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Smaller genitals at school age in boys whose mothers were exposed to non-persistent pesticides in early pregnancy

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Summary

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Endocrine disrupting chemicals are believed to play a role in the development of the testicular dysgenesis syndrome. Many pesticides are known to have endocrine disrupting abilities. In a previous study, sons of women who were occupationally exposed to non-persistent pesticides in early pregnancy showed signs of impaired reproductive function (reduced genital size and altered serum hormone concentrations) at three months of age. To assess the possible long-term effects of prenatal pesticide exposure, the boys were re-examined at 6–11 years. The 94 boys (59 exposed, 35 unexposed) underwent genital examinations including ultrasound of testicular volumes, puberty staging (Tanner), anthropometry, and blood sampling. Only a few of the boys had reached puberty ($n = 3$). Among prepubescent boys, testicular volume and penile length (age- and weight-adjusted) were reduced if mothers were exposed to pesticides. The effects were associated with the maternal exposure levels, so that high-exposed boys had smaller genitals than medium-exposed boys, who had smaller genitals than those who were unexposed. Boys of mothers in the high exposure group ($n = 23$) had 24.7% smaller testes (95% CI: -62.2 ; -10.1) and 9.4% shorter penile length (95% CI: -16.8 ; -1.1) compared with the unexposed. The testicular volume and penile length at school age could be tracked to measures from the same boys made at 3 months, e.g. those that had small testes at school age also had small testes at 3 months. Pituitary and testicular hormone serum concentrations did not differ between exposed and unexposed boys. Eight prenatally exposed boys had genital malformations (no unexposed). These boys had smaller testis, shorter penile length and lower inhibin B concentrations than prepubertal boys without genital malformations. The findings support the results obtained at three months of age and indicate that prenatal pesticide exposure has long-term effects on reproductive function in boys.

Introduction

Endocrine disrupting chemicals are suspected of contributing to the high prevalence of genital malformations and low testicular volumes in Danish boys and the low semen quality in Danish men. (Wohlfahrt-Veje *et al.*, 2009b). Many pesticides are known to possess endocrine disrupting abilities. In vitro studies of some modern non-persistent pesticides suggest that they may exert both oestrogenic and anti-androgenic activity and disturb steroidogenic enzymes like CYP19 (aromatase) and

CYP17 (Andersen *et al.*, 2002, 2006; Kjaerstad *et al.*, 2010; Orton *et al.*, 2011). In rats, different fungicides administered during gestation, disturbed the reproductive development in male offspring (Vinggaard *et al.*, 2005; Taxvig *et al.*, 2007). Mixtures of anti-androgenic compounds, including some fungicides, caused a reduction in anogenital distance (AGD) and sex organ weights in male offspring, at low dosages equal to the 'no observed adverse effect' levels for the individual compounds (Christiansen *et al.*, 2009). The potential effects of prenatal exposure to modern pesticides on human reproductive

development are not known. Thus, in 1996, a cohort of children whose mothers worked in greenhouses was established to investigate possible effects of occupational exposure to pesticides on reproductive and endocrine function as well as growth of the children. At three months of age, the boys of exposed mothers had an increased risk of congenital cryptorchidism. In addition, they had shorter penile length, smaller testes, decreased serum testosterone and inhibin B, increased LH/inhibin B ratio and increased concentrations of SHBG and FSH compared with boys of unexposed mothers (Andersen *et al.*, 2008). For individual parameters, only the decreased penile length was statistically significant, but a joint multivariate test of the combined results was significant, suggesting adverse effects on gonadal development. These findings at 3 months may predict future reproductive function as this age is a window with brief activation of the pituitary-gonadal axis (Andersson *et al.*, 1998; Grumbach, 2005; Sharpe, 2006). We therefore re-examined the children at an early school age to assess the possible long-term effects of prenatal pesticide exposure on their reproductive development.

