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ORIGINAL ARTICLE ***Reproductive epidemiology***

The association between *in-utero* exposure to stressful life events during pregnancy and male reproductive function in a cohort of 20-year-old offspring: The Raine Study

E.V. Bräuner[1,2], Å. M. Hansen[3,4], D. A. Doherty[5], J. E. Dickinson[5], D. J. Handelsman[6], M. Hickey[7], N. E. Skakkebæk[1,2], A. Juul[1,2], R. Hart[5,8,*]

[1]Department of Growth and Reproduction, Rigshospitalet, University of Copenhagen Denmark [2]The International Research and Research Training Centre in Endocrine Disruption of Male Reproduction and Child Health (EDMaRC), Rigshospitalet, University of Copenhagen, Denmark [3]Department of Public Health, University of Copenhagen, Copenhagen, Denmark [4]The National Research Centre for the Working Environment, Copenhagen Denmark [5]Division of Obstetrics and Gynaecology, University of Western Australia, Perth, Western Australia, Australia [6]ANZAC Research Institute, University of Sydney, Sydney NSW Australia [7]Department of Obstetrics and Gynaecology, University of Melbourne, Melbourne, Victoria, Australia [8]Fertility Specialists of Western Australia, Bethesda Hospital, Claremont, Western Australia, Australia

* Correspondence address. Medicine Medical Director of Fertility Specialists of Western Australia UWA School of Women's and Infants' Health University of Western Australia King Edward Memorial Hospital 374 Bagot Road Subiaco, Australia. WA 6008. Tel: (+61) 08-9340-1328; Fax: (+61) 08-9381- 3031; E-mail: roger.hart@uwa.edu.au

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STUDY QUESTION: Is exposure to gestational stress in the critical time window for the normal differentiation and growth of male reproductive tissue associated with male reproductive function in offspring in later life?

SUMMARY ANSWER: Exposure to stressful life events (SLEs) in early, but not late gestation, are associated with reduced adult male reproductive function, consistent with the hypothesis that events during early prenatal life programme adult male reproductive function.

WHAT IS ALREADY KNOWN: Animal studies suggest that gestational stress may impact on the reproductive function of male offspring, but human evidence is sparse.

STUDY DESIGN, SIZE, DURATION: Using a prospective longitudinal cohort, we examined the association between number and type of maternal stressors during pregnancy in both early and late gestation and reproductive function in 643 male Generation 2 (offspring) at age 20 years. Mothers and their male Generation 2 (offspring) from The Raine Study participated. Mothers prospectively reported SLEs during pregnancy recorded at gestational weeks 18 and 34 using a standardized 10-point questionnaire.

PARTICIPANTS/MATERIALS, SETTING, METHODS: The 643 male Generation 2 (offspring) underwent testicular ultrasound examination and semen analysis and provided serum for reproductive hormone analysis. Multivariate linear regression analysis was used to examine associations.

MAIN RESULTS AND ROLE OF CHANCE: Of 643 recruited males, 407 (63%) were exposed to at least one SLE in early gestation. Fewer SLEs were reported in late gestation ($n = 343$, 53%). Maternal SLE exposure in early gestation was negatively associated with total sperm count ($\beta = -0.31$, 95% CI −0.58; −0.03), number of progressive motile sperm ($\beta = -0.15$, 95% CI −0.31; 0.00) and morning serum testosterone concentration ($\beta = -0.04$, 95% CI −0.09; −0.00). No similar effects of maternal SLE exposure in late pregnancy were detected. The large sample size and an objective detailed direct assessment of adult male reproductive function with strict external quality control for sperm quality,

as well as detailed prospectively collected information on prenatal SLEs in two distinct time windows of pregnancy reported by the women in early and late gestation along with other risk factors, imply minimal possibility of recall, information bias and selection bias. When assessing our results, we adjusted for *a priori* chosen confounders, but residual confounding or confounding by factors unbeknown to us cannot be ruled out.

LIMITATIONS, REASONS FOR CAUTION: It is not possible to measure how SLEs impacted differently on the mother's experience or perception of stress. Resilience (coping) gradients may alter cortisol levels and thus modify the associations we observed and the mothers' own perception of stress severity may have provided a more precise estimate of her exposure.

WIDER IMPLICATIONS OF THE FINDINGS: Our findings suggest that exposure to SLEs in early, but not late gestation, are associated with reduced adult male reproductive function. Improved support for women with exposure to SLEs during pregnancy, particularly during the first trimester, may improve the reproductive health of their male offspring in later life. Intervention studies of improved pregnancy support could provide more insight into this association and more information is needed about the potential specific epigenetic mechanisms underlying this association.

STUDY FUNDING/COMPETING INTEREST(S): The male fertility sub-study was funded by NHMRC Grant 634 457. The core management of the Raine Study is funded by University of Western Australia, Curtin University, Telethon Kids Institute, Women and Infants Research Foundation, Edith Cowan University, Murdoch University, The University of Notre Dame Australia and Raine Medical Research foundation. Dr Bräuner's salary was supported by Læge Sofus Carl Emil Friis og Hustru Olga Doris Friis foundation in Denmark. All authors declare no competing interests.

