

1 **Impaired semen quality associated with environmental DDT exposure in young men living in a**
2 **malaria area in the Limpopo Province, South Africa**

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26 **Short running title:** DDT and seminal parameters

27

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30

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40 study.

41

42 **ABSTRACT**

43 The pesticide DDT [1,1,1-trichloro-2,2-bis(chlorodiphenyl)ethane] is one of the 12 persistent
44 organic pollutants (POPs) that were under negotiation at the Stockholm Convention, to restrict or
45 ban their production and use because of their toxicity, resistance to breakdown, bioaccumulation
46 and potential for being transported over long distances. DDT has estrogenic potential and the main
47 metabolite *p,p'*-DDE is a potent anti-androgen. In response to mounting evidence on the endocrine
48 disrupting influence of environmental chemicals on human health, this epidemiological study was
49 initiated to test the hypothesis that non-occupational exposure to DDT affects male reproductive
50 parameters. In a cross sectional study healthy male subjects (n=311) aged between 18 and 40 years
51 (23±5) were recruited from three communities in an endemic malaria area where DDT is sprayed

52 annually. A semen analysis according to the World Health Organization (WHO) (1999) standards
53 was performed. The Hamilton Thorne Computer Assisted Sperm Analysis (CASA) system was
54 simultaneously used to determine additional sperm motility parameters. Blood plasma samples were
55 assayed for *p,p'*-DDT and metabolites as a measure of exposure. The exposure levels were
56 expressed as lipid adjusted *p,p'*-DDT and *p,p'*-DDE values. The mean *p,p'*-DDT and *p,p'*-DDE
57 concentrations were 90.23 µg/g (±102.4) and 215.47 µg/g (±210.6), respectively. The multivariate
58 linear regression analyses indicated that: the mean CASA motility was lower with a higher *p,p'*-
59 DDE concentration (beta = -0.02; p =0.001) and the CASA parameter, beat cross frequency (BCF)
60 was higher with a higher *p,p'*-DDT concentration (beta= 0.01; p =0.000). There was also a
61 statistically significantly positive association between the percentage sperm with cytoplasmic
62 droplets and *p,p'*-DDT concentration (beta =0.0014; p =0.014). The ejaculate volume (mean:
63 1.9±1.33mL) was lower than the normal range (≥2.0mL) for the WHO, and a significant decrease
64 with increasing *p,p'*-DDE values was seen for both square rooted volume (beta =-0.0003; p= 0.024)
65 and count (beta =-0.003; p= 0.04). Although there were no associations between either *p,p'*-DDT or
66 *p,p'*-DDE concentrations and the rest of the seminal parameters, the incidence of teratozoospermia
67 (% Normal sperm <15%) (99%) was high. Twenty-eight percent of the study group presented with
68 oligozoospermia (< 20 million sperm/mL) which had a significantly positive association with *p,p'*-
69 DDE (OR:1.001; p = 0.03). There was a significantly positive association between participants with
70 asthenozoospermia (32%) and *p,p'*-DDT (OR:1.003, p = 0.006) and *p,p'*-DDE (OR:1.001, p = 0.02).
71 The results imply that non-occupational exposure to DDT is associated with impaired seminal
72 parameters in men. The high exposure levels of *p,p'*-DDT and *p,p'*-DDE are of concern, as these
73 levels may have far reaching implications for reproductive and general health.

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76

77 **INTRODUCTION**

78 The Stockholm Convention resulted from a decision made in 1995 by the United Nations
79 Environment Program Governing Council (UNEP) to develop a legally binding instrument to
80 control certain chemicals. The convention initially targeted 12 chemicals known as persistent
81 organic pollutants (POPs), arguing that those chemicals pose major and increasing threats to human
82 health and the environment (<http://www.pops.int/documents/background/gcdecision/18-32/gc1832en.html>).
83 The Stockholm Convention on POPs became legally binding on 17 May 2004.
84 The Convention is global multilateral agreement with the aim of protecting human and
85 environmental health from the effects of exposure to specific POPs. Restricting the use and
86 production of these chemicals or banning them, will, when the measures of the convention are
87 successfully implemented, reduce the hazards posed by these pollutants. Although South Africa
88 ratified the Convention on 4 September 2002 (Bouwman, 2004), it has applied for exemption as far
89 as using DDT for malaria vector control is concerned.

90
91 POPs are organic compounds that to a varying degree resist photolytic, biological and chemical
92 degradation. These compounds are often halogenated and characterized by low water solubility and
93 high lipid solubility. They are also semi-volatile, enabling them to move long distances in the
94 atmosphere before deposition occurs. POPs, which are noted for their persistence and
95 bioaccumulative characteristics, include DDT (1,1,1-trichloro-2,2-bis(chlorodiphenyl)ethane),
96 dieldrin, toxaphene and chlordane and several industrial chemical products and byproducts including
97 polychlorinated biphenyls (PCBs), dioxins and furans (<http://www.chem.unep.ch>).

98
99 DDT and similar stable chlorinated compounds can be transported via air, rivers (Rawn et al, 1999;
100 Buehler et al, 2004), and ocean currents (Bidleman et al, 1995) over long distances and have been

101 detected in the Antarctic and other areas, far from their production sites or regions of use
102 (Bouwman, 2004). While DDT is targeted by the treaty, exemptions are available for countries that
103 are still using DDT to combat malaria. The treaty mobilizes much needed funding to help countries
104 shift to safer alternatives for malaria control, which has drawn attention and resources to the ongoing
105 and long-ignored tragedy of malaria, particularly in Africa. A small group of United States (U.S.)
106 conservatives continues to push to re-establish DDT as a “safe” chemical for use against malaria,
107 despite a clear decision by the international community that DDT should be targeted for ultimate
108 elimination (<http://www.panna.org/resources/newsroom>). All these factors have made the continued
109 use of DDT for malaria vector control in South Africa, Africa and all the other countries that have
110 applied for exemption a matter of global interest.