Materials and methods

The cohort and the exposure

From July 1996 to October 2000, pregnant women working in greenhouses in Funen, Denmark, and referred to the department of occupational medicine at Odense University Hospital, Denmark, were recruited (Andersen *et al.*, 2008). Detailed information about working conditions, pesticide use, lifestyle, social factors, reproductive history and health parameters was obtained through interviews at enrolment. Information about pesticide use was confirmed and supplemented by telephone contact to the employers. Then, the women were categorized as occupationally exposed or unexposed (controls). The total pesticide exposure level was categorized independently by two toxicologists into three groups: none/low (controls), medium and high as previously described (Andersen *et al.*, 2002; Wohlfahrt-Veje *et al.*, 2011). The exposure rating was performed before the first examination of the children at 3 months independently (with agreement in all cases) by two toxicologists with special expertise in working conditions in greenhouses. The type and number of pesticides were not taken into consideration in this rating. Approximately 200 different pesticide formulations, representing 121 various, active pesticide ingredients (11 growth regulators, 40 fungicides, 59 insecticides and 11 herbicides), were used in the working areas. In general, the elapsed time between pesticide treatment and the handling of the plants (re-entry interval) was several days for the insecticides, but often only few hours for growth

regulators and fungicides. Therefore, the main exposure for most of the women was assessed to be growth regulators and fungicides.

After medical counselling, women exposed to pesticides were either moved to other work functions with either no or minimal pesticide exposure, or paid leave. Hence, the maternal exposure classification of the women relates to the period before enrolment. Time from conception to removal of the women from an exposed work situation was calculated for all exposed women. Pregnant women with medium exposure worked a median of 39 days, (5–95 percentiles: 15–118 days) and high exposure 40 days (5–95 percentiles: 4–62 days after conception).

At three months, 113 boys were examined (Andersen *et al.*, 2008). At follow-up when the children were between 6 and 11 years of age (2007–2008), 94 boys (59 exposed, 35 unexposed) were examined. Among these were 21 newly recruited unexposed (boys whose mothers had not been occupationally exposed to pesticides during pregnancy), recruited among siblings, friends and classmates of the participants to increase statistical power (Wohlfahrt-Veje *et al.*, 2011).

Questionnaire

Information about maternal lifestyle (including smoking, and alcohol consumption during pregnancy) was obtained from a questionnaire at enrolment in early pregnancy (Andersen *et al.*, 2008; Wohlfahrt-Veje *et al.*, 2011) or at the follow-up examination for the newly recruited unexposed children. Information on birth weight and length were obtained from obstetric records.

Information about maternal smoking in pregnancy (before enrolment) was evaluated (yes/no, mean number of cigarettes) and coded as no = 0, 1–9 cigarettes daily as 1, and ≥ 10 as 2.

Social class of the family was coded based on the parents' education and occupation according to the standards of the Danish National Institute of Social Research (Hansen, Hansen), into five social classes, from 1 (high) to 5 (low). The social class of the highest-ranking parent currently living together with the child was used.

The clinical examination

Ninety-four boys underwent a general physical examination including weight, height, and skin fold measurements. Pubertal staging was performed according to Marshall & Tanner (1970): Genital stage G1–G5 and pubic hair stage PH1–PH5. Boys were also examined with the standardized procedure used by The Nordic Cryptorchidism Study Group including measurements of penile

length (from the pubic bone to the tip of the penis (Boas *et al.*, 2006), testicular position (Boisen *et al.*, 2004), testicular volume by ultrasound (Main *et al.*, 2006), and evaluation of position of the urethral opening/hypospadias (Boisen *et al.*, 2005) in the boys who did not have physiological phimosis. Gynecomastia was assessed by inspection and palpation of the chest.

Eight of the 94 boys did not consent to genital examination. One boy's testicular volume measurement was not feasible due to severely retractile testis.

A single paediatrician (CWV) did all the examinations and was blinded as to information about pesticide exposure.

The study was conducted according to the Helsinki II Declaration and was approved by the Regional Danish Ethics Committee and the Danish Data Protection Agency. All parents gave written consent.

Blood samples and hormone assays

Blood samples were obtained from 84 of 94 boys. Serum was stored at -20°C until analysis. Serum concentrations of FSH, LH, SHBG, oestradiol, testosterone, inhibin B and AMH were measured at the University Department of Growth and Reproduction, Rigshospitalet, Copenhagen.

Serum FSH, LH and SHBG were measured by time-resolved immunofluorometric assays (Delfia Wallas Inc., Turku, Finland) with detection limits (LOD) of 0.06 and 0.05 U/L for FSH and LH respectively, and 0.23 nmol/L for SHBG. Intra and interassay coefficients of variation were less than 5% for gonadotropins assays and less than 6% for SHBG. Total serum oestradiol was measured by RIA (Pantex Corp; Immunodiagnostic Systems Limited, Santa Monica, CA, USA). The detection limit was 18 pmol/L. AMH concentrations were determined using the immunotech Coulter (Immunotech, Beckman Coulter Ltd., Marseille, France) enzyme immunometric assay (LOD of 2.0 pmol/L) (Hagen *et al.*, 2010).

