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Sexually dimorphic impact of preconceptional and gestational exposure to a real-life environmental chemical mixture (biosolids) on offspring growth dynamics and puberty in sheep

Neil P. Evans (0000-0001-7395-3222)a, Michelle Bellingham (0000-0002-3646-8989) a, Christopher S. Elcombe (0000-0002-7869-0123)a, Mohammad Ghasemzadeh-Hasankolaei (0000-0003-2053-5566) a, Richard G. Lea (0000-0002-6793-3601)b, Kevin D. Sinclair (0000-0002-6375-215X)b, Vasantha Padmanabhan (0000-0002-8443-7212)c

a School of Biodiversity One Health and Veterinary Medicine, College of Medical, Veterinary and Life Sciences, University of Glasgow, UK
b University of Nottingham, Sutton Bonington Campus, Loughborough, UK
c Department of Pediatrics, University of Michigan, Ann Arbor, MI, USA

email addresses:
Neil P. Evans – Neil.evans@glasgow.ac.uk
Michelle Bellingham - Michelle.Bellingham@glasgow.ac.uk
Christopher S. Elcombe - CElcombe001@dundee.ac.uk
Mohammad Ghasemzadeh-Hasankolaei - Mohammad.GhasemzadehHasankolaei@glasgow.ac.uk
Richard Lea - richard.lea@nottingham.ac.uk
Kevin Sinclair - kevin.sinclair@nottingham.ac.uk
Vasantha Padmanabhan - vasantha@umich.edu

Corresponding author: Neil P. Evans

1 Present address - Drug Discovery Unit, University of Dundee, UK
Abstract

Humans are ubiquitously exposed to complex mixtures of environmental chemicals (ECs). This study characterised changes in post-natal and peripubertal growth, and the activation of the reproductive axis, in male and female offspring of sheep exposed to a translationally relevant EC mixture (in biosolids), during pregnancy. Birthweight in both sexes was unaffected by gestational biosolids exposure. In contrast to females (unaffected), bodyweight in biosolids males was significantly lower than controls across the peripubertal period, however, they exhibited catch-up growth eventually surpassing controls. Despite weighing less, testosterone concentrations were elevated earlier, indicative of early puberty in the biosolids males. This contrasted with females in which the mean date of puberty (first progesterone cycle) was delayed. These results demonstrate that developmental EC-mixture exposure has sexually dimorphic effects on growth, puberty and the relationship between body size and puberty. Such programmed metabolic/reproductive effects could have significant impacts on human health and wellbeing.

Keywords (3-5)

Developmental toxicity; Environmental chemicals; sexually dimorphic; puberty; bodyweight

1. Introduction

As a result of human activity, a large number of chemicals are ubiquitously present in the environment, albeit at low individual concentrations [1] [2]. This mixture of environmental chemicals (ECs) contains heavy metals, pharmaceuticals and chemicals associated with industrial, medical and agricultural activity [3-11]. Many of these ECs have endocrine disrupting properties which, if they enter the body, can alter normal patterns of endogenous hormone release and/or action. Exposure to ECs during critical periods of development and/or system activation [12], such as during in utero development, can lead to pronounced
physiological effects, the expression of which can be delayed until later in life [13, 14] demonstrating that ECs can play an important role in the developmental origins of disease [15]. Human epidemiological studies and animal models have also demonstrated that EC exposure effects are sexually dimorphic [16].