111
112 A number of reports have indicated that, in addition to being toxic organochlorine pesticides,
113 including DDT and its metabolites, may act as endocrine disruptors (Turusov et al, 2002).
114 Endocrine disrupting chemicals (EDCs) can be defined as compounds that influence normal
115 hormone function generally causing adverse effects (Godduhn and Duffy, 2003). Technical grade
116 DDT is a mixture of *p,p'*-DDT (~85%), *o,p'*-DDT (~15%) and *o,o'*-DDT (trace amounts) with both
117 *p,p'*-DDT and *o,p'*-DDT having estrogenic activity. *p,p'*-Dichlorodiphenyl-dichloroethylene (*p,p'*-
118 DDE), a persistent metabolite of *p,p'*-DDT, is a widespread environmental contaminant (Turusov et
119 al, 2002). The *p,p'*-DDE isomer is anti-androgenic by inhibitive binding to androgen receptors
120 (Rogan and Chen, 2005) and has been shown to inhibit the action of testosterone (Danzo, 1997;
121 Kelce et al, 1995; Bhatia et al, 2005). The hypothesis has been advanced that *p,p'*-DDE may interact
122 in an additive or multiplicative way with other endocrine-disruptive environmental pollutants
123 (Turusov et al, 2002). Serum levels of *p,p'*-DDE are an integrated measure of internal dose,

124 reflecting exposure from all sources over the previous years (Hauser et al, 2003). Reproductive
125 disorders were among the first adverse impacts linked to organochlorine exposure (Beard, 2005).

126
127 Reproductive abnormalities attributed to DDE exposure after a major pesticide spill in 1980 were
128 found in reptiles inhabiting Lake Apopka in Florida in the 1990s. The types of deformities found
129 were ambiguous gonads (ovotestes) in turtles, and abnormal sex hormone levels, poorly organized
130 testes and small penises in male alligators (Guillette et al, 1995). The Great Lakes fish found to be
131 contaminated with organochlorine compounds such as PCBs, dioxins, DDT metabolites and
132 dibenzofuran have exhibited reproductive and other endocrine abnormalities. Wildlife (birds, turtles
133 and mammals) that have consumed these fish have also exhibited various abnormalities which
134 include impaired reproduction, same sex pairing, feminization, ambiguous genitalia and reduced
135 fertility (Fry, 1995; Colborn et al, 1993, 1996). In mice the uterotrophic effect of DDT increased the
136 weight of the uterus and the development of a pseudouterus (Morozova et al, 1997). A permanent,
137 functional male-to-female sex reversal following a single exposure of eggs to *o,p'*-DDT was
138 observed in medaka fish (Edmunds et al, 2000; Turosov et al, 2002). There is also evidence that
139 DDT may act as a promoter of mammary tumors in rats and that it can inhibit gap junctional
140 intercellular communication (Snedeker, 2001). Other evidence of hormone-disrupting effects of
141 DDT and its metabolites has included reproductive defects and eggshell thinning in avian species
142 (Fry, 1995; Snedeker, 2001). DDT or *p,p'*-DDE may alter sex hormone metabolism, reducing
143 available testosterone to tissues (Guillette et al, 1995).

144
145 In humans a trend in decreasing human sperm counts may have occurred in several European
146 regions during the last 50 years (Auger et al, 1995; Irvine, 1994; Toft et al, 2004). The decrease in
147 sperm count is paralleled by a rise in the trend of testicular cancer and malformations of the male

148 reproductive organs such as hypospadias and cryptorchidism (Toppari et al, 1996). Skakkebaek et
149 al (2001) presented a hypothesis that poor semen quality, testicular cancer, cryptorchidism and
150 hypospadias are all indicative of one underlying entity: testicular dysgenesis syndrome (TDS), with
151 an origin in fetal life. The cause of TDS is unclear, but, owing to the rapid temporal changes in
152 symptoms over the last few decades, it is suspected to be at least partly linked to environmental and
153 lifestyle factors. In addition, genetic polymorphisms or aberrations may render some individuals
154 particularly susceptible to potential environmental disrupters (Bay et al, 2006). Since technical
155 grade DDT comprises estrogenic molecules, and because its major metabolite is a potent anti-
156 androgen, it has been hypothesized that exposure to DDT is involved in the increase in male
157 reproductive tract anomalies (Guillette et al, 1995; de Jager et al, 2006).

158
159 In Africa, indoor residual spraying of DDT has become part of the national Roll Back Malaria
160 strategic plan in several countries (Hougard et al., 2002; Rogan and Chen, 2005). In South Africa
161 DDT is sprayed in the low-altitude parts of Limpopo Province, Mpumalanga Province, and
162 KwaZulu Natal. Currently, of the approximately 40 million people in South Africa, 10 per cent, or 4
163 million, live in a malaria risk area (Rogan and Chen, 2005). This puts the inhabitants of the rural
164 communities in these areas at risk of being exposed to high concentrations of DDT and DDE. The
165 exposure occurs through inhalation (indoor air spraying of dwellings and outdoors), dermal contact
166 (soil and house dust), and ingestion of contaminated foods and water. In South Africa there is no
167 information on the health effects of environmental DDT exposure. In light of the above, this study
168 aimed to assess the effects of non-occupational exposure to DDT and semen parameters in young
169 healthy men in a rural area in the Limpopo Province, South Africa, where DDT is still sprayed.

170

171 **MATERIALS AND METHODS**

172 ***Study design and population***

173 In a cross sectional study design, the participants were volunteer, non-occupationally exposed Venda
174 men. The participants recruited were between 18 and 40 years old and had been living in the
175 communities for at least a year. Participants were excluded if they presented with a history of,
176 testicular trauma, orchitis, urinary infection, sexually transmitted diseases, use of hormonal
177 medication, exposure to known gonadotoxins, or had neuropsychiatric disorders.

178
179 ***Study area***

180 The Limpopo Province is situated in the northeastern corner of South Africa and is divided into six
181 districts, with the study area lying within the Vhembe district. After consultation with the regional
182 Department of Health and Social Development, three rural communities, Dididi, Tshiulungoma, and
183 Tshikhudini, near Thoyohandou were selected from a malaria endemic area. The housing in these
184 communities consists of traditional mud dwellings with thatch (grass) roofs, or brick and cement
185 houses. DDT is sprayed inside unpainted brick, cement and mud houses annually, but not inside the
186 painted houses.

187
188 ***Recruitment and sampling***

189 The Ethics Committee of the Faculty of Health Sciences, University of Pretoria (Reference no:
190 43/2003) and the Limpopo Provincial Government's Department of Health approved the research
191 protocol (07-11-2002). An initial visit to the proposed study area took place in October 2003. The
192 project team approached the village Chiefs and Elders for permission to address the community
193 about the proposed study. Meetings were held at all three villages to inform the residents about the
194 study and the procedures that would be followed. A representative was selected from each village
195 (Dididi, Tshiulungoma, and Tshikhudini) to assist with the recruitment of participants. The
196 representative was also trained to assist with the study questionnaire. After being properly

197 informed, any man who volunteered to participate and met the inclusion criteria was included in the
198 study.

