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Occupational exposure to pesticides, reproductive hormone levels and sperm quality in young Brazilian men

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ABSTRACT

The association of occupational exposure to current-use pesticides with reproductive hormones, semen quality, and genital measures was investigated among young men in the South of Brazil. A cross-sectional study was conducted in 99 rural and 36 urban men aged 18–23 years. Information on pesticide use was obtained through questionnaire. Serum and semen samples were analyzed for sex hormones and sperm parameters, respectively, and measurement of anogenital distance (AGD) and testis volume (TV) were performed. Associations were explored using multivariate linear regression. Rural men had poorer sperm morphology, higher sperm count, and lower LH levels relative to urban subjects. Lifetime use of pesticides, especially herbicides and fungicides, was associated with poorer morphology and reduced LH and prolactin, with evidence of a linear pattern. Maternal farming during pregnancy was associated with larger AGD and TV. Chronic occupational exposure to modern pesticides may affect reproductive outcomes in young men.

1. Introduction

Endocrine disrupting chemicals (EDCs) are compounds that alter the normal functioning of the endocrine system of both wildlife and humans [1]. Increasing human exposure to EDCs has been hypothesized to play a causative role in some of the observed adverse trends in male reproductive health, such as undescended testes, hypospadias, decreased sperm quality, and testicular cancer [2].

Many modern pesticides possess hormonal activity and have thus been classified as EDCs. *In vitro* studies of a variety of non-persistent pesticides suggest that they may exert estrogenic and anti-androgenic activity and disturb sex steroid-synthesizing enzymes [3,4]. Given their endocrine-disrupting potential, several epidemiological studies have explored the association between exposure to currently-used pesticides and altered sperm quality [5–8]. Most data are from occupational studies linking exposure to moderate-high levels of multiple pesticides and poor semen quality, based on decreased concentration and motility, low percentage of morphologically normal spermatozoa, and changes in sperm DNA [6,9]. Because pesticides are a heterogeneous group of substances with diverse chemical structures and mechanisms of action, some pesticides may directly affect spermatogenesis by damaging or destroying Sertoli cells and other spermatogenesis support cells, whereas others may act indirectly by altering hormonal signaling [10]. Accordingly, a growing number of human studies have demonstrated an association between exposure to certain non-persistent pesticides and changes in circulating lev-

Abbreviations: AChE, acetylcholinesterase; AGD, anogenital distance; BChE, butyrylcholinesterase; EDCs, endocrine disrupting chemicals; FAI, free androgen index; FSH, follicle-stimulating hormone; LH, luteinizing hormone; OP, organophosphate; SHBG, sex hormone-binding globulin; TDS, testicular dysgenesis syndrome; TV, testicular volume.

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Table 1
Sociodemographic and occupational characteristics, lifestyle, gestational and birth factors of study population.

N (%)	Total N = 135	Rural N = 99	Urban N = 36	p-value[a]
Sociodemographic and occupation				
Age (years)				
18–20	77 (57.0)	63 (63.6)	14 (38.9)	0.01
21–23	58 (43.0)	36 (36.4)	22 (61.1)	
Years of education				
≤8	12 (8.9)	12 (12.1)	0	<0.01
9–11	81 (60.0)	64 (64.6)	17 (47.2)	
≥12	42 (31.1)	23 (23.2)	19 (52.8)	
Occupation				
Non farmer	65 (48.1)	29 (29.3)	30 (100)	<0.01
Farmer	70 (51.9)	70 (70.7)	0	
Employed in the last 3 months				
No	9 (6.7)	3 (3.0)	6 (16.7)	0.01
Yes	126 (93.3)	96 (97.0)	30 (83.3)	
Lifestyle				
Current smoking status				
Non-smokers	127 (94.1)	91 (91.9)	36 (100)	0.11
Smokers	8 (5.9)	8 (8.1)	0 (0)	
Regular alcohol intake in the last 30 days				
No	54 (40.0)	32 (32.2)	22 (61.1)	<0.01
Yes	81 (60.0)	67 (67.7)	14 (38.9)	
Regular physical activity in the last 3 months				
No	53 (39.3)	34 (34.3)	19 (52.8)	0.05
Yes	82 (60.7)	65 (65.7)	17 (47.2)	
Stress in the last 3 months				
No	100 (74.1)	69 (69.7)	31 (86.1)	0.05
Yes	35 (25.9)	30 (30.3)	5 (13.9)	
Body mass index (BMI)				
Eutrophic (<25 kg/m^2)	91 (67.4)	68 (68.7)	23 (63.9)	0.60
Overweight or obese (≥25 kg/m^2)	44 (32.6)	31 (31.3)	13 (36.1)	
Gestation and birth				
Maternal occupation during pregnancy				
Non farmer	66 (48.9)	33 (33.3)	33 (91.7)	<0.01
Farmer	69 (51.1)	66 (66.7)	3 (8.3)	
Maternal smoking during pregnancy				
No	120 (88.9)	87 (87.9)	33 (91.7)	0.76
Yes	15 (11.1)	12 (12.1)	3 (8.3)	
Premature birth				
No	106 (78.5)	82 (82.8)	26 (72.2)	0.17
Yes	27 (21.5)	17 (17.2)	10 (27.8)	
Birth weight (g)				
<2500	11 (8.1)	8 (8.1)	3 (8.3)	1.00
≥2500	124 (91.9)	91 (91.9)	33 (91.7)	
Birth length (cm)				
<50	55 (40.7)	40 (40.4)	15 (41.7)	1.00
≥50	80 (59.3)	59 (59.6)	21 (58.3)	

SD: standard deviation.
[a] Chi-square or Fisher test.

els of reproductive hormones, particularly among occupationally exposed males [11–16]. Despite this, epidemiological evidence on the effects of pesticides in current use on male reproductive system remains inconclusive.

Male anogenital distance (AGD), which is the distance from anus to scrotum, has been used as a measure of androgen status in experimental animals [10]. In human males, testicular volume (TV) and penile length have traditionally been used as indicators of androgenicity [17], and use of AGD has been rare [18]. However, recent epidemiological studies suggest that *in utero* exposure to anti-androgenic compounds may be associated with shortened AGD [18,19], whilst shorter AGD may predict poorer testicular function in adult men [20,21].

The effects of environmental and occupational exposure to pesticides on male reproductive system are of continuing concern. Developing countries account for one third of global pesticide consumption. In 2008, Brazil became the world's largest consumer [22], with continued use of active ingredients already banned in other countries. Serra Gaúcha is a mountainous region in the South of Brazil settled by German and Italian immigrants characterized by family farms producing fruit, mostly grapes for wine production. The present study aimed to: 1) investigate the association of occupational exposure to modern pesticides with reproductive hormone levels, sperm quality, and TV among rural and urban young men residents from this region, and 2) examine reproductive hormone levels, sperm quality, and AGD and TV measurements in relation to potential gestational exposures, including maternal farming and smoking during pregnancy.

2. Materials and methods

2.1. Study design and population

A cross-sectional study was conducted between 2012 and 2013 with a random sample of men aged 18–23 years from the agricultural population of the municipality of Farroupilha, in Rio Grande do Sul, the southernmost state of Brazil. A control group of the same age was selected from the urban area of the town. Considering a population of about 800 men in the age group of 18–23 years residing in the rural area of Farroupilha, a prevalence of acute pesticide

exposure in the rural population of 7%, a confidence level of 95%, and a margin of error of 5%, the minimum sample size for the study was estimated at 90 young rural men.