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Key words: maternal stress / testicular volume / semen analysis / sperm concentration / reproductive hormones / pregnancy / *in-utero* exposures / The Raine Study

Introduction

Infertility, defined as the absence of pregnancy after a year of unprotected regular intercourse, affects around 15% of couples trying to conceive, and in approximately half of these couples a male-related factor may be implicated as the underlying cause (Inhorn and Patrizio, 2015). Apart from genetic and direct spermatogenic damage, the cause of male infertility is largely unexplained, but there is growing evidence that male reproductive function may be regulated by the early life environment (Juul *et al.*, 2014; Skakkebaek *et al.*, 2016). The critical prenatal window for the normal differentiation and growth of male reproductive tissue (male programming window) is estimated to be around 8 to 14 weeks of gestation (Welsh *et al.*, 2008; Sharpe, 2010). Several early prenatal exposures, mainly chemicals with anti-androgenic properties, have been proposed to disrupt prenatal reproductive development in males (Juul *et al.*, 2014). Maternal stress has also been shown to impact on the prenatal endocrine environment, potentially impacting male reproductive function (Barrett and Swan, 2015). Extensive animal data supports the role of maternal stress in male reproductive function, including reduced fertility, decreased sexual activity, fewer ejaculations, reduced testicular weight, delayed testicular descent and delayed puberty (Ward, 1972; Dahlof *et al.*, 1978; Crump and Chevins, 1989; Gerardin *et al.*, 2005). Additionally, male offspring exposed to maternal stress during early prenatal life have reduced anogenital distance compared to controls (Ward and Weisz, 1980; Ward and Weisz, 1984), indicative of reduced exposure to prenatal androgens (Swan *et al.*, 2005; Eisenberg *et al.*, 2011, 2012a,b; Thankamony *et al.*, 2016). However, human evidence regarding the association between maternal stressful life events (SLE) and male reproductive function is very limited. One study has reported that maternal bereavement before and during pregnancy is significantly associated with a composite outcome of male reproductive disorders including congenital genital malformations, testicular cancer and infertility (Plana-Ripoll *et al.*, 2017). However, that study was limited by the uncertainty of the timing of the exposure, and only considered one SLE, namely bereavement, and found no associations with infertility considered as an independent individual outcome. Small studies of medical students during exam periods (Eskiocak *et al.*, 2005; Eskiocak *et al.*, 2006), healthy young men from the general population and adult males attending infertility clinics suggest that adult exposure to psychological stress may be weakly associated with reduced paternity and abnormal semen parameters (Nargund, 2015). However, the data are limited by small sample sizes and reverse causation, which is likely to occur in populations recruited from infertility clinics. Furthermore, the effects of psychological stress on the adult male are distinct from the potential effects of maternal psychological stress on the developing male fetus and the potential subsequent deleterious effects in later life.

The premise of the present study is that maternal exposure to external psychological SLEs in pregnancy are associated with subsequent decreased reproductive function in adult male offspring. No previous studies have prospectively measured this using direct measures of male reproduction, such as semen parameters, testicular volume or reproductive hormones. We set out to examine the number and type of maternal SLEs in both early and late pregnancy, and their potential association with reproductive function in male Generation 2 (offspring) at age 20 years, using the unique longitudinal data from the Western Australian pregnancy Raine Cohort.

Materials and Methods

The Raine Study

The Western Australian Pregnancy Cohort (Raine) Study, formed from a pregnancy cohort study, was designed to measure the relationships between early life events and subsequent health and behaviour. The study recruited almost 3000 women in their 18th gestational week (GW) in the period from May 1989 to November 1991. The 2868 children (including 1454 boys) born to 2804 mothers were retained to

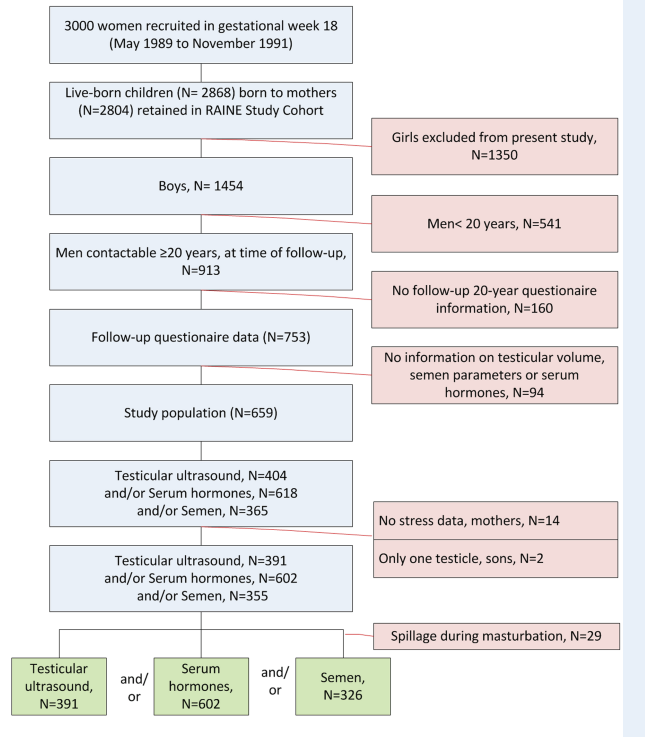

Figure 1 Study population and exclusions.

form The Raine Study (Straker *et al.*, 2017). The present prospective longitudinal cohort study included 643 young Generation 2 male offspring aged 20 years who participated in the follow-up study and provided semen samples ($n = 326$) and/or testicular ultrasound examination ($n = 391$) and/or reproductive hormones ($n = 602$) (Fig. 1).

Exposure variables

SLEs

Data were collected at 18- and 34-weeks' gestation using a 10-item questionnaire based on the validated Life Stress Inventory (Tennant and Andrews, 1976; Tennant, 1977). At 18 weeks gestation, women were asked to record SLEs experienced since confirmation of their pregnancy and at 34 weeks they were asked about SLEs only in the preceding four months of pregnancy, to ensure that the same SLE was not reported twice. SLEs included death of a close relative, death of a close friend, separation or divorce, marital problems, problems with children, own involuntary job loss, partner's involuntary job loss, money problems, pregnancy concerns, residential move and other problems (Table I). Each response to each of the 10-item questionnaire were recorded as 'yes/no' once, in accordance with previous studies to maximize recall (Carmichael *et al.*, 2007). There was a minimum to maximum of zero to 10 SLEs per women in each survey.