Prolactin was measured with an immuno-fluorometric assay (LOD 5 μL). Serum inhibin B was measured in double antibody enzyme immunometric assay using monoclonal antibodies raised against either the β_{A} or β_{B} -subunit respectively in combination with a labelled antibody against the inhibin α -subunit (Serotec, UK). The detection limit was 20 pg/mL and intra- and interassay variation was less than 13 and 19%.

Total testosterone, DHT, Androstendione and DHEAS were measured at Statens Serum Institut, Copenhagen by tandem mass spectrometry and immunoassay (DHEAS). Detection limits were 0.1, 0.1, 0.1 and 81.4 respectively.

Technicians were blinded to exposure status. All samples were analysed in one batch to reduce assay variation

Statistics

Differences in characteristics between the exposed and unexposed boys were tested by chi square or Fisher's exact test (two categories) for categorical variables and Mann-Whitney *U*-test for continuous variables. Difference in pubertal stage was tested with logistic regression (probability of puberty for age).

Covariates were identified from a priori considerations of relevant factors that might influence the outcome variables. For testicular volume and penile length, age and weight at examination were considered as obligatory covariates. Social class and maternal smoking during pregnancy were considered confounding factors, but were not significantly associated with outcomes and did not change estimates. Hence, they were not included in final models. Differences in testicular volume and penile length between the exposed and unexposed prepubertal boys (boys without clinical signs of puberty and testis volume below 1000 mm³) were tested by multivariate regression analyses. In model 1, only age and weight were adjusted for. The same analysis was repeated with exposure groups (medium and highly exposed) as dummy variables (model 2) and with exposure groups and \pm genital malformation (model 3).

To obtain the best normal distribution of residuals, the variables were log-transformed, and the estimates are expressed as differences between unexposed (reference) and exposed in percentages. Correlations between measures of testicular size and penile length at 3 months and at 6–11 years were tested with multivariate regression adjusting for age and weight at 6–11 years.

Serum hormone concentrations in exposed and unexposed boys were compared using multivariate regression analysis adjusting for age. Log-transformed variables were used in regression analysis when necessary to obtain normal distribution of residuals. Serum concentrations of hormones below LOD were set to $\frac{1}{2}$ LOD. For oestradiol, inhibin B, testosterone, DHT, a non-parametric test (Mann-Whitney) was used to compare exposed and unexposed. We compared the percentages of measurable hormone levels and the percentage of boys with genital malformations in exposed and unexposed using Chi square test (Fisher's test).

Differences in testis volume, penile length and serum hormone concentrations in boys with and without genital malformations were tested by multivariate regression adjusting for age and weight in prepubertal boys.

Results

In Table 1, birth information and characteristics of the children are shown. Among the 86 boys who underwent a

Table 1 Population characteristics. For scale variables: medians (5–95 percentiles), and for categorical variables: number of children (% of group)

| | Unexposed (<i>n</i> = 35) | Pesticide exposed (<i>n</i> = 59) | <i>p</i> value ^a |
|---|--------------------------------|------------------------------------|-----------------------------|
| Birthweight (g) | 3800 (2850; 4720) | 3525 (2321; 4400) | 0.790 |
| Gestational age (days) | 282 (263; 297) | 281 (252–296) | 0.650 |
| Weight for gestational age (% deviation) | 1 (–18; 22) | –4 (–27; 19) | 0.332 |
| Twin or triplet pregnancy | 0 (0) | 7 (7.7) | 0.153 |
| Small for gestational age | 0 (0) | 7 (7.7) | 0.291 |
| Large for gestational age | 3 (7.0) | 1 (1.1) | 0.144 |
| Smoking in pregnancy | 12 (27.9) | 29 (32.6) | 0.352 |
| Social group: 1–3, 4, 5 | 13 (37.1), 15 (42.9), 7 (20.0) | 11 (18.6), 32(54.2), 16 (27.1) | 0.137 |
| Ethnicity other than Caucasian | 3 (8.6) | 3 (5.1) | 0.667 |
| Age at examination (years) | 8.71 (6.39; 11.01) | 8.39 (6.98; 10.32) | 0.519 |
| Weight (kg) | 29 (23; 40) | 30 (24; 44) | 0.462 |
| Height (cm) | 134.9 (120.8; 151.0) | 133.3 (122.6; 148.7) | 0.684 |
| BMI (kg/m ²) | 15.7 (14.3; 19.7) | 16.8 (14.0; 21.8) | 0.054 |
| Body fat % | 16.6 (10.1; 30.4) | 18.4 (11.4; 32.2) | 0.099 |
| In puberty ^b | 3(9.4) (<i>n</i> = 32) | 0 (0) (<i>n</i> = 54) | 0.997 |
| Penile length (cm) ³ | 5.5 (4.1; 6.9) | 5.5 (4.2; 6.3) | 0.311 |
| Testicular volume (mm ³) ^c | 538.4 (339.9; 2252.3) | 479.4 (267.7; 827.5) | 0.051 |
| Cryptorchidism or hypospadias ^d | 0 | 8 (14.3) | 0.047 |