Firstly, all rural male residents in the age group under study were identified from the list of rural households of the municipal agriculture office. Among the 180 subjects identified, a sample of 80 men was randomly selected and contacted to participate in the study. An additional sample of 30 rural males was randomly selected from the list of members of the military service for the period between 2009 and 2013. Of the 110 invited rural subjects, 3 (2.7%) refused to participate. The control group was composed of men of the same age residing in the urban area of Farroupilha, randomly selected from the same military service list. From a sample of 50 urban men contacted, 4 (8%) refused to participate in the study. The main reason for the refusal was lack of availability to go to the clinic for semen collection, as the sample collection occurred during work hours. Subjects that previously had a vasectomy, infertility diagnosis or confirmed paternity, endocrine or reproductive disorders such as testicular cancer, and men without physical or mental capacity to answer the questionnaire were excluded from the study (8 rural men and 5 urban men). Finally, urban residents with a history of agricultural work or pesticide use were also excluded from the study (5 individuals), leaving a final sample of 99 rural men and 36 urban men.

The study was approved by the Ethics Committee of the National School of Public Health, Oswaldo Cruz Foundation (ENSP/FIOCRUZ), and all participants read and signed an informed consent form.

2.2. Questionnaire and self-reported exposure variables

Information on demographics, occupation, lifestyle, gestation and birth, agricultural work practices, pesticide use, and medical history was obtained through a structured questionnaire. Trained research staff administered the questionnaires through face-to-face interviews at the participants' residences. The following variables were used in this study: age (continuous and grouped into 18–20 and 21–23 years), years of education (continuous and categorized into ≤8, 9–11 and ≥12 years), occupation (farmer; non farmer), employed in the last 3 months (no; yes), current smoking status (non-smoker; smoker), regular alcohol intake in the last 30 days (no; yes), practiced physical activity regularly in the last 3 months (no; yes), felt stress in the last 3 months (no; yes), current weight (kg) and height (cm), maternal farming during pregnancy (no; yes), maternal smoking during pregnancy (no; yes), premature birth (no: ≥37 weeks; yes: <37 weeks), low birth weight (no: ≥2500 g; yes: <2500 and), and birth length (categorized into <50 and ≥50 cm). Body mass index (BMI) was calculated by dividing weight in kg by height in meters squared and categorized as lower than 25 kg/m^2 (eutrophic) and equal to or greater than 25 kg/m^2 (overweight or obese).

Variables related to agricultural work and pesticide use were years of agricultural work (categorized into <6 and ≥6 years), years mixing or applying pesticides (categorized into ≤1 and >1 year), frequency of mixing or applying pesticides (categorized into <5 and ≥5 days per year), season of interview and blood sample collection (low pesticide use season: from March to September; high pesticide use season: from April to August), use of full personal protective equipment (PPE) (yes; no), and current use of pesticides (all) (no; yes). Age at starting farm work was calculated by subtracting the number of years of agricultural work from the current age, and categorized into two groups: 9–14 years (*i.e.* normal ages of puberty in males) and ≥15 years or never farming. Participants also reported on their current and former use of specific pesticides from a list of products obtained from the Brazilian Entity for Technical Assistance and Rural Extension (EMATER), which provided the trade name of commercial formulations most commonly used in the study area

Table 2
Agricultural work-related characteristics and pesticide use among young rural men (N = 99).

	N (%)
Agricultural work	
Years of agricultural work	
<6	38 (38.4)
≥6	61 (61.6)
Years mixing or applying pesticides	
≤1	20 (20.2)
>1	79 (79.8)
Days per year mixing or applying pesticides	
<5	20 (20.2)
≥5	79 (79.8)
Sampling season	
Low pesticide use season	61 (61.6)
High pesticide use season	38 (38.4)
Use of personal protective equipment (PPE)	
Yes	43 (43.4)
No	56 (56.6)
Current use of pesticides (all)	
No	81 (81.8)
Yes	18 (18.2)
Age at starting farm work	
≥15 years or never farming	48 (48.5)
9–14 years	51 (51.5)
Current pesticide use by functional class	
Fungicides	
No	90 (90.9)
Yes	9 (9.1)
Insecticides	
No	89 (89.9)
Yes	10 (10.1)
Herbicides	
No	85 (85.9)
Yes	14 (14.1)
Current pesticide use by chemical class	
OP insecticides	
No	91 (91.9)
Yes	8 (8.1)
Dithiocarbamate fungicides	
No	93 (93.9)
Yes	6 (6.1)
Carbamates	
No	99 (100)
Yes	0 (0)
Synthetic pyrethroids	
No	96 (97.0)
Yes	3 (3.0)
Other chemical classes	
No	84 (84.8)
Yes	15 (15.2)
Lifetime years of pesticide use by class	
All pesticides	
<1	18 (18.2)
1–5	43 (43.4)
≥6	38 (38.4)
Fungicides	
<1	27 (27.3)
1–5	37 (37.4)
≥6	35 (35.4)
Insecticides	
<1	46 (46.5)
1–5	28 (28.3)
≥6	25 (25.3)
Herbicides	
<1	23 (23.2)
1–5	39 (39.4)
≥6	37 (37.4)
OP insecticides	
<1	48 (48.5)
1–5	28 (28.3)
≥6	23 (23.2)
Dithiocarbamate fungicides	
<1	36 (36.4)
1–5	33 (33.3)
≥6	30 (30.3)
Other chemical classes[a]	
<1	18 (18.2)

Table 2 (*Continued*)

	N (%)
1–5	43 (43.4)
≥6	38 (38.4)
Lifetime years of use of specific pesticides	
Mancozeb	
Never	43 (43.4)
1–5	28 (28.3)
≥6	28 (28.3)
Glyphosate	
Never	35 (35.4)
1–5	26 (26.3)
≥6	38 (38.3)
Paraquat	
Never	36 (36.4)
1–5	31 (31.3)
≥6	32 (32.3)

[a] Synthetic pyrethroids and carbamates included.

(Table S1). Participants were also asked to report on the use of any pesticide not included in this list. Active ingredients of commercial products were then grouped into chemical and functional classes, *i.e.*: fungicides, insecticides, herbicides, organophosphate (OP) insecticides, dithiocarbamate fungicides, carbamates, synthetic pyrethroids, and others chemical classes. Lifetime years of overall pesticide use and for each functional and chemical class were calculated as the difference between starting and finishing years of use, regardless of simultaneous use of different pesticides of the same class, and categorized as less than 1 year, 1–5, and 6 or more years. Variables of lifetime years of use of mancozeb, glyphosate, and paraquat were also determined and categorized into never, 1–5, and ≥6 years. These three pesticides are potential EDCs [23–27] and were those most often used among participants (Table S1). Particularly, mancozeb is widely used in grape vines and peach and plum trees grown in the study area.

2.3. Biological samples

2.3.1. Blood collection and analysis

During home visits to participants, an intravenous blood sample (15 mL) was collected in heparin tube between 8:30 and 10:00 in the morning after 12 h overnight fast. Plasma and serum were separated from whole blood by centrifugation. Specimens were stored at −20 °C in vacutainer tubes containing EDTA.

Serum concentrations of total testosterone, luteinizing hormone (LH), follicle-stimulating hormone (FSH), sex hormone-binding globulin (SHBG), and prolactin were measured by electrochemiluminescence immunoassay using Roche® kit. Free androgen index (FAI) was calculated as the percentage molar ratio of total testosterone to SHBG levels. The ratio of testosterone to LH, which represents a measure of Leydig cell function, was calculated by dividing testosterone (ng/dL) by LH (IU/dL). Normal laboratory reference range was 249–836 ng/dL for total testosterone, 1.7–8.6 IU/L for LH, 1.5–12.4 IU/L for FSH, 13–71 nmol/L for SHBG, 4.04–15.02 ng/mL for prolactin, and 30–150% for FAI.