The primary exposure of interest

The primary exposure was SLE exposures during early pregnancy (as reported at 18 weeks' gestation) according to *a priori* evidence estimating that period as critical for fetal male reproductive masculinization and organ development (Sharpe, 2010; van den Driesche *et al.*, 2017).

Number and timing of SLE exposure

Separate continuous variables including the total number of maternal SLEs reported at 18 weeks' gestation and at 34 weeks' gestation were created (O'Connor *et al.*, 2003; Ronald *et al.*, 2010; Whitehouse *et al.*, 2010; Robinson *et al.*, 2011; Grace *et al.*, 2016a,b). Then, three categories based on the number of maternal stressors, weighting each stressor equally were developed for each gestational survey: (i) no SLE exposure (NS) (ii) moderate number of SLEs (MS; <3) or (iii) high number of SLEs (HS; ≥3).

Outcome variables

Detailed descriptions of the methods of testicular examination, semen sample collection and analysis, as well as the quantification of reproductive hormones have previously been reported (Evenson and Jost, 2000; Harwood and Handelsman, 2009; Hart *et al.*, 2015; Hart *et al.*, 2016).

Testicular examination

Testicular volume was assessed by ultrasound of the scrotum conducted by a single experienced operator with the men positioned supine and the scrotum supported by a rolled towel. All examinations were performed with a light direct transducer contact using a 9–3 MHz linear array transducer (Philips IU22, Philips Healthcare, Cincinnati, Ohio, USA). Each testis was measured by estimating its maximal dimensions in three planes, excluding the epididymis, using electronic callipers and testis volume was calculated using the formula for a prolate ellipsoid (length (L) × width (W) × height (H) × 0.52) (Lenz *et al.*, 1993; Sakamoto *et al.*, 2007). One value for L, W and H was recorded per examination. Venous diameter was measured in the supine position with Valsalva maneuver and varicocele was defined as present when the maximal venous diameter was over 3 mm and increased with the Valsalva maneuver (Oyen, 2002; Pilatz *et al.*, 2011).

Semen samples

Each man visited the IVF unit and provided semen samples by masturbation as previously described in detail (Hart *et al.*, 2015; Hart *et al.*, 2016). The men were encouraged to have the recommended abstinence intervals (2–7 days) as per World Health Organization (WHO) guidelines (Cooper *et al.*, 2010). All participants provided information on the time and date of the semen sample and spillage during semen sample collection. Standard semen assessment and reporting was performed according to the WHO semen manual(WHO, 1999) and parameters (Cooper *et al.*, 2010) as well as in accordance with Björndahl guidelines (Bjorndahl *et al.*, 2016), except that sperm concentration was determined by using a Makler chamber and semen volume was determined using a graduated, volumetric pipette. Sperm output was characterized as both sperm concentrations (million sperm per ml of ejaculate) and total sperm output (million per ejaculate). Examination of 92.1% of the samples was initiated within the first hour, where it has been shown that the motility is stable (Makler *et al.*, 1979), and examination of 99.7% of the samples was initiated within 2 hours.

For sperm concentration, morphology and motility, inter-laboratory and intra-laboratory results were plotted against the mean with strict warning (two standard deviations from the mean) and action (three

Table I Type and number of stress life events (SLEs) experienced by the 643 women at each measured time point[a], during pregnancy, 1989–1991.

Life event	Timing of events	
	GW18	GW34
	N (%)[b]	N (%)[b]
Number of women reporting ≥1 event	407 (63.3)	343 (53.3)
Stress event reported		
Death of a relative	42 (6.5)	20 (3.1)
Death of a friend	13 (2.0)	11 (1.7)
Your own job loss (not voluntary)	14 (2.2)	9 (1.4)
Your partner's job loss (not voluntary)	35 (5.4)	33 (5.1)
Pregnancy concerns	168 (26.1)	104 (16.2)
Separation or divorce	19 (3.0)	14 (2.2)
Residential move	105 (16.3)	116 (18.0)
Marital problems	53 (8.2)	39 (6.1)
Problems with your children	40 (6.2)	37 (5.8)
Money problems	150 (23.3)	141 (21.9)
Other problems	111 (17.3)	62 (9.6)
Total events (N)	*750*	*586*
Number of SLEs [c]		
None (NS: 0 SLEs)	236 (36.7)	300 (46.7)
Moderate (MS: <3 SLEs)	320 (49.8)	285 (44.3)
High (HS: ≥3 SLEs)	87 (13.5)	58 (9.0)

[a]The questionnaire at GW18 related to the period since becoming pregnant, and on the GW34 questionnaire the women were asked whether any of the SLEs had been experienced during the past four months, ensuring that the same event was not counted twice.
[b]Percentages calculated based on total ($n = 643$) number of mothers the total sum of percentages is over 100% due to some mothers reporting more than one stressor.
[c]Categories based on the unweighted number of SLEs experienced.

standard deviations from the mean) control limits enforced, according to those recommended by the EQASRM programme (Palacios *et al.*, 2012).