Exposure to ECs has been implicated as a contributory factor to changes in human reproductive health, including a decline in fertility and alterations to the timing (advancement and delay) and duration of the pubertal transition [17, 18]. The nature of EC effects on puberty appears to be dependent on which chemicals are measured, and often differ sexually between males and females [18, 19]. Many reports have linked earlier puberty in females with exposure to ECs such as BPA, phthalates, organohalogen [20] and PCBs with altered puberty in both males and females [21]. While the effects of EC exposure on male puberty have not been as well characterized, reports have linked Polybrominated diphenyl ether (PBDE), and phthalates with earlier puberty but others have found no or inconsistent effects of phthalates and BPA on male puberty [19]. These effects of ECs on human reproductive development and health have significant financial [22, 23] and psychological [24] consequence as a result of infertility but also affect psychosocial, behavioural, and physical health of young adults [25]. EC exposure has also been implicated in changes in metabolic health; i.e., the control of energy balance, adiposity, metabolic function [17], growth and development [26, 27]. Data from human studies suggest that prenatal exposure to a number of ECs including PFASs and PCBs [28], phthalates and phenols [29-32], PAHs [33], and EC mixtures [27, 34] can all decrease birth weight, an established risk factor for adult reproductive and metabolic pathologies (reviewed in [35]). ECs have also been found to have a negative impact on growth in the first year of life (prenatal PFAS and PCB exposure [36]), the first two years of life (PAH exposure [33]), and over both the first 19 months [37] and 6 years [27], after exposure to EC mixtures. Conversely, prenatal exposure to organochlorine pesticides has been linked with an increased
growth rate in the first year of life [38, 39]. Growth rate in early life has been linked with later health outcomes [40, 41] and alterations in childhood growth and metabolism are also associated with financial and social impacts [42]. Of course, these two effects of ECs on reproductive and metabolic health may also be linked as growth, nutrition and energy metabolism are important factors which can influence puberty onset and fertility [43, 44].

Much of the existing literature that describes the effects of EC exposure has been conducted using single or simple mixtures of ECs. This poses a significant problem because when considering possible health effects of real-life exposure, which is chronic, low-level exposure to mixtures whereby component ECs within a mixture can exhibit additive, synergistic or antagonistic effects [45, 46]. One of the few animal models of real-life EC exposure is provided by the biosolids-treated pasture (BTP) grazed sheep. Biosolids are the solid waste resulting from wastewater treatment which is widely used in land remediation or as an agricultural fertiliser [47, 48]. Given their derivation, biosolids contain a complex mixture of thousands of ECs including heavy metals, brominated flame retardants (BFRs), PCBs, pharmaceuticals, polyfluorinated hydrocarbons, personal care products (PPCPs), antibiotics, dioxins and metabolites [5, 49-54] the profile of which broadly reflects the human exposome. Sheep are also a useful translational model as they are an outbred species (similar to humans) and have a longer lifespan compared to rodents, on which many EC studies have been conducted. Sheep are also born with greater prenatal brain development and have steroid synthesis pathways and neuroendocrine regulatory pathways which are more similar to humans than rodents [55, 56]. Previous work with the BTP model has shown multiple effects of maternal biosolids exposure on offspring including perturbations in timing of development [57-59], hypothalamic-pituitary reproductive regulatory systems [60, 61], testis phenotype [59, 62, 63], thyroid function [64] and adult liver function [65]. However, the effects of maternal
exposure to biosolids on post-natal and peri-pubertal growth and pubertal timing in offspring, all risk factors for later adverse health outcomes, have not been characterised previously.

With the foregoing discussion in mind, the aim of the current study was to characterise temporal changes in post-natal and peripubertal growth and the activation of the reproductive system (timing of puberty) in both male and female offspring born to mothers exposed to the translationally relevant mixture of ECs found in biosolids during pregnancy.

2. Materials and Methods

2.1. Ethics statement

The experiment was conducted under the United Kingdom’s Animals (Scientific Procedures) Act 1986, under the specific authority of Project Licence PF10145DF. All animals were humanely treated throughout the study, with due consideration to alleviation of pain, suffering, distress and lasting harm.