In addition, blood acetylcholinesterase (AChE) and plasma butyrylcholinesterase (BChE) enzyme activities were determined following an optimized protocol that has been previously described in detail [28]. Laboratory reference cutoff values for enzymatic inhibition were ≤0.56 μmol/min/mg of protein for AChE and ≤2.29 μmol/min/mL of plasma for BChE.

2.3.2. Semen collection and analysis

All participants were asked to abstain from ejaculation for at least 48 h. Collection, handling, preparation, and analysis of sperm samples manually followed the standard methods established by the World Health Organization in 2010 [29]. Semen specimens were collected by masturbation and stored in polystyrene tubes. Samples were kept on a hot plate at 37 °C (90 °F) for 30 min to obtain full liquefaction and were subsequently analyzed by the same embryologist.

The macroscopic parameters analyzed were semen volume (mL) and pH. Microscopic parameters included: sperm concentration (million per mL) and total number of sperm (sperm concentration × semen volume); progressive sperm motility, as the percentage of motile sperm (number of motile sperm/total number of sperm); and sperm morphology, as the percentage of sperm with normal morphology.

The volume was measured by suctioning the entire sample using a pipette. Sperm pH was checked with an indicator strip presenting a range going from 7.0 to 8.0, on which an aliquot of semen was deposited and the color obtained through comparing it with colorimetric pH calibration tape. For microscopic analysis of sperm concentration and sperm motility, a volume of 5 μL liquefied semen was deposited in the center of the Makler® chamber with the aid of a micropipette. The analysis was performed under phase microscopy at 200-fold increase (ocular 2000). Sperm morphology was evaluated according to Kruger's criteria [30]. Assessment of sperm morphology was performed by the Panoptic staining method using smears of 5 μL aliquots of fresh semen with frost-ended blades. The methodology consisted of passing of the following reagents: alcohol 75°, plunging the blade 5× and allowing it to dry, xanthene solution 0.1%, dipping 3× and allowing it to dry, thiazine 0,1%, dipping 5× and allowing it to dry. This procedure was carried out by reading through an immersion objective (ocular 1000) and sperm morphology was classified as having at least 100 sperm. It should be noted that the Kruger methodology uses Papanicolau stain and the laboratory responsible for sperm analysis uses slides that are stained with Panoptic stain.

According to laboratory reference values, an abnormal semen sample was defined by one of the following criteria: sperm concentration below 15×10^6/mL, percentage of motile sperm below 32%, and percentage of sperm with normal morphology below 2%. According to a study conducted by the laboratory in patients undergoing vasectomy, the reference value for morphology was set at 2% and not 4% because the coloration used was different from the originally described by Kruger (unpublished data).

2.4. Genital measurements

After semen collection, an urologist specialist in reproductive medicine measured width, length, and height (cm) of both testicles and AGD (cm), using a digital caliper. AGD is the distance from the posterior aspect of the scrotum to the anal verge. Testicle volume (TV) was calculated using the formula: width × length × height (cm^3) and afterwards, the arithmetic mean of left and right TV was computed. Measurements were performed in the supine, frog-legged position with the legs abducted allowing the soles of the feet to meet.

2.5. Statistical analysis

Frequency distribution, medians, and interquartile ranges were used to describe the characteristics of the participants. The normal distribution of continuous variables was examined using the Kolmogorov-Smirnov test. Comparisons between rural and urban subjects for categorical variables were performed through chi-square and Fisher tests. Differences in distribution of hormone levels, sperm parameters, genital measures, and cholinesterase activity between groups were explored using *t*-test and non-parametric tests.

Table 3
Hormonal profile, semen parameters, genital measures, and cholinesterase activities in urban and rural young men.

	Median (25th–75th percentiles)			% Reduced value[a]			% Elevated value[b]		
	Rural (N = 99)	Urban (N = 36)	p-value[c]	Rural	Urban	p-value[d]	Rural	Urban	p-value[d]
Reproductive hormones									
Testosterone (ng/dL)	542.5 (453.5–671.6)	495.8 (440.0–617.6)	0.55	0	0	1.00	5.1	2.8	1.00
LH (IU/L)	4.4 (3.4–5.7)	5.4 (4.2–7.1)	**0.01**	1.0	2.8	0.40	4.0	13.9	0.09
FSH (IU/L)	3.6 (2.3–5.4)	3.4 (2.0–5.9)	0.57	7.1	8.3	0.90	0	2.8	0.24
SHBG (nmol/L)	25.0 (21.0–34.2)	28.0 (18.9–32.9)	0.89	5.3	5.6	0.96	0	0	1.00
Prolactin (ng/mL)	18.1 (10.4–25.9)	20.6 (16.1–27.4)	**0.05**	2.1	0	0.12	63.9	80.6	0.16
FAI (%)	72.3 (62.5–86.4)	64.6 (55.8–88.9)	0.85	0	0	1.00	0.1	0.1	1.00
T:LH (ng/IU)	1263 (931–1602)	1071 (744–1212)	**0.01**	–	–	–	–	–	–
Semen parameters									
Seminal volume (mL)	2.3 (1.3–3.0)	2.0 (1.9–2.6)	0.64	–	–	–	–	–	–
pH	7.5 (7.5–7.5)	7.5 (7.5–7.5)	0.57	–	–	–	–	–	–
Concentration (x10^6/mL)	88.0 (36.0–160.0)	76.5 (28.0–114.0)	**0.04**	11.1	11.1	1.00	–	–	–
Motility (%)	56.0 (42.6–69.5)	64.6 (51.9–72.9)	**0.01**	12.1	0	0.04	–	–	–
Morphology (%)	1.0 (0.0–2.0)	2.5 (1.0–4.0)	**<0.01**	62.6	30.6	<0.01	–	–	–
Genital measures									
Anogenital distance (AGD) (cm)	1.8 (1.8–2.1)	1.8 (1.6–1.8)	**<0.01**	–	–	–	–	–	–
Testicular volume (TV) (cm^3)									
Right	27.6 (21.7–34.3)	23.2 (16.2–28.4)	**0.02**	–	–	–	–	–	–
Left	25.0 (20.0–30.0)	17.2 (16.0–24.9)	**<0.01**	–	–	–	–	–	–
Mean TV	26.3 (21.0–31.9)	19.2 (16.1–25.7)	**<0.01**						
Cholinesterase activity									
AChE (μmol/min/mg)	0.65 (0.55–0.72)	0.76 (0.70–0.82)	**<0.01**	31.3	2.8	<0.01	–	–	–
BChE (μmol/min/mL)	3.43 (2.74–4.08)	3.33 (2.81–4.02)	0.79	5.2	0	0.32	–	–	–

FAI: Free androgen index; T:LH: Testosterone to LH ratio.
Bold values show statistically significant p-values ($p < 0.05$).
[a] For reproductive hormones and sperm parameters, level below laboratory lower reference limit (testosterone: 249 ng/dL; LH: 1.7 IU/L; FSH: 1.5 IU/L; SHBG: 13 nmol/L; prolactin: 4.04 ng/mL; FAI: 30%; sperm concentration: 15×10^6/mL; motility: 32%; morphology: 2%). For AChE and BChE, frequency of inhibition.
[b] Level above laboratory upper reference limit (testosterone: 836 ng/dL; LH: 8.6 IU/L; FSH: 12.4 IU/L; SHBG: 71 nmol/L; prolactin: 15.02 ng/mL; FAI: 150%).
[c] *T*-test or non-parametric test.
[d] Chi-square or Fisher test.