The sperm chromatin structural assay (SCSA) was performed as previously described (Evenson and Jost, 2000) with slight modifications as previously described in detail (Hart *et al.*, 2015; Hart *et al.*, 2016) and in accordance with Björndahl guidelines (Bjorndahl *et al.*, 2016). External quality control samples were sourced from the DNA fragmentation (by SCSA), quality assurance programme (FertAid, NSW) and were performed prior to each sample batch. Assay controls were run in duplicate with an intra-assay average quality control coefficient of variation (CV) of 13.0.

The DNA fragmentation index represents the percentage of sperm within the sample with fragmented or damaged DNA. The percentage of sperm with high DNA stainability represents the percentage of sperm within the sample with incomplete chromatin condensation.

All analyses were carried out by experienced clinical staff who undergo regular external and internal quality control assessment (EQASRM, Australia) (Palacios *et al.*, 2012).

Reproductive hormones

Blood samples were drawn from an antecubital vein in the morning, centrifuged and serum was stored for later measurement of reproductive hormones. Testosterone concentration was determined by liquid chromatography–mass spectrometry (Harwood and Handelsman, 2009), with the following limits of quantification: 0.025 ng/ml. Reproducibility was 10 for testosterone in male serum. Serum inhibin B concentrations were measured in duplicate by an Inhibin B Gen II ELISA from Beckman Coulter Inc. (Brea, CA, USA), which had a limit of detection of 2.6 pg/ml. Luteinizing Hormone (LH) and Follicle Stimulating Hormone (FSH) levels were determined in duplicate using ELISA kits (IBL International, Hamburg, Germany). The limit of detection of the LH assay was 0.4 IU/l (calibrated against WHO International Reference Preparation (IRP) 80/552), while for FSH assay it was 0.2 IU/l (calibrated against National Institute for Biological Standards and Control (NIBSC) 92/510). The intra-assay precision (CV) of the ELISAs ranged from 8 to 11% based on the mean values for low and high value quality control samples from $n = 16$–17 assays (Hart *et al.*, 2015).

Statistical strategy

First, we calculated descriptive statistics of the young men at 20 years of age with a testicular examination and/or semen analysis and/or serum hormone analysed, stratified by level of maternal stressors in GW18, which was considered our primary exposure.

Estimations of the association between exposure to prenatal SLEs (timing, number and type) and male reproductive function in Generation 2 offspring were performed using linear regression analyses. Main

Table II Anthropometric variables and untransformed outcomes (testicular volume, semen parameters and reproductive hormones) of the participants, stratified by early *in-utero* stress exposure (number of SLEs reported in week 18, main exposure time window).

	NS: 0 SLEs	MS: <3 SLEs	HS: ≥3 SLEs
	Median (25th, 75th ‰) /N (%)	Median (25th, 75th ‰) /N (%)	Median (25th, 75th ‰) /N (%)
Mothers[a,b]			
Total mothers (N)	*236*	*320*	*87*
Gravidity (1st pregnancy)	52 (22.0)	60 (18.8)	23 (26.4)
Body Mass Index (BMI, kg/m^2)	22.9 (21.2, 25.1)	23.0 (21.0, 25.4)	23.8 (21.6, 26.8)
Socio-economic status[c]	63 (26.3)	128 (40.0)	40 (40.0)
Sons[a]			
Testicular examination[d]			
Sons with testicular examination (N)	*152*	*194*	*47*
Mean volume of left and right testicle, ml	15.2 (12.6, 17.3)	14.9 (13.0,17.1)	14.5 (11.8,16.7)
Semen parameter[e,f]			
Sons providing a semen sample (N)	*129*	*157*	*40*
Total sperm count, million	130 (54,224)	114 (53, 190)	73 (21, 161)
Semen volume, mL	2.7 (1.9, 3.7)	3.0 (2.2, 3.8)	1.9 (1.3, 3.1)
Sperm concentration, million/ml	47 (27,77)	42 (20, 68)	38 (15,62)
DNA Fragmentation Index (DFI)%[g]	3.4 (1.9, 5.0)	3.0 (1.8, 5.1)	2.7 (1.8, 5.4)
High DNA Stainability (HDS)%[h]	1.5 (1.2, 2.4)	2.1 (1.3, 3.3)	1.8 (1.0, 4.5)
Morphology normal, %	5.0 (3.0,7.0)	5.0 (3.5, 7.0)	4.8 (3.8, 6.5)
Progressive motility (a + b grade), %	60 (46,68)	57 (40, 65)	57 (34, 66)
Spillage, yes	14 (9.7)	12 (7.1)	3 (6.9)
Duration of abstinence, days	2 (2, 3)	2 (2, 3)	2 (1, 3)
<2 days	28 (19.5)	40 (23.6)	13 (30.2)
2–5 days	108 (75.5)	120 (71.0)	26 (60.5)
>5 days	5 (3.5)	7 (4.1)	4 (9.3)
Time from ejaculation to analysis, min	35 (30,50)	40 (25,50)	37 (25,50)
Serum hormones			
Sons with blood for hormone analysis (N)	*221*	*299*	*82*
Testosterone, nmol/L	16 (12.7, 21.0)	16 (12.6, 19.9)	14 (11.5, 18.5)
Luteinizing hormone (LH), IU/L	11 (8.3, 12.9)	11 (8.3, 13.2)	11 (8.1, 12.0)
Follicle stimulating hormone (FSH), IU/L	4.2 (2.9, 6.5)	4.3 (2.8, 5.8)	4.5 (3.4, 6.5)
Inhibin B, pg/ml	218 (178, 273)	217 (171, 267)	218 (166, 258)
Testosterone/LH ratio	1.69 (1.13, 2.13)	1.54 (1.14, 1.99)	1.42 (1.08, 1.88)
Inhibin B/FSH ratio	50.0 (29.6, 85.8)	50.0 (34.0, 87.1)	44.5 (28.2, 72.5)