199
200 The Tshilidzini Hospital near Thoyohandou was used as a central laboratory point. Samples were
201 collected between November 2003 and July 2005. The participants produced semen samples in
202 specially provided rooms adjacent to the onsite laboratory. In addition to the semen samples, blood
203 samples were collected and all participants signed an informed consent form and completed a
204 questionnaire. During this period 362 participants were recruited, of which 51 were unable to
205 produce a semen sample or did not meet the inclusion criteria.

206
207
208 ***Questionnaire***
209 The questionnaire included questions on the general health history, DDT exposure source (whether
210 houses were sprayed with DDT for malaria control or not), diet, fertility history and other potential
211 spermatotoxic exposures. Exposures studied included: physical agents (exposure to heat or
212 radiations and history of testicular trauma); biological agents (genito-urinary tract infections, history
213 of STDs, orchitis and epididimitis) and chemical agents (recreational and/or occupational exposure
214 to drugs, pollutants, other pesticides, or any other chemical agent, and smoking and drinking habits).

215
216 ***Exposure assessment***
217 Blood samples were collected from each participant. The samples were centrifuged at 2000 rpm for
218 ten minutes at room temperature. Plasma was stored at -20°C on site and then transferred to a
219 -70°C freezer until analysed. The Agricultural Research Council, Veterinary Institute, Residue
220 Laboratory in Pretoria, South Africa determined DDT and its metabolites, using a Shimatzu GCMS
221 -QP2010. Concentrations of DDT compounds in the plasma were expressed on a lipid-adjusted
222 basis ($\mu\text{g/g}$). The detection limit for *p,p'*-DDT and *p,p'*-DDE was $0.02\mu\text{g/g}$ lipid adjusted. Total

223 cholesterol and triglycerides were determined by enzymatic methods and the total plasma lipid
224 concentration was calculated according to the formula proposed by Rylander et al (2006).

225

226 *Semen analyses*

227 Semen samples were obtained from 311 participants after the prescribed three-day period of sexual
228 abstinence. Semen specimens were produced by masturbation directly into a sterile wide-mouthed
229 container. The semen sample was then incubated at 37°C until liquefied. Trained researchers
230 performed semen analyses and additional andrological tests according to the standards and
231 procedures of the World Health Organization (WHO, 1999), and quality control (QC) procedures
232 were adhered to (WHO, 1999; ESHRE, 1998).

233

234 After liquefaction the following seminal physical characteristics were assessed: appearance,
235 liquefaction, viscosity, ejaculate volume, and semen pH (Mortimer, 1994). Sperm concentration
236 was determined using a haemocytometer (WHO, 1999). Sperm motility was assessed manually on a
237 wet preparation, using the WHO (1999) motility classification. This classification uses the class *a-d*
238 sperm progression rating (*a* = rapid progressive motility; *d* = immotile sperm) (WHO, 1999; NAFA
239 and ESHRE-SIGA 2002). The viable sperm were assessed using the eosin-nigrosin method
240 (Mortimer, 1994). The presence of leukocytes, erythrocytes, bacteria, and agglutinates was also
241 noted. The presence of immunoglobulin on the sperm surface was assessed with the IgG test
242 (SperMar test) on all fresh samples with motility < 40%. Immunological infertility can be
243 considered when 50% or more of the motile sperm have IgG antibodies. Sperm morphology slides
244 were stained using the Papanicolau method and scored according to the WHO (1999) classification.
245 The morphology assessment was performed by the same technologist in the Andrology laboratory at
246 the University of Pretoria. This laboratory ensures that the technologists follow strict quality control

247 (QC) and quality assurance (QA) procedures. The Andrology laboratory also takes part in an
248 international external QC program with the European Society for Human Reproduction and
249 Embryology (ESHRE), and all observations fall within ± 1 SD of the reference results.

250
251 ***Computer Assisted Sperm Analysis (CASA)***
252 Sperm motility was further evaluated using a Hamilton Thorne sperm motion analyzer (HTM-IVOS,
253 Version 12, Beverly, MA) at 60Hz. Twelve microlitres of semen were placed into Leja slides (Leja,
254 Cat no. SC 20-01-C, Calicom Trading (PTY) Ltd, South Africa) with a chamber depth of 20 μ m.
255 Thirty frames were captured for analysis; a minimum of 150 sperm were analyzed in duplicate at
256 37°C (Mortimer and Fraser, 1996; Schrader et al, 1992). Samples having an estimated count of more
257 than 40x10⁶/ml spermatozoa were diluted with cell-free seminal plasma from the same individual.
258 The percentages of motile sperm, progressive motility, linear velocity and curvilinear velocity were
259 measured.

260
261 ***Statistical analyses***
262 Exploratory data analysis was conducted on the final database to detect missing or outlier values.
263 Tabulation and graphical univariate analysis was done to describe the distribution of each variable
264 and identify the necessary transformation to normalize variables. In each case, after exploring
265 several transformations and the raw form, the closer to normal distribution was used in linear
266 regression analysis. The distribution of variables describing sperm morphology defects: head, mid-
267 piece and tail defects percentage, required negative binomial regression analysis. Information
268 obtained from the questionnaire as well as the participant's *p,p'*-DDT and *p,p'*-DDE levels were
269 compared between different categories using ANOVA or regression analyses. Bivariate analyses
270 using regression models were conducted between the different reproductive outcomes and
271 questionnaire variables to determine the risk factors and to identify confounding factors.

272 Multivariate models were examined to evaluate the effect of DDE/DDT in the different reproductive
273 outcomes. A saturated multivariate model was produced for each dependent variable (semen
274 parameter), including all independent variables with a p value ≤ 0.15 in the bivariate analyses. A
275 manual stepwise elimination was used until every variable in the multivariable model had a p value
276 ≤ 0.05 or was capable of altering the other coefficients by at least 10%. *p,p'*-DDT and *p,p'*-DDE
277 plasma levels were used as continuous as well as categorical variables in multivariate analysis. All
278 final regression models were adjusted by age. Final model sensitivity to individual observations was
279 done by plotting the residuals versus fitted values; leverage versus normalized residuals squared;
280 and residuals versus predicted values. dfbetas were also estimated. Models were tested without
281 detected influential observations with dfbetas $> 2\sqrt{n}$.