Association between exposure variables (*i.e.* urban/rural residence, agricultural work-related variables, cholinesterase activity, current and cumulative pesticide use, and gestational/birth-related variables) and male reproductive outcomes (hormone levels, sperm endpoints, AGD, and mean TV) was explored by multivariate linear regression in the pooled sample of rural and urban men, using natural-logarithm transformed (log-transformed) outcome variables, which fitted normal distributions. Regardless of their statistical significance, all models were adjusted for age, smoking status, alcohol consumption, physical activity, years of education, stress, and current BMI, which are variables identified in the literature as potential confounders. The estimated regression coefficients (β) and 95% confidence intervals (CI) were transformed back [exp(β)] on the original scale and expressed as percentage change in dependent variable per one-unit change in exposure variable, i.e. if exposure variable changes by 1 (unit), dependent variable is expected to change by 100·β%. Interaction between maternal farming during pregnancy and subsequent occupational exposures of male participants was also explored. Further multivariate analyses were conducted restricting the sample to rural men, which may be a more homogeneous group. Lastly, given that two different recruitment sources were used for the rural group, sensitivity analysis was performed in the pooled sample by excluding rural subjects recruited from the military service list.

A significance level of 0.05 was established. SPSS version 20.0 (SPSS Inc., Chicago, IL, US) was used for the analysis.

3. Results

Rural men were younger than urban men (64% of rural men were 18–20 years old *versus* 39% of urban men), while urban ones were more likely to have a higher education (Table 1). Most rural men were farmers at the time of the interview and had been employed the last three months. Very few participants reported current smoking, all from the rural area. About 25% of subjects reported feeling stressed, mostly those from the urban area, and one third of both rural and urban males were overweight or obese. Rural men were more likely to drink alcohol and practice physical activity regularly than urban residents. Around two thirds of rural men were born of mothers working in agriculture during pregnancy, whereas frequency among urban subjects was only 8%. Otherwise, gestation and birth characteristics were similar: taking urban and rural men all together, 11% had been exposed to cigarette smoke *in utero*, 21% were born prematurely, 8% were born with low birth weight, and 40% had a birth length <50 cm (Table 1).

Agricultural work-related characteristics and pesticide use among men residing in the rural area are shown in Table 2. Over 60% of subjects reported working for more than 5 years as a farmer, most of them had mixed or applied pesticides for more than 1 year, with a frequency of at least 5 days per year, and half of them had started farm work at the age of 9–14 years old. More than half of the rural participants did not use full PPE regularly, and less than 20% were using pesticides at the time of the interview. Herbicides were the most commonly used type of pesticides by rural men, followed by insecticides and fungicides. Regarding chemical classes, few men reported current use of OP insecticides and dithiocarbamates, very few were using pyrethroids, and none of them were using carbamates. Over one third of rural men had handled pesticides for 6 or more years (range: <1–14), with 37% having used herbicides for the same period of time, followed by fungicides (35%), and insecticides (25%). In regards to individual pesticides, more than half of the rural men reported having used mancozeb, glyphosate, or paraquat at some point.

None of the participants showed low testosterone or androgen insufficiency (FAI <30%) (Table 3). LH was significantly lower in rural relative to urban men, among which 14% had elevated

Table 4
Adjusted[a] regression coefficients (95% confidence intervals) for percentage change[b] in hormone levels associated with exposure variables.