[a]Frequencies and percentages based on total population.
[b]As reported by mothers in questionnaire data at GW 18.
[c]Average annual family income level per annum below $24 000 reflecting the minimum income level in 1989–1991, according to the Australian Government guidelines.
[d]Frequencies and percentages based on population of sons who underwent testicular examination.
[e]Frequencies and percentages based on population of sons who provided a semen sample for analysis.
[f]Calculated based on men reporting no spillage during masturbation.
[g]Percentage of sperm within the sample with fragmented or damaged DNA.
[h]Percentage of sperm within the sample with incomplete chromatin condensation.

outcomes included total sperm output, sperm concentration and the total number of motile spermatozoa per ejaculate, mean testicular volume and morning serum testosterone concentration. Secondary endpoints included other semen sample parameters (semen volume, percentage morphologically normal spermatozoa, percentage high DNA stainability (%HDS) and percentage DNA fragmentation index (%DFI)) and reproductive hormones (LH, FSH and inhibin B). Further, the T/LH- and FSH/Inhibin-B-ratios were investigated to independently

Table III Linear regression analyses of main transformed outcome variables as a function of number of stressors in EARLY (GW18) and LATE (GW34) GESTATION.

	Model	No stress (NS = 0 SLEs)	Moderate stress (MS: <3 SLEs)	High stress (HS: ≥3 SLEs)	*Linear trend*
GW18					
Testicular examination		*Back transformed adjusted estimated marginal means (95% CI)*			β (95% CI)[§]
Mean volume, ml, $n = 391$	Crude	14.82 (14.3, 15.4)	14.76 (14.3, 15.3)	14.23 (13.3, 15.2)	−0.02 (−0.05, 0.02)
	Adjusted,[b,c]	14.83 (14.2, 15.4)	14.70 (14.2, 15.2)	14.25 (13.3, 15.3)	−0.02 (−0.05, 0.02)
Semen parameter[a]					
Total sperm count, million, $n = 326$	Crude[d]	124 (103, 147)	106 (89,126)	75 (50, 107)	−0.34 (−0.62, −0.06)*
	Adjusted,[b–e]	131 (103, 165)	114 (90,142)	84 (56, 122)	−0.31 (−0.58, −0.03)*
Sperm concentration,million/ml, $n = 326$	Crude[d]	44.3 (37.7, 51.7)	37.4 (32.0, 43.4)	35.0 (25.3, 47.0)	−0.15 (−0.33, 0.03)
	Adjusted,[b–e]	44.9 (35.7, 55.6)	37.9 (30.4, 46.5)	36.4 (25.6, 49.9)	−0.14 (−0.32, 0.04)
Progressive motility (a + b grade), %, $n = 321$	Crude[d,f]	56.9 (52.9, 60.7)	51.4 (47.8, 55.0)	49.8 (42.6, 57.0)	−0.17 (−0.32, −0.01)*
	Adjusted,[b–f]	54.8 (49.1, 60.4)	49.6 (44.4, 54.8)	48.4 (40.4, 56.5)	−0.15 (−0.31, 0.00)*
Serum hormones					
Testosterone (T), nmol/L, $n = 602$	Crude	15.8 (15.1, 16.6)	15.7 (15.0, 16.3)	14.0 (13.0, 15.2)	−0.05 (−0.09, −0.00)*
	Adjusted,[b,c]	15.7 (14.9, 16.5)	15.6 (15.0, 16.3)	14.0 (12.9, 15.2)	−0.04 (−0.09, 0.00)
GW34[£]					
Testicular examination		*Back transformed adjusted estimated means (95% CI)*			β (95% CI)[§]
Mean volume, ml, $n = 391$	Crude	14.55 (14.1, 15.1)	14.87 (14.3, 15.4)	15.06 (13.7, 16.5)	0.02 (−0.02, 0.06)
	Adjusted,[b,c]	14.54 (14.0, 15.1)	14.86 (14.3, 15.4)	15.04 (13.7, 16.5)	0.01 (−0.01, 0.04)
Semen parameter[a]					
Total sperm count, million, n = 326	Crude[d]	109 (91, 129)	114 (95,135)	74 (41, 121)	−0.11 (−0.43, 0.22)
	Adjusted,[b–e]	114 (89, 145)	121 (95,152)	103 (59, 166)	0.01 (−0.18, 0.21)
Sperm concentration, million/ml, $n = 326$	Crude[d]	38.4 (32.7, 44.6)	41.8 (35.6, 48.7)	36.9 (23.2, 55.1)	0.04 (−0.17, 0.25)
	Adjusted,[b–e]	37.9 (29.9, 47.3)	42.2 (33.7, 52.1)	41.1 (25.2, 62.6)	0.08 (−0.12, 0.29)
Progressive motility (a + b grade), %, $n = 321$	Crude[d,f]	52.6 (48.9, 56.3)	55.7 (51.9, 59.5)	43.6 (34.0, 53.6)	−0.03 (−0.21, 0.15)
	Adjusted,[b–f]	50.5 (45.0, 56.0)	53.2 (47.8, 58.5)	44.2 (33.7, 55.3)	−0.01 (−0.19, 0.18)
Serum hormones					
Testosterone (T), nmol/L, $n = 602$	Crude	15.9 (15.2, 16.7)	15.2 (14.5, 15.9)	14.7 (13.3, 16.2)	−0.04 (−0.09, 0.01)
	Adjusted,[b,c]	15.8 (15.1, 16.6)	15.7 (15.0, 16.3)	14.0 (13.0, 15.2)	−0.03 (−0.08, 0.02)

[§] β-coeefficient effect estimate and 95% CI for SLE strata modelled as a continuous explanatory variable in the multivariate linear regression models of transformed outcomes [cubic root (sperm concentration, total sperm count), natural logarithm (testicular volume and serum hormones), logit-transformation (%progressively motile spermatozoa (class a + b))] with adjustments for co-variates as indicated below.

