Aberrant fetal growth and development after *in vitro* culture of sheep zygotes

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The effects of *in vitro* culture systems for sheep zygotes on subsequent fetal growth and development to day 61 and day 125 of gestation were studied. Zygotes recovered from superovulated Scottish Blackface ewes approximately 36 h after intrauterine insemination using semen from a single Suffolk sire were cultured for 5 days in (a) a granulosa cell co-culture system (co-culture); (b) synthetic oviductal fluid medium without serum (SOF-); and (c) synthetic oviductal fluid medium supplemented with human serum (SOF+). Control embryos were recovered from superovulated donor ewes at day 6 after oestrus. Embryos were transferred at day 6 to synchronous Scottish Blackface recipient ewes. In total, 146 gravid uteri were recovered, comprising 97 at day 61 (20 co-culture, 27 SOF-, 25 SOF+ and 25 control) and 49 at day 125 (13 co-culture, 8 SOF-, 6 SOF+ and 22 control) of gestation. Fetuses derived from co-cultured embryos were 14% heavier ($P < 0.01$) by day 61 of gestation than those derived from control embryos. Growth coefficients derived from the linear allometric equation $\log_e y = \log_e a + b \log_e x$ (where y = organ mass; x = fetal mass) were significantly greater ($P < 0.05$) for liver, heart, kidneys and plantaris muscle in fetuses derived from co-cultured embryos, and for liver in fetuses derived from SOF+ embryos than those for control fetuses. Fetuses derived from co-cultured embryos were 34% heavier ($P < 0.001$) and fetuses derived from SOF+ embryos were 18% heavier ($P < 0.01$) by day 125 of gestation than those derived from control embryos. Growth coefficients for liver and heart for fetuses derived from co-culture and SOF+ embryos were also significantly greater ($P < 0.05$) at this stage of gestation than those for control group fetuses. In contrast, allometric coefficients for these organs in fetuses derived from embryos cultured in SOF without serum supplementation were not different from those for controls. Excessive volumes of amniotic fluid (polyhydramnios) were observed in 23% of conceptuses derived from co-cultured embryos. *In vitro* embryo culture can significantly influence fetal growth and this study provides quantitative evidence of major shifts in the patterns of organ and tissue development.

Introduction

Advanced reproductive technologies can deliver substantial benefits to livestock production through their ability to increase the rate of genetic gain within genetic improvement programmes and to enhance the rate of dissemination of improved genetic material (Woolliams and Wilmut, 1989; Brash *et al.*, 1996; Sinclair and Broadbent, 1996). New industries are being generated on the basis of the production of transgenic animals with the ability, for example, to produce novel proteins for use in human medicine (Wilmut and Whitelaw, 1994). An integral part of these new technologies is *in vitro* embryo culture. However, reports of unusually large

offspring in ruminants after the transfer to recipients of embryos cloned by nuclear transfer or cultured *in vitro*, with associated complications at birth resulting in high perinatal morbidity and mortality (for review, see Walker *et al.*, 1996; Kruip and den Daas, 1997; Young *et al.*, 1998), are impeding the adoption of such technologies in these species.

Field-scale trials with cattle have provided quantitative information on the effects on birth weight of nuclear transfer (Willadsen *et al.*, 1991; Wilson *et al.*, 1995) and *in vitro* embryo production (Hasler *et al.*, 1995; van Wagtenonk-de Leeuw *et al.*, 1998). However, to date, information on fetal development has been largely anecdotal with detailed physical, physiological and metabolic studies being limited to the neonate (Garry *et al.*, 1996). The gross changes described are sporadic in their incidence and may disguise

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more subtle but fundamental shifts in organ and tissue development than have been reported. These developmental shifts may occur at a much higher frequency and could, in addition to influencing neonatal morbidity and mortality, impinge on postnatal growth and longevity. Therefore, the purpose of this study was to describe any such changes in the growth and development of sheep fetuses derived from *in vitro* cultured embryos.

Materials and Methods

The experiment described in this paper was reviewed by the Animals and Welfare Committees of the Scottish Agricultural College and was conducted under the auspices of, and in accordance with the requirements of, the Home Office Animals (Scientific Procedures) Act, 1986.

Animals and experimental design

Mature Scottish Blackface ewes, with no overt disease or reproductive abnormalities, were assembled in August 1995 and assigned randomly to be either zygote donors, embryo donors or recipients. The donor ewes were superovulated and artificially inseminated using semen from the single Suffolk sire used by Sinclair *et al.* (1998). Zygotes recovered from these donors were cultured *in vitro* for 5 days on one of three treatments: (a) a granulosa cell co-culture system (co-culture); (b) unsupplemented synthetic oviductal fluid medium (SOF-); or (c) synthetic oviductal fluid medium supplemented with serum (SOF+). Control embryos were recovered from superovulated donor ewes at day 6 after oestrus (day 0). All embryos were transferred singly at day 6 to synchronous recipients. Gravid uteri were recovered at day 61 and day 125 of gestation.

Embryo production and transfer

Oestrous cycles were synchronized in both donor and recipient ewes using progestogen-releasing intravaginal sponges (30 mg fluorogestone acetate, Chronogest, Intervet, Cambridge) for 12 days. For recipient ewes, the same sponge remained in position for 12 days whereas, for donor ewes, it was removed and a second sponge was inserted after 7 days. Recipients received 400 iu pregnant mares' serum gonadotrophin (Intervet, Cambridge) at sponge withdrawal. Oestrous observations began 24 h after progestogen withdrawal and were carried out at 08:00, 12:00, 16:00 and 24:00 h during the next 3 days, using raddled vasectomized rams.

Superovulation was induced in donor ewes using a total dose of 9 mg oFSH (Ovagen, ICP Ltd, Auckland) administered twice daily in equal doses over 4 days beginning on day 10 of the 12 day progestogen priming period. Laparoscopic intrauterine insemination was carried out 44 h after sponge withdrawal using fresh semen from a single Suffolk sire diluted 1:3 in PBS containing 1000 iu

sodium penicillin and 1 mg streptomycin sulphate ml⁻¹. The diluted semen was held at 30°C before insemination and approximately 100 × 10⁶ motile spermatozoa were placed in each uterine horn.