Exposure variables	Testosterone	LH	FSH	SHBG	Prolactin	FAI	T:LH
Rural (ref = urban)	1.05 (0.94–1.16)	**0.81 (0.68–0.95)**	0.94 (0.72–1.21)	1.02 (0.87–1.21)	0.94 (0.74–1.17)	1.03 (0.90–1.17)	**1.30 (1.09–1.55)**
Farmer (ref = non-farmer)	1.03 (0.94–1.13)	0.87 (0.76–1.02)	0.86 (0.70–1.07)	0.99 (0.87–1.13)	1.17 (0.97–1.40)	1.04 (0.94–1.15)	**1.16 (1.01–1.33)**
Years of agricultural working ≥6 (ref= <6)	0.95 (0.87–1.05)	1.13 (0.99–1.28)	1.02 (0.83–1.27)	1.08 (0.95–1.25)	0.95 (0.79–1.15)	0.89 (0.79–1.15)	**0.83 (0.73–0.97)**
Years mixing or applying pesticides >1 (ref = ≤1)	1.02 (0.92–1.12)	**0.84 (0.71–0.96)**	0.84 (0.68–1.05)	0.93 (0.84–1.11)	1.02 (0.83–1.25)	1.05 (0.94–1.16)	**1.22 (1.05–1.42)**
Days/year mixing or applying pesticides ≥5 (ref = <5)	0.99 (0.90–1.09)	**1.21 (1.04–1.39)**	1.15 (0.92–1.43)	1.02 (0.87–1.17)	1.00 (0.83–1.22)	0.97 (0.87–1.08)	0.83 (0.71–1.10)
High pesticide use season (ref = low use season)	0.98 (0.88–1.07)	1.13 (0.97–1.30)	0.95 (0.76–1.19)	0.93 (0.81–1.07)	**1.43 (1.20–1.72)**	1.05 (0.94–1.16)	0.87 (0.75–1.01)
Not using PPE (ref = use of PPE)	**0.89 (0.80–0.98)**	1.02 (0.87–1.19)	1.10 (0.87–1.35)	0.95 (0.83–1.10)	0.98 (0.80–1.20)	0.94 (0.84–1.05)	0.87 (0.75–1.03)
AChE inhibited (ref = normal)	1.04 (0.94–1.15)	0.90 (0.77–1.08)	0.95 (0.73–1.23)	0.97 (0.83–1.14)	0.94 (0.76–1.17)	1.07 (0.95–1.21)	1.15 (0.97–1.36)
BChE inhibited (ref = normal)	1.07 (0.86–1.34)	0.86 (0.61–1.21)	0.95 (0.55–1.63)	1.09 (0.79–1.52)	0.91 (0.57–1.46)	0.80 (0.75–1.28)	1.25 (0.85–1.80)
Lifetime years of pesticide use (ref = <1 year)							
All pesticides							
1–5	0.95 (0.86–1.06)	0.86 (0.73–1.02)	0.93 (0.72–1.21)	0.95 (0.81–1.10)	1.03 (0.83–1.28)	1.01 (0.88–1.15)	1.10 (0.92–1.31)
≥6	0.92 (0.83–1.03)	**0.63 (0.65–0.89)**	0.90 (0.68–1.17)	0.86 (0.73–1.02)	**0.76 (0.60–0.95)**	1.06 (0.93–1.22)	**1.21 (1.02–1.46)**
p–trend	0.14	**0.002**	0.44	0.08	**0.02**	0.35	**0.03**
Fungicides							
1–5	0.95 (0.85–1.06)	0.94 (0.79–1.10)	0.95 (0.74–1.24)	0.91 (0.77–1.06)	1.25 (0.99–1.55)	1.06 (0.94–1.20)	1.03 (0.85–1.22)
≥6	0.96 (0.85–1.06)	**0.82 (0.68–0.97)**	0.94 (0.72–1.22)	0.87 (0.74–1.03)	0.89 (0.72–1.13)	1.08 (0.95–1.25)	1.16 (0.97–1.38)
p–trend	0.32	**0.03**	0.63	0.09	0.53	0.19	0.13
Insecticides							
1–5	1.02 (0.91–1.13)	1.00 (0.84–1.19)	1.01 (0.77–1.30)	0.97 (0.83–1.13)	**1.31 (1.05–1.63)**	1.05 (0.93–1.20)	1.02 (0.84–1.21)
≥6	0.92 (0.83–1.04)	0.85 (0.71–1.03)	1.05 (0.79–1.38)	**0.84 (0.71–0.99)**	0.91 (0.72–1.16)	1.07 (0.93–1.23)	1.08 (0.89–1.31)
p–trend	0.27	0.14	0.81	0.08	0.95	0.23	0.44
Herbicides							
1–5	0.97 (0.87–1.08)	0.85 (0.72–1.01)	0.91 (0.69–1.18)	0.95 (0.81–1.12)	1.15 (0.92–1.43)	1.01 (0.88–1.15)	1.13 (0.95–1.35)
≥6	0.93 (0.84–1.04)	**0.75 (0.64–0.90)**	0.94 (0.71–1.24)	0.89 (0.76–1.05)	**0.76 (0.63–0.97)**	1.04 (0.91–1.19)	**1.22 (1.02–1.46)**
p-trend	0.21	**0.002**	0.64	0.18	**0.05**	0.53	**0.03**
OP insecticides							
1–5	1.02 (0.91–1.13)	1.00 (0.84–1.19)	0.99 (0.76–1.29)	0.97 (0.83–1.14)	**1.31 (1.04–1.63)**	1.05 (0.91–1.18)	1.01 (0.85–1.21)
≥6	**0.89 (0.79–0.98)**	**0.83 (0.68–0.99)**	1.05 (0.79–1.40)	**0.83 (0.69–0.99)**	0.88 (0.68–1.12)	1.07 (0.92–1.20)	1.08 (0.88–1.31)
p–trend	0.14	**0.04**	0.75	0.06	0.86	0.28	0.48
Dithiocarbamate fungicides							
1–5	0.99 (0.88–1.10)	0.89 (0.76–1.06)	0.84 (0.65–1.08)	0.90 (0.77–1.06)	**1.39 (1.12–1.73)**	1.08 (0.96–1.22)	1.10 (0.93–1.32)
≥6	0.99 (0.89–1.12)	**0.83 (0.69–0.98)**	0.93 (0.71–1.22)	0.89 (0.76–1.07)	0.98 (0.78–1.23)	1.10 (0.97–1.26)	**1.19 (1.01–1.45)**
p–trend	0.94	**0.03**	0.48	0.19	0.69	0.10	**0.04**
Other chemical classes[c]							
1–5	0.95 (0.84–1.06)	0.86 (0.73–1.02)	0.84 (0.65–1.09)	0.94 (0.81–1.11)	1.03 (0.83–1.28)	1.01 (0.88–1.15)	1.09 (0.92–1.31)
≥6	0.92 (0.83–1.03)	**0.76 (0.63–0.90)**	0.93 (0.68–1.20)	0.86 (0.73–1.02)	**0.76 (0.60–0.95)**	1.06 (0.93–1.22)	1.21 (0.99–1.46)
p–trend	0.14	**0.002**	0.89	0.09	**0.02**	0.35	**0.03**
Mancozeb (ref = Never)							
1–5	1.02 (0.90–1.14)	0.92 (0.77–1.10)	0.99 (0.75–1.29)	0.93 (0.79–1.10)	**1.32 (1.05–1.67)**	1.09 (0.96–1.25)	1.10 (0.91–1.32)
≥6	1.01 (0.89–1.13)	0.86 (0.71–1.03)	0.92 (0.70–1.22)	0.92 (0.78–1.10)	0.95 (0.75–1.20)	1.08 (0.95–1.25)	1.17 (0.97–1.42)
p–trend	0.84	0.08	0.60	0.33	0.89	0.15	0.08
Glyphosate (ref = Never)							
1–5	1.10 (0.90–1.14)	0.97 (0.81–1.17)	0.88 (0.67–1.17)	0.99 (0.84–1.18)	1.23 (0.97–1.58)	1.02 (0.89–1.17)	1.04 (0.86–1.26)
≥6	0.96 (0.87–1.06)	**0.83 (0.70–0.96)**	0.99 (0.78–1.27)	0.90 (0.78–1.07)	0.89 (0.73–1.10)	1.05 (0.93–1.18)	1.16 (0.99–1.38)
p-trend	0.50	**0.02**	0.88	0.28	0.45	0.41	0.07
Paraquat (ref = Never)							
1–5	1.05 (0.93–1.17)	0.96 (0.81–1.16)	0.98 (0.75–1.29)	1.02 (0.86–1.20)	**1.36 (1.08–1.71)**	1.03 (0.90–1.17)	1.08 (0.89–1.30)
≥6	0.96 (0.86–1.08)	**0.83 (0.69–0.99)**	1.04 (0.79–1.37)	0.88 (0.74–1.05)	0.83 (0.66–1.04)	1.08 (0.95–1.25)	1.16 (0.97–1.40)
p–trend	0.63	**0.05**	0.80	0.20	0.35	0.22	0.11

PPE: Personal protective equipment; FAI: Free androgen index; T:LH: Testosterone to LH ratio; ref: reference category.
Bold values show statistically significant p-values ($p < 0.05$).

[a] Adjusted for age, smoking status, alcohol intake, physical activity, years of education, stress, and BMI (overweight/obese or eutrophic).

[b] An estimate of 1 equals 100%.

[c] Synthetic pyrethroids and carbamates included.

Table 5
Adjusted[a] regression coefficients (95% confidence intervals) for percentage change[b] in sperm parameters and genital measures associated with exposure variables.