[a] Men who reported spillage ($n = 29$) during masturbation were excluded from analyses.

Models adjusted for:

[b] Maternal BMI

[c] Maternal socioeconomic status [total household annual income: dichotomized to reflect a minimum income level (<$24 000 p.a. or ≥$24 000 p.a.) according to the Australian Government guidelines at the time (1989–1991)]

[d] Abstinence time (categorical: <2 days, 2–5 days, and >5 days).

[e] Parity (continuous).

[f] Time from ejaculation to semen analysis (continuous, minutes).

[£] Models in GW34 adjusted for stressors reported at GW18.

address effects of prenatal stress on Leydig- and Sertoli cell function. All outcomes were transformed by cubic root for semen volume, sperm concentration, total sperm count or by natural logarithm for testicular volume and serum hormones or by logit-transformation (progressively motile spermatozoa (class a + b)), before statistical linear regression analyses to meet linear regression model assumptions of normal distribution and constant variance (homoscedasticity) in residuals. The percentages of progressively motile spermatozoa (class a + b), %DFI and %HDS were logit-transformed. Morphology (% normal spermatozoa) and the residuals of this variable were close to normally distributed and entered the model untransformed. We evaluated the fit of the regression models by visually inspecting the residual plots and by testing the residuals for normality (the Shapiro–Wilk W test).

For the transformed outcomes, the crude and adjusted associations were evaluated. The multivariable linear models were adjusted for potential confounders based on the literature supporting their association with prenatal stress (fetal period) and a reduction in semen parameters and testicular volume, including maternal BMI (continuous), parity (continuous) and socio-economic status (SES) as reported by the mother (total household annual income: dichotomised to reflect a minimum income level (<$24 000 p.a. or ≥$24 000 p.a.) according to the Australian Government guidelines at the time of the pregnancies: 1989–1991). These three variables were selected as the mothers own BMI, SES and parity were specifically relevant as they represented a common cause of the indirectly measured stress hormones in the fetal period and male reproductive function in offspring. Co-variates, close in time and clinically associated with the outcome but not exposure, were also included to improve model precision and all semen variables (crude and adjusted) were adjusted for abstinence time (categorical: <2 days, 2–5 days and > 5 days) and the progressive spermatozoa motility model (both crude and adjusted) was adjusted for time from ejaculation to semen analysis (continuous, minutes). Data on participants who reported spillage were excluded ($n = 29$, 8% of men who provided semen). Causal directed acyclic graphs were used to explore different causal scenarios between the maternal stress exposure and primary outcomes and to reduce conditional associations and confounding bias (Shrier and Platt, 2008).

The associations with prenatal SLE exposures was assessed by separately examining the effects of early gestational exposures (SLEs reported at GW18, critical exposure time window) and late gestational exposure (SLEs reported at GW34, negative control). The exposures in each time window were included in separate models as categorically coded explanatory variables (none, <3, ≥3 SLEs). Models addressing effects of SLEs in late gestation (GW34) were mutually adjusted for SLEs reported at GW18. Linear trend was assessed by entering the stress strata, into the model as a continuous explanatory variable.

Collinearity between GW18 and GW34 was addressed by assessing pair-wise correlations ($rho = 0.457$) of the continuous variables as well as testing for collinearity in the models for tolerance values and there was no threat of collinearity (tolerance = 1 and variance inflation <10).

All statistical analyses were performed by SAS, using the GLM (version 9.4; SAS Institute, Cary, NC version 9.4). β-coefficient effect estimates and 95% CI are presented in the multivariate linear regression models of the transformed outcomes. The estimated marginal means are not directly interpretable. Thus, the estimated marginal means and CIs are also presented as back-transformed values according to stress strata.

Ethics

Research was conducted in accordance with principles of the Declaration of Helsinki. The Raine Study was approved by the University of Western Australia Human Research Ethics Committee and written informed consent was obtained from all participants prior to enrollment. The study is reported according to the STROBE (Strengthening the Reporting of Observational Studies) guidelines and checklist (checklist available in the online supplement).

Results

Of 643 male Generation 2 study participants, 407 (63%) were exposed to at least one SLE in utero in early gestation (reported at GW18), including 87 (13.5%) who were exposed to three or more SLEs. Pregnancy concerns was the most frequently reported (26%) individual SLE in early gestation (Table I). The men were exposed to fewer SLEs reported exclusively in late gestation ($n = 343$ (53%), at least one SLE).

Anthropometric variables and untransformed reproductive outcomes of the 20-year-old men, stratified according to number of individual early *in-utero* SLEs (none, <3 and ≥3 SLEs), are reported in Table II. The mothers reporting no stress events in early gestation had higher incomes and lower BMI compared to mothers reporting at least one stressor. Women experiencing their first pregnancy were more likely to report three or more SLEs than women who had experienced previous pregnancies (Table II).