Zygotes were collected during mid-ventral laparotomy after induction of general anaesthesia (halothane; oxygen; nitrous oxide) 36–42 h after insemination. Each oviduct was flushed with 20 ml Hepes-buffered synthetic oviductal fluid, derived from the formulation of Tervit *et al.* (1972), and containing 20.0 mmol Hepes buffer l⁻¹ (pH 7.4), 5.0 mmol sodium bicarbonate l⁻¹, 1.5 mmol glucose l⁻¹, 3.3 mmol sodium lactate l⁻¹, 0.33 mmol sodium pyruvate l⁻¹, 1.0 mmol L-glutamine l⁻¹ and 30 mg bovine serum albumin ml⁻¹. All zygotes with normal morphology were allocated to one of three culture systems. Allocation to treatments aimed to ensure that, in so far as zygote yields permitted, each donor and each stage of development (range = 1-cell to 4-cell) were represented equally across treatments, thus avoiding any pre-culture bias.

The co-culture medium used was M199 with Earle's salts (Life Technologies, Paisley) together with 50 iu penicillin ml⁻¹, 50 µg streptomycin sulphate ml⁻¹ and 10% (v/v) heat-inactivated (56°C; 30 min) steer serum (Globepharm Ltd, Esher). The medium was at pH 7.3 and 290–310 mOsm. Both SOF media, derived from the original formulation of Tervit *et al.* (1972) and excluding Hepes buffer, included 25.0 mmol sodium bicarbonate l⁻¹, 9.9 mmol sodium lactate l⁻¹, 0.99 mmol sodium pyruvate l⁻¹, 1.5 mmol glucose l⁻¹, 1.0 mmol L-glutamine l⁻¹, 50 iu penicillin ml⁻¹ and 50 µg streptomycin sulphate ml⁻¹. SOF- also contained 1% (v/v) non-essential amino acids (catalogue number M7145; Sigma, Poole) and 2% (v/v) essential amino acid (catalogue number B6766; Sigma) solutions, together with 0.4% (w/v) bovine serum albumin (catalogue number 81-001; Miles Pentex; Bayer Diagnostics, Basingstoke). In contrast, SOF+ was supplemented with heat-inactivated human serum to form a 20% (v/v) serum culture medium. Both media were at pH 7.4 and 270–280 mOsm.

In the co-culture treatment, up to four day 1 zygotes were introduced to 3-day-old bovine granulosa cell layers, which had been established previously in 50 µl droplets of co-culture medium placed in tissue culture dishes (60 mm diameter; Bibby Sterilin, Stone) overlaid with mineral oil (catalogue number M8410; Sigma). The culture medium was refreshed (50% replacement, by volume) just before the zygotes were introduced and thereafter at 48 h intervals during the culture period, which proceeded for 5 days in a humidified atmosphere (5% CO₂, 95% air) at 38.5°C.

Cell- and serum-free SOF- droplets and cell-free but serum-supplemented SOF+ droplets, each of 20 µl under oil, were prepared (eight droplets per culture dish) on the day of zygote collection. Up to four zygotes were cultured per SOF- or SOF+ drop in a reduced-oxygen humidified atmosphere (5% CO₂, 5% O₂, 90% N₂) at 38.5°C. At 48 h intervals thereafter, zygotes were moved to freshly prepared 20 µl droplets incubated in identical conditions.

Steer serum was used to supplement co-culture media because in a small scale trial it was observed that this culture system resulted in fetal oversize in sheep after transfer of embryos cultured from *in vivo* produced zygotes. This culture

system also produced oversize in large scale cattle field trials involving embryos produced totally *in vitro* from abattoir-derived oocytes. The SOF medium was supplemented with human serum because Walker *et al.* (1992a) consistently observed fetal oversize when *in vivo* produced sheep zygotes were cultured in this medium-serum combination and the resultant embryos were transferred. There was insufficient evidence to show whether the culture system, the type of serum or both the culture system and serum were the causative factors resulting in fetal oversize. Consequently, the two systems with their respective serum supplements were tested in the present study.

On day 6 of culture, morula and blastocyst stage embryos with quality grades 1 to 3 (1 = excellent, 5 = degenerate; Lindner and Wright, 1983) were removed from their respective culture environments, placed into ovum culture medium (catalogue number 1-150-07; Imperial Laboratories, Andover), and promptly transferred to the waiting recipient ewe. Day 6 *in vivo* or *in vitro* produced embryos were transferred singly to recipient ewes. Transfer of an embryo was preceded by induction of general anaesthesia in the recipient. The transfer technique involved temporary exposure of the tip of the uterine horn through a small ventral incision of the abdominal wall by laparoscopically-guided pickup (McMillan and Hall, 1994).

Conceptus recovery

Pregnancy was determined at days 55–60 of gestation by transabdominal ultrasound examination. Pregnant recipient ewes were killed at day 61 and day 125 of gestation by i.v. administration of 25 ml 20% (w/v) pentobarbitone sodium (Euthatal, Rhône Mérieux Ltd, Harlow) and bled from the main vessels of the neck. The gravid uterus of each ewe was then recovered and weighed. The fetus was removed, weighed and its curved crown–rump length measured. Fetal membranes, cotyledons and uterine caruncles were separated and weighed. Viscera, including the liver, heart and kidneys, and individual plantaris and tibialis anterior muscles were excised and weighed, and the humerus length was measured.

Data analysis

Within and between treatment differences in blastocyst production and pregnancy rate were analysed using the chi-squared test. Differences in the mass of the products of conception, and of fetal organs and muscle tissue, and differences in humerus length among treatments at both day 61 and day 125 of gestation were analysed by the ANOVA directive within GENSTAT 5 (Genstat, 1993). The sex ratio of the fetuses, the stages of development and grades of embryos from which the fetuses were derived did not differ significantly among the treatments and, therefore, these factors were not included in the final statistical model. Data are presented as simple means $\pm$ SEM.

During normal growth, the fetus, its organs and tissues increase in size and mass at different rates. Consequently, larger fetuses, which are developing normally but at a faster

rate, will have different proportions of each body part and component to those of smaller fetuses, which are developing normally but at a slower rate. Therefore, if growth is perturbed but the proportions of each organ and tissue are commensurate with those observed in an animal of the same size that has been achieved by normal growth, then development is normal. However, if the proportion of a body component does not show the relationship to body size associated with normal growth, then development is altered.

The relationship between body size (growth) and proportions of organs and tissues (development) can be assessed using the allometric equation of Huxley (1932) $y = ax^b$ (where y = the mass of the organ or tissue, x = the mass of the whole body, b is the organ or tissue growth coefficient, and a is a constant). Where the values of b differ from those observed during normal growth, then the fundamental patterns of development have been altered and the differences in body proportions are not the same as those observed during normal growth. In the present study, the allometric equation was used in its linear form: $\log y = \log a + b \log x$, to assess fetal development at day 61 and day 125 and the values for the allometric growth coefficient (b) of fetuses derived from cultured embryos were compared with those observed during normal growth from the data of Sinclair *et al.* (1998).