Exposure variables	Sperm parameters			TV
	Concentration	Motility	Morphology	
Rural (ref = urban)	**1.58 (1.01–2.48)**	0.88 (0.67–1.16)	**0.71 (0.55–0.93)**	**1.31 (1.14–1.54)**
Farmer (ref = non-farmer)	1.19 (0.83–1.73)	0.90 (0.73–1.14)	0.85 (0.68–1.07)	**1.20 (1.05–1.34)**
Years of agricultural working ≥6 (ref = <6)	0.81 (0.56–1.20)	0.99 (0.78–1.23)	1.19 (0.93–1.54)	**0.84 (0.75–0.96)**
Years mixing or applying pesticides > 1 (ref = ≤1)	1.25 (0.84–1.88)	1.01 (0.78–1.27)	**0.71 (0.56–0.90)**	**1.22 (1.07–1.39)**
Days/year mixing or applying pesticides ≥ 5 (ref= <5)	0.85 (0.58–1.27)	0.99 (0.78–1.26)	**0.80 (0.75–0.98)**	0.84 (0.73–1.01)
High pesticide use season (ref = low use season)	0.87 (0.59–1.30)	1.10 (0.87–1.40)	1.14 (0.88–1.43)	0.88 (0.78–1.01)
Nor using PPE (ref = use of PPE)	0.74 (0.50–1.11)	1.05 (0.82–1.34)	1.15 (0.88–1.46)	**0.85 (0.75–0.97)**
AChE inhibited (ref = normal)	0.77 (0.50–1.21)	0.95 (0.73–1.26)	1.03 (0.75–1.39)	1.13 (1.00–1.36)
BChE inhibited (ref = normal)	0.75 (0.29–1.91)	1.06 (0.60–1.89)	0.95 (0.50–1.79)	1.10 (0.79–1.54)
Current pesticide use (ref = non-use)				
All pesticides	0.76 (0.44–1.31)	0.92 (0.67–1.27)	**0.75 (0.52–0.99)**	1.06 (0.88–1.27)
Fungicides	0.80 (0.37–1.73)	0.67 (0.43–1.04)	0.93 (0.58–1.51)	0.92 (0.72–1.18)
Insecticides	0.73 (0.36–1.43)	1.05 (0.68–1.58)	0.73 (0.49–1.11)	0.99 (0.78–1.26)
Herbicides	0.91 (0.49–1.68)	0.88 (0.62–1.27)	**0.71 (0.50–0.98)**	1.04 (0.85–1.27)
OP insecticides	0.63 (0.29–1.32)	1.05 (0.66–1.66)	0.73 (0.46–1.17)	1.11 (0.85–1.43)
Dithiocarbamate fungicides	0.80 (0.31–2.09)	0.62 (0.99–1.05)	0.88 (0.51–1.58)	0.90 (0.67–1.22)
Other chemical classes[c]	0.95 (0.52–1.73)	0.86 (0.60–1.22)	0.76 (0.54–1.07)	1.04 (0.85–1.26)
Lifetime years of pesticide use (ref = < 1 year)				
All pesticides				
1–5	1.08 (0.68–1.71)	0.95 (0.73–1.26)	**0.75 (0.58–0.98)**	1.15 (0.99–1.34)
≥6	1.23 (0.76–1.99)	1.15 (0.87–1.55)	**0.55 (0.42–0.73)**	**1.17 (1.01–1.39)**
p-trend	0.39	0.33	**<0.001**	**0.04**
Fungicides				
1–5	0.74 (0.47–1.16)	0.94 (0.72–1.25)	0.82 (0.62–1.07)	1.15 (0.99–1.33)
≥6	0.99 (0.62–1.59)	1.10 (0.83–1.46)	**0.54 (0.41–0.73)**	**1.19 (1.03–1.40)**
p-trend	0.87	0.55	**<0.001**	**0.02**
Insecticides				
1–5	0.69 (0.44–1.10)	0.85 (0.64–1.13)	0.90 (0.68–1.21)	1.07 (0.91–1.24)
≥6	1.05 (0.64–1.72)	0.97 (0.73–1.31)	**0.60 (0.45–0.80)**	1.13 (0.96–1.34)
p-trend	0.77	0.62	**0.001**	0.12
Herbicides				
1–5	1.07 (0.76–1.68)	0.85 (0.64–1.13)	**0.76 (0.58–0.99)**	1.13 (0.97–1.32)
≥6	1.21 (0.76–1.93)	0.90 (0.69–1.20)	**0.58 (0.44–0.77)**	1.17 (1.00–1.38)
p-trend	0.44	0.45	**<0.001**	**0.04**
OP insecticides				
1–5	0.71 (0.45–1.12)	0.88 (0.66–1.16)	0.92 (0.70–1.23)	1.06 (0.89–1.25)
≥6	1.15 (0.69–1.89)	1.17 (0.86–1.58)	**0.61 (0.45–0.84)**	1.14 (0.96–1.36)
p–trend	0.99	0.55	**0.004**	0.11
Dithiocarbamate fungicides				
1–5	0.73 (0.46–1.14)	0.92 (0.69–1.21)	0.97 (0.73–1.30)	**1.17 (1.01–1.36)**
≥6	0.98 (0.61–1.60)	1.07 (0.80–1.42)	**0.61 (0.45–0.82)**	**1.19 (1.03–1.42)**
p-trend	0.75	0.79	**0.003**	**0.01**
Other chemical classes[c]				
1–5	1.08 (0.68–1.71)	0.95 (0.73–1.26)	**0.75 (0.58–0.98)**	1.16 (0.99–1.34)
≥6	1.23 (0.76–1.99)	3.15 (0.87–1.55)	**0.55 (0.41–0.73)**	1.08 (1.00–1.38)
p-trend	0.39	0.33	**<0.001**	**0.04**
Mancozeb (ref = Never)				
1–5	0.66 (0.41–1.05)	0.89 (0.67–1.19)	0.99 (0.74–1.34)	**1.19 (1.02–1.40)**
≥6	0.87 (0.54–1.42)	1.01 (0.75–1.37)	**0.66 (0.47–0.90)**	1.12 (0.95–1.32)
p-trend	0.36	0.91	**0.02**	0.08
Glyphosate (ref = Never)				
1–5	1.02 (0.63–1.68)	1.02 (0.76–1.38)	0.91 (0.68–1.23)	1.14 (0.96–1.34)
≥6	1.08 (0.70–1.67)	1.13 (0.87–1.47)	**0.68 (0.52–0.89)**	1.13 (0.97–1.30)
p-trend	0.74	0.37	**0.01**	0.09
Paraquat (ref = Never)				
1–5	0.79 (0.49–1.28)	0.90 (0.67–1.21)	0.90 (0.66–1.21)	1.09 (0.92–1.29)
≥6	1.13 (0.69–1.83)	1.12 (0.84–1.50)	**0.66 (0.48–0.92)**	1.08 (0.91–1.28)
p-trend	0.80	0.56	**0.02**	0.28

PPE: personal protective equipment; TV: testicular volume; ref: reference category.
Bold values show statistically significant p-values (p < 0.05).
[a] Adjusted for age, smoking status, alcohol intake, physical activity, years of education, stress, and BMI (overweight/obese or eutrophic).
[b] An estimate of 1 equals 100%.
[c] Synthetic pyrethroids and carbamates included.

LH. Consequently, a greater testosterone:LH ratio was observed in rural males. Prolactin was elevated in 64% of rural *versus* 81% of urban men. Differences in sperm parameters between groups were observed for concentration, motility, and morphology: rural men had higher sperm concentration but poorer motility and morphology. Among rural subjects, 12 and 63% had abnormal motility and morphology, respectively. None of the urban men had abnormal motility and 31% had poor morphology. Regarding genital measures, rural men had larger AGD and TV. AChE levels were significantly lower among rural residents, while BChE values did not differ between groups.

In multivariate analysis for adult exposures and reproductive hormones (Table 4), testosterone showed a significant reduction of 11% in men not using full PPE. LH levels were reduced by 19 and 16%, respectively, in rural men and those mixing or applying pesticides for more than 1 year. Use of pesticides (all), fungicides, herbicides, OP insecticides, dithiocarbamates, other chemical classes, glyphosate, and paraquat for 6 or more years was significantly associated with reduced LH by 17–37%, showing significant linear trends of decrease with increasing number of years. Conversely, no association was observed between adult exposures and FSH. SHBG was significantly decreased by 16 and 17% in men that had used insecticides and OPs for more than 5 years, respectively. Although prolactin was universally increased, especially in urban participants, levels were 24% lower in rural men with greater number of years using pesticides, herbicides, and other chemical classes, with evidence of linear trends. Otherwise, prolactin was 31–43% higher in men sampled in the high exposure season and those reporting use of insecticides, OPs, dithiocarbamates, mancozeb, and paraquat for 1–5 years. Testosterone:LH ratio was positively associated with rural living, working as a farmer, and duration of mixing or applying pesticides, showing positive dose-response relationships with lifetime use of all pesticides, herbicides, dithiocarbamates, and other chemical classes. Cholinesterase activities were not associated with reproductive hormones (Table 4), nor did reported current use of pesticides (data not shown).

Regarding sperm quality (Table 5), morphology was inversely associated with living in the rural area, mixing or applying pesticides for more than one year and a frequency of at least 5 days per year, and current use of pesticides (all) and herbicides, with reductions of 20–29%. Furthermore, use of pesticides (all), pesticide classes, and individual pesticides for 6 or more years was significantly associated with lowered morphology by 32–46%, showing significant linear trends in decreasing morphology with increasing cumulative use. Sperm count was 58% greater in rural relative to urban men, although it did not show significant association with pesticide use. Motility was not associated with adult pesticide exposure either. As observed for reproductive hormones, cholinesterase inhibition was not associated with sperm parameters. None of the exposure variables showed statistically significant associations with seminal volume or pH (data not shown). In regard to testicular size, TV was 31% larger in rural men, and was also increased in farmers and men mixing or applying pesticides for more than one year. Additionally, TV showed positive dose-response relationships with increasing lifetime use of pesticides (all), fungicides, herbicides, dithiocarbamates, and other chemical classes. As TV, AGD was increased by 9% (95%CI: 4–15%) in rural compared to urban men (data not shown).

