to be more sensitive to the actions of exogenous leptin and leptin
353 receptor antagonism than female fetuses. The mechanisms responsible, and the consequences
354 for bone structure and mechanical strength in later life, remain to be determined. Different
355 patterns in circulating testosterone concentration have been reported in male and female sheep
356 fetuses from mid-gestation (39) and there may be sex-specific expression of endocrine and
357 other signalling pathways in developing bone. Treatment of pregnant rats with leptin in mid-
358 gestation led to a lower birthweight, and greater longer term reductions in skeletal growth and
359 bone mineral content, in male compared with female offspring (33). It is possible that a
360 longer duration of exposure to exogenous leptin and leptin receptor antagonism, and/or at
361 different time points in bone development, would have led to more profound effects on the
362 developing ovine skeleton in both sexes.

363

364 In postnatal life, leptin is known to have an important role in the physiological adaptations to
365 fasting: low circulating levels of leptin, due to reductions in body fat mass, lead to enhanced
366 appetite and impaired fertility and body, including bone, growth (20). In mice, leptin
367 treatment has been shown to correct the reduction in tibial bone length induced by calorie

368 restriction, independent of IGF-I levels (16). In addition, the effects of calorie restriction on
369 bone formation are bone site-specific, with bone mineral content decreased in the femur and
370 increased in the vertebra of mice undernourished over a six-month period (5). Before birth,
371 the role of leptin in the response to changes in nutrient availability is less clear. In the sheep
372 fetus, maternal undernutrition appears to have little effect on leptin production, although
373 adipose leptin mRNA abundance and plasma leptin concentration are sensitive to levels of
374 glucose, insulin, oxygen and glucocorticoids *in utero* (14).

375

376 **Perspectives and Significance**

377 This study has shown a role for leptin in the growth and development of the ovine fetal
378 skeleton which is dependent on the leptin concentration, bone site and sex of the fetus.
379 Further longer term studies are required to determine the extent to which physiological
380 changes in leptin contribute to the endocrine control of bone growth during normal and
381 suboptimal nutrition *in utero*. In addition, it will be important to assess whether the changes
382 observed in bone structure induced by variations in leptin activity before birth have
383 consequences for bone function across the life-course.

384

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580 **Figure legends**

581 1. Mean (\pm SEM) bone volumes (A) and percentage bone volumes (B) in a fixed length of
582 midshaft femur from fetuses infused for five days with either saline, leptin (LEP1 and LEP2)
583 or leptin antagonist (SOLA). For comparisons between saline and leptin treatment groups,
584 columns with different letters are significantly different from each other; uppercase letters
585 indicate differences in the total volume, and lowercase letters at the SEM bars indicate
586 differences in volume compartments (one-way ANOVA, $p < 0.05$). Compartments with no
587 letters at the SEM bars are not significantly different from each other ($p > 0.05$).

588

589 2. Mean (\pm SEM) bone volumes (A) and percentage bone volumes (B) in a fixed length of
590 midshaft metatarsal from fetuses infused for five days with either saline, leptin (LEP1 and
591 LEP2) or leptin antagonist (SOLA). For comparisons between saline and leptin treatment
592 groups, compartments with different letters at the SEM bars are significantly different from
593 each other (one-way ANOVA, $p < 0.05$). Compartments with no letters at the SEM bars are
594 not significantly different from each other ($p > 0.05$).

595

596 3. Mean (\pm SEM) porosity (A) and connectivity density (B) in the femur, and trabecular
597 thickness (C) in the lumbar vertebra, of fetuses infused for five days with either saline or
598 leptin (LEP1 and LEP2). Columns with different letters are significantly different from each
599 other (one-way ANOVA, $p < 0.05$).

600

601 4. Mean (\pm SEM) trabecular number (A), trabecular spacing (B), degree of anisotropy (C)
602 and connectivity density (D) in the lumbar vertebra of fetuses infused for five days with either
603 saline or leptin antagonist (SOLA). *, significantly different from saline-treated fetuses
604 (Student's unpaired t-test, $p < 0.05$).

605

606 5. Mean (\pm SEM) porosity in the lumbar vertebra of fetuses infused for five days with either
607 saline or leptin antagonist (SOLA). *, significantly different from saline-treated fetuses of the
608 same sex (two-way ANOVA, $p < 0.05$); †, significantly different from male fetuses in the same
609 treatment group (two-way ANOVA, $p < 0.05$).