Results

Overall blastocyst production on day 6 from *in vivo* produced zygotes (1–4-cell stages) was 28.9%, 13.2% and 28.3% (chi-squared = 29.8; df = 2; $P < 0.001$), and the proportions of grade 1 and 2 embryos produced from zygotes were 13.5%, 6.4% and 10.7% (chi-squared = 9.484; df = 2, $P < 0.01$) from co-culture, SOF– and SOF+, respectively. In contrast, 73.4% of *in vivo* embryos recovered at day 6 were blastocysts and 82.3% of embryos recovered were graded 1 and 2. Morula stage embryos as a percentage of morulae plus blastocysts transferred were: co-culture, 44.7%; SOF–, 27.4%; SOF+, 40.0%; and control, 11.2% (chi-squared = 26.9; df = 3; $P < 0.001$). The proportion of grade 1 and 2 embryos among those transferred was also significantly different (co-culture, 42.0%; SOF–, 27.1%; SOF+, 29.2%; control, 63.0%; chi-squared = 30.5; df = 3; $P < 0.001$) with a significant difference between co-culture and the two SOF treatments (chi-squared = 5.498; df = 1; $P < 0.05$).

The proportion of fetuses produced as a consequence of transfer of a grade 1 or 2 embryo did not differ significantly among treatments (co-culture, 56.3%; SOF–, 50.0%; SOF+, 45.2%; control, 57.4%; chi-squared = 1.399; df = 3; $P > 0.05$). The transfer of morulae resulted in fewer pregnancies (13.7%) compared with the transfer of zona-intact unexpanded and expanded blastocysts (55.5%; chi-squared = 23.9; df = 1; $P < 0.001$; Table 1). The method of embryo production had no significant effect on the percentage of fetuses produced or of embryos transferred.

Pregnancy determination at days 55–60 meant that early pregnancy losses could not be observed and there were no losses from day 61 to day 125. In total, 146 fetuses were recovered, with 97 fetuses recovered at day 61 and 49 fetuses

Table 1. Stage at transfer and pregnancy outcome at day 61 and day 125 of gestation for Suffolk × Scottish Blackface sheep embryos

	Co-culture		SOF-		SOF+		Control		Total	
	Transferred	Pregnant	Transferred	Pregnant	Transferred	Pregnant	Transferred	Pregnant	Transferred	Pregnant
Embryo stage:										
Morula	34	7/34 (20.6%)	26	1/26 (3.8%)	32	1/32 (3.1%)	10	5/10 (50.0%)	102	14/102 (13.7%)
Blastocyst	27	19/27 (70.4%)	42	21/42 (50.0%)	22	14/22 (63.6%)	21	13/21 (61.9%)	112	67/112 (59.8%)
Expanded blastocyst	15	10/15 (66.7%)	27	10/27 (37.0%)	26	16/26 (61.5%)	58	29/58 (50.0%)	126	65/126 (51.6%)
Total	76	36/76 (47.4%)	95	32/95 (33.7%)	80	31/80 (38.8%)	89	47/89 (52.8%)	340	146/340 (42.9%)

Co-culture: bovine granulosa cell layers with added serum; control: *in vivo* derived from superovulated donor ewes; SOF-: synthetic oviductal fluid medium without serum; SOF+: synthetic oviductal fluid medium supplemented with human serum.

Table 2. Products of conception (mean ± SEM) at day 61 of gestation for Suffolk × Scottish Blackface sheep fetuses derived from day 6 *in vivo* produced embryos (controls) or day 6 *in vitro* produced embryos from *in vivo* produced zygotes

	Co-culture	SOF-	SOF+	Control
Number	20	27	25	25
Gravid uterus (g)	1354 ± 67	1390 ± 81	1351 ± 75	1281 ± 70
Fluids (g)	530 ± 66	538 ± 64	555 ± 56	481 ± 46
Chorioallantois (g)*	419 ± 26	455 ± 23	413 ± 20	417 ± 24
Uterus + caruncles (g)	320 ± 15	319 ± 12	307 ± 10	308 ± 11
Fetal weight (g)	85.1 ± 2.7 ^b	77.4 ± 2.0 ^a	75.8 ± 1.9 ^a	74.2 ± 1.7 ^a
Fetal CRL (mm)	162.4 ± 2.2	158.3 ± 1.1	158.3 ± 1.3	157.2 ± 1.5

Means with different superscripts within rows are significantly different ($P < 0.01$).

*Includes amnion.

For description of treatments, see Table 1.

SOF-: synthetic oviductal fluid medium without serum; SOF+: synthetic oviductal fluid medium supplemented with human serum; CRL: curved crown-rump length.

recovered at day 125 of gestation. The overall fetal sex ratio was exactly 1:1, and there was no significant difference in this ratio among the experimental treatments. Male fetuses were approximately 5% heavier than female fetuses but this difference was not significant at either stage of gestation.

By day 61 of gestation, fetuses derived from co-culture embryos were, on average, 10.9 g or 14.7% heavier ($P < 0.01$) than fetuses derived from control embryos (Table 2), but those derived from embryos produced by the other two culture treatments were not significantly different from controls at this time point. Culture treatment had no significant effect on chorioallantois mass at day 61 of gestation. There was a weak but significant ($r = 0.46$; $P < 0.001$) overall relationship between cotyledon mass and fetal mass at this time point.

Organ (liver, heart and kidneys) and muscle (plantaris and tibialis anterior) masses were significantly greater ($P < 0.01$) in fetuses derived from the co-culture treatment than in fetuses derived from the control treatment at day 61 of gestation (Table 3). Kidneys were also significantly heavier in fetuses derived from the SOF+ treatment than in controls (1.20 g versus 0.98 g; $P < 0.05$), but the tibialis anterior muscle was significantly lighter in SOF+ fetuses than in

fetuses derived from the control treatment (36.6 mg versus 44.7 mg; $P < 0.05$).

Allometric coefficients, relating component mass to fetal mass, were significantly greater ($P < 0.05$) for liver, heart, kidneys and plantaris muscle in fetuses derived from the co-culture treatment than for those components derived from control treatment; and were greater for liver in fetuses derived from SOF+ than for liver derived from the control treatment (Table 4; Fig. 1).