2.2. Experimental animals

The experiment was conducted at the University of Glasgow Cochno Farm and Research Centre (55.94, -4.41) where specific plots are available that have been fertilised with either biosolids at conventional rates (2.25 tonnes dry matter/ha, twice annually since 2014) or inorganic fertiliser containing equivalent amounts of nitrogen (225kg nitrogen/ha/year; Control), as previously described [60]. EasyCare ewes (n=320), not previously grazed on biosolids treated pasture, were blocked for bodyweight, and randomly allocated to either the control (C) or biosolids (B) exposure group. Ewes were grazed on their respective pastures for approximately one month prior to mating by artificial insemination with semen collected from four EasyCare rams which were maintained on control pastures. Following AI, ewes were
maintained on their respective biosolids or control pastures until approximately two weeks prior to the expected date of lambing when they were moved indoors. While indoors, ewes were fed forage supplemented with concentrates as per normal husbandry practice. BTP exposed ewes received forage harvested from biosolids treated pasture while indoors. After lambing, control and BTP exposed animals (mothers and lambs) were maintained outdoors, as a single flock, on conventionally fertiliser treated pasture (i.e., the difference between control and BTP exposed lambs was increased maternal (placental and lactation) exposure to ECs via biosolids in the B group). There was little or no growth of clover or other oestrogenic plant species in any of the pastures, hence animals were not exposed to additional phytoestrogens.

The median date of parturition/birth of the lambs was the 17th April 2020 (range 4th-26th). At parturition, litter size, sex and birthweight were recorded for all lambs which were then given an individual identification (ear tag) to allow longitudinal monitoring relative to maternal and paternal ancestry and exposure group.

2.3. Morphometric Data

All of the F1 lambs (Female C n=94, B n=105; Male C n=105, B n=108) were weighed as part of normal husbandry at 9.5 and 16 weeks of age. For detailed tracking of peripubertal growth and aspects of reproductive development, a focal group of male (n= 21C, 19B) and female (n=10C, 11B) lambs were followed ensuring only one offspring per mother was included for twin and triplet births. Male and female focal cohorts were weighed monthly from September 2020 through February 2021 and their girth (measured at the last rib) recorded. Females were blood sampled twice weekly from 6th October 2020 to 19 March 2021 for progesterone assay. For males in the focal cohort, scrotal length and circumference (at the widest point) was also recorded (by the same person for consistency) and monthly blood samples were collected for measurement of testosterone. Testosterone concentrations
were also assessed in a single blood sample collected from an additional cohort of male lambs (n= 11C, 11B) at 8 weeks of age as part of a separate study. Blood samples were collected by jugular venepuncture using lithium-heparin Vacutainers (BD Diagnostics - 367885), plasma was harvested and stored at -20°C until assay.

2.4. Hormone Assays

Testosterone was assayed using a commercially available testosterone ELISA (Parameter, R&D systems, KG010). Samples were assayed neat (8 weeks of age) or were diluted with the supplied assay buffer 1:30 or 1:100 fold, prior to assay according to the manufacturer’s instructions. Assay sensitivity averaged 0.027ng/ml and inter- and intra-assay coefficients of variation were 7.4 and 4.8% respectively.

Progesterone was assayed using a commercially available progesterone ELISA (Enzo Progesterone ELISA kit Cat# ADI-901-011). Samples were diluted with the supplied assay buffer 1:30 fold, prior to assay as per the manufacturer’s instructions. Assay sensitivity averaged 4.95pg/ml and inter- and inter-assay coefficients of variation were 6.9 and 5.6% respectively.

2.5. Statistical Analyses

All statistical analyses were performed in R (version 4.1.1). Lamb bodyweight at birth was analysed using a linear mixed model with ram, sex, treatment and interactions between these factors as variables, with non-significant factors being removed in a stepwise manner. Birth weight was analysed for all F1 offspring and the focal cohort on which ongoing morphometric and/or pubertal data were collected. Bodyweight, girth, testes size and testosterone concentrations across the 55 weeks of the study were analysed using liner mixed models with sire, sex, treatment and interactions between these factors as variables, with non-
significant factors being removed in a stepwise manner. Where age-related changes in the data were nonlinear, the data was analysed in blocks of time defined by the inflection points within that variable. Girth in the males at 49 weeks of age was compared between groups using a t-test.