610 **Table 1.** Mean (\pm SEM) plasma hormone and metabolite concentrations in the fetuses before (basal) and five days after infusion with saline,
611 leptin (LEP1, LEP2) or leptin receptor antagonist (SOLA). Basal = mean of days 0, -1 and -2. In comparisons between saline and leptin
612 treatment groups, values with different superscript letters are significantly different from each other (one-way ANOVA, $p < 0.05$); † significant
613 difference between fetuses treated with saline or leptin receptor antagonist (Student's unpaired t-test, $p < 0.05$); * significant difference from basal
614 values (paired t-test, $p < 0.05$).

615

		Saline (n=9-11)	LEP1 (n=9-10)	LEP2 (n=7)	SOLA (n=6)
Leptin (ng/ml)	Basal	0.69 \pm 0.05	0.85 \pm 0.03	0.90 \pm 0.07	0.59 \pm 0.03
	Day 5	0.72 \pm 0.07^a	4.66 \pm 1.11^{*b}	8.19 \pm 1.73^{*c}	7.93 \pm 1.10^{*†}
	Change	+0.03 \pm 0.04^a	+3.81 \pm 1.05^b	+7.29 \pm 1.76^c	+7.35 \pm 1.09[†]
IGF-I (ng/ml)	Basal	17.4 \pm 1.7	14.0 \pm 2.3	11.3 \pm 1.3	16.1 \pm 1.2
	Day 5	19.5 \pm 2.4	14.8 \pm 1.7	14.6 \pm 2.8	14.9 \pm 2.5
	Change	+2.1 \pm 1.7	+0.9 \pm 1.2	+3.3 \pm 3.6	-1.2 \pm 2.0
Osteocalcin (ng/ml)	Basal	10.15 \pm 0.44	11.95 \pm 0.65	11.20 \pm 0.55	10.95 \pm 0.45
	Day 5	10.11 \pm 0.39	11.86 \pm 0.43	10.05 \pm 1.13	10.16 \pm 0.47
	Change	-0.04 \pm 0.41	-0.09 \pm 0.41	-1.15 \pm 0.87	-0.80 \pm 0.40
Calcium (mM)	Basal	2.91 \pm 0.03	2.86 \pm 0.05	2.81 \pm 0.07	2.89 \pm 0.04
	Day 5	2.94 \pm 0.05	2.93 \pm 0.07	3.02 \pm 0.17	2.85 \pm 0.14

	Change	+0.03 ± 0.05	+0.08 ± 0.08	+0.23 ± 0.20	-0.04 ± 0.17
Inorganic phosphate (mM)	Basal	2.23 ± 0.09	2.40 ± 0.09	1.95 ± 0.10	2.19 ± 0.13
	Day 5	2.12 ± 0.10	2.21 ± 0.08	2.13 ± 0.11	1.99 ± 0.14
	Change	-0.12 ± 0.07	-0.19 ± 0.09	+0.18 ± 0.15	-0.20 ± 0.12
Alkaline phosphatase (U/l)	Basal	172 ± 20	156 ± 15	122 ± 11	215 ± 16
	Day 5	201 ± 24*	190 ± 22*	166 ± 22*	244 ± 10*
	Change	+28 ± 12	+34 ± 14	+44 ± 17	+30 ± 11

616

617 **Table 2.** Mean (\pm SEM) measurements of bodyweight and morphometry in the fetuses on the fifth day after infusion with saline, leptin (LEP1,
618 LEP2) or leptin receptor antagonist (SOLA).

619

	Saline (n=13)	LEP1 (n=10)	LEP2 (n=7)	SOLA (n=6)
Sex of fetuses (female:male)	7F:6M	5F:5M	4F:3M	3F:3M
Bodyweight (kg)	2.76 \pm 0.16	2.74 \pm 0.12	2.32 \pm 0.19	2.67 \pm 0.14
Crown-rump length (cm)	43.0 \pm 1.0	43.5 \pm 0.7	41.4 \pm 1.1	44.6 \pm 1.1
Fore-limb lengths (cm)				
Humerus	9.2 \pm 0.4	8.8 \pm 0.1	8.4 \pm 0.2	9.2 \pm 0.8
Radius	10.3 \pm 0.3	10.5 \pm 0.2	9.8 \pm 0.3	10.5 \pm 0.4
Metacarpal	12.5 \pm 0.5	12.5 \pm 0.2	12.0 \pm 0.4	11.8 \pm 0.7
Hind-limb lengths (cm)				
Femur	10.0 \pm 0.5	10.2 \pm 0.4	9.4 \pm 0.3	10.8 \pm 1.0
Tibia	13.2 \pm 0.4	13.5 \pm 0.3	12.6 \pm 0.4	12.9 \pm 0.3
Metatarsal	15.1 \pm 0.5	15.0 \pm 0.2	14.5 \pm 0.4	14.0 \pm 1.1

620

621 **Table 3.** Structural properties of femur, metatarsal and lumbar vertebra bones in fetuses infused for five days with saline or leptin (LEP1,
622 LEP2). In comparisons between saline and leptin groups, values with different superscript letters are significantly different from each other
623 (two-way ANOVA, p<0.05). † significantly different from male fetuses in same treatment group (two-way ANOVA, p<0.05).