At day 61 of gestation, the incidence of oversize fetuses (mass > 3 SD heavier than control mean) was: co-culture, 20.0% (4/20); SOF-, 0%; and SOF+, 0%. The masses of these oversized co-culture fetuses were significantly greater (105.0 ± 3.8 g, $n = 4$, $P < 0.001$) than the control mean (74.2 ± 1.7 g). The magnitude of the shift in organ development for these fetuses is illustrated (Table 5) and the mean organ masses predicted from the allometric equations of Sinclair *et al.* (1998) (normal allometric growth) are compared with those predicted from the co-culture equations using, in both cases, the observed co-culture fetal masses, which were > 3 SD above the mean of control fetuses. This analysis allowed treatment effects on organ mass to be compared in the absence of the confounding effects of fetal mass, and

Table 3. Organ and muscle masses, and humerus length (means $\pm$ SEM) for Suffolk $\times$ Scottish Blackface sheep fetuses recovered at day 61 of gestation

	Co-culture	SOF-	SOF+	Control
Number	20	27	25	25
Liver (g)	6.91 $\pm$ 0.37 ^b	5.96 $\pm$ 0.23 ^a	6.19 $\pm$ 0.26 ^{ab}	5.50 $\pm$ 0.16 ^a
Heart (g)	0.831 $\pm$ 0.057 ^b	0.628 $\pm$ 0.027 ^a	0.667 $\pm$ 0.033 ^a	0.605 $\pm$ 0.024 ^a
Kidneys (g)	1.25 $\pm$ 0.08 ^b	1.10 $\pm$ 0.04 ^{ab}	1.20 $\pm$ 0.08 ^b	0.98 $\pm$ 0.03 ^a
Humerus (mm)	16.8 $\pm$ 0.7 ^a	15.4 $\pm$ 0.3 ^{ab}	14.9 $\pm$ 0.3 ^b	15.9 $\pm$ 0.4 ^{ab}
Plantaris (mg)	36.2 $\pm$ 2.4 ^b	29.4 $\pm$ 1.6 ^a	28.1 $\pm$ 1.6 ^a	30.8 $\pm$ 1.3 ^a
Tibialis anterior (mg)	55.3 $\pm$ 2.7 ^b	39.2 $\pm$ 2.6 ^{ac}	36.6 $\pm$ 2.2 ^c	44.7 $\pm$ 2.1 ^a

Means with different superscripts within rows are significantly different ($P < 0.05$).

For description of treatments, see Table 1.

SOF-: synthetic oviductal fluid medium without serum; SOF+: synthetic oviductal fluid medium supplemented with human serum.

Table 4. Allometric coefficients ($b \pm$ SEM) relating organ or muscle tissue mass, or humerus length to fetal mass at day 61 of gestation (from the equation $\log_e y = \log_e a + b \log_e x$, where y = organ mass and x = fetal mass) for Suffolk $\times$ Scottish Blackface sheep fetuses

	Co-culture	SOF-	SOF+	Control
Number	20	27	25	25
Liver (g)	1.379 $\pm$ 0.199 ^{bc}	0.850 $\pm$ 0.204 ^{ab}	1.442 $\pm$ 0.183 ^c	0.773 $\pm$ 0.183 ^a
Heart (g)	1.700 $\pm$ 0.246 ^b	0.982 $\pm$ 0.226 ^a	1.537 $\pm$ 0.235 ^{ab}	1.042 $\pm$ 0.229 ^a
Kidneys (g)	1.547 $\pm$ 0.195 ^b	0.807 $\pm$ 0.214 ^a	1.306 $\pm$ 0.347 ^{ab}	0.984 $\pm$ 0.199 ^a
Humerus (mm)	0.757 $\pm$ 0.261	0.230 $\pm$ 0.142	0.309 $\pm$ 0.137	0.537 $\pm$ 0.209
Plantaris (mg)	1.832 $\pm$ 0.307 ^b	1.119 $\pm$ 0.331 ^a	1.497 $\pm$ 0.378 ^{ab}	0.921 $\pm$ 0.349 ^a
Tibialis anterior (mg)	1.297 $\pm$ 0.314	0.882 $\pm$ 0.506	1.884 $\pm$ 0.519	1.106 $\pm$ 0.367

Means with different superscripts within rows are significantly different ($P < 0.05$).

For description of treatments, see Table 1.

SOF-: synthetic oviductal fluid medium without serum; SOF+: synthetic oviductal fluid medium supplemented with human serum.

indicated that organ masses of oversized fetuses were greater than predicted and that organ development had been fundamentally altered.

At day 125 of gestation, both the gravid uterus and chorioallantois were significantly heavier for the co-culture and SOF+ treatments than they were for the control and SOF- treatments ($P < 0.05$; Table 6). Mean conceptus fluid mass was significantly greater ($P < 0.01$) for the co-culture treatment than for the other treatments. The mass of fluid for three conceptuses (3/13 or 23%) from this treatment was > 3 SD above the mean of the control treatment. The mass of fluid for five conceptuses (5/13 or 38%) from the same treatment was greater than that for the heaviest conceptus from the control treatment. Fetuses derived from the co-culture treatment were, on average, 1121 g or 34% heavier ($P < 0.001$), and 47 mm or 9% longer ($P < 0.001$) than fetuses from the control treatment. Fetuses derived from the SOF+ treatment were 585 g heavier (18%; $P < 0.05$), and were associated with a larger chorioallantois ($P < 0.05$) and total gravid uterus mass ($P < 0.05$) than fetuses derived from the control treatment. There was a weak but significant ($r = 0.55$; $P < 0.01$) correlation between cotyledon mass and the mass of fetuses derived from embryos cultured in each of the three *in vitro* systems.

All organ and muscle masses, and humerus lengths at day 125 of gestation were greater ($P < 0.001$) for fetuses derived from the co-culture treatment than those from the control treatment (Table 7). Both liver and kidney masses were

greater ($P < 0.05$) for co-culture and SOF+ fetuses than controls. The number of fetuses recovered from the three culture treatments at this time point were too few to establish meaningful allometric relationships within culture treatment. By combining data from the co-culture and SOF+ treatments, however, allometric coefficients (mean $\pm$ SEM) of 1.203 ± 0.213 and 1.461 ± 0.280 were obtained for fetal liver and heart at this stage of gestation. These coefficients, together with those obtained at day 61 of gestation were significantly greater ($P < 0.05$) than those of 0.850 and 0.921 established for liver and heart respectively in normally developing fetuses between day 60 and day 135 of gestation in the study of Sinclair *et al.* (1998).