There was a significant association between the number of unweighted SLEs in early gestation after appropriate transformation of outcome variables in crude linear regression analyses for total sperm count ($\beta = -0.34$, 95% CI −0.62; −0.06), progressive sperm motility ($\beta = -0.17$, 95% CI −0.32; −0.01) and morning serum testosterone levels ($\beta = -0.05$, 95% CI −0.09; −0.00). These significant associations persisted after adjustment in the multivariate linear regression analyses for total sperm count ($\beta = -0.31$, 95% CI −0.58; −0.03), progressive sperm motility ($\beta = -0.15$, 95% CI −0.31; 0.00) and morning serum testosterone levels ($\beta = -0.04$, 95% CI−0.09; 0.00) (Table III). Non-significant negative trends were observed in association with sperm concentration (Table III) and the testosterone/LH-ratio after adjustment for covariates (not shown in tables). The corresponding back-transformed adjusted estimated means of the multivariate regression analyses according to number of SLEs were reported. The back-transformed mean of total sperm counts, sperm motility and serum testosterone concentration of men exposed to ≥3 SLEs in early gestation were 84 mill, 48.4% and 14.0 nmol/L, respectively, which corresponds to 36%, 12% and 11% lower values than those observed in men exposed to no SLEs in early pregnancy.

No similar associations were noted in the adjusted multivariate linear regression analyses when repeating analyses for the effects of number of unweighted SLEs reported exclusively in late gestation (reported at GW34) (Table III). No significant influence of the number or the timing of the SLEs *in utero* on testicular volume (Table III) or on any of the other secondary reproductive outcomes (data not shown) was noted.

When considering effects of individual SLEs, the strongest negative significant effect estimates were noted in association with problems with children and total sperm count ($\beta = -0.84$, 95% CI: −1.63; −0.07), with strong negative non-significant associations with sperm

Table IV Linear regression analyses of transformed semen outcome variables as a function of individual stressors in EARLY GESTATION (GW18).

Individual event in GW18	Total sperm count, million[a-e]	Sperm concentration, million/ml [a-e]	Progressive motility (a + b grade), % [a–f]
	β (SE)[§]	β (SE)[§]	β (SE)[§]
Death of family	0.68 (−0.06, 1.43)	0.11 (−0.38, 0.60)	0.08 (−0.36, 0.52
Death of close friend	0.44 (−0.89, 1.78)	0.03 (−0.84, 0.90)	0.31 (−0.45, 1.07)
Own job loss	0.25 (−1.00, 1.49)	0.40 (−0.41, 1.21)	0.05 (−0.66, 0.76)
Partner's job loss	0.02 (−0.93, 0.98)	0.16 (−0.46, 0.78)	−0.20 (−0.74, 0.35)
Pregnancy concerns	−0.28 (−0.78, 0.22)	−0.16 (−0.49, 0.16)	0.02 (−0.26, 0.31)
Separation or divorce	0.04 (−1.00, 1.09)	−0.24 (−0.92, 0.44)	0.22 (−0.38, 0.81)
Residential move	−0.39 (−1.00, 0.20)	−0.02 (−0.41, 0.38)	−0.01 (−0.36, 0.34)
Marital problems	0.70 (−0.03, 1.42)	0.47 (0.00, 0.95)	0.11 (−0.30, 0.53)
Problems with your children	−0.84 (−1.63, −0.07)	−0.39 (−0.90, 0.12)	−0.42 (−0.86, 0.02)
Money problems	−0.08 (−0.64, 0.48)	−0.11 (−0.48, 0.25)	−0.15 (−0.47, 0.18)

[§] β-coefficient effect estimates and 95% CI, for the stress strata modelled as a categorical (yes/no) explanatory variable in the multivariate linear regression models of transformed outcomes [cubic root (sperm concentration, total sperm count), natural logarithm (testicular volume and serum hormones), logit-transformation (%progressively motile spermatozoa (class a + b))] with mutual adjustment for other stressors in GW18 and adjustments for co-variates as indicated below.

[a] Men who reported spillage ($n = 29$) during masturbation were excluded from analyses.

Models adjusted for:

[b] Maternal BMI

[c] Maternal socioeconomic status [total household annual income: dichotomized to reflect a minimum income level (<$24 000 p.a. or ≥ $24 000 p.a.) according to the Australian Government guidelines at the time (1989–1991)]

[d] Abstinence time (categorical: <2 days, 2–5 days, and >5 days).

[e] Parity (continuous).

[f] Time from ejaculation to semen analysis (continuous, minutes).

concentration and progressive sperm motility. Pregnancy concerns were also strongly associated with declines in total sperm counts and sperm concentration, with no statistical significance (Table IV).

Discussion

This is the first study to examine the prospective relationships between exposures to early and late gestational SLEs and reproductive function using direct assessments of male reproductive function in young adult men. We found significant negative associations between maternal SLEs in early pregnancy (18 weeks gestation) and reduced total sperm count and borderline negative associations with progressive motility and morning serum testosterone levels. No similar effects of maternal stress in late pregnancy were detected. The strongest negative effect estimates for the individual items on the 10-point (yes/no) questionnaire were noted for problems with children on reduced total sperm counts and progressive sperm motility and for pregnancy concerns and on reduced total sperm counts.

These potential associations could provide important insight into the decline of total sperm count in Western men (Levine *et al.*, 2017), which has been, apart from genetic and direct spermatogenic damage, largely unexplained. Our finding supports the hypothesis that exposure to SLEs in early pregnancy within the window considered vulnerable for the development of male reproductive organs may have important lifelong adverse effects on male reproductive function. This contrasts with the absence of any significant effect of exposure to maternal stress in late gestation. The independent reductions in morning serum testosterone and possible reductions in the testosterone/LH-ratio that we note in this study are compatible with negative effects of early gestational SLE exposure on mature Leydig cell function.