624

Bone property	Bone type	Saline (n=13)		LEP1 (n=10)		LEP2 (n=7)		Effect of leptin infusion (p-value)		
		Male (n=6)	Female (n=7)	Male (n=5)	Female (n=5)	Male (n=3)	Female (n=4)	Treatment	Sex	Interaction
Midshaft total diameter (mm)	Femur	7.50 ± 0.26^a		7.24 ± 0.14^{ab}		6.58 ± 0.22^b		0.010	NS	0.014
		8.13 ± 0.23^a	6.95 ± 0.33[†]	7.16 ± 0.25^b	7.32 ± 0.17	6.31 ± 0.31^b	6.79 ± 0.30			
	Metatarsal	7.11 ± 0.20		7.11 ± 0.16		6.64 ± 0.23		NS	(0.076)	NS
		7.59 ± 0.12	6.64 ± 0.27	7.26 ± 0.27	6.95 ± 0.19	6.59 ± 0.36	6.68 ± 0.34			
Midshaft lumen diameter (mm)	Femur	3.61 ± 0.18		3.37 ± 0.21		3.40 ± 0.24		NS	NS	(0.059)
		3.83 ± 0.26	3.43 ± 0.24	2.93 ± 0.15	3.81 ± 0.27	3.24 ± 0.37	3.52 ± 0.36			
	Metatarsal	4.34 ± 0.17		4.25 ± 0.10		4.14 ± 0.17		NS	NS	0.009
		4.78 ± 0.11^a	3.89 ± 0.18[†]	4.20 ± 0.18^b	4.30 ± 0.10	4.09 ± 0.22^b	4.17 ± 0.27			
Midshaft wall thickness (mm)	Femur	1.94 ± 0.12		1.93 ± 0.12		1.59 ± 0.09		(0.075)	NS	NS
	Metatarsal	1.39 ± 0.06		1.43 ± 0.06		1.25 ± 0.09		NS	NS	NS
Body length (mm)	Vertebrae	7.83 ± 0.18		7.91 ± 0.13		7.31 ± 0.15		(0.091)	NS	NS
Total bone volume (mm ³)		394.7 ± 29.9		398.0 ± 28.6		311.6 ± 36.6		NS	NS	NS
Bone volume/total volume (%)	Femur	28.8 ± 2.5		30.0 ± 3.0		31.7 ± 3.9		NS	NS	NS
	Metatarsal	28.7 ± 1.8		30.0 ± 1.3		29.5 ± 1.8		NS	NS	NS
	Vertebra	31.6 ± 2.8		38.4 ± 3.5		41.8 ± 6.5		NS	NS	NS
Bone surface/bone	Femur	32.6 ± 1.4		37.0 ± 1.4		35.1 ± 1.4		(0.088)	NS	NS