^aDifference between exposed and unexposed tested with Chi square or Fisher's exact test for categorical variables and Mann–Whitney *U*-test for continuous variables.

^b*n* = 86, tested with logistic regression (probability of puberty for age).

^c*n* = 85.

^d*n* = 88.

genital examination, three had entered puberty [testis volume above 1000 mm³ and genital stage 2 (Tanner G2)]. One boy (unexposed, 9.6 years) had pubic hair (PH2), but no other signs of pubertal development (infantile testis, scrotum and penis) and was rated as prepubertal. One boy (exposed, 8.2 years) had gynecomastia.

Eight boys (all prenatally pesticide exposed) had genital malformations (Table 1). Four had high scrotal testis, hereof one with bilateral inguinal testis at 3 months and previous orchidopexy and three boys who had normal scrotal testis at 3 months and therefore classified as cases of acquired cryptorchidism (Wohlfahrt-Veje *et al.*, 2009a). Three boys had spontaneously resolved congenital cryptorchidism (two unilateral, one bilateral). One boy had glandular hypospadias, which had not been diagnosed at 3 months due to phimosis.

Testicular volumes were lower in exposed boys (Table 1), even after excluding pubertal boys and adjusting for weight and age (Table 2, model 1). Reductions in testicular volumes and penile length were related to the prenatal exposure level and were significantly lower in boys of mothers with high exposure level (Table 2, model 2). Testicular volume and penile length at age 6–11 correlated significantly (*p* < 0.01) with measures from same boy at 3 months (adjusted for age and weight at 6–11 years), for example, boys with low volumes at school age also had low volumes at 3 months (Fig. 1).

The differences between exposed and unexposed were partly, but not entirely, caused by the boys with genital malformations (Table 2, model 3). After excluding the boys with genital malformations, the exposure estimates were reduced [testis volume: –10.3% (95% CI: –21.5; 2.6), penile length: –2.7 (95% CI: –8.8; 3.8)]. However, testis volume was still significantly reduced among boys whose mothers were in the high exposure group [–25.2% (95% CI: –36.9; –11.3)]. Boys with genital malformations had shorter penile length, smaller testis volume, lower inhibin B, higher FSH/inhibin B ratio and higher DHEAS than the healthy boys (Table 3). Social grouping, smoking in pregnancy, birth weight and being SGA was not significantly associated with having a genital malformation.

There was a non-significant trend towards higher hormone concentrations of DHEAS and Androstenedione and lower concentrations of SHBG in exposed boys. Apart from this, serum hormone concentrations were not significantly different between the exposed and unexposed boys (Table 4).

Discussion

Boys who were prenatally exposed to modern non-persistent pesticides in early pregnancy had smaller testes at school age than unexposed boys. Boys with mothers in the high exposure group also had significantly shorter penile lengths. There was a dose-dependent association,

Table 2 Reduction in genital size (relative difference in percent (95% CI)) in prepubertal boys prenatally exposed to pesticides from multiple regression analyses

| Testis volume (log-transformed) | Model 1 ^a B (5;95 CI) | Model 2 ^b B (5;95 CI) | Model 3 ^c B (5;95 CI) |
|---------------------------------|----------------------------------|----------------------------------|----------------------------------|
| Exposure | -14.1 (-25.4; -1.1)* | | |
| Medium exposure | | -8.0 (-20.6; 6.6) | -3.6 (-16.4; 11.2) |
| High exposure | | -24.7 (-62.2; -10.1)* | -21.3 (-33.6; -6.9)* |
| Genital malformation | | | -31.6 (-46.2; -12.9)* |
| Penile length (log-transformed) | | | |
| Exposure | -4.1 (-10.5; 2.6) | | |
| Medium exposure | | -1.1 (-8.1; 6.1) | 0.9 (-6.0; 8.4) |
| High exposure | | -9.4 (-16.8; -1.1)* | -8.0 (-15.3; 0)* |
| Genital malformation | | | -13.5 (-22.7; -3.4)* |

* $p < 0.005$.