Two-thirds of mothers in the study reported at least one SLE during their pregnancy, which is more than the 42% of pregnant women reported by The Australian Bureau of Statistics (ABS, 2018). Pregnancy concerns constituted the most commonly reported individual item and was identified as a specific SLE strongly negatively correlated with the outcomes. The inclusion of maternal pregnancy concerns as an SLE is considered important in this selected population, as psychological stress caused by nausea, discomfort, complications or pregnancy thoughts about the actual birth, implications for relationships, work and finance can be viewed as stressful *per se* (Saunders *et al.*, 2006). However, this inclusion may also have indirectly brought into consideration other non-stress related pregnancy risk factors that may in themselves lead to reduced male reproductive health in offspring, leading to the possibility of residual confounding of our statistical models.

Although we noted that women experiencing their first pregnancy were more likely to report SLE exposures, we could not attribute the prevalence of the item 'pregnancy concerns' to this group as this item was equally distributed among women in first pregnancy and women who had experienced at least one previous pregnancy (not shown in tables). Further, we detected no significant effect modification of the association between early gestational stress and main semen outcomes, by number of pregnancies (not shown in tables).

The SLE relating to problems with children was likewise strongly negatively correlated with the outcomes; there are several reasons why

having children at home could be stressful, such as arguments, teenage problems, problems in school etc. However, the question may have likewise indirectly brought into consideration non-stress reactions such as concerns about the future child or risk of an anomaly that may in themselves lead to reduced male reproductive health in offspring via biological mechanisms, leading to the possibility of residual confounding of our statistical models.

Possible mechanisms

The biology of pregnancy and the background for the associations between early gestational exposure to SLEs and male reproductive function is complex and not fully understood. Within the critical male reproductive developmental period (early gestation 8–14 weeks) sufficient androgen exposure is needed to ensure subsequent normal differentiation and growth of male reproductive organs. Recent evidence suggests that exposure to maternal SLEs may reduce androgen activity (Barrett and Swan, 2015), and evidence from previous epidemiological studies indicates that reduced net fetal androgen actions may be associated with male infertility, poor semen quality and reduced testosterone levels in adulthood (Dean and Sharpe, 2013; Thankamony *et al.*, 2014), which could explain the biological mechanisms behind of the associations we report in the present study.

There could also be some form of epigenetic inheritance such that genes affected by stress in the mother carry epigenetic markers that are inherited by the son. Future studies identifying these potential pathways are needed and could explain the associations we observed.

Strengths and limitations

We benefited from a large sample size and an objective detailed direct assessment of adult male reproductive function including ultrasound techniques for testicular volume and high quality clinical methodology for semen parameters and serum hormones, with strict external quality control for sperm quality, as well as detailed prospectively collected information on prenatal SLEs in two distinct time windows of pregnancy reported by the women in early and late gestation, along with other risk factors. Consequently, there was minimal possibility of recall, information bias and selection bias, as the included men had little knowledge of their own reproductive status and we do not expect that their participation was motivated by any knowledge of the number of SLEs reported by their mothers twenty years earlier. The collection of exposure data from two windows allowed us to determine the effects of the critical time window and compare it to within-individual negative controls in late gestation without counting the same event twice.

There were also challenges in this study. It is not possible to measure how SLEs impacted differently on the mothers' experience or perception of stress. Resilience (coping) gradients may alter cortisol levels and thus modify the associations we observed, and the mothers' own perception of stress severity may have provided a more precise estimate of her exposure. While the mothers experience of items including bereavement, job loss (partner and own), separation/divorce and marital and financial problems are specific, we have no idea what the question regarding problems with children encompasses. Although this affects the opportunity of discussion, it would not affect the results we present here.

The associations between maternal SLE exposures (via the unmeasured stress hormone response) and outcomes may have been non-linear, which can be obscured by standard regression techniques. When assessing our results, we adjusted for several confounders, but unmeasured factors during the pre- and post-natal periods could have occurred and influenced the outcomes of interest. These may have impacted on the developing fetus via biological mechanisms during the prenatal period and/or the infant during breast feeding. In addition, there are numerous potential mechanisms by which maternal factors may have influenced the fetus and neonate via non-biological (e.g. psychological and psychosocial) mechanisms. It is not possible to account for all these potential factors; thus, residual confounding or confounding by these factors unbeknown to us cannot be ruled out. Lifetime exposures of these men are also suspected to play an important role. According to the Australian Bureau of Statistics(ABS, 2018), a greater proportion (49%) of young people aged 20–25 years of age are still living in the parental home, and young men even more likely. This would imply that many of the men included in the present study had not moved out of their parental home. The lifetime shared environment could imply lifetime stressful exposures from mother to son, which may have modified the associations we report here.

Finally, we performed multiple tests, thus increasing the chance of a false positive.

Perspectives

Improved support for women with exposure to SLEs during pregnancy, particularly during the first trimester, may improve the reproductive health of their male offspring in later life.

Conclusion

These novel data support the hypothesis that maternal exposures to SLEs in early gestation may be negatively associated with male reproductive function. Intervention studies of improved pregnancy support could provide more insight and more information is needed about the potential epigenetic mechanisms underlying this association.

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Authors' roles

E.V.B. drafted the manuscript and performed the statistical analyses. A.J., Å.M.H., N.E.S., R.H., M.H. and D.D. contributed to the manuscript preparation. E.V.B., A.J. and R.H. contributed to the concept and design for the study. J.E.D. organized and reviewed all testicular ultrasound

measurements. D.D. prepared data for analyses and E.V.B. contributed with data clean-up. D.D. and E.V.B. collaborated on the statistical strategy. R.H. and D.D. provided all The Raine Study data. All authors contributed to critical interpretation of data and the final draft of the manuscript.

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Conflict of interest

None declared.

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