Age of puberty onset in female lambs was identified by analysis of biweekly serum progesterone concentrations for patterns of progesterone indicative of cycles of ovarian activity. As there was inter-animal variation in baseline progesterone concentrations, to be considered a cycle, progesterone concentration for an individual had to increase <1.5 fold over the lowest of the previous two samples and remain above the lowest sample concentration for the next three sampling occasions (<7 days). The date of the first cycle was then converted to a Julian date and the effects of biosolids exposure on date of puberty and age (in days) compared using Student's t test (P<0.05 considered significant).

3. Results

3.1. Morphometry

3.1.1. Lamb bodyweight at birth: Across the complete cohort of F1 lambs (n=412), birth weight was not affected by sire or maternal biosolids exposure in either male (B 4.10 ± 0.17, C 4.11 ± 0.16) or female (B 4.46 ± 0.27, C 4.21 ± 0.19) lambs.

3.1.2 Lamb body Morphometrics between 9 and 55 weeks of age: Bodyweight in female lambs increased progressively during the study period but in males the pattern of change in bodyweight was nonlinear as these animals underwent a short period of weight loss at approximately 30 weeks of age. Given these differences, the morphometric data from males and females were analysed separately.
3.1.3. Bodyweight changes between 9 and 55 weeks of age in female lambs: Bodyweight increased (P<0.001) with age but was not significantly affected by either sire or biosolids exposure and there was no significant interaction between any factors (Figure 1A).

3.1.4. Girth between 9 and 49 weeks of age in female lambs: In both C and B lambs, girth did not increase linearly with age, however, between 17-33 weeks of age the average girth of the B lambs was consistently lower than the C group (P=0.09). None of the variables tested had a statistically significant effect on girth. In both C and B groups, girth increased (P<0.001) with age between 9 and 29 weeks but in the period from 29 weeks to 33 weeks, girth decreased (P<0.001) in both groups. After 37 weeks, girth showed a progressive increase until the end of the study (Figure 2).

3.1.5. Bodyweight changes between 9 and 55 weeks of age in male lambs: Both B and C male lambs showed a period of relatively linear increase (P<0.001) in bodyweight with age between 9 and 29 weeks of age (Figure 1C). Across this period, the bodyweight of C lambs was greater (P<0.01) than that of B lambs.

During the period from 29 to 45 weeks of age, average bodyweight decreased (P<0.001) with age in both B and C groups. While the average bodyweight of the controls remained higher than the B group, the difference was not statistically significant, and bodyweight was not affected by sire (4 time points).

After 45 weeks of age, average bodyweight increased (P<0.001) with age in both B and C lambs. During this period there were no significant effects of sire or biosolids exposure on bodyweight, but it should be noted that, in contrast to the previous weeks, the average bodyweight of the C lambs was lower than for B lambs (time point week 49 and 55).
3.1.6. Girth between 21 and 49 weeks of age in male lambs: Girth increased (P<0.001) between 21 to 29 weeks of age and there was a trend (P=0.07) for girth to increase between 29 to 45 weeks of age (Figure 2). Girth was not affected by sire but between 21 and 29 weeks of age there was a trend (P=0.09) for girth to be lower in biosolids exposed animals. At 49 weeks of age, there was a trend for the mean girth of biosolid-exposed males to be higher than that of the controls (P=0.06).

3.1.7. Testes morphology: For both C and B lambs, testes length and circumference were measured between 21 and 49 weeks of age. As a proxy for testes size, testes ‘volume’ was calculated assuming the testes to be cylindrical. Changes in the overall profile of testes length circumference and volume were similar across time. Patterns of change in all variables were similar so only mean testis volume is shown in Figure 3. In both groups, age-related growth was seen between 21 and 33 weeks for testes length (P<0.001), and between 21 and 29 weeks for testes circumference and the calculated variable, testes volume (P<0.001). During this initial period of testes growth, testes length, circumference and volume were smaller (P<0.05) in B compared to C lambs.