282

283 **RESULTS**

284 The mean age of the participants was 23 ± 4.7 (mean \pm SD). Participants were Venda men from a
285 rural area and a low socio-economic status who had never been occupationally exposed to the
286 pesticide DDT. A selection bias affecting results is not probable, since participants were not aware
287 of the study hypothesis. Since this study's design controlled for sexual abstinence time, and this
288 variable was not significant, there was no need to control for it in analysis. Other explored exposures
289 did not prove to be sufficiently present or intense to cause sperm alterations.

290

291 The mean serum concentration of *p,p'*-DDT was 529.67 ± 617.7 $\mu\text{g/l}$ and the mean lipid adjusted
292 *p,p'*-DDT concentration was 90.23 ± 102.4 $\mu\text{g/g}$ (Table 1). The *p,p'*-DDE level had a mean serum
293 concentration of 1259.10 ± 1297.0 $\mu\text{g/l}$. When expressed as a lipid adjusted concentration, the mean
294 *p,p'*-DDE concentration was 215.47 ± 210.6 $\mu\text{g/g}$ (Table 1). The source of *p,p'*-DDT or *p,p'*-DDE

295 exposure was found to be statistically significantly higher between participants whose houses were
296 sprayed with DDT (n = 249) (i.e. mud and thatch roof dwellings) when compared to those whose
297 houses weren't sprayed (n = 48) (p = 0.000, *p,p'*-DDE; p = 0.000, *p,p'*-DDT) (Table 1).

298
299 The distribution of the semen parameters and their age adjusted regression associations with the
300 serum lipid *p,p'*-DDT and *p,p'*-DDE are shown in Table 2. Diagnostic tests on final regression
301 models showed an influential observation that by itself consistently and considerably altered the
302 statistical significance, but not so the coefficients. Although *p,p'*-DDT and *p,p'*-DDE and semen
303 results were measured correctly in this case, the subject was excluded, since he was not
304 representative of the studied population. Excluding him only changed previously borderline
305 significant associations. Volume and count p values with and without the subject in the final models
306 changed from 0.05 to 0.02 and from 0.1 to 0.04, respectively. Parameters showing a significantly
307 positive association with continuous *p,p'*-DDT levels were the round cells (beta = 0.0013; p =
308 0.000) and the cytoplasmic droplets (beta = 0.0014; p = 0.014). The significantly negative
309 associations with continuous *p,p'*-DDT levels were: volume (square rooted) (beta = -0.0003; p =
310 0.024) and count (square rooted) (beta = -0.003; p = 0.04). Semen volume and sperm count (both
311 square root transformed) were also significantly reduced when the first three quartiles of lipid
312 adjusted *p,p'*-DDE (0-345µg/g) were compared to the fourth quartile (346-997 µg/g): volume beta =
313 -0.16, p = 0.01; sperm count beta = -1.16, p = 0.04. The CASA parameters that had a statistically
314 significantly negative association with *p,p'*-DDT were the VSL (beta = -0.002; p = 0.03), ALH (beta
315 = -0.0003; p = 0.03) and the BCF (-0.01; p = 0.000). The CASA mean motility (cubed) had a
316 significantly negative association with *p,p'*-DDE (beta = -0.02; p = 0.001) (Table 2). In comparing
317 the first three *p,p'*-DDE quartiles against the fourth, the coefficient for cubed motility was found to
318 be = -8.79 (p = 0.001).

319

320 The participant's semen characteristics were classified according to dichotomous abnormal semen
321 categories (WHO, 1999) and these were expressed as percentages (Table 3). Twenty eight percent of
322 all the participants were classified with oligozoospermia, 99.5% with teratozoospermia and 32%
323 with asthenozoospermia. The distribution and crude regression of the dichotomous abnormal semen
324 categories indicated by the Odds Ratio (OR) showed that those participants with oligozoospermia
325 were significantly associated with *p,p'*-DDE (OR = 1.001; *p* = 0.03) (Table 3). The distribution of
326 oligozoospermia was statistically significantly associated with the lipid adjusted *p,p'*-DDE
327 percentile concentrations, as is shown in Figure 1. The distribution of asthenozoospermia was also
328 significantly associated with the lipid adjusted *p,p'*-DDT percentile concentrations (OR = 1.003; *p* =
329 0.006) and *p,p'*-DDE (OR = 1.001; *p* = 0.02). The graph shows that the higher the *p,p'*-DDE
330 concentration, the greater the incidence of oligozoospermia and asthenozoospermia (Figure 1).

331

332 **DISCUSSION**

333 The finding that both *p,p'*-DDT and *p,p'*-DDE values were statistically significantly higher in men
334 living in sprayed houses than in men from non-sprayed houses (102.0µg/g *p,p'*-DDT and
335 239.0µg/g *p,p'*-DDE vs 31.0µg/g *p,p'*-DDT and 100.0µg/g *p,p'*-DDE, *p*=0.0000) highlighted an
336 important route of exposure. This may be an indication that indoor residual spraying could
337 contribute to increased exposure to DDT.

338

339 The mean lipid adjusted *p,p'*-DDT levels (90.23 ± 102.4 µg/g) and *p,p'*-DDE levels (215.47 ± 210.6
340 µg/g) in the non-occupationally exposed population of this study can be considered very high when
341 compared to another study in the same province. Dalvie et al (2004a, b) assessed the reproductive
342 effects of long-term DDT exposure in malaria vector-control workers (n=47) in Limpopo Province.

343 The mean lipid adjusted *p,p'*-DDT levels in that study were $26.1 \pm 13.7 \mu\text{g/g}$ and mean *p,p'*-DDE
344 levels were $65.0 \pm 48.8 \mu\text{g/g}$. Those levels are almost three and a half times' lower than those found
345 in this study, which is to be expected as the malaria vector-control workers wear protective clothing
346 and take the necessary safety precautions when working with DDT. In a similar non-occupational
347 exposure study done in 2000-2001 by de Jager et al (2006) in Chiapas, Mexico, the *p,p'*-DDE level
348 ($45 \pm 32 \mu\text{g/g}$) was found to be almost five times lower than in the present study: however this was
349 after DDT had been phased out by 2000. It is known that *p,p'*-DDE concentration in lipids is used
350 as a surrogate for chronic exposure to technical DDT, a mixture that comprises estrogenic
351 compounds such as *o,p'*-DDT and *p,p'*-DDT and the androgen antagonist *p,p'*-DDE (de Jager et al,
352 2006). The high *p,p'*-DDT level in this study indicates that there is current acute exposure to DDT
353 and the high *p,p'*-DDE levels indicate chronic long term exposure. These levels are much higher
354 than the levels in the abovementioned studies that found reproductive effects due to DDT exposure
355 (Ayotte et al, 2001; Dalvie et al, 2004a, b; de Jager et al, 2006). The levels in this study are
356 supported by the fact that semen volume, total sperm count, progressive motility and viability were
357 lower with higher levels of *p,p'*-DDT. Higher levels of *p,p'*-DDE also resulted in a lower semen
358 volume, total sperm count, progressive motility, and viability.