The incidence of oversized fetuses at day 125 of gestation, defined as those heavier than the control mean plus 3 SD, was: co-culture, 69.2% (9/13); SOF-, 0%; and SOF+, 16.7% (1/6). The mean mass of the oversized fetuses on the basis of this criterion was significantly greater (4623 ± 193 g, $n = 10$, $P < 0.001$) than the control mean (3282 ± 65 g). The magnitude of the shift in the development of key organs was predicted from the observed fetal masses of co-culture and SOF+ treatments using the combined allometric equation for those treatments and comparing the data with those predicted from the allometric equations of Sinclair *et al.* (1998) for fetuses derived from *in vivo* produced embryos (Table 8). This analysis allowed treatment effects on organ size to be compared in the absence of confounding effects of

Fig. 1. Differential growth of the sheep fetal liver (a) and heart (b) at day 61 of gestation after *in vitro* embryo culture. Allometric plots for fetuses derived from embryos co-cultured in the presence of bovine granulosa cells (co-culture; ●); and cultured in a synthetic oviductal fluid medium with added serum (SOF+; ○). Control fetuses are represented by the solid lines. Points A and B represent the minimum and maximum masses, respectively, of control fetuses at this stage of gestation.

fetal mass and, as for day 61, organ masses of oversized fetuses were greater than those expected had these fetuses been developing normally.

Discussion

The results from this study clearly indicate that the system of *in vitro* embryo culture can significantly influence both fetal growth and development. *In vivo* derived sheep zygotes cultured in the presence of bovine granulosa cells or in a synthetic oviductal fluid medium with added serum gave rise to a number of fetuses that were significantly larger than those derived from *in vivo* produced embryos or zygotes cultured in a synthetic oviductal fluid medium with no added serum. Moreover, these differences in size were proportionally greater at day 125 of gestation than at day 61, indicating a divergent growth pattern arising from treatment differences in specific growth during early fetal life. Larger

fetuses exhibited altered development and, on occasion, were associated with excessive volumes of amniotic fluid (polyhydramnios). Therefore, these findings are consistent with those of others who showed that *in vitro* embryo culture significantly affects fetal growth and birth mass in both cattle (for example, Behboodi *et al.*, 1995; Farin and Farin, 1995; Hasler *et al.*, 1995) and sheep (for example, Thompson *et al.*, 1995; Holm *et al.*, 1996; Brown and Radziewicz, 1998), but add to these observations by showing that the fundamental patterns of organ and tissue development have also been altered.

The present study has provided a formal mathematical description of alterations in organ and tissue development for fetuses derived from *in vitro* produced embryos. Furthermore, this study is the first demonstration, to our knowledge, of any *in vitro* embryo culture treatment creating an increase in the allometric relationships of key organs and tissues to the whole animal. Organs and tissues grow differentially during normal development. Thus, simple ratios of organ:fetal mass provide little indication of alterations in fetal development because such ratios change during the normal growth process. Consequently, the calculation of these ratios or simple adjustments for body weight, as in previous studies (for example, Farin *et al.*, 1997; Hartwich *et al.*, 1997), are inappropriate indicators of changes in fetal development. In contrast, allometry implicitly recognizes that the ratio of the component to the whole inevitably changes as the fetus grows and, therefore, provides a means of assessing whether the ratio is equal to, or different from, the expected ratio at the observed mass. The data presented here clearly demonstrate that this ratio was profoundly altered in co-culture compared with control fetuses at day 61 of gestation for the key organs, liver and heart, and that these differences were still present and significant at day 125 of gestation. Moreover, these effects were more pronounced for those fetuses classified as oversized than for fetuses of average mass.

It is generally accepted that shifts in the development of fetal organs and tissues can only be observed in the most extreme circumstances (Everitt, 1968); and even then, apart from the liver which is highly responsive to transitory shifts in nutritional status, other organs and structural components of body show only minor shifts in their allometric growth coefficients (Robinson, 1981).

Allometric coefficients established for each of the visceral organs and muscle tissues recovered from fetuses derived from co-cultured embryos indicate that their development was altered. Large fetuses from this treatment group had disproportionately large visceral organs and muscles. Histological examination of the fetal plantaris muscle at days 61 and 125 of gestation in the present study (Maxfield *et al.*, 1998) showed that the increased muscle development arising from co-culturing embryos with granulosa cells was due to an increase in primary muscle fibre hypertrophy and an increase in secondary muscle fibre hyperplasia and hypertrophy, resulting in increases in the secondary:primary muscle fibre ratio and muscle mass.

A similar picture is apparent for fetuses derived from embryos that were cultured in synthetic oviductal fluid with added serum. Although the effect of this treatment was

Table 5. Expected versus actual organ masses for Suffolk × Scottish Blackface sheep fetuses derived from co-cultured embryos at day 61 of gestation

	<i>n</i>	Organ mass (g)		SED	Significance ¹
		Expected ²	Actual ³		
Co-culture fetal masses > control mean ⁴ + 3 SD:					
Liver	4	8.62	10.16	0.28	<i>P</i> < 0.01
Heart	4	1.030	1.167	0.039	<i>P</i> < 0.05

The expected values are based on the allometric relationships of Sinclair *et al.* (1998) (normal allometric growth) and the actual values are based on allometric relationships obtained from fetuses derived from co-culture embryos in the present study.

¹Paired *t* test; ²control (Sinclair *et al.*, 1998) allometric equations (y = organ mass; x = fetal mass) were liver, $\log_e y = -2.17 + 0.857 \log_e x$; heart, $\log_e y = -4.30 + 0.922 \log_e x$; ³co-culture allometric equations (y = organ mass; x = fetal mass) were liver, $\log_e y = -4.10 + 1.379 \log_e x$; heart, $y = -7.76 + 1.70 \log_e x$; ⁴control: masses of fetuses derived from *in vivo* produced embryos in present experiment.

Table 6. Products of conception (mean ± SEM) at day 125 of gestation for Suffolk × Scottish Blackface sheep fetuses derived from day 6 *in vivo* produced embryos (control) or day 6 *in vitro* produced embryos from *in vivo* produced zygotes

	Co-culture	SOF–	SOF+	Control
Number	13	8	6	22
Gravid uterus (g)	8230 ± 426 ^c	6274 ± 179 ^a	7205 ± 372 ^b	6056 ± 132 ^a
Fluids (g)	2268 ± 321 ^b	1369 ± 114 ^a	1266 ± 101 ^a	1454 ± 88 ^a
Chorioallantois* (g)	905 ± 54 ^b	720 ± 78 ^a	932 ± 88 ^b	637 ± 29 ^a
Uterus + caruncles (g)	717 ± 22	736 ± 41	728 ± 50	678 ± 17
Fetal mass (g)	4403 ± 180 ^c	3454 ± 127 ^{ab}	3867 ± 164 ^b	3282 ± 65 ^a
Fetal CRL (mm)	570 ± 12 ^b	527 ± 9 ^a	539 ± 9 ^a	523 ± 6 ^a

Means with different superscripts within rows are significantly different (*P* < 0.05).

*Includes amnion.

For description of treatments, see Table 1.