There then followed a period (up until 41 weeks of age) in both B and C lambs, when testes length and circumference decreased (P<0.001). There were no statistically significant effects of the other tested variables on length, circumference or volume as the testes declined in size, but length and volume were consistently smaller in B compared to C lambs, during this period. Testes length, circumference, and volume increased (P<0.001) between 41 and 49 weeks of age, but no significant effects of biosolids exposure or the other tested factors were observed. At 49 weeks of age, length, circumference and volume were greater in B compared to C lambs.

3.2. Pubertal hormonal changes:
3.2.1. **Testosterone in male lambs:** An increase (P<0.001) in circulating testosterone concentrations occurred between 21 and 33 weeks of age in both C and B lambs (Figure 3 B). There was a trend (P=0.053) for testosterone concentrations to be higher in B than in C lambs during this period. After 33 weeks of age there were no significant changes in testosterone concentrations with age and no statistically significant effects of the other explanatory variables. When compared each month, the mean testosterone concentration tended to be higher in B lambs in August (P=0.06), September (P=0.07), and October (P=0.08) but were not different from the C group from November through to March.

3.2.2. **Circulating progesterone in female lambs:** All animals demonstrated at least one increase in progesterone concentrations that met the criteria for definition as an estrous cycle. Over the period of sample collection (6th Oct 2020- 9th Feb 2023), repeated cycles of progesterone secretion were observed in 9 of 10 biosolids and 10 of 11 control females (Figure 4A). The mean date of the first cycle (i.e., onset of puberty), was 13th November in C females which was 13 days earlier (P<0.05) than that of B females (26th November). When analysed relative to birth date, B females were two weeks older (P<0.05) than C females at the onset of ovarian cyclicity (biosolids, 225 days; controls, 211 days, Figure 4B).

4. **Discussion**

This study demonstrates that maternal exposure to a complex mixture of ECs prior to and during pregnancy is associated with sexually differentiated effects on both pre/peripubertal growth dynamics and the timing of the pubertal transition in offspring. With regards to male offspring, although there were no differences in birth weight between the lambs from control and biosolids-exposed mothers, by 9 weeks of age biosolids exposed offspring weighed less than controls, and this difference persisted throughout the first year of life encompassing the pubertal transition. Importantly, later in the first year of life there was evidence of ‘catchup’
and ‘overshoot’ growth in offspring from biosolids-exposed mothers relative to controls. Concurrently, the testes of the male B lambs were smaller than those of the controls, until around the middle of the breeding season (33 weeks of age, early December) after which they became more similar to C lambs. Despite having lower bodyweights and smaller testes, B rams tended to have higher concentrations of circulating testosterone, compared to C rams, from August through December, suggestive of earlier testicular activation and earlier puberty onset. In contrast, the bodyweight of B females did not differ from controls across the first year of life. These findings emphasise the sexually dimorphic effects of exposure to the ECs from biosolids on postnatal bodyweight, there being a selective impact on growth dynamics in male but not female offspring. The effect of EC mixture exposure on female puberty was diametrically opposite to that in males; while males showed earlier testicular activation indicative of advanced puberty, in the females puberty (i.e., the first detected progesterone cycle) was delayed. The relevance of the sexually dimorphic effects on growth and puberty are addressed below.

4.1. Sexually dimorphic perturbation in early growth dynamics: The regulation of fetal growth is influenced by a variety of factors including the maternal environment, endocrine influences, placental transfer of nutrients (and chemicals) and fetal factors [66]. We reported previously that biosolids exposure results in changes in the maternal milieu (in ewes from the same study) including the maternal metabolome [67], increased steroids and reactive oxygen metabolites [68]. While changes in the maternal milieu can influence fetal growth, they did not result in differences in birth weight in animals in the current study. The lack of an effect on lamb birth weight may be the result of compensatory changes that acted to protect fetal growth. In this regard, reported effects of EC exposure on fetal growth are inconclusive, while some studies have indicated a negative relationship [34, 69-72], others have reported a positive association, and a review (human and animal studies) concluded that this diversity of effect
may be a consequence of differences in the ECs assessed, their mechanisms of action, and the models tested [71, 73].