359
360 In order to assess reproductive function a basic semen analysis was carried out and in addition
361 CASA motility parameters were evaluated. This included semen volume, pH, and viscosity. The
362 epididymal epithelium is androgen-dependent and has both absorptive and secretory functions. The
363 epididymal plasma in which the sperm are suspended within the epididymis is also secreted by the
364 epididymal epithelium. It is a complex fluid that changes along the length of the epididymis. The
365 spermatozoa experience a series of sophisticated microenvironments that regulate their maturation

366 (Mortimer, 1994). Under physiological conditions the various components of the ejaculate originate
367 in different parts of the male reproductive tract and are emitted in a definite order (Mann and
368 Lutwak-Mann, 1982). The vesicular fluid is the last fraction of semen ejaculated and contributes to
369 70% of the ejaculate volume, while the prostate contributes the other 30% (Mortimer, 1994). It has
370 been hypothesized that toxicants or their metabolites may act directly on accessory glands by
371 altering the quality or quantity of their secretions and that this could influence semen volume (Mann
372 and Lutwak-Mann, 1982; Pant et al, 2004). As both the prostate and seminal vesicles are also
373 androgen-dependent organs (Mann and Lutwak-Mann, 1982), the anti-androgen properties of *p,p'*-
374 DDE could have an influence on the functions of the organs. This could account for the mean
375 semen volume ($1.88 \pm 1.3\text{ml}$) which was slightly lower than the reference value of the WHO (1999)
376 and similar to the Chiapas study (mean volume = 1.84ml) (de Jager et al, 2006). The study showed a
377 strong and significant negative association between *p,p'*-DDE and volume; a similar association was
378 found in the Chiapas study. When *p,p'*-DDE was analysed by quartiles, results showed that the
379 expected semen volume of the most exposed men (*p,p'*-DDE = 346-997 $\mu\text{g/g}$) would be
380 approximately 1.38ml. Pant et al (2004) showed that *p,p'*-DDE and *p,p'*-DDD were higher in the
381 semen of infertile men when compared to fertile men. The levels of gamma-glutamyl transpeptidase
382 and acid phosphatase activity were also lower in infertile men, while the high fructose level
383 observed could suggest “non-utilization of the enzyme by sperms due to some biochemical defects”,
384 although this was not studied (Pant et al, 2004). It would have been in the interest of the study to
385 assess the accessory gland markers such as fructose and α -glucosidase, but unfortunately because of
386 the low volume this could not be done.

387
388 Although the mean total sperm count was within the WHO (1999) reference range, there was a
389 significantly negative association with *p,p'*-DDT and *p,p'*-DDE. Analyzing *p,p'*-DDE by quartiles

390 showed that the expected sperm count in the highest quartile (p,p' -DDE = 346-997 μ g/g) would be
391 approximately 56.3 million. The participants were divided into Oligo- and Normozoospermic
392 categories according to the WHO (1999). The crude regression associations showed a statistically
393 significant positive dose-dependent association between participants with oligozoospermia (28%)
394 and p,p' -DDE concentration (OR:1.001; $p = 0.03$). This indicates that participants with high
395 concentrations of p,p' -DDE are at risk of presenting with oligozoospermia. In support, the
396 distribution of oligozoospermia shows that the higher the p,p' -DDE concentration the greater the
397 incidence of oligozoospermia. This trend is similar to the findings of a study by Rozati et al (2002),
398 which suggested that there was a significant deterioration in semen parameters, including sperm
399 count in infertile men with PCBs in their seminal plasma compared to the control group. This trend
400 is also in agreement with a report citing an inverse correlation of PCBs and sperm motility in men
401 with oligozoospermia (Bush et al, 1986).

402
403 There were negative associations between the progressive, total motility and viability and the p,p' -
404 DDT and p,p' -DDE concentrations. Some animal data suggest that p,p' -DDE may be hormonally
405 active and therefore adversely affect semen parameters. The compounds that readily pass the blood-
406 testis barrier may directly affect spermatogenesis (Hauser et al, 2003). Effects at the mitotic or
407 meiotic level may lead to decreased sperm production, whereas the targeting of the postmeiotic
408 processes and epididymal sperm maturation may lead to impaired sperm motility (Hauser et al,
409 2003). Despite the fact that the mean motility was within the WHO normal range (WHO, 1999),
410 32% ($n = 285$) of the participants presented with asthenozoospermia ($>50\%$ $a+b$ or $>25\%$ grade a).
411 Although there was no significant association between sperm motility and p,p' -DDE the distribution
412 of asthenozoospermia shows that the higher the p,p' -DDE concentration the greater the incidence of
413 asthenozoospermia. The multivariate logistic regressions OR showed a statistically significant

414 positive dose-dependent association between participants with asthenozoospermia and *p,p'*-DDT
415 concentrations (OR:1.003; $p = 0.006$) and *p,p'*-DDE concentrations (OR:1.001; $p = 0.02$). This
416 result was similar to a finding of a study by Hauser et al (2003), which showed that PCB-138, which
417 is also an organochlorine compound, was inversely associated with sperm motility and morphology.