volume (mm ² /mm ³)	Metatarsal	30.0 ± 0.8		27.8 ± 1.1		29.7 ± 1.0		NS	NS	NS
	Vertebra	27.0 ± 1.1		23.1 ± 1.4		23.4 ± 2.7		(0.081)	NS	(0.057)
		29.4 ± 1.0	25.1 ± 1.6	20.9 ± 1.7	25.2 ± 1.9	20.6 ± 4.1	25.6 ± 3.7			
Trabecular thickness (mm)	Femur	0.116 ± 0.003		0.110 ± 0.003		0.112 ± 0.004		NS	NS	NS
	Metatarsal	0.127 ± 0.003		0.137 ± 0.003		0.128 ± 0.003		(0.073)	NS	NS
Trabecular number (/mm)	Femur	2.44 ± 0.16		2.69 ± 0.21		2.78 ± 0.27		NS	NS	NS
	Metatarsal	2.26 ± 0.13		2.18 ± 0.07		2.30 ± 0.12		NS	NS	NS
	Vertebra	2.20 ± 0.13		2.24 ± 0.10		2.44 ± 0.20		NS	NS	NS
Trabecular spacing (mm)	Femur	0.26 ± 0.02		0.22 ± 0.02		0.22 ± 0.02		NS	NS	NS
	Metatarsal	0.27 ± 0.02		0.27 ± 0.01		0.27 ± 0.02		NS	NS	NS
	Vertebra	0.30 ± 0.02		0.30 ± 0.02		0.27 ± 0.04		NS	NS	NS
Trabecular pattern factor (/mm)	Femur	3.97 ± 1.09		2.23 ± 1.75		0.99 ± 2.50		NS	NS	NS
	Metatarsal	5.08 ± 0.82		4.97 ± 0.40		4.81 ± 0.58		NS	NS	NS
	Vertebra	2.96 ± 0.83		-1.04 ± 1.54		-0.84 ± 2.22		(0.069)	NS	NS
Porosity (%)	Metatarsal	0.007 ± 0.002		0.005 ± 0.001		0.004 ± 0.002		NS	NS	NS
	Vertebra	0.007 ± 0.001		0.029 ± 0.012		0.045 ± 0.033		NS	NS	NS
Structural model index	Femur	1.32 ± 0.13		1.37 ± 0.15		1.22 ± 0.17		NS	NS	NS
	Metatarsal	1.61 ± 0.08		1.68 ± 0.06		1.55 ± 0.08		NS	NS	NS
	Vertebra	1.29 ± 0.12		0.89 ± 0.15		0.69 ± 0.35		(0.077)	NS	NS
Degree of anisotropy	Femur	2.15 ± 0.05		1.99 ± 0.06		2.01 ± 0.12		NS	NS	NS
	Metatarsal	1.43 ± 0.07		1.65 ± 0.07		1.64 ± 0.09		(0.080)	NS	NS
	Vertebra	1.53 ± 0.06		1.44 ± 0.06		1.50 ± 0.14		NS	NS	NS
Connectivity density (/mm ³)	Metatarsal	78.1 ± 14.1		65.6 ± 7.3		69.7 ± 7.7		NS	NS	NS
	Vertebra	52.9 ± 6.8		72.7 ± 19.1		66.0 ± 15.9		NS	NS	NS

625

626 **Table 4.** Structural properties of femur, metatarsal and lumbar vertebra bones in fetuses infused for five days with saline or a leptin receptor
 627 antagonist (SOLA). † significantly different from male fetuses in same treatment group (two-way ANOVA, p<0.05).

Bone property	Bone type	Saline (n=13)		SOLA (n=6)		Effect of SOLA infusion (p-value)		
		Male (n=6)	Female (n=7)	Male (n=3)	Female (n=3)	Treatment	Sex	Interaction
Midshaft total diameter (mm)	Femur	7.50 ± 0.26		7.62 ± 0.23		NS	0.006	NS
		8.13 ± 0.23	6.95 ± 0.33†	8.06 ± 0.10	7.17 ± 0.24†			
	Metatarsal	7.11 ± 0.20		6.98 ± 0.30		NS	0.001	NS
		7.59 ± 0.12	6.64 ± 0.27†	7.61 ± 0.25	6.35 ± 0.07†			
Midshaft lumen diameter (mm)	Femur	3.61 ± 0.18		3.26 ± 0.16		NS	NS	NS
	Metatarsal	4.34 ± 0.17		4.25 ± 0.20		NS	0.001	NS
		4.78 ± 0.11	3.89 ± 0.18†	4.66 ± 0.19	3.84 ± 0.03†			
Midshaft wall thickness (mm)	Femur	1.94 ± 0.12		2.18 ± 0.10		NS	NS	NS
	Metatarsal	1.39 ± 0.06		1.37 ± 0.08		NS	NS	NS
Body length (mm)	Vertebra	7.83 ± 0.18		8.06 ± 0.40		(0.091)	NS	NS
Total bone volume (mm ³)		394.7 ± 29.9		454.7 ± 54.7		NS	NS	NS
Bone volume/total volume (%)	Femur	28.8 ± 2.5		33.1 ± 1.7		NS	NS	NS
	Metatarsal	28.7 ± 1.8		23.8 ± 1.5		NS	NS	NS
	Vertebra	31.6 ± 2.8		38.1 ± 3.3		NS	(0.097)	NS
		26.6 ± 2.6	35.9 ± 4.2	34.9 ± 3.2	41.4 ± 5.8			
Bone surface/bone volume (mm ² /mm ³)	Femur	32.6 ± 1.4		29.6 ± 1.3		NS	NS	NS
	Metatarsal	30.0 ± 0.8		32.1 ± 1.5		NS	NS	NS
	Vertebra	27.0 ± 1.1		28.0 ± 1.3		NS	0.041	NS
		29.4 ± 1.0	25.1 ± 1.6†	29.6 ± 1.4	26.3 ± 1.9			

Trabecular thickness (mm)	Femur	0.116 ± 0.003		0.121 ± 0.004		NS	NS	NS
	Metatarsal	0.127 ± 0.003		0.120 ± 0.003		NS	NS	NS
	Vertebra	0.142 ± 0.005		0.142 ± 0.008		NS	NS	NS
Trabecular number (/mm)	Femur	2.44 ± 0.16		2.73 ± 0.06		NS	NS	NS
	Metatarsal	2.26 ± 0.13		1.99 ± 0.09		NS	NS	NS
Trabecular spacing (mm)	Femur	0.26 ± 0.02		0.23 ± 0.01		NS	NS	NS
	Metatarsal	0.27 ± 0.02		0.29 ± 0.02		NS	NS	NS
Trabecular pattern factor (/mm)	Femur	3.97 ± 1.09		2.41 ± 0.60		NS	NS	NS
	Metatarsal	5.08 ± 0.82		7.77 ± 1.21		(0.099)	NS	NS
	Vertebra	2.96 ± 0.83		1.68 ± 0.82		NS	(0.058)	NS
		4.50 ± 0.90	1.64 ± 1.16	2.79 ± 1.17	0.57 ± 0.88			
Porosity (%)	Femur	0.005 ± 0.002		0.003 ± 0.001		NS	NS	NS
	Metatarsal	0.007 ± 0.002		0.002 ± 0.001		NS	NS	NS
Structural model index	Femur	1.32 ± 0.13		1.13 ± 0.07		NS	NS	NS
	Metatarsal	1.61 ± 0.08		1.77 ± 0.14		NS	NS	NS
	Vertebra	1.29 ± 0.12		1.38 ± 0.16		NS	0.037	NS
1.52 ± 0.12		1.09 ± 0.17	1.60 ± 0.27	1.16 ± 0.10				
Degree of anisotropy	Femur	2.15 ± 0.05		2.24 ± 0.06		NS	NS	NS
	Metatarsal	1.43 ± 0.07		1.36 ± 0.05		NS	NS	NS
Connectivity density (/mm ³)	Femur	68.5 ± 7.9		63.5 ± 2.65		NS	NS	NS
	Metatarsal	78.1 ± 14.1		53.2 ± 7.8		NS	NS	NS

Figure 1. Mean (\pm SEM) bone volumes (A) and percentage bone volumes (B) in a fixed length of midshaft femur from fetuses infused for five days with either saline, leptin (LEP1 and LEP2) or leptin antagonist (SOLA). For comparisons between saline and leptin treatment groups, columns with different letters are significantly different from each other; uppercase letters indicate differences in the total volume, and lowercase letters at the SEM bars indicate differences in volume compartments (one-way ANOVA, $p < 0.05$). Compartments with no letters at the SEM bars are not significantly different from each other ($p > 0.05$).

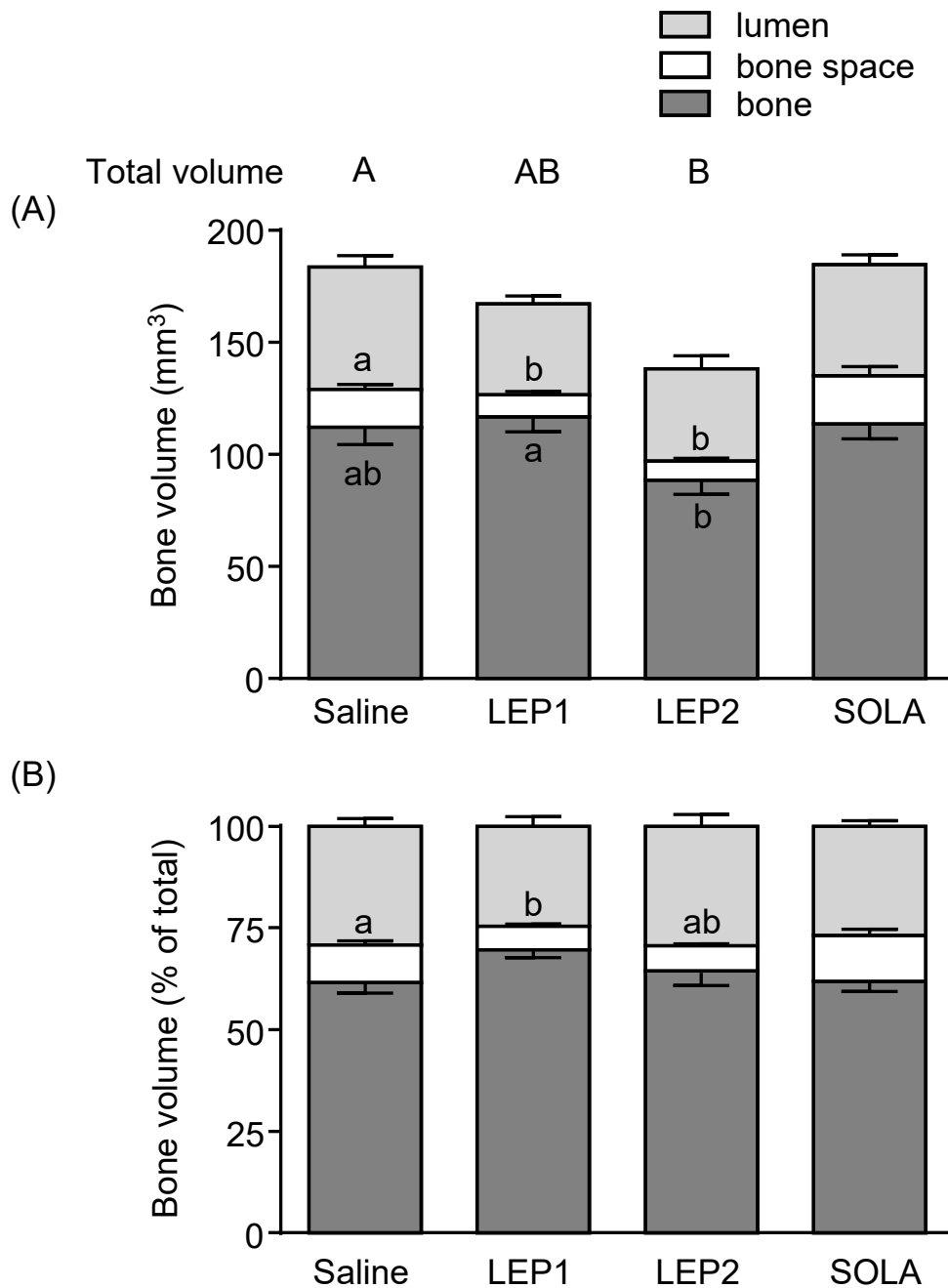
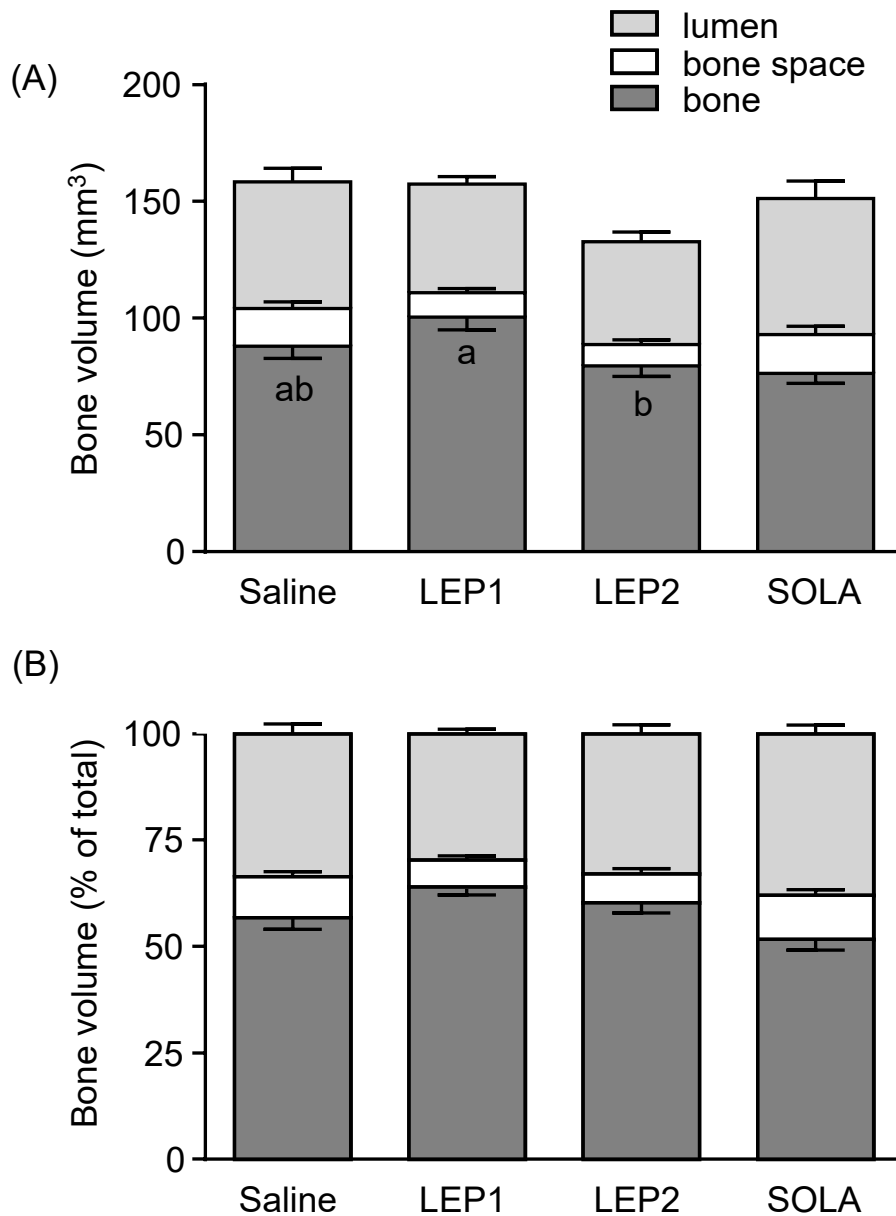
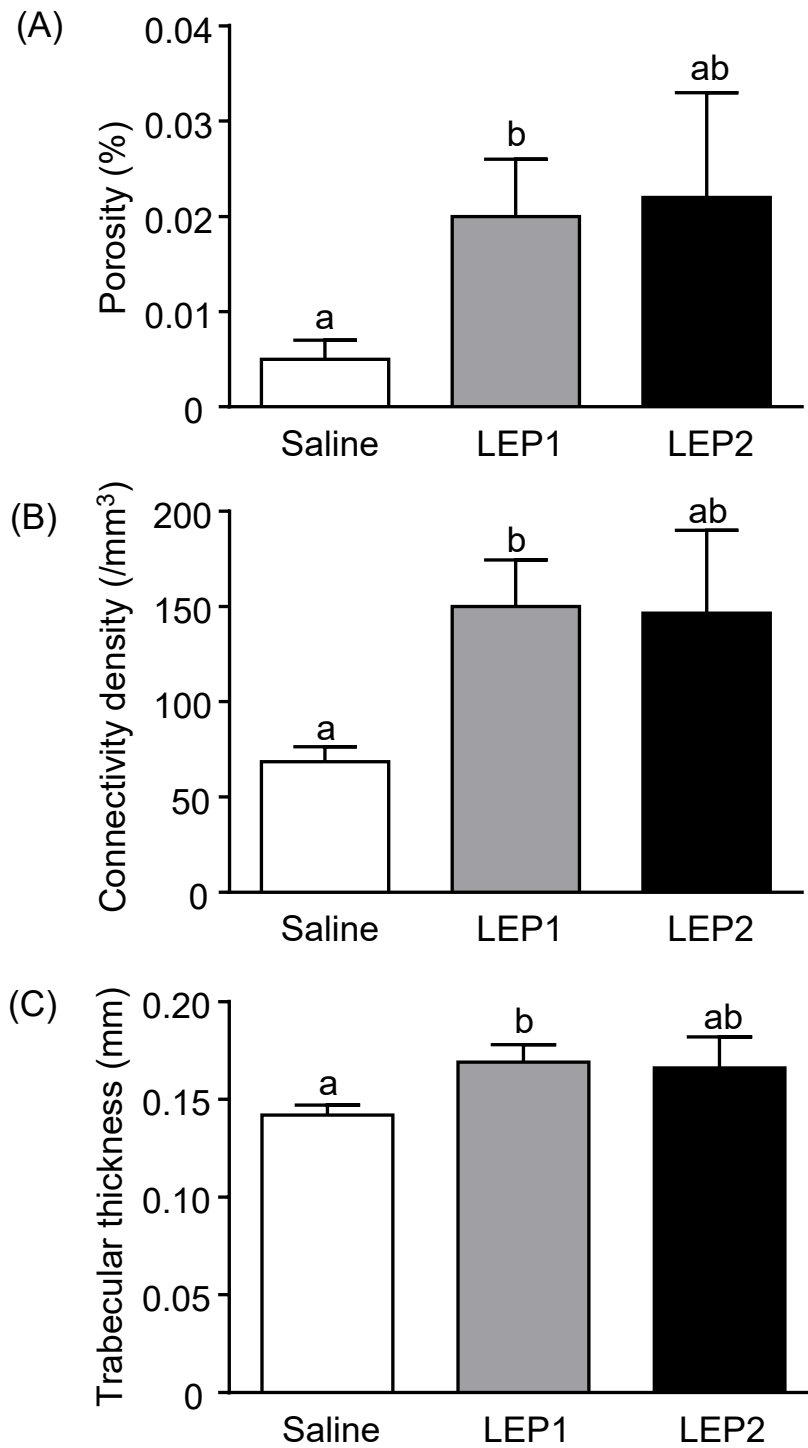


Figure 2. Mean (\pm SEM) bone volumes (A) and percentage bone volumes (B) in a fixed length of midshaft metatarsal from fetuses infused for five days with either saline, leptin (LEP1 and LEP2) or leptin antagonist (SOLA). For comparisons between saline and leptin treatment groups, compartments with different letters at the SEM bars are significantly different from each other (one-way ANOVA, $p < 0.05$). Compartments with no letters at the SEM bars are not significantly different from each other ($p > 0.05$).



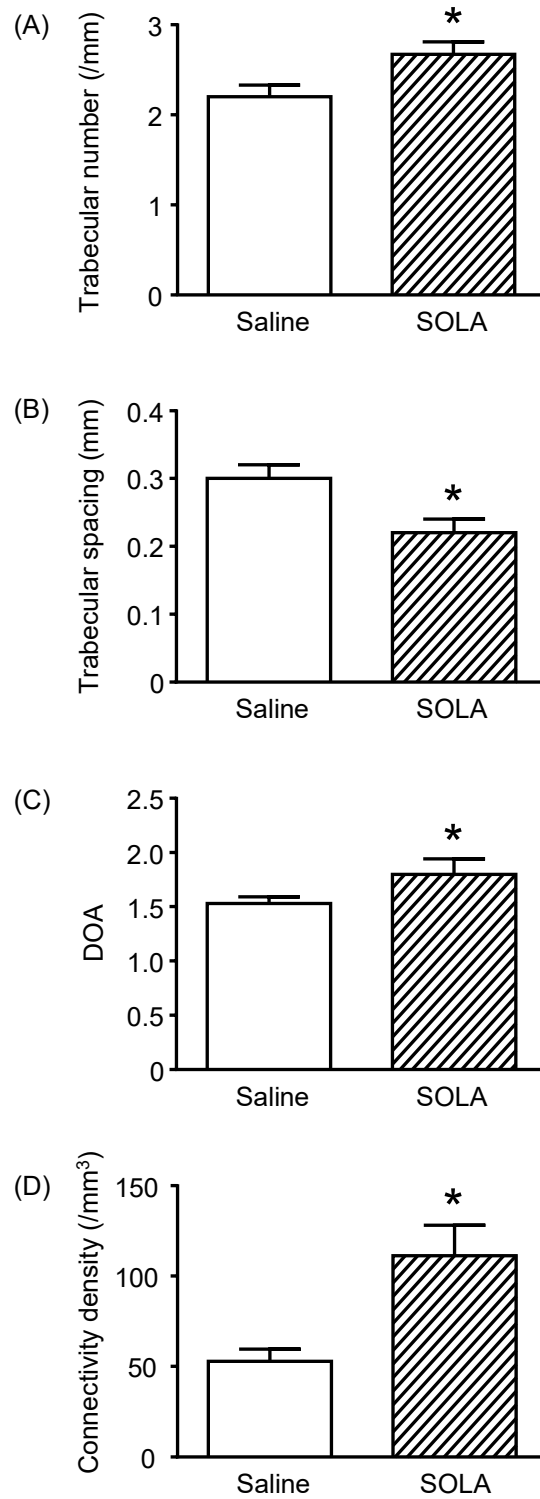
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Figure 3. Mean (\pm SEM) porosity (A) and connectivity density (B) in the femur, and trabecular thickness (C) in the lumbar vertebra, of fetuses infused for five five days with either saline or leptin (LEP1 and LEP2). Columns with different letters are significantly different from each other (one-way ANOVA, $p < 0.05$).



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Figure 4. Mean (\pm SEM) porosity in the lumbar vertebra of fetuses infused for five days with either saline or leptin antagonist (SOLA). *, significantly different from saline-treated fetuses of the same sex (two-way ANOVA, $p < 0.05$); †, significantly different from male fetuses in the same treatment group (two-way ANOVA, $p < 0.05$).



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Figure 5. Mean (\pm SEM) porosity in the lumbar vertebra of fetuses infused for five days with either saline or leptin antagonist (SOLA). *, significantly different from saline-treated fetuses ($p < 0.05$); †, significantly different from male fetuses in the same treatment group ($p < 0.05$).