^aPrenatal pesticide exposure (yes/no), age and weight in model.

^bPrenatal pesticide exposure (medium/ high as dummy variables), age and weight in model.

^cPrenatal pesticide exposure (medium/ high as dummy variables) genital malformation (yes/no), age and weight in model.

Examples:

Testicular volume is 21.3% and penile length is 8.0% lower in boys with highly exposed mother after adjustment for age, weight and genital malformation.

Boys with genital malformations have 31.6% lower testicular volume and 13.5% lower penile length after adjustment for age, weight and exposure status.

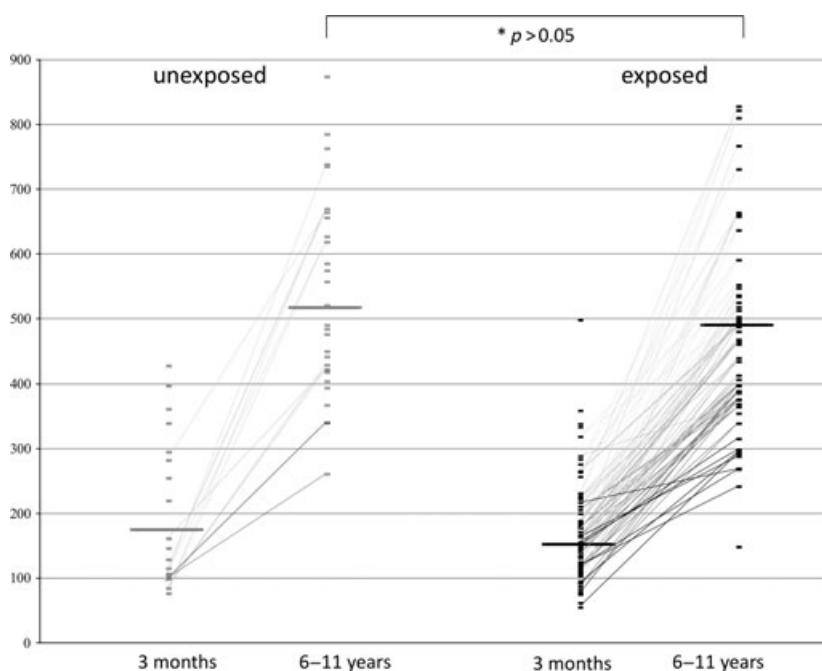


Figure 1 Testicular volumes by ultrasound (mm^3) at 3 months and at 6–11 years (prepubertal) in boys prenatally exposed and unexposed to modern pesticides. Examinations of the same boy are connected with lines (black lines for those with lowest volume <300 at 6–11 years, grey >300 and <400 , light grey >400). Geometric means are shown as horizontal lines. *Testicular volumes are significantly lower in exposed at age 6–11 compared with unexposed after adjustment for age and weight with multiple regression analysis.

i.e. sons of women in the high exposure group had smaller genital size compared with sons of women who had medium exposure who, in turn, had smaller genital size than those who were unexposed. This was only partly

explained by a smaller testis volume in boys with genital malformations which was more frequent in the exposed group. There was a correlation between the testicular volumes and penile length at 3 months and school age

Table 3 Reduction in genital size and differences in hormone concentrations [relative difference in percent (95% CI)] in prepubertal boys with genital malformations ($n = 7$) compared with all other prepubertal boys in the cohort without genital malformations ($n = 76$). Multiple regression analyses were performed adjusting for age and weight on log-transformed outcomes