418
419 The mean CASA motility ($48.53 \pm 18.6\%$) compared well with the manual mean motility ($50.1 \pm$
420 15.8%). CASA is being used in reproductive toxicology, as some of the motility parameters are
421 sensitive to toxins (ESHRE, 1998). The significant association with *p,p'*-DDE and the cubed CASA
422 mean motility ($\beta = -0.02$; $p = 0.001$) in this study compares well to a study by Hirano et al (2001),
423 which compared the CASA parameters of “good” (fertilization rate $> 50\%$) and “poor” (fertilization
424 rate $\leq 50\%$) fertilization groups. The “poor” fertilization group was found to be $48.9 \pm 22.1\%$ as
425 opposed to the “good” fertilization group $59.9 \pm 16.5\%$ (Hirano et al, 2001). This indicates that the
426 fecundity of this exposed Limpopo population may be compromised. CASA parameters showing a
427 significant negative association with *p,p'*-DDT were the VSL ($\beta = -0.002$; $p = 0.03$), ALH ($\beta =$
428 -0.0003 ; $p = 0.03$) and BCF ($\beta = -0.01$; $p = 0.000$). The VSL ($26.98\mu\text{m/s}$) and ALH ($2.54\mu\text{m}$)
429 values in this study are similar to a study by Guo et al (2000), which found that the VSL ($25.4\mu\text{m/s}$)
430 and ALH ($2.9\mu\text{m}$) were lower in a PCB exposed population than in an unexposed population (VSL
431 $= 33.0\mu\text{m/s}$ and ALH $= 3.3\mu\text{m}$). The BCF is useful in determining changes in the flagellar beat
432 pattern (Mortimer, 2000). The negative association with *p,p'*-DDT indicates that higher levels of
433 DDT cause an increase in the flagellar beat pattern with an adverse effect on sperm motility. In the
434 study by Guo et al (2000), for the BCF value of participants prenatally exposed to PCBs and
435 dibenzofurans, the BCF (17.4 Hz) was lower, but was comparable to this study (BCF $= 29\text{ Hz}$).
436 These findings were similar to those in animals, in which *in-utero* exposure to similar toxic levels of

437 these chemicals reduced daily sperm production and increased the percentage abnormal sperm
438 (Faqui et al, 1998; Guo et al, 2000). The findings of this study indicate that DDT exposure could
439 have a negative effect on sperm motility. Sperm motility is commonly believed to be one of the
440 most important characteristics correlated with fertility (Eimers et al, 1994; Hirano et al, 2001).

441

442 This study population had a high percentage of round cells ($1.13 \pm 1.3 \times 10^6/\text{ml}$) that had a
443 statistically significant positive association with both *p,p'*-DDT (beta = 0.0013; p = 0.000) and *p,p'*-
444 DDE (beta = 0.0005; p = 0.000). Indications are that round cells occur frequently in infertile patients
445 and are associated with poor semen quality (Arata de Bellabarba et al, 2000). A study carried out in
446 Austria showed a significant increase in round cells in the semen of smokers compared to non-
447 smokers (Trummer et al, 2002). In this study, smoking was taken into account as a possible
448 confounder and did not affect the outcome of the data.

449

450 During spermatogenesis, spermatids are transformed into sperm by different processes, including
451 condensation and structural shaping of the cell nucleus and the formation of the flagellum.
452 Disruption at this stage of development can cause impairment of sperm condensation, motility, and
453 morphology (Parvinen, 1998; de Jager et al, 2006). A significant proportion of the sperm in each
454 sample may be morphologically abnormal, but, if the proportion is above 5%, it may account for
455 impaired fertility (Menkveld et al, 1990). The WHO (1999) states that below 15% normal forms the
456 fertilization rates *in vitro* will be reduced. The study investigating exposure to DDT in malaria
457 vector-control workers had a mean normal morphology score of $2.5 \pm 1.8\%$, with 84% of the
458 morphology scores being below the WHO (1992) and Tygerberg strict criteria. The Tygerberg strict
459 criteria could be said to be inappropriately strict for epidemiological settings in which the aim is to

460 detect more subtle effects (Dalvie et al, 2004c). A significantly high proportion of the participants
461 in this study presented with teratozoospermia (99.5%) and the mean percentage normal morphology
462 was $4.13 \pm 2.70\%$, which is well below the WHO (1999) reference range of 15% normal forms.
463 Cytotoxic effects such as the production of superoxide anion and activation of various intracellular
464 signal transduction pathways may explain the significant decrease in normal morphology (Rozati et
465 al, 2002).

466
467 During spermiation residual cytoplasm is shed from the neck of the mature spermatid and a small
468 residual cytoplasmic droplet remains attached to the testicular sperm, which is lost through
469 epididymal transit and sperm maturation (Hess et al, 2001). The mean prevalence of cytoplasmic
470 droplets is 2.2% (Belsey et al, 1980; Mortimer, 1994) whereas in this study it was 11.5%. Fisher et
471 al (1998) demonstrated that neonatal exposure of rats to diethylstilboestrol (DES) caused permanent
472 distention of the rete testis and efferent ducts, with loss of epithelial height through to adulthood.
473 Sharpe (1998) argues that owing to the loss of the apical portion of the cell, the endocytotic
474 apparatus might be dysfunctional. This could imply that, similar to DES, prenatal DDT (estrogenic)
475 exposure could have affected the development of these epididymal cells and subsequently may
476 contribute to the high number of cytoplasmic droplets.

477
478 Not only are cytoplasmic droplets associated with immature spermatozoa, their presence is
479 correlated with oxidative damage (Chantler and Abraham-Peskir, 2004; Gergely et al, 1999,
480 Mortimer, 1994). A study by Gomez et al (1996) correlated a specific morphological defect of
481 human sperm with reactive oxygen species (ROS). The residual cytoplasm present in the midpiece
482 of the human sperm revealed a significant correlation between excess residual cytoplasm in the
483 midpiece and the enhanced generation of ROS. The study by Aziz et al (2004) supported this

484 finding and additionally showed a positive correlation of ROS with the percentage sperm with
485 cytoplasmic droplets (8%) and tail defects (12%). This study showed a statistically significant
486 positive association between cytoplasmic droplets (11%) and *p,p'*-DDT (beta = 0.0014; p = 0.014);
487 although there was no association with the tail defects (13%) these percentages were similar to the
488 study by Aziz et al (2004). Oxidative stress or ROS generation can be induced in the testes by
489 exposure to common xenobiotics such as nonylphenol and dioxin (Chitra and Mathur, 2004; Aitken
490 et al, 2004). Subchronic exposure to DDT is associated with an increase in free radical generation by
491 lipid peroxidation (Koner et al, 1998). It is well known that ROS generation impairs sperm motility
492 (Aitken et al, 1998). This means that there is a strong possibility of increased ROS generation in
493 this exposed group of men which will influence morphology and sperm motility parameters
494 negatively.

495
496 The aetiology of TDS is suspected to be related to genetic and/or environmental factors, including
497 endocrine disruptors. Both *p,p'*-DDT and *p,p'*-DDE are considered to be hormonally active, with
498 *p,p'*-DDT having estrogenic activity via binding and activation of the estrogen receptor and *p,p'*-
499 DDE being anti-androgenic (Kelce et al, 1995). Irrespective of the exact mechanism, which remains
500 to be elucidated in these cases, either reduced testosterone production by Leydig cells (via DDT's
501 estrogenic suppression of the hypothalamic-pituitary-testicular axis) or by impeded androgen action
502 (via DDE's effect on the androgen receptor), the physiological consequence would be impaired
503 Sertoli cell function (Parvinen, 1998). The primary role of these cells is to support spermatogenesis
504 (de Jager et al, 2006). The increased serum *p,p'*-DDT and *p,p'*-DDE levels could be exerting an
505 effect on the Sertoli cells, preventing normal spermatogenesis and resulting in abnormal sperm
506 function as observed in this study.