SOF–: synthetic oviductal fluid medium without serum; SOF+: synthetic oviductal fluid medium supplemented with human serum; CRL: curved crown–rump length.

Table 7. Organ and muscle masses, and humerus length (means ± SEM) for Suffolk × Scottish Blackface sheep fetuses recovered at day 125 of gestation

	Co-culture	SOF–	SOF+	Control
Number	13	8	6	22
Liver (g)	139.6 ± 7.4 ^b	115.9 ± 8.7 ^{ac}	135.5 ± 10.6 ^{bc}	107.5 ± 4.5 ^a
Heart (g)	35.6 ± 2.4 ^b	20.7 ± 1.8 ^a	25.8 ± 1.9 ^a	24.6 ± 1.3 ^a
Kidneys (g)	34.8 ± 2.9 ^b	22.7 ± 3.1 ^{ac}	29.8 ± 3.2 ^{bc}	20.0 ± 0.7 ^a
Humerus (mm)*	80.8 ± 1.2 ^b	–	–	72.8 ± 1.1 ^a
Plantaris (g)	3.67 ± 0.35 ^b	2.64 ± 0.11 ^a	2.71 ± 0.13 ^a	2.42 ± 0.09 ^a
Tibialis anterior (g)	5.35 ± 0.37 ^b	3.97 ± 0.27 ^a	4.11 ± 0.11 ^a	3.88 ± 0.13 ^a

Means with different superscripts within rows are significantly different (*P* < 0.05).

*Samples restricted to control and co-culture treatments.

For description of treatments, see Table 1.

SOF–: synthetic oviductal fluid medium without serum; SOF+: synthetic oviductal fluid medium supplemented with human serum.

statistically significant only for fetal liver at day 61 of gestation, significant differences existed for both liver and heart at day 125 of gestation when allometric coefficients for these organs were compared to those established by Sinclair *et al.* (1998). The allometric lines at day 61 for fetuses derived from co-cultured embryos and embryos that were cultured in synthetic oviductal fluid with added serum were virtually identical, but gradient coefficients were significantly greater than those for fetuses developing normally. This means that,

in the absence of significant differences in fetal size at day 61 of gestation, significant differences in fetal development were detectable between control fetuses and those derived from embryos cultured in synthetic oviductal fluid with added serum. Given the central role of the viscera in supporting the metabolic requirements of growth, it is possible that deviations in the development of key organs during early fetal life may have a bearing on growth during later fetal life.

Table 8. Expected versus actual organ masses for Suffolk × Scottish Blackface sheep fetuses derived from *in vitro* produced embryos (co-culture and synthetic oviductal fluid medium plus serum (SOF+)) at day 125 of gestation

	<i>n</i>	Organ mass (g)		SED	Significance ¹
		Expected ²	Actual ³		
Co-culture fetal masses > control mean ⁴ + 3 SD:					
Liver	10	139.4	151.2	3.51	<i>P</i> < 0.01
Heart	10	32.2	36.1	1.07	<i>P</i> < 0.01

The expected values are based on the allometric relationships of Sinclair *et al.* (1998) (normal allometric growth) and the actual values are based on allometric relationships obtained from fetuses derived from co-culture and SOF+ embryos in the current study.

¹Paired *t* test; ²control (Sinclair *et al.*, 1998) allometric equations (*y* = organ mass; *x* = fetal mass) were liver, $\log_e y = -1.27 + 0.736 \log_e x$; heart, $\log_e y = -4.20 + 0.909 \log_e x$; ³*in vitro* allometric equations (*y* = organ mass; *x* = fetal mass) were liver, $\log_e y = -5.11 + 1.20 \log_e x$; heart, $\log_e y = -8.74 + 1.46 \log_e x$; ⁴control: masses of fetuses derived from *in vivo* produced embryos in present experiment.

There have been previous reports of gross abnormalities, albeit at a relatively low frequency, of several key organs and tissues following *in vitro* embryo culture, including head and limb deformities (Walker *et al.*, 1992b) and cerebellar dysplasia (Schmidt *et al.*, 1996). Although only 2/35 calves born as a consequence of *in vitro* embryo culture and transfer showed any signs of gross abnormalities in the study of Van Soom *et al.* (1994), it is perhaps noteworthy that these abnormalities included a ventricular septum defect and a cirrhotic liver.

In contrast to the systems discussed above, allometric coefficients established for the same organs and tissues in control fetuses and those derived from embryos cultured in synthetic oviductal fluid with no added serum are in close agreement with those established for normally growing and developing fetuses of the same genotype in the study of Sinclair *et al.* (1998), and with those established in the early observations on fetal growth made by Wallace (1948). This comparison implies that development was normal for the SOF- fetuses in the present study, thus supporting earlier observations that lamb birth weight and the incidence of developmental abnormalities are not increased when zygotes are cultured in synthetic oviductal fluid supplemented with bovine serum albumin and amino acids only (Walker *et al.*, 1992a; Thompson *et al.*, 1995). While such semi-defined culture systems may avoid the problem of aberrant fetal growth and development, the lower blastocyst yields and pregnancy rates reported by these authors and observed in the present study need to be improved for commercial application. It is necessary to ensure that such improvements are not accompanied by perturbations of growth and development in some of the fetuses produced. However, the incidence and severity of fetal oversize observed after the transfer of embryos produced in co-culture or SOF+ systems were of such magnitude as would make their use in practice inadvisable on ethical and welfare grounds.

A further observation from the present study was the high incidence of polyhydramnios ($\geq 23\%$) in conceptuses derived from co-cultured embryos. The frequency with which this condition appears naturally in sheep is unknown but, in cattle, Hasler *et al.* (1995) reported a natural incidence of 1 per 7500 calvings, and an increased incidence to 1 per 200 calvings in calves derived from *in vitro* produced embryos. In humans, the natural incidence of polyhydramnios is higher

at around 1% and is normally associated with pregnancies complicated by maternal diabetes mellitus or congenital anomalies involving the gastrointestinal tract, central nervous and cardiovascular systems, and the urinary tract (Moise, 1997). The mechanisms that produce such fluid imbalances are not known, but the condition is associated with fetal hypoxia and increased fetal lactate which, in sheep, has been shown to act as a powerful osmotic agent for the accumulation of fluid from the maternal compartment by the conceptus (Powell and Brace, 1991). Since placental size in sheep reaches a plateau towards the end of the second third of pregnancy (Robinson *et al.*, 1977) and, as the present data show, large fetuses from embryo culture do not necessarily have large placentae, it is postulated that precocious fetal growth during the final trimester of pregnancy may induce fetal hypoxia and an associated increase in fetal plasma lactate concentrations, which are predisposing conditions for the development of polyhydramnios.

The mechanisms which cause these marked changes in growth and development in the fetal oversize syndrome are the subject of much speculation and have been reviewed recently (Walker *et al.*, 1996; Farin and Farin, 1997; Young *et al.*, 1998). Candidate perturbing agents and/or facilitating factors include ammonia (Lane and Gardner, 1994; McEvoy *et al.*, 1997), progesterone (Kleemann *et al.*, 1994; Carolan *et al.*, 1997) and growth factors (Watson *et al.*, 1992, 1994) which may be provided by serum or granulosa cells. Ammonia, or ammonium, and growth factors are potential causative agents in the present study although some role for progesterone cannot be overlooked because of its presence, and promotion by serum growth factors, in the co-culture system (Kuran *et al.*, 1996). It is believed that the causative agent(s) alter(s) the expression of a gene or genes associated with growth and development. Imprinted genes, including H19, Igf2, Igf2r and Insulin-2, have been identified as candidates for this role due to their involvement in early embryo development (Leighton *et al.*, 1996) and because similar phenotypes to those observed in the present study exist in both humans and mice when expression of such genes has been altered (Young *et al.*, 1998). Their role, however, remains to be confirmed since patterns of imprinting have not been established in ruminant embryos.

In the present experiment, predicted mean birth weights (from the data of Sinclair *et al.*, 1998) were approximately 6

kg for control and 8 kg for co-culture and SOF+ treatments, with the heaviest lamb from these treatments predicted to be about 9 kg at birth. However, these predictions for lambs derived from cultured embryos are underestimates because specific growth rates appropriate to controls were used to calculate all the birth weights whereas fetuses on the culture treatments had higher specific growth rates that could not be quantified. All fetuses heavier than the control mean plus 3 SD would probably have required a Caesarean delivery and this proportion (52.6%, 10/19) is similar to that observed by Goodhand *et al.* (1997) for the delivery of calves (46.0%, 12/26) derived from *in vitro* embryos produced using the co-culture system described here.

The long-term effects of perturbed fetal growth and development have not been reported but, in the medium term, at about 1 year of age, both lambs and calves that are oversize at birth have similar weights to those with normal birth weights (Wilson *et al.*, 1995; Walker *et al.*, 1996; McEvoy *et al.*, 1998). However, McEvoy *et al.* (1998) showed that oversized calves produced from *in vitro* co-cultured embryos had abnormally large hearts compared with their control counterparts when killed at the same weight at about 13 months of age. In view of the extent and degree of the prenatal and perinatal problems associated with the fetal oversize syndrome, it is probable that there are some consequences for the long term health, well-being, welfare, productivity and life span of the animal that would be of profound significance if they occurred in human subjects (Barker and Clark, 1997) born as a consequence of assisted reproductive technologies.

In conclusion, this work extends the observation that the presence of serum (SOF+) or somatic cells (co-culture), but not their absence (SOF-), during the *in vitro* culture of embryos influences fetal growth. It demonstrates that oversized fetuses recovered during late gestation arise as a consequence of a divergent growth pattern, due to treatment differences in specific growth rate during early fetal life. Furthermore, this study provides the first formal evidence of altered fetal development in fetuses arising from embryo culture as demonstrated by deviations in allometric relationships between organ and fetal masses. The sporadic occurrence of this problem has made it difficult to study (Farin and Farin, 1997; Young *et al.*, 1998). However, the co-culture and SOF+ systems in the present study provide robust, repeatable and relatively efficient models for the study of the fetal oversize syndrome.

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References

- Barker DJP and Clark PM (1997) Fetal undernutrition and disease in later life *Reviews of Reproduction* 2 105–112
- Behboodi E, Anderson GB, BonDurant RH, Cargill SL, Kreuscher BR, Medrano JF and Murray JD (1995) Birth of large calves that developed from *in vitro* derived bovine embryos *Theriogenology* 44 227–232
- Brash LD, Wray NR and Goddard ME (1996) Use of MOET in Merino breeding programmes: a practical and economic appraisal *Animal Science* 62 241–254
- Brown BW and Radziewicz T (1998) Production of sheep embryos *in vitro* and development of progeny following single and twin embryo transfers *Theriogenology* 49 1525–1536
- Carolan C, McEvoy TG, Gebbie FE, Sinclair KD, Robinson JJ and Broadbent PJ (1997) Steer sera from different nutritional backgrounds alter ammonia and progesterone concentrations in a bovine granulosa cell culture system *Journal of Reproduction and Fertility Abstract Series* 19 126
- Everitt GC (1968) Prenatal development of uniparous animals with particular reference to the influence of maternal nutrition in sheep. In *Growth and Development of Mammals* pp 131–157 Eds GA Lodge and GE Lamming. Butterworths, London
- Farin PW and Farin CE (1995) Transfer of bovine embryos produced *in vivo* or *in vitro*: survival and fetal development *Biology of Reproduction* 52 676–682
- Farin PW and Farin CE (1997) Perspectives on large calves following transfer of embryos produced *in vitro*. *IETS Embryo Transfer Newsletter* 15 15–19
- Farin PW, Farin CE and Mungal SA (1997) Measurements of bovine fetuses and placentas at 63 days after transfer of embryos produced *in vivo* or *in vitro*. *Theriogenology* 47 319 (Abstract)
- Garry FB, Adams R, McCann JP and Odde KG (1996) Postnatal characteristics of calves produced by nuclear transfer cloning *Theriogenology* 45 141–152
- Genstat 5 Committee (1993) *Genstat 5 Release 3 Reference Manual* Oxford Science Publications, Clarendon Press, Oxford
- Goodhand KL, Dolman DF, Watt RG, Higgins LC, Staines ME and Broadbent PJ (1997) Calving outcome following transfer of *in vivo* and *in vitro* produced bovine embryos *Proceedings of the British Society of Animal Science* 34
- Hartwich KM, Kleeman DO, Seamark RF and Walker SK (1997) Fetal development in the sheep following manipulation of the embryo or its environment *Theriogenology* 47 322 (Abstract)
- Hasler JE, Henderson WB, Hurtgen PJ, Jin ZQ, McCauley AD, Mower SA, Neely B, Shuey S, Stokes JE and Trimmer SA (1995) Production, freezing and transfer of bovine IVF embryos and subsequent calving results *Theriogenology* 43 141–152
- Holm P, Walker SK and Seamark RF (1996) Embryo viability, duration of gestation and birth weight in sheep after transfer of *in vitro* matured and *in vitro* fertilized zygotes cultured *in vitro* or *in vivo*. *Journal of Reproduction and Fertility* 107 175–181
- Huxley JS (1932) *Problems of Relative Growth* Methuen, London
- Kleemann DO, Walker SK and Seamark RF (1994) Enhanced fetal growth in sheep administered progesterone during the first three days of pregnancy *Journal of Reproduction and Fertility* 102 411–417
- Kruip ThAM and den Daas JHG (1997) *In vitro* produced and cloned embryos: effects on pregnancy, parturition and offspring *Theriogenology* 47 43–52
- Kuran M, Hutchinson JSM and Broadbent PJ (1996) The response of bovine granulosa cells to different gonadotrophins in culture *Animal Reproduction Science* 45 1–12
- Lane M and Gardner DK (1994) Amino acids increase post-implantation development of cultured mouse embryos while ammonium induces exencephaly and fetal retardation *Journal of Reproduction and Fertility* 102 305–312
- Leighton PA, Saam JR, Ingram RS and Tilghman SM (1996) Genomic imprinting in mice: its function and mechanism *Biology of Reproduction* 54 273–278
- Lindner GM and Wright RW, Jr (1983) Bovine embryo morphology and evaluation *Theriogenology* 20 407–416
- McEvoy TG, Robinson JJ, Aitken RP, Findlay PA and Robertson IS (1997) Dietary excesses of urea influence the viability and metabolism of preimplantation sheep embryos and may affect fetal growth among survivors *Animal Reproduction Science* 47 71–90
- McEvoy TG, Sinclair KD, Goodhand KL, Broadbent PJ and Robinson JJ (1998) Post-natal development of Simmental calves derived from *in vivo* or *in vitro* embryos *Gametes: Development and Function* p 573 Eds A Lauria, F Gandolfi, G Enne and L Gianaroli. Tabloid S.r.l, Rome
- McMillan WH and Hall DRH (1994) Laparoscopic transfer of ovine and cervine embryos using the transpic technique *Theriogenology* 42 137–146
- Maxfield EK, Sinclair KD, Broadbent PJ, McEvoy TG, Robinson JJ and Maltin CA (1998) Short term culture of ovine embryos modifies fetal