| | B (95% CI) | <i>P</i> value |
|--------------------------------------|----------------------|----------------|
| Testicular volume (mm ³) | -34.2 (-48.5; -15.9) | 0.001 |
| Penile length (cm) | -13.7 (-22.7; -3.4) | 0.011 |
| Inhibin B (pg/mL) | -27.7 (-47.8; 0.4) | 0.053 |
| FSH (U/L) | 13.5 (-23.6; 68.5) | 0.524 |
| FSH/inhibinB ratio | 59.6 (1.6; 150.0) | 0.042 |
| SHBG (nmol/L) | 6.7 (-23.6; 40.3) | 0.646 |
| Prolactin (miu/L) | -22.08 (-56.7; 40.6) | 0.402 |
| Androstenedione (nmol/L) | 26.2 (-22.6; 106.1) | 0.346 |
| DHEAS (nmol/L) | 80.7 (3.0; 217.7) | 0.039 |
| AMH (pmol/L) | -18.0 (-42.5; 16.9) | 0.271 |

among the same boys i.e. those that had small testes at school age also had small testes at 3 months.

The small number of boys who had entered puberty at the time of examination does not allow us to make conclusions about effects on pubertal timing of the boys. We found no evidence of early pubertal development among the exposed boys as reported for the exposed girls from the same cohort (Wohlfahrt-Veje *et al.*, 2012). No significant differences were found in pituitary and testicular hormones between the exposed and unexposed boys. However, Leydig cell function cannot be evaluated as serum concentrations of LH and Testosterone in prepubertal boys are typically below LOD.

In line with the findings in the girls of the same cohort (Wohlfahrt-Veje *et al.*, 2012), the exposed boys had slightly (not significantly) higher Androstenedione and

DHEAS concentrations compared with the unexposed, indicating an earlier or more pronounced adrenarche. We are not able to discern whether this may be a direct pesticide effect on the adrenals by interference with enzymes in steroidogenesis (Andersen *et al.*, 2002), or by indirect effects on body composition (Sorensen *et al.*, 2007) as prenatal pesticide exposure was also associated with higher body fat at school age (Wohlfahrt-Veje *et al.*, 2011). SHBG concentrations were slightly lower in exposed boys which could also be secondary to higher body fat percentage (Sorensen *et al.*, 2007).

As cryptorchidism is quite frequent in the Danish population (Wohlfahrt-Veje *et al.*, 2009a), we would have expected to find mild forms of genital malformation among both unexposed and exposed boys. However, in our study group, all genital malformations were among boys prenatally exposed to pesticides. The majority were mild cases. As previously found on a population level (Suomi *et al.*, 2006), these boys had shorter penile length, smaller testicular volume, lower inhibin B and higher FSH/inhibin B ratio than healthy boys, indicating a mild degree of testicular dysgenesis. Although testicular volume is weakly associated with sperm parameters in adult men (Mendiola *et al.*, 2011), and perinatal factors are known to influence adult testis function (Sharpe, 2006), as of date, we have no certain indication whether our findings are predictive of reduced adult testis function in these boys.

Testicular volume was measured with ultrasound as it is a more sensitive and accurate measure than orchidometry (Lenz *et al.*, 1994; Kollin *et al.*, 2006; Main *et al.*, 2006). Genital size increases slightly during childhood as the boys grow (Tomova *et al.*, 2010), and therefore results were adjusted for chronological age and body size. In

Table 4 Serum concentrations (geometric means, 5–95 percentiles) of reproductive hormones in prenatally pesticide unexposed and exposed boys in greenhouse cohort. *p* values are from chi square Fishers test (below or above LOD), Mann–Whitney test, multivariate regression analysis correcting levels for age

| | Unexposed ($n = 31$) | Below LOD | | Below LOD n (%) | Chi square <i>p</i> value | Mann–Whitney <i>U</i> -test <i>p</i> value | Age-adjusted <i>p</i> value |
|--|------------------------|-----------|----------------------|----------------------|------------------------------|---|--------------------------------|
| | | n (%) | Exposed ($n = 53$) | | | | |
| Free testosterone ^a (nmol/L) | 0.0005 (0.0005–0.008) | 16 (52%) | 0.001 (0.0005–0.01) | 28 (52%) | | 0.671 | |
| Total testosterone ^a (nmol/L) | <0.1 (<0.1–0.87) | 16 (52%) | <0.1 (<0.1–1.45) | 28 (52%) | 1 | 0.527 | |
| SHBG (nmol/L) | 110 (52–196) | 0 | 99 (43–169) | 0 | | | 0.115 |
| Inhibin B (pg/mL) | 90 (35–273) | 0 | 85 (46–154) | 0 | | | 0.425 |
| LH (U/L) ^a | 0.05 (<0.05–0.44) | 14 (45%) | 0.01 (<0.05–0.26) | 28 (53%) | 0.651 | 0.338 | |
| FSH (U/L) | 0.65 (0.27–1.99) | 0 | 0.55 (0.24–1.48) | 0 | | | 0.315 |
| Prolactin (miu/L) | 101 (45–187) | 0 | 107 (8–213) | 0 | | | 0.733 |
| DHT (nmol/L) ^a | 0.05 (0.05–0.47) | 17 (57%) | 0.05 (0.05–0.45) | 27 (52%) | 0.819 | 0.619 | |
| Androstenedione (nmol/L) | 0.43 (0.14–1.55) | 0 | 0.48 (0.12–1.41) | 0 | | | 0.145 |
| DHEAS (nmol/L) | 1029 (263–4170) | 0 | 1131 (227–5330) | 0 | | | 0.236 |
| AMH (pmol/L) | 521 (177–1040) | 00 | 594 (250–1219) | 0 | | | 0.254 |