507

508 **CONCLUSIONS**

509 The data on seminal parameters from 311 participants makes this study one of the largest studies to
510 look at the effects of DDT exposure. The study found evidence that indicated that non-occupational
511 exposure to *p,p'*-DDT and its metabolite *p,p'*-DDE has an effect on seminal parameters of young
512 men living in the Limpopo Province. The mean age of the participants was 23 years, which is young
513 when compared to other similar studies. This is of concern, as it has been found that semen volume,
514 motility, and morphology decrease with age (Kidd et al, 2001). It is impossible to know what the
515 effects will be on the fertility potential of this population in five to ten years with the continued use
516 of DDT for malaria vector control. This data and the Mexico data (de Jager et al, 2006), together
517 with well-documented effects of DDT on animals (Toppari et al, 1996), should provide sufficient
518 evidence to be concerned about the impact of this pesticide and its metabolite *p,p'*-DDE on human
519 health.

520

521 Long-term exposure to small amounts of organochlorine contaminants leads to the accumulation of
522 considerable burdens in animal and human tissue (de Jager et al, 2006). The young are the most
523 vulnerable. It is not necessarily the amount of DDT to which the mother is exposed during
524 pregnancy that is critical but rather her lifetime exposure and bioaccumulation that determines the
525 level of exposure of the fetus and breastfed infant (Korrick et al, 2001; Longnecker et al, 2000).
526 Many of the infants in these rural areas in South Africa are breast-fed. Other effects may occur; in a
527 study by Longnecker et al (2002), there was a modest-to-moderate association in boys with maternal
528 levels of DDE greater or equal to 85.6 µg/l with the development of cryptorchidism, hypospadias
529 and polythelia. Sunyer et al (2005) found that prenatal exposure to DDE residues (geometric mean
530 in cord serum: 1.06 µg/l) may contribute to the development of asthma. Prenatal exposure to DDT,

531 and to a lesser extent DDE, was associated with neurodevelopmental delays during early childhood
532 (Eskenazi et al, 2006).

533

534 Of concern in this non-occupationally exposed population is that these high levels of *p,p'*-DDT and
535 *p,p'*-DDE appear to have adverse effects on the seminal parameters, supporting the findings by de
536 Jager et al (2006). The elevated levels of exposure indicate a high degree of chronic exposure and
537 imply an urgent need for continued epidemiologic studies in the Limpopo Province to study the
538 potential adverse effects of these pesticides. In conclusion, these findings are not only applicable to
539 Limpopo Province, but also to other malaria areas in South Africa, Africa and other parts of the
540 world where DDT is used for malaria control. The feasibility of cost-effective and environmentally
541 safe alternative methods for pest control needs to be considered.

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801 **Table 1. Exposure data indicating the *p,p'*-DDE and *p,p'*-DDT serum levels (n=303)**
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Metabolite	Mean (±SD)	Median	Minimum	Maximum	Mean (±SD) Participant's house sprayed**	
					No (n=48)	Yes(n=249)
<i>p,p'</i> -DDE (µg/L)	1259.10 (±1297.0)	697.0	ND*	6621.0	529.7 (658)	1409.8 (1339)
<i>p,p'</i> - DDE (µg/g) lipid adjusted	215.47 (±210.6)	134.0	ND*	997.0	99.5 (123)	239.0 (215)***
<i>p,p'</i> -DDT (µg/L)	529.67 (±617.7)	249.0	ND*	2644.0	167.0 (339)	602.4 (630)
<i>p,p'</i> -DDT (µg/g) lipid adjusted	90.23 (±102.4)	46.0	ND*	519.0	30.5(58)	101.9 (104)***

803 *ND = Non-detectable (detection limit = 0.02µg/g)
 804 **DDT sprayed in the last year. Six participants did not know
 805 *** p = 0.000

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834 **Table 2. Distribution of seminal parameters and age adjusted regression associations* with**
 835 **serum lipid *p,p'*-DDT and *p,p'*-DDE**
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Semen parameter	n	Mean (± SD)	Median	<i>p,p'</i> -DDE		<i>p,p'</i> -DDT	
				beta	95% CI	beta	95% CI
Volume (ml) ^a	303	1.88(1.3)	1.5	-0.0003	-0.0006, -0.00004	-0.0005	-0.001, 0.00004
Total count (ml/ejac) ^a	295	101.6 (159.3)	59	-0.003	-0.006, -0.0002	-0.001	-0.007, 0.005
Sperm concentration (10 ⁶ /ml) ^a	296	51.76(48.2)	39	-0.0003	-0.0020, 0.0014	0.0022	-0.0014, 0.0057
pH ^b	300	7.46(0.3)	7.5	0.0033	-0.0226, 0.0291	0.0195	-0.0337, 0.0727
Progressive motility (%) (sum of grades <i>a+b</i>) ^c	298	48.14(21.1)	55	-0.2807	-1.099, 0.5379	-0.8514	-2.533, 0.8302
Motility (%) (sum of grades <i>a+b+c</i>) ^b	299	50.1(15.8)	57	-1.56	-63.90, 60.74	-27.63	-155.8, 100.5
Immotility (%) (grade <i>d</i>) ^a	299	49.26(20.7)	43	0.0006	-0.0002, 0.0013	0.0013	-0.0003, 0.0028
Viability (%) ^c	267	54.13(21.8)	59	-0.6571	-1.756, 0.4417	-1.7258	-3.993, 0.5415
Normal morphology (%)	282	4.13(2.70)	4	0.00006	-0.0003, 0.0004	0.0002	-0.0006, 0.0009
Head defects (%)	282	95.13(3.3)	96	-0.00009	-0.00007, 0.00005	-0.00002	-0.0001, 0.0001
Neck/Mid-piece defects (%)	282	15.01(5.4)	15	-0.00009	-0.0003, 0.0001	-0.0004	-0.0008, 0.00004
Tail defects (%)	282	12.92(7.7)	11	-0.0002	-0.0005, 0.0001	-0.0007	-0.001, -0.00004
Round cells (10 ⁶ /ml) ^a	291	1.13(1.3)	1	0.0005	0.0002, 0.0008	0.0013	0.0007, 0.0019