- myogenesis *American Journal of Physiology* **274** (Endocrinology and Metabolism 37) E1121–E1123
- Moise KJ, Jr (1997) Polyhydramnios *Clinical Obstetrics and Gynecology* **40** 266–279
- Powell TL and Brace RA (1991) Elevated fetal plasma lactate produces polyhydramnios in the sheep *American Journal of Obstetrics and Gynecology* **165** 1595–1607
- Robinson JJ (1981) Prenatal growth and development in the sheep and its implications for the viability of the newborn lamb *Livestock Production Science* **8** 273–281
- Robinson JJ, McDonald I, Fraser C and Crofts RMJ (1977) Studies on reproduction in prolific ewes I. Growth of the products of conception *Journal of Agricultural Science Cambridge* **88** 539–552
- Schmidt M, Greve T, Avery B, Beckers JE, Sulon J and Hansen HB (1996) Pregnancies, calves and calf viability after transfer of *in vitro* produced bovine embryos *Theriogenology* **46** 527–539
- Sinclair KD and Broadbent PJ (1996) Increasing the efficiency of suckled calf production using embryo transfer technology *Veterinary Record* **139** 409–412
- Sinclair KD, Dunne LD, Maxfield EK, Maltin CA, Young LE, Wilmot I, Robinson JJ and Broadbent PJ (1998) Fetal growth and development following temporary exposure of day 3 ovine embryos to an advanced uterine environment *Reproduction, Fertility and Development* **10** 263–269
- Tervit HR, Whittingham DG and Rowson LEA (1972) Successful culture *in vitro* of sheep and cattle ova *Journal of Reproduction and Fertility* **30** 493–497
- Thompson JG, Gardner DK, Pugh A, McMillan WH and Tervit HR (1995) Lamb birth weight is affected by culture system utilized during *in vitro* pre-elongation development of ovine embryos *Biology of Reproduction* **53** 1385–1391
- Van Soom A, Mijten P, Van Vlaenderen I, Van den Branden J, Mahmoudzadeh AR and de Kruif A (1994) Birth of double-muscled Belgian Blue calves after transfer of *in vitro* produced embryos into dairy cattle *Theriogenology* **41** 855–867
- van Wagtenonck-de Leeuw AM, Aerts BJG and den Daas JHG (1998) Abnormal offspring following *in vitro* production of bovine pre-implantation embryos: a field study *Theriogenology* **49** 883–894
- Walker SK, Heard TM and Seamark RF (1992a) *In vitro* culture of sheep embryos without co-culture: success and perspectives *Theriogenology* **37** 111–126
- Walker SK, Heard TM, Bee CA, Frensham AB, Warnes DM and Seamark RF (1992b) Culture of embryos of farm animals. In *Embryonic Development and Manipulation in Animal Production* pp 77–92 Eds A Lauria and F Gandolfi. Portland Press Ltd, London
- Walker SK, Hartwich KM and Seamark RF (1996) The production of unusually large offspring following embryo manipulation: concepts and challenges *Theriogenology* **45** 111–120
- Wallace LR (1948) The growth of lambs before and after birth in relation to the level of nutrition *Journal of Agricultural Science Cambridge* **38** 92–153; 367–401
- Watson AJ, Hogan A, Hahnel A, Weimer KE and Scultz GA (1992) Expression of growth factor ligand and receptor genes in the preimplantation bovine embryo *Molecular Reproduction and Development* **31** 87–95
- Watson AJ, Watson PH, Arcellana-Panlilo M, Warnes D, Walker S, Schultz GA, Armstrong DT and Seamark RF (1994) A growth factor phenotype map for ovine preimplantation development *Biology of Reproduction* **50** 725–733
- Wilmot I and Whitelaw CBA (1994) Strategies for production of pharmaceutical proteins in milk *Reproduction, Fertility and Development* **6** 625–630
- Wilson JM, Williams JD, Bondioli KR, Looney CR, Westhusin ME and McCalla DF (1995) Comparison of birth weight and growth characteristics of bovine calves produced by nuclear transfer (cloning), embryo transfer and natural mating *Animal Reproduction Science* **38** 73–83
- Willadsen SM, Janzen RE, McAlister RJ, Shea BF, Hamilton G and McDermand D (1991) The viability of late morulae and blastocysts produced by nuclear transplantation in cattle *Theriogenology* **35** 161–170
- Woolliams JA and Wilmot I (1989) Embryo manipulation in cattle breeding and production *Animal Production* **49** 3–30
- Young LE, Sinclair KD and Wilmot I (1998) Large offspring syndrome in cattle and sheep *Reviews of Reproduction* **3** 155–163