While maternal biosolids exposure did not significantly affect lamb birth weight, sexually differentiated effects were seen on growth dynamics across the first year of life. Males, but not females, born to biosolids exposed mothers were significantly smaller than controls between 9 and 29 weeks of age. Previous studies of single EC exposure have reported effects of early life exposure on postnatal bodyweight. For instance, combined gestational and lactational BPA exposure is positively associated with post-natal bodyweight in male and female rats [74] and mice [75], and gestational BPA exposure is associated with higher postnatal growth in female mice [76]. While few studies have documented effects of exposure to EC mixtures on postnatal growth, prenatal exposure to a mixture of 31 persistent EDCs has been reported to be associated with decreased bodyweight across the first 18 months of life in British girls [37]. The sexually dimorphic nature of the effects of biosolids exposure on growth may not be a surprise given that growth itself is sexually differentiated, with adult males being larger than females in many species, including sheep [77]. However, it is of note that the effects of biosolids exposure on the metabolome of exposed mothers was influenced by fetal sex [67]. This could reflect an inherent sex difference but could also have contributed to programmed differences in post-natal growth.

A decrease was observed in the bodyweight of male but not female B and C groups between 29 and 45 weeks of age. This difference in the growth trajectory between the males and females is most likely the result of an artifact of differing environmental conditions. C and B males were maintained outside until early-December after which time they were moved indoors and experienced a change in diet, whereas the focal females were housed indoors since August to permit regular blood sample collection. After this period, the treatment-related
difference in male bodyweight declined and at the last two timepoints (49 and 55 weeks of age), biosolids males were on average heavier than the controls. This contrasts with the earlier ages when they were weighed less. Catchup growth, which has long been recognised following intrauterine growth retardation, is often associated with disturbances of the maternal environment (, as a result of poor nutrition, disease, intrauterine infection, placental dysfunction, endocrine disturbances and in utero exposure to EDCs [78-83] [84]) and may have contributed to the offspring reproductive pathologies evidenced in biosolids males [63, 85]. It is of note, however, that rapid postnatal (and catchup) growth following in utero growth restriction has been linked with negative health outcomes such as metabolic dysfunction and cardiovascular disease in later life [86] [87]. While the long term effects of post-natal changes in growth in the absence of intrauterine growth restriction (IUGR) have not been as well characterised, there is evidence that rapid infant weight gain is also associated with later life adverse metabolic changes and obesity [88, 89]. Thus, the pattern of growth seen in the biosolids rams in the current study may put them at greater risk of metabolic disease in later life.

4.2. Sexually dimorphic effects of biosolids on puberty: Previous work with the biosolids model has investigated possible effects of EC exposure on a reproductive parameters at single time points in late gestation [57-61, 90], neonatal [62] and adult animals [85]. In the current study, animals were studied temporally which allowed assessment of the effect of maternal biosolids exposure on puberty. Puberty is complex, as it is can be affected by factors including genetic and epigenetic influences, as well as nutrition, photoperiod, circadian rhythms, olfactory cues and EDCs [91]. In the current study, control and biosolids exposed lambs were maintained under the same conditions throughout post-natal life and thus observed effects must be the result of gestational EC mixture exposure via biosolids. When considering possible EC
effects on puberty, an additional complication is that effects can occur at multiple points in the hypothalamo-pituitary-gonadal (HPG) axis and that EC mixtures can act through several mechanisms and/or at multiple levels within the HPG axis. It is likely that the mixed EC exposure resulting from biosolids exposure, acts at multiple levels within the HPG axis, and we have previously documented changes in the hypothalamus, pituitary [60, 61] and ovaries/testes [57, 58, 85, 92] with this model. Many human studies have reported that female puberty is advanced and occurs quicker in girls exposed to ECs [13, 93-96] [97]. While less conclusive, studies have also reported an association between EC exposure and earlier puberty in boys [98-100]. While others have reported a delay in puberty in both boys [100, 101] and girls [102] lack of effect [103-106] or no consistent picture [97]. The results of this study indicated that puberty was altered in both sexes, being advanced in males and delayed in females.