Cytoplasmic droplets (%) ^a	282	11.47(6.5)	10	0.0005	-0.0008, 0.0010	0.0013	0.0002, 0.0024
Teratozoospermic Index (TZI) ^d	282	1.39(0.1)	1.4	8.94e-06	-0.00003, 0.00005	0.00003	-0.00005, 0.00014
CASA parameters:	n	Mean (± SD)	Median	p,p'-DDE		p,p'-DDT	
				beta	p-value	beta	p-value
Average path velocity $\mu\text{m/s}$ (VAP) ^e	241	36.51(15.2)	36.0	-0.0065	-0.0154, 0.0024	-0.0166	-0.0354, 0.0022
Straight line velocity $\mu\text{m/s}$ (VSL) ^a	241	26.98(13.0)	26.5	-0.0006	-0.0014, 0.0001	-0.0018	-0.0034, -0.0002
Amplitude of lateral head displacement (ALH) ^a	241	2.54(0.8)	2.5	-0.0001	-0.0003, 0.00003	-0.0003	-0.0007, -0.00003
Beat cross frequency (BCF) ^e	239	28.68(6.3)	29.0	0.0064	0.0028, 0.0100	0.0138	0.0062, 0.0213
Mean motility (%) ^e	240	48.53(18.6)	52.0	-0.0175	-0.0283, -0.0068	-0.0494	-0.0720, -0.0268

837 * Dependent variables were transformed when required to normalize their distribution for
838 linear regression analysis. Negative binomial regression was used for morphology
839 parameters because of their distribution.

840 ^a Square root transformation

841 ^b Cubed transformation

842 ^c Squared transformation

843 ^d Reciprocal transformation

844 ^e No transformation (raw)

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854 **Table 3. Oligo, astheno and teratozoospermia distribution and their age adjusted association**
 855 **to lipid adjusted *p,p'*-DDE and *p,p'*-DDT**
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<i>Parameter</i>	n	Percentage		OR	p	IC 95%
		No	Yes			
Oligozoospermia (< 20 million sperm/mL)	295	72	28	DDE: 1.001	0.03	(1.0001, 1.0025)
Asthenozoospermia ($< 50\%$ Grades a + b motility)	285	68	32	DDT: 1.003	0.006	(1.0007, 1.0055)
Teratozoospermia ($< 15\%$ normal morphology)	291	0.5	99.5	*	*	*

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 858 * Not computable because of variable's distribution
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891 **Figure 1: The distribution of oligozoospermia and asthenozoospermia associated with the**
892 **dose-dependent lipid adjusted *p,p'*-DDE percentile concentrations (1= 25th percentile; 2= 50th**
893 **percentile; 3= 75th percentile; 4= 100th percentile). The graph shows that the higher the *p,p'*-**
894 **DDE concentration the greater the incidence of oligozoospermia (OR:1.001; p=0.03) and**
895 **asthenozoospermia(OR: 1.001; p= 0.02).**
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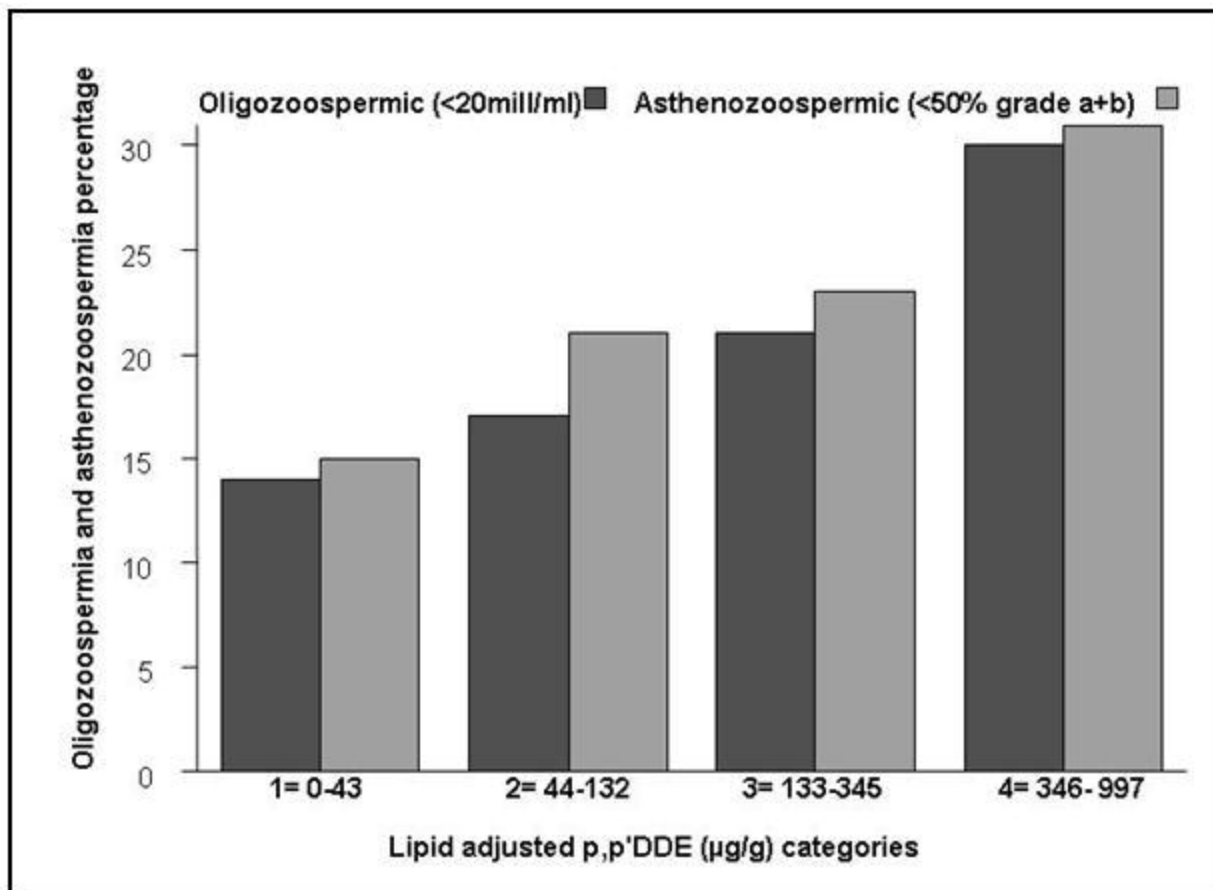


Figure 1: