

The effect of litter size upon foetal growth rate and the placental transfer of calcium and phosphorus in superovulated Scottish half-bred ewes

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1. The ovulation rate of forty-eight Scottish half-bred ewes was increased by using pregnant mare's serum gonadotrophin thus inducing litters of one to four foetuses.
2. The effects of increased litter size upon the foetal growth rate and upon the rate of transfer of calcium and phosphorus across the placenta were studied at 108-112, 122-126 and 136-140 d gestation.
3. The number of foetuses had little effect upon foetal weight at 112 d, the mean weights of a singleton, twin, triplet or quadruplet being similar. However, by 140 d mean foetal weight decreased markedly as litter size increased.
4. Failure of individual quadruplets to grow as fast as a singleton was associated with a limitation in the capacity of the placenta for transferring minerals. The maximum rates of transfer of Ca and P, whatever the number of foetuses, were approximately 2.8 and 1.4 g/d respectively. These rates were attained by 112 d when quadruplets were being carried, by 126 d for triplets, and by 140 d for twins.

Modern methods of sheep husbandry are frequently based on improving the lamb crop by increasing the number of lambs born per ewe. This can be achieved either by increasing the frequency of pregnancies per year or by increasing the number of lambs per gestation. Such exploitation of the ewe's production potential must not only impose extra demands upon her metabolism but also affect foetal growth, for it is known that as the number of foetuses *in utero* increases there is a corresponding reduction in the birth weight and also possibly in the viability of the lambs. The metabolic limits of superfoetation have not been determined. This paper reports an investigation of the ability of the placenta to transfer calcium and phosphorus to the foetuses of superfoetated ewes and of whether this ability constitutes a physiological limit to litter size. A preliminary report of this work has been published (Twardock, Symonds & Sansom, 1971).

EXPERIMENTAL

Animals and diet

Oestrus was synchronized in batches of Scottish half-bred ewes (Border Leicester ram × Cheviot ewe), 6-8 years of age, by means of a progestin sponge inserted into the vagina for 2 weeks. Pregnant mare's serum gonadotrophin was given before the second oestrus after synchronization. Rams were kept with the ewes from 15 d after

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Table 1. *Composition of sheep meal*

Ingredient	g/kg
Barley meal	400
Bran	250
Flaked maize	250
Groundnut cake	100

32 g of a mineral supplement for sheep was added to each 1 kg of the meal.

Table 2. *Numbers of ewes carrying one, two, three, four, five or six foetuses at 112, 126 and 140 d gestation*

Period of gestation (d)	One foetus	Two foetuses	Three foetuses	Four foetuses	Five* foetuses	Six* foetuses
112	1	5	4	2	—	1
126	4	4	4	4	1	—
140	4	5	6	2	1	—

* Values for ewes carrying five and six foetuses are not included in the statistical analysis.

removal of the sponge through two succeeding oestrous cycles. Five batches of ewes were treated in this way at intervals of 1 month between September 1969 and January 1970. The number of foetuses carried by each ewe was estimated by X-ray examination at 90 d gestation.

The ewes were kept on grass pasture and given a supplement of grass-nuts until 3 weeks after tupping, when they were moved to a covered enclosure and fed *ad lib.* on timothy-lucerne high-dry-matter silage and sheep meal (Table 1). One week before use in experiments, they were moved to individual indoor pens with peat-moss bedding and were given 700 g of meal and 700 g high-dry-matter silage twice daily; unconsumed meal and silage were weighed before the next feeding. Ca and P intake varied between animals according to their consumption of meal and silage. Mean intakes were 9.7 g Ca/d and 9.5 g P/d during the 12 d during which feed consumption was measured.

Experimental procedure

The transfer of Ca and P from dam to foetus was measured in forty-eight ewes between 2 February and 8 May 1970. Measurements were made over approximately 108–112, 122–126 or 136–140 d gestation, with approximately the same number of ewes in each of these three groups. Ewes were assigned to these groups according to X-ray estimates of the numbers of foetuses they carried, in an effort to obtain similar numbers of ewes carrying one, two, three and four foetuses in each group. The distribution of foetuses is given in Table 2.

The technique used for measuring rates of transfer of Ca and P across the placenta was that described by Symonds, Manston, Payne & Sansom (1966), except that intravenous injections of radionuclides were used instead of subcutaneous depots. Three hundred μCi ^{32}P as sodium orthophosphate and 150 μCi ^{45}Ca as CaCl_2 (Radiochemical Centre, Amersham, Bucks.) in approximately 10 ml isotonic saline (9 g NaCl/l) containing 0.1 mg P/ml and 0.2 mg Ca/ml were injected through an

indwelling catheter in the maternal jugular vein. The exact dose given to each ewe was determined by weighing each syringe before and after injection. Fifteen samples of heparinized maternal blood were obtained from each ewe by venepuncture of the jugular vein not used for radionuclide injection, at the following times after injection: 5, 15, and 30 min, and 1, 2, 4, 8, 11, 23, 30, 47, 54, 71, 78, and 96 h.

Immediately after the final blood sample was taken each ewe was killed by intravenous injection of pentobarbitone sodium and her foetuses were removed through incisions in the abdominal and uterine walls. The foetuses were stripped of excess fluid and mucus, placed in polyethylene buckets, partly dismembered, weighed, and covered with 16 M-nitric acid. Digestion for 2–4 weeks in a room at outdoor temperature produced an acid digest of sufficient fluidity and uniformity to allow representative sampling.

Chemical and radiochemical analyses

Blood plasma Ca and inorganic P concentrations were determined by auto-analytical methods: Ca by the method of Kessler & Wolfman (1964) and P by the AutoAnalyzer method (Technicon Method Sheet N-4b). Plasma inorganic ^{32}P and ^{45}Ca were counted with a Beckman DPM 100 liquid scintillation spectrometer. Cerenkov radiation from ^{32}P was measured in filtrates from plasma precipitated with trichloroacetic acid. Plasma (2 ml) was mixed with 5 ml trichloroacetic acid (200 g/l) for the first eight samples from each ewe, and 5 ml plasma with 5 ml trichloroacetic acid for the last seven samples; 4 ml and 5 ml samples of filtrates were counted from the first eight and last seven samples respectively. Plasma ^{45}Ca was assayed by the resin absorption method described by Gibbons & Sellwood (1968). Radioactivity standards were prepared by adding known fractions of the dose solutions to blood plasma obtained from non-radioactive sheep and were radioassayed by the same methods.

The polyethylene buckets containing foetuses digested in nitric acid were weighed and the contents stirred vigorously while duplicate samples of 400–600 g were obtained. From each of the duplicates, approximately 40 g were weighed into an evaporating dish, evaporated slowly to dryness on a hot plate, and then dry-ashed at 650° overnight. The resultant ash was dissolved in 10 ml 2M-HCl, transferred to a 25 ml or 50 ml volumetric flask, and diluted to volume. P concentrations were measured in 1–50 dilutions of the ash solutions by the same automated method used for plasma inorganic P; Ca concentrations were determined by atomic absorption spectrometry using lanthanum chloride to suppress phosphate interference. The P and Ca contents of each foetus were calculated from the total weight of nitric acid digestate and subsequent weighings and dilutions from it. Foetal ^{32}P was measured by Cerenkov (Parker & Elrick, 1966) counting of the ash solutions, using internal standards to correct for quenching differences caused by variations in ash content among samples. Foetal ^{45}Ca was determined by adding 0.5 ml foetal ash solution to 9 ml liquid scintillator (Braithwaite, Glascock & Riazuddin, 1969) and counting in a Beckman DPM 100 spectrometer at least 20 weeks after radionuclide injection into the ewe. Standards were prepared from aqueous dilutions of the dose solution. To ensure equal counting efficiency in standards and foetal solutions, 0.5 ml of ash solution prepared from normal non-radioactive foetuses was added to all standards. The presence

of the acid ash solution enhanced the count rate by approximately 1%. The delay of 20 weeks ensured that ^{32}P radioactivity was negligible.

Calculations

For each ewe the specific radioactivities of blood plasma Ca and inorganic P were calculated as % dose/g. Ca and P in each of the fifteen samples taken between 5 min and 96 h after intravenous injection. The mean specific activities of blood plasma for the 96 h period of the experiment were determined from the areas under the curves obtained by plotting the specific activities (C_n) of ^{45}Ca and ^{32}P against time (t_n). The areas under these curves, expressed as (% dose/g) \times h, were divided by the duration of the experiment expressed in h. The areas were computed by assuming that between each pair of experimental points (t_n, C_n), (t_{n+1}, C_{n+1}), the specific activity declined exponentially as follows:

$$C_{n+1} = C_n e^{-k(t_{n+1}-t_n)}.$$

The concentration at the time of dosing with radionuclide, C_0 , was calculated by assuming that C_0 lay on the extrapolation to t_0 of the exponential line joining the first two experimental points obtained at 5 and 15 min after injection. These assumptions having been made, the area under each curve was calculated by computer. The results for two sets of experimental results were compared with those obtained by two other methods. These methods were to draw a curve free-hand on paper and to measure the area (*a*) by cutting out the area, weighing it, and comparing the weight with that of a known area of the same piece of paper, and (*b*) by applying Simpson's (1743) rule to the same curve after dividing it into ninety-five equal time-intervals. The three methods gave results within 1% of each other, and the computer method was therefore used because of its speed and simplicity.

Ca or P transfer from dam to foetus during the 96 h was calculated as:

$$\frac{\text{Percentage of dose } ^{32}\text{P or } ^{45}\text{Ca in foetus}}{\text{Mean specific activity of maternal plasma } ^{32}\text{P or } ^{45}\text{Ca}}$$

Statistical analysis

For practical reasons the experiments on the forty-eight ewes were carried out on eight batches of ewes. It was therefore likely that batch effects (due, for example, to analyses being done at different times, and to uncertainties in the stage of gestation of individual ewes) could influence the results. Adjustments of the results for these batch effects was therefore made by the method of least squares.

Since the increases in some variables from 112 to 140 d gestation were associated with increased ewe-to-ewe variation, for the purposes of litter size comparison for a particular gestation day the adjusted values for 112, 126 and 140 d gestation were analysed separately.

Table 3. Mean maternal plasma calcium and inorganic phosphorus concentrations and mean foetal total Ca and P content, Ca and P transfer rate and foetal weight in ewes carrying one to four foetuses over 108-112, 122-126 and 136-140 d gestation (values adjusted for batch effects; see p. 440)

No. of foetuses in uterus	Total no. of foetuses	Stage of gestation (d)	Plasma concentration (mg/l)		Foetal stable content (g)		Transfer rate (g/d)		Foetal wt (kg)	Transfer rate/kg foetal body-wt	
			Ca	P	Ca	P	Ca	P*		Ca	P
1	1	108-112	98	41	14.8	8.8	0.72	0.40	2.08	0.346	0.192
2	10		101	42	14.8	8.8	0.78	0.39	2.01	0.388	0.194
3	12		100	47	14.0	8.1	0.66	0.33	1.88	0.351	0.176
4	8		100	41	13.5	7.7	0.61	0.31	1.73	0.353	0.179
SE			2	3	1.0	0.5	0.08	0.04	0.19		
1	4	122-126	98	46	31.4	16.9	1.06	0.57	3.55	0.299	0.161
2	8		92	46	24.6	14.8	0.88	0.45	3.35	0.262	0.134
3	12		92	41	28.0	14.5	0.82	0.43	3.12	0.262	0.138
4	16		93	45	24.6	13.5	0.65	0.33	3.01	0.216	0.110
SE			2	2	3.3	1.2	0.08	0.04	0.19		
1	4	136-140	95	43	50.0	26.3	1.52	0.75	5.66	0.269	0.132
2	10		92	44	42.9	23.1	1.30	0.61	4.86	0.267	0.125
3	18		90	42	34.2	19.4	0.82	0.42	4.16	0.197	0.100
4	8		91	43	31.8	17.8	0.69	0.34	3.57	0.193	0.095
SE			3	2	4.1	2.2	0.14	0.06	0.31		

SE, standard error of any of the four means for four ewes per mean.
Significance of litter size: *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$.

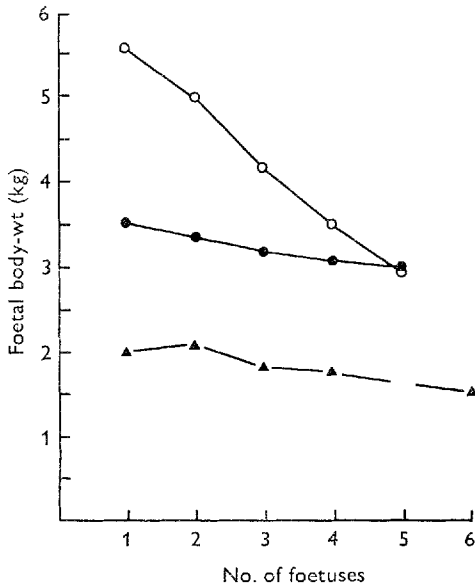


Fig. 1

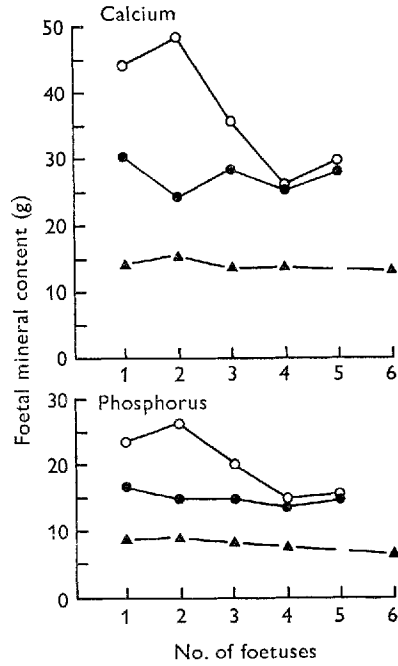


Fig. 2

Fig. 1. Change in mean foetal body-weight with increase in the number of foetuses in the uterus of ewes at 108-112 (▲—▲), 122-126 (●—●) and 136-140 (○—○) d of gestation.

Fig. 2. Change in the mean foetal total calcium and phosphorus content with increase in the number of foetuses in the uterus of ewes at 108-112 (▲—▲), 112-126 (●—●) and 136-140 (○—○) d of gestation.

RESULTS

Table 3 lists the mean content of stable Ca and P within a foetus, the mean rate of transfer of Ca and P to a foetus, and the mean foetal body-weights at 112, 126 and 140 d gestation, all adjusted for experimental batch effects as described. The unadjusted results are plotted in Figs 1, 2 and 3 and include values for two ewes carrying five foetuses and one ewe carrying six.

Increasing the number of foetuses in the uterus affected the variables measured as follows.

Effect upon foetal body-weight. The single foetus increased its body-weight rapidly over the last 5 weeks of gestation. The mean weight at 112 d was 2.08 kg and at 140 d, 5.66 kg. Increasing the litter size resulted in a lower mean foetal body-weight, the comparative effect being greatest at the later stages of gestation. At 126 d the effect of foetal numbers on mean foetal body-weight was significant at the 5% level, at 140 d it was significant at the 1% level. At all stages, the total litter weight was always in the order quadruplets > triplets > twins > singletons.

Effect upon stable Ca and P content of foetuses. The effect of increase in litter size upon mineral content was similar to the effect upon body-weight. Statistically the effect was significant only at 140 d for Ca ($P < 0.01$) and P ($P < 0.01$).

Effect upon transfer rate. The rate of transfer of Ca and P to singletons and twins

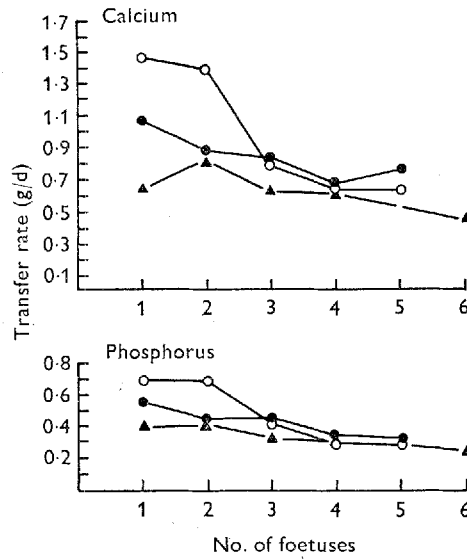


Fig. 3. Change in mean rate of transfer of calcium and phosphorus to a fetus with increase in the number of fetuses in the uterus of the ewe at 108-112 (▲—▲), 122-126 (●—●) and 136-140 (○—○) d of gestation.

Table 4. Total calcium and phosphorus (g) transferred daily to fetuses in ewes carrying singletons, twins, triplets and quadruplets over 108-112, 122-126 and 136-140 d gestation

Foetuses	108-112 d		122-126 d		136-140 d	
	Ca	P	Ca	P	Ca	P
Singleton	0.72	0.40	1.06	0.57	1.52	0.75
Twins	1.56	0.78	1.76	0.90	2.60	1.22
Triplets	1.98	0.66	2.46	1.29	2.46	1.26
Quadruplets	2.44	1.24	2.60	1.34	2.76	1.36

continued to increase from 112 d up to 140 d. For triplets the transfer rate did not increase after 126 d and for quadruplets the rate changed little from 112-140 d. At each stage, the increase in litter size was associated with a decrease in transfer rate, and this decrease was significant at all stages except for Ca at 112 d (Table 3). There appeared to be a maximum rate of transfer. Thus if the total amount of mineral supplied daily to all foetal tissue within a uterus is considered (Table 4) then there was no significant difference in the amount transferred by those ewes carrying four foetuses at 112, 126 and 140 d, those ewes carrying three foetuses at 126 and 140 d, and those ewes carrying twins at 140 d. This rate was between 2.44 and 2.76 g/d for Ca, and between 1.22 and 1.36 g/d for P.

Effect upon blood mineral concentrations. There was no consistent relationship between the maternal plasma inorganic P concentrations and the number of foetuses carried or stage of gestation. However, mean plasma Ca concentrations were significantly lower ($P < 0.01$) at 126 and 140 d of pregnancy than at 112 d. The fall in plasma Ca with increase in litter size was not significant.

DISCUSSION

There is no obvious reason why the intrinsic capacity for growth of each foetus in a multifoetate ewe should not be similar to that of a singleton. However, it is well known, and it is confirmed in the present work, that the larger the number of lambs in a litter the lower is their birth weight. Some factor or factors must therefore prevent the achievement of full growth potential by multifoetate lambs. The observations reported here suggest that the sum of the effects of these factors is to set an upper limit to the transfer rate across the ewe's placenta of nutrients essential for the lamb's growth. For the minerals Ca and P such a maximum transfer rate was observed, under the experimental conditions described, at approximately 2.8 and 1.4 g/d respectively.

The time during gestation at which the maximum rate of transfer is reached will markedly affect birth weight since, once it is reached, there will be no further increase in the rate of foetal growth. As would be expected, the larger the litter size the earlier in gestation was the maximum rate reached. When quadruplets were carried, maximum transfer of minerals had been achieved by (or before) 112 d gestation, when triplets were carried it was reached between 112 and 126 d gestation, and when twins were carried it was reached between 126 and 140 d. Transfer rates to the single foetus continued to increase throughout gestation and did not attain the maximum over the measured period.

Large litters mean that a high transfer rate is reached earlier in gestation. The ewe carrying several foetuses will therefore need its diet to be improved earlier in gestation. This is emphasized by considering the total foetal weight contained within the ewe at the three stages of gestation. The greatest weight of foetal tissue was carried when quadruplets were present and at 140 d gestation averaged 14.3 kg. But even at 112 d the average total weight of quadruplets, 6.9 kg, was greater than the average weight of the singleton at 140 d, 5.8 kg.

The factors producing this limit to the transfer rate must lie either in the placenta (in terms of available blood supply, area for transfer, or transfer capacity), in the ewe's ability to take in food and supply minerals and nutrients, or in the uterine capacity for distension.

The area of placenta available for each foetus in large litters would appear to be less than that available for the singleton. Foetal growth is affected by placental development in the cow. When twins are carried the number of placental cotyledons developing and the birth weight of calves are greater when each foetus occupies a uterine horn than when both develop in the same horn (Rowson, Lawson & Moore, 1971).

Another possible factor limiting the mineral transfer rate is the ewe's capacity to supply sufficient Ca and P. The significant fall in plasma Ca concentrations from 112 to 126 and 140 d gestation suggests that the ewe's Ca homeostasis was not entirely coping with the demands of the foetuses. However, metabolic disease in the ewe is likely to intervene before the transfer rate is decreased. In fact it seems more probable that the limit on placental transfer will have a protective action and prevent excessive demands upon the ewe. This is important because the ewe carrying single or twin foetuses is reported to be unable to absorb sufficient Ca from the diet by 100 d gestation to main-

tain the supply to the foetus without some reliance upon body reserves (Braithwaite & Riazuddin, 1971).

The use of radioisotopes as a measure of placental transfer is open to two criticisms. (1) Radioisotope may return from the foetus to the ewe in large amounts allowing only net accretion to the foetus to be measured. This value would still be acceptable as an indicator of net mineral loss to the ewe each day but it would not indicate the total placental capacity to transfer mineral. In sheep, however, back-transfer is small. In Scottish half-bred ewes at stages of gestation similar to those of the ewes in the present experiment, not more than 0.4 and 7% respectively of the Ca and P transferred to the foetus daily, returned to the dam (Symonds, Sansom & Twardock, 1972). (2) The specific activity of Ca and inorganic P in jugular blood may not be the same as the specific activities in uterine arterial blood. This fact would introduce errors in the estimation of transfer rates because of the method used for their calculation. However, since foetus-to-dam transfer of mineral is very small it is unlikely that specific activity changes across the maternal placental circulation were sufficiently large to affect the calculations.

The low rate of back-transfer of Ca and P from foetus to dam indicates that deposition rates in foetal tissue must approximate to the transfer rate, that is there is no over-supply or 'safety factor'. This fact is also shown by comparing the amounts of Ca transferred over the periods 112-126 and 126-140 d gestation with the increase in foetal Ca content over the same period. Over the period 112-126 d the mean foetal Ca increased by 12.9 g, and the mean amount transferred may be calculated to be 10.9 g; similarly over the period 126-140 d the mean foetal Ca increased by 13.0 g and the amount transferred may be calculated to be 13.7 g. In having no 'safety factor' the ewe differs from the rabbit and monkey, in which transfer of Ca is reported to be much greater than the amounts accreted by the foetuses (Wasserman, Comar, Nold & Lengemann, 1957; MacDonald, Hutchinson, Hepler & Flynn, 1965). This difference may be due to the differences in placental structure between the species, the syndesmochorial placenta of the ewe forming more of a barrier to the passage of mineral back from foetus to ewe than the haemochorial placenta of the rabbit and monkey.

Recent work indicates that the use of pregnant mare's serum gonadotrophin to increase litter size may be of practical value only in those breeds of sheep which naturally ovulate at rates below the capacity of their uterus to support foetuses (Rowson, 1971). The present work appears to confirm that, unless viability and growth potential of the lambs remain good at markedly lower birth weights, the value of increasing litter size by increasing ovulation rate is limited.

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