Multivariate analysis for gestational and birth-related variables (Table 6) revealed a significant reduction of 21% in sperm morphology and significant increases of 5 and 16%, respectively, in AGD and TV among men born to women who worked in agriculture during pregnancy. Sperm concentration was not associated with gestational exposures, and motility only showed an inverse association with premature birth. Significant reductions of 16 and 33% in testosterone and SHBG levels, respectively, among men exposed to cigarette smoke *in utero* were also found. Additionally, men whose mothers smoked during pregnancy had higher FAI and lower testosterone:LH ratio. LH was reduced by 15% in males with low birth length, and an increase in FSH and a decrease in SHBG were observed among subjects born prematurely.

Interaction analysis showed that the association of lifetime use of pesticides (overall use and across classes) with poorer sperm morphology was strengthened among men exposed to pesticides during gestation. However, the interaction was not statistically significant. No substantial variation was noted in regression estimates for hormone levels, sperm concentration, and motility between men born to women farming during pregnancy and those not exposed to pesticides prenatally (data not shown).

Results of multivariate analysis in rural men were quite similar to those of the pooled sample (Tables S2–S4). Nonetheless, lifetime pesticide use was associated with decreased testosterone and SHBG in the rural sample, while associations of maternal farming during pregnancy with lower sperm morphology and higher genital measures (AGD and TV) did not remain significant. In addition, starting farm work at a younger age was not associated with any of the reproductive outcomes (data not shown). Finally, although some associations with hormone levels lost their statistical significant after excluding the 24 rural subjects recruited from the military service list, sensitivity analysis did not lead to substantial change in the main findings of the study (data not shown).

4. Discussion

The present study investigated reproductive hormone levels, sperm quality, and genital measures in rural compared to urban young men in the South of Brazil, examining their association with occupational exposure to agricultural pesticides. The main findings were 1) the linear dose-response relationship poorer sperm morphology and lifetime use of all pesticides, fungicides, insecticides, herbicides, OP insecticides, dithiocarbamates and other chemical classes, mancozeb, glyphosate, and paraquat; 2) the association of lifetime use of pesticides, particularly herbicides and fungicides, with reduced levels of LH and prolactin; and 3) the association of rural living and maternal farming during pregnancy with larger AGD and TV, but poorer sperm morphology.

The observed reduced levels of LH in rural men and the inverse association between LH and lifetime years of pesticide use could be explained by inappropriate blunted LH in response to a testicular distress. Moreover, positive association of testosterone:LH ratio with rural living, farming, and duration of pesticide mixing/applying suggests that occupational pesticide exposure might have contributed to uncompensated Leydig cell dysfunction. On the other hand, males sampled in the high exposure season experienced greater prolactin levels. The study area has a large seasonal difference in pesticide-use intensity, where the harvesting period occurs from September to March. This finding is not in agreement with a study among Mexican farmers showing no difference in prolactin levels between the heavy and the low pesticide spraying seasons [16]. Seasonal variation in prolactin may depend on the estrogenicity of the used chemicals, and the acute increase observed here is consistent with the negative linear trends seen in LH and prolactin with cumulative pesticide use, suggesting a suppressive effect of chronic pesticide exposure at the pituitary level. In general, results from prior human studies examining prolactin levels in relation to pesticides are conflicting [11,13,14,31].

Despite the fact that no overall association was found between pesticide exposure and testosterone or androgen status in the pooled sample, when the analysis was restricted to rural men, lifetime use of pesticides, particularly herbicides and OP insecticides, seemed to have a detrimental effect on testosterone levels. Inverse association with lifetime OP use is in partial agreement with two previous studies that found urinary OP metabolites to be related to reduced testosterone [11,15] and one study showing lower testosterone in OP sprayers compared to non-exposed males [8]. These findings are supported by experimental studies demonstrating that certain OPs can elicit anti-androgenic activity [3,4] and reduce steroidogenic acute regulatory (StAR) protein expression, resulting in reduction of testosterone production by Leydig cells [32]. Other human studies, however, have shown equivocal results with regard to testosterone or FAI and pesticide exposure [12,15,16,31,33].

Table 6
Adjusted[a] regression coefficients (95% confidence intervals) for percentage change[b] in reproductive outcomes associated with gestation and birth-related variables.

Exposure variables	Reproductive hormones							Sperm parameters			Genital measures	
	Testosterone	LH	FSH	SHBG	Prolactin	FAI	T:LH	Concentration	Motility	Morphology	AGD	TV
Maternal farming	1.05 (0.96–1.14)	0.92 (0.80–1.06)	0.99 (0.91–1.04)	0.98 (0.86–1.12)	0.99 (0.83–1.20)	1.06 (0.96–1.17)	1.13 (0.98–1.30)	0.97 (0.67–1.39)	0.97 (0.77–1.21)	**0.79 (0.63–0.98)**	**1.05 (1.01–1.09)**	**1.16 (1.02–1.30)**
Maternal smoking during pregnancy	**0.84 (0.73–0.95)**	1.10 (0.88–1.38)	1.08 (0.77–1.51)	**0.67 (0.55–0.82)**	**0.72 (0.54–0.94)**	**1.22 (1.04–1.43)**	**0.76 (0.60–0.94)**	0.72 (0.39–1.30)	0.99 (0.69–1.42)	0.84 (0.58–1.23)	0.97 (0.90–1.05)	0.84 (0.68–1.02)
Preterm birth	0.90 (0.81–1.00)	1.06 (0.88–1.26)	**1.31 (1.02–1.70)**	**0.83 (0.70–0.96)**	0.99 (0.79–1.23)	1.09 (0.96–1.23)	0.85 (0.72–1.02)	0.65 (0.41–1.03)	**0.85 (0.64–0.13)**	0.92 (0.69–1.21)	0.99 (0.94–1.05)	0.91 (0.78–1.06)
Low birth weight	0.93 (0.80–1.05)	0.82 (0.64–1.06)	1.11 (0.77–1.63)	0.97 (0.77–1.22)	1.14 (0.83–1.58)	0.95 (0.79–1.15)	1.13 (0.87–1.46)	0.93 (0.48–1.80)	1.18 (0.79–1.75)	0.94 (0.56–1.57)	0.97 (0.90–1.06)	0.88 (0.71–1.12)
Birth length <50 cm	0.95 (0.87–1.04)	**0.85 (0.74–0.98)**	1.16 (0.94–1.42)	1.00 (0.88–1.14)	1.12 (0.94–1.35)	0.95 (0.85–1.05)	1.10 (0.96–1.27)	0.85 (0.59–1.22)	1.02 (0.82–1.27)	1.09 (0.84–1.35)	0.99 (0.95–1.05)	0.95 (0.84–1.07)

FAI: Free androgen index; T:LH: Testosterone to LH ratio; AGD: Anogenital distance; TV: Testicular volume.
Bold values show statistically significant p-values ($p < 0.05$).
[a] Adjusted for age, smoking status, alcohol intake, physical activity, years of education, stress, and BMI (overweight/obese or eutrophic).
[b] An estimate of 1 equals 100%.

Association between cumulative OP use and reduced LH observed in the pooled sample is in agreement with previous studies among farmers [8,12,16]. If this association represents a cause-effect, OP-induced inhibition of LH release could have contributed to reduce testicular testosterone production, which might explain the negative association of OP exposure with testosterone. In addition, association between OP exposure and depleted LH along with variable prolactin levels is partially consistent with experimental data suggesting the presence of dose-dependent stimulatory and inhibitory effects of OPs on the pituitary function [34]. Lack of association with cholinesterase activity and current OP use in turn suggests that changes in sex hormones induced by OPs may be permanent rather than acute or reversible. However, whether endocrine-disrupting effects of any pesticide are transient or sustained may depend on the nature of the dose-time relationship.