^aSerum levels of free testosterone, total testosterone, LH, and DHT were unmeasurable in many boys (hence medians are limits of detection). Here, medians are presented and *p* values for these are from Mann–Whitney *U*-test (unadjusted).

addition, only a few boys had entered puberty and, as this profoundly changes genital size, analyses were performed with prepubertal boys only. We did not exclude the boys with genital malformations from the analyses of genital size as the pesticide-related associations are a continuum. Some boys have smaller genitals, some have genital malformations and some have both. However, reduced genital size was also found after adjustment for, or the exclusion of, boys with genital malformations.

The mothers were exposed to a variety of different pesticide combinations, which makes it difficult to identify the specific compounds responsible for the effects. The main exposure for most of the women was assessed to be fungicides and growth regulators due to a shorter elapsed time between treatment and handling of plants for these pesticides. However, also inert ingredients in the pesticide formulations may have contributed to the found associations.

Our findings are supported by other studies that have found reproductive effects in male rats after gestational exposure to non-persistent pesticides, including increased frequency of hypospadias, nipple retention, reduced anogenital distance, reduced reproductive organ weights, reduced testosterone levels and delayed pubertal development (Noriega *et al.*, 2005; Vinggaard *et al.*, 2005; Laier *et al.*, 2006; Blystone *et al.*, 2007; Orton *et al.*, 2011). In combinations, both cumulative and synergistic effects of the tested pesticides have been found (Hass *et al.*, 2007; Christiansen *et al.*, 2008, 2009; Rider *et al.*, 2009). Furthermore, several human studies have found that genital malformations may be linked to parental pesticide exposure (Garcia-Rodriguez *et al.*, 1996; Kristensen *et al.*, 1997; Weidner *et al.*, 1999; Damgaard *et al.*, 2006; Rocheleau *et al.*, 2009). Most recently, Gaspari *et al.* observed an OR of 4.41 for genital malformations in humans (cryptorchidism, hypospadias and micropenis) when parents were exposed to pesticides (Gaspari *et al.*, 2011).

As our study group was small and some unexposed boys were lost to follow-up since infancy, new unexposed children were recruited at school age to gain statistical power. This introduced small differences in social class between exposed and unexposed, thereby causing a possible selection bias. However, none of the outcomes was associated with maternal smoking in pregnancy or social class in this study and estimates did not change if these were included in models.

The classification of mothers as unexposed or medium/highly exposed was done in early pregnancy completely blinded to subsequent child outcomes and is, to our best belief, a valid classification. This is supported by the fact that we found an exposure-dependent trend related to impaired reproductive function. All clinical

examinations and analyses of serum samples were likewise blinded to exposure information.

It is our experience that inter-observer variation may have a considerable impact on measures of testes volume and penile length (Boas *et al.*, 2006; Main *et al.*, 2006), and the fact that all boys were seen by the same paediatrician is therefore an important strength of this study.

In conclusion, our study supports the previous findings of signs of impaired reproductive function in boys prenatally exposed to modern pesticides. The exposure was associated with smaller genital size and higher frequency of genital malformations in boys at school age. Testicular volumes and penile length at school age could be tracked to measurements from the same boys made at 3 months, e.g. those who had small testes at school age, also had small testes at 3 months, suggesting a persisting reproductive impairment in prenatally exposed boys. Thus, current protection measures for pregnant women appear to be insufficient to prevent adverse reproductive effects in the male offspring.

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