In the current study, changes in testicular morphometry and testosterone concentrations were used as a proxy of puberty which may be more accurately defined by the presence of sperm. Relative to the advancement of puberty (testosterone concentrations) seen in the current study, similar results have been reported with regard to some single EC exposure paradigms. Postnatal exposure of male rats to Diethylhexylphthalate (DEHP) (10 mg/kg), a phthalate plasticiser results in earlier male puberty (testosterone production and preputial separation), however, a higher dose of DEHP (750 mg/kg) resulted in delayed puberty [107]. Other animal based studies have also not shown a dose-dependent stimulatory effect of ECs on the male pubertal transition, and gestational exposure to PCB mixtures and dioxins was reported to result in delayed puberty in exposed males [108-110]. A delay in puberty has also been reported in a variety of rodent models after exposure to estrogenic ECs [111, 112] but the nature of the effects can be dependent on dose and the timing (gestational, lactational etc) and specific periods of exposure [113-116]. This diversity may reflect the fact that ECs can have
androgenic, antiandrogenic, estrogenic and antiestrogenic effects depending on their mechanism of action. The earlier increase in testosterone in the current study could be interpreted as showing that biosolids have predominantly androgenic actions, however, the effects on the morphometric measurements (testes size and body weight) would argue for antiandrogenic actions. It is likely, therefore that there is a complex interplay between ECs from biosolids with different mechanisms of action which cannot be differentiated in this study. When considering the advanced timing of puberty in the B males, it is of note that they weighed less than the controls. This is in opposition to the critical weight hypothesis for puberty put forth by Frisch and Revelle (1970) [117] although this has since been challenged, and it has been suggested that the timing of puberty is more closely linked with energy reserves, adiposity [118] or BMI [119].

In female lambs in the current study, maternal exposure to biosolids resulted in a delayed onset of puberty. These data are counter to the majority of human studies that suggest that puberty in girls is advanced due to a variety of factors including EC exposure [120]. In humans, the advancement of female puberty is occurring in association with an obesity pandemic and it has been suggested that changes in the timing of puberty could be secondary to changes in energy availability [121, 122]. While bodyweight was not affected by biosolids exposure in the females in the current study, girth was consistently, but not significantly, lower in biosolids exposed animals. As abdominal girth is often used as a proxy for visceral fat, this could suggest that biosolids-exposed females had a lower energy reserve. There is evidence that prenatal exposure to ECs such as genistein can result in a pubertal delay in female rats, , but this effect was reversed when exposure was applied during postnatal development [111, 112].

The results of this study demonstrate that maternal biosolids exposure results in sexually differentiated effects on both postnatal growth dynamics and timing of the pubertal transition. While this exposure scenario is translationally relevant, the observed changes are not what may
have been expected based on changes in human bodyweight and pubertal timing, where an increase in bodyweight and an advancement of puberty has been reported. These differences may be related to the fact that the exposure scenario used in the current study only encompassed the period preceding mating and gestation whereas human exposure is throughout life. While the use of a mixtures model is critical for risk determination and management, the interpretation of findings from such studies is difficult due to the fact that the mixtures are likely to contain chemicals that exert both antagonistic and agonistic actions on growth and the HPG axis and these effects may be different depending on the dose [123] and the profile [46] of chemicals present, which do not remain consistent across all life history stages. While the exact EC composition of the biosolids applied to the pasture used in the current study is not known, given the likely variation in biosolids EC composition between batches, and across time, its relevance may not be critically important. However, we have previously reported that biosolids contain a large array of ECs [124, 125] and that concentrations of some ECs are raised in soil after a single application of biosolids and that some ECs may accumulate in the soil over time [9, 126]. A further consideration when assessing the results of this study would be differences in the nutritional value of the grass from the biosolids and control pastures. In this regard, the nitrogen content of the biosolids and organic fertiliser applied to the pasture were matched and the body condition score of ewes was randomised at the start of the study and was not different at parturition, which would suggest no major differences.