Regarding exposure to herbicides and fungicides, to date, no epidemiological study has demonstrated an association with male sex hormones. Despite this, herbicides glyphosate and paraquat and fungicide mancozeb are EDCs with potential effects on the male reproductive system [23–27]. Particularly, glyphosate is a potent reproductive toxicant, which is known to inhibit StAR protein expression and an aromatase enzyme, causing reduction in testosterone and estradiol synthesis *in vitro* [25]. Nonetheless, glyphosate exposure was not associated with testosterone levels in the present study, and potential mechanisms of action explaining the effect of long-term use of glyphosate, as well as paraquat, on LH are not clear at this time.

The present findings suggest that chronic exposure to agricultural pesticides can affect semen quality in young men, as indicated by inverse dose-response relationships between lifetime pesticide use and morphology. According to this, working in agriculture for more than 10 years was associated with poor semen quality among farmers in Vietnam [35]. As far as we know, other previous studies did not examine semen quality or reproductive hormones in relation to cumulative exposure to pesticides. However, finding of higher sperm count in rural men is not consistent with the study by Swan et al. [7] showing reduced sperm concentration and motility in men residents in a U.S. agricultural region compared to men from urban areas. It should be noted that differences in sperm quality between rural and urban men could be explained by differential exposure to unmeasured environmental factors, as discussed below. Several epidemiological studies are in partial agreement with associations reported here between individual pesticides or classes and sperm quality [5,8,36–40]. All prior studies examining OP exposure found negative relationships with morphology, but also with motility and sperm count [5,8,38–40]. Concerning fungicides, the ethylene-bis-dithiocarbamate mancozeb has demonstrated anti-androgenic activity *in vitro* [23]. According to our results, a study in rats observed decreased epididymis and testicular sperm counts, weight of testis, epididymis, seminal vesicle, and ventral prostate following oral administration of mancozeb [24]. Other fungicides such as carbendazim and benomyl also caused alterations in Sertoli cells in rat testis [41,42]. To our best knowledge, no human study has examined exposure to fungicides specifically in relation to semen quality. Results regarding herbicides are supported by experimental data showing that glyphosate causes disturbances in the reproductive development of male rats, including changes in the morphology of the testis [26]. For its part, paraquat, administered dermally to rats, resulted in decreased sperm count, reduced motility, and increased sperm abnormalities [27]. Accordingly, reduction in sperm concentration, motility, and morphology was found among farmers exposed to paraquat in Malaysia [36].

Pesticides can affect sperm quality through different mechanisms, including induction of testicular apoptosis, interference with Sertoli cells and other spermatogenesis support cells, disruption of androgenic signaling, and sperm DNA damage [10,41–43]. Contrary to most previous human studies, in which decrease in sperm concentration and motility were the most frequent effects associated with pesticide exposure [6,9], our main finding was a reduction in morphology, which may be indicative of problems during spermatogenesis and spermiogenesis and may be produced by the direct loss of developing spermatocytes [10], even at embryogenesis due to maternal exposure.

Prenatal tobacco exposure has been associated with reduced sperm quality [44,45]. In the present study, men exposed to cigarette smoke *in utero* showed decreased total testosterone and SHBG, and higher FAI. Similar results were obtained in a recent study of young Danish men which suggested that prenatal tobacco exposure may lead to increased free testosterone in adulthood [44]. Another study suggested that cigarette smoking has no significant effect on the biologically active fraction of male testosterone, but may influence the levels of total and free testosterone through changes in SHBG levels [46]. The observed inverse association with total testosterone could thus be attributed to the effect of maternal tobacco smoke on congenitally determined SHBG levels. However, whether fetal exposure to tobacco smoke, which contains more than 4000 chemical compounds, has adverse effects on reproductive hormones later in life is controversial and remains largely unexplored.

Association between maternal farming and poorer sperm morphology could be explained in terms of the hypothesis of the testicular dysgenesis syndrome (TDS). This hypothesis supports the concept that hypospadias, undescended testis, poor semen quality, and testicular cancer may be a result of the disruption of foetal programming and gonadal development by intrauterine exposure to EDCs [2]. In addition, the effect of cumulative pesticide use on sperm morphology was somewhat stronger among men exposed to pesticides *in utero*, suggesting that adult exposures may be modified by pesticide exposure during critical period of development. By contrast, maternal farming was associated with increased AGD and TV in our study, suggesting an androgen effect rather than anti-androgenic action during fetal life among sons of women working with pesticides, though it could also be the result of higher exposure to unmeasured anti-androgenic chemicals among men not exposed to pesticides prenatally. Very few epidemiological studies have explored the association between intrauterine pesticide exposure and genital measures in the male offspring [47–49]. Only one of these studies focused on non-persistent pesticides, showing lower TV and penile length in prepubescent boys born to women working in greenhouses during pregnancy [49]. Finding of larger AGD and TV in rural men also warrants cautious interpretation in light of possible residual confounding, since prenatal exposure to other EDCs not accounted for may have led to reduced genital measures among urban males. On the other hand, while AGD is primarily determined *in utero*, TV can be affected by adult exposures [50,51] and, therefore, the observed association between cumulative use of pesticides, particularly fungicides, and increased TV could be explained by inflammation of the testicles induced by damaged germ cells, as observed in adult rats treated with the fungicides carbendazim [50] and diuron [51].

One limitation of this study may be the small number of subjects within each stratum, which may have reduced statistical power. Secondly, we cannot rule out the possibility that some of the associations may have resulted from interaction between pesticides or that residual confounding due to unmeasured variables and multiple comparisons may have led to biased associations, as discussed earlier. Despite this, we adjusted for education as a proxy measure for socioeconomic status to partially control these unmeasured risk factors. Moreover, consistent and statistically significant associations were found with reproductive endpoints in

the pooled analysis and the rural sample that seem unlikely to be the result of chance or residual confounding, particularly the dose-response relationships observed with LH and sperm morphology. Another limitation is that we did not measure exposure to individual compounds directly but instead we were able to assess cumulative exposure to pesticide classes and three relevant chemicals, examining linear trends. Finally, more detailed information about exposures (*e.g.* combined exposure to multiple pesticides or maternal use of specific pesticides) might provide further insight into the relationship between occupational pesticide exposure and reproductive outcomes.

Strengths of the study include the random sampling of rural men, who were selected to be representative of young males residing in the rural area of Farroupilha, and the narrow age range of participants. Although results cannot be generalized to agricultural workers in the whole country, this is the first epidemiological study providing evidence concerning the impact of agricultural pesticides on sperm quality and reproductive hormone levels among male farm workers in Brazil, and one of the very few studies that have been performed regarding AGD in adults and its relationship with *in utero* pesticide exposure.

5. Conclusion

The present data suggest that chronic occupational exposure to modern pesticides, particularly herbicides and fungicides, may adversely affect semen quality in young male farmers in the South of Brazil, potentially leading to poorer morphology. Data also suggest that exposure to agricultural pesticides may acutely increase prolactin and chronically alter sex hormone levels acting at the pituitary level through prolactin and LH suppression, hampering compensatory responses to testicular dysfunction. Whether pesticide exposure in the rural area under study can reduce sperm quality sufficiently to impair male fertility and affect pituitary function to produce clinical effects is unknown, but given that poor knowledge about pesticide risks and unsafe handling practices among small farmers in Brazil is common, implications for public health and agricultural work could be considerable.

Conflict of interest

None.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.reprotox.2017.01.001.

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