This is the first study to demonstrate that maternal exposure to a “real-life” low-level mixture of chemicals during pregnancy, can alter both growth patterns and pubertal timing in a sheep model. While the mechanisms underlying these changes remain to be determined, these results suggest that EC exposure may alter the interplay between body size and puberty and add to the increasing body of evidence that exposure to some environmental chemicals can perturb
metabolic and reproductive function and that this may be associated with the parallel declining metabolic and reproductive health observed in humans.

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CRediT authorship contribution statement

Neil P. Evans: Conceptualization, Methodology, Writing – original draft. Michelle Bellingham: Conceptualization, Methodology, Writing – writing, review & editing. Chris S. Elcombe: Methodology, Writing - review & editing. Kevin D. Sinclair: Conceptualization, Writing – review & editing. Richard Lea: Conceptualization, Writing – review & editing, Mohammad Ghasemzadeh-Hasankolaei: Writing – review & editing. Vasantha Padmanabhan, Conceptualization, Writing – writing, review & editing.

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Declaration of Competing Interest

The authors have no conflicts of interest to declare and have not participated in, nor anticipate participation in, any legal, regulatory, or advocacy proceedings related to the contents of the paper. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.
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**Figure 1.** Mean±SEM bodyweight between 9 and 55 weeks of age in female (A) and male (B) control and biosolids lambs. As growth was nonlinear in male lambs, as a result of environmental conditions, panels C-E show the changes in male growth between 9 - 29, 33 - 45 and 45 - 50 weeks of age, with statistically significant effects of age and treatment noted for each time period.

**Figure 2.** Mean±SEM girth (measured at the last rib) between 17 and 49 weeks of age in female (A) and male (B) control and biosolids lambs. The female data were statistically analysed in three periods, 17-29, 29-33 and 33-49 weeks of age; significant effects are detailed in the figure. The male data were statistically analysed in three periods, 17-29, 29-45 and 49 weeks of age; significant effects are detailed in the figure.

**Figure 3.** Mean±SEM testes volume (A) and circulating concentrations of testosterone (B), between 21 and 49 weeks of age in male control and biosolids lambs. The data for testes volume were statistically analysed for effects of age and treatment in three periods, 21-29, 33-41 and 41-49 weeks of age, and significant effects are detailed in the figure.

**Figure 4.** A) Circulating progesterone concentration from representative control and biosolids exposed lambs (lamb ID shown). Vertical arrows indicate the date of puberty onset. B) mean±SEM age of puberty onset for control and biosolids exposed female lambs.
CRediT authorship contribution statement

Neil P. Evans: Conceptualization, Methodology, Writing – original draft. Michelle Bellingham: Conceptualization, Methodology, Writing – writing, review & editing. Chris S. Elcombe: Methodology, Writing - review & editing. Kevin D. Sinclair: Conceptualization, Writing – review & editing. Richard Lea: Conceptualization, Writing – review & editing, Mohammad Ghasemzadeh-Hasankolaei: Writing – review & editing. Vasantha Padmanabhan, Conceptualization, Writing – writing, review & editing.

Declaration of interests

☐ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☒ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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Highlights

- Puberty was delayed in female offspring of gestational biosolids exposed sheep.
- Testicular activation was advanced in male offspring of biosolids exposed sheep.
- Maternal biosolid exposure did not affect birthweight of male and female lambs.
- Male offspring of biosolids exposed mothers weighed less prior to adulthood.
- Maternal biosolid exposure had no impact on bodyweight of female lambs.