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SCHOLARLY REVIEW

Pesticides, Sexual Development, Reproduction, and Fertility: Current Perspective and Future Direction

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ABSTRACT

Improvements in chemical analytical technology and non-invasive sampling protocols have made it easier to detect pesticides and their metabolites at very low concentrations in human tissues. Monitoring has revealed that pesticides penetrate both maternal and paternal reproductive tissues and organs, thus providing a pathway for initiating harm to their offspring starting before fertilization throughout gestation and lactation. This article explores the literature that addresses the parental pathway of exposure to pesticides. We use DDT/DDE as a model for chemicals that oftentimes upon exposure have no apparent, immediate health impacts, or cause no obvious birth defects, and are seldom linked with cancer. Their health effects are overlooked because they are invisible and not life threatening—but might have significant health, social, and economic impacts at the individual and population levels. The purpose of this article is to demonstrate the necessity to develop new approaches for determining the safety of pesticides and the need for innovative regulatory policy to protect human and environmental health.

Key Words: adverse effects, agriculture, endocrine disruption, exposure, fetal origin, pesticides.

INTRODUCTION

More than 15 years ago it was revealed that traditional toxicological testing protocols to determine chemical safety had missed vast numbers of diverse chemicals that enter the womb and interfere with the construction and programming of developing animals, including humans (Colborn and Clement 1992). Called endocrine disruptors, even prior to fertilization these chemicals interfere with gene-controlled signaling systems that control prenatal and postnatal development and function throughout life (Chapin *et al.* 1996). As a result of recent research in the growing

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discipline of endocrine disruption, knowledge has accumulated rapidly about the biological systems that control reproduction, sexual development, and fertility and their vulnerability. This body of knowledge has evolved from many disciplines and encompasses molecular and cellular *in vitro* studies, whole animal studies, and human epidemiology.

However, when testing synthetic chemicals for regulatory purposes, traditional testing has not focused on testing at ambient exposure concentrations nor taken into consideration the extremely low concentrations of naturally produced chemicals that control the endocrine system. In contrast to this approach, endocrine disruption testing focuses on the covert developmental and functional effects of synthetic chemicals at environmentally relevant exposure concentrations that target various organ systems at selected stages of development prior to birth and through adulthood. The expression of the results of low dose exposure during early development is unlike that at much higher doses during adult life stages. These differences in dose-response relationships, overt versus covert biological endpoints, and what they imply in terms of life-long human health outcomes as a result of pesticide exposures, have been ignored in many instances by regulators.

Pesticides, including their presumed inert ingredients, are among a growing list of chemicals demonstrated to interfere with sexual development, reproduction, and fertility when exposure occurs during vulnerable life stages. To date, pesticide toxicological testing requirements, risk assessment models, and exposure standards have not seriously nor rigorously responded to the threat posed by endocrine disruptors. Despite passage of the U.S. Food Quality Protection Act (FQPA) in 1996 and its clear mandate and provisions calling for the testing of pesticides for their potential to impair endocrine system function, the U.S. Environmental Protection Agency (USEPA) has yet to develop and adopt a series of standardized assays to meet the mandate. Consequently, pesticides continue to be registered and reregistered based on traditional toxicological assays, ignoring the growing evidence in the open literature that some widely dispersed pesticides interfere with development and function at ambient exposure concentrations. In what follows, we will address the need to broaden the scope of pesticide testing to include endocrine disruption and to rethink regulatory strategies in terms of pesticide impacts on human sexual development, reproductive success, and fertility.

In order to do this, let's turn first to some population statistics that are relevant to our discussion. Human population statistics indicate that fertility, reproductive success, and male/female live birth ratio are declining in the industrialized world. Globally, the fertility rate (the number of live births per female of reproductive age) since the 1970s has dropped below the population replacement number of 2.1 children per female in most of the major developed countries. The United States is no exception, with a fertility rate of 2.08 in 2005 compared with 3.65 in 1960. Germany's fertility rate was 1.37 and Japan's was 1.39 in 2005 (U.S. Bureau of the Census 2005). Birth rates per thousand population are 14.14 (US), 8.3 (Germany), and 9.47 (Japan) (CIA 2005). These statistics do not reveal how much of this decrease is by choice and how much might be due to contaminants such as pesticides.

The expected normal male/female sex ratio at birth is 1.06/1.00. Any substantial shift away from this could signal that something is disturbing fetal development. A

clear shift in the ratio toward fewer male births began to appear in 1970 in Canada, the U.S., the U.K., and Europe (Davis *et al.* 1998; Marcus *et al.* 1998). Between 1970 and 1995, U.S. figures show a highly significant loss of 1.0 male per 1,000 births ($p < 0.001$). In Canada, where statistical data go back as far as 1930, the shift also commenced in 1970. Since then there was a cumulative loss of 2.2 males per 1,000 births across Canada, whereas the eastern Atlantic region's loss was 5.6 male births per 1,000 births over the same 25 years (Allan *et al.* 1997). In Japan, the only country that records the sex of aborted and stillborn babies, the percentage of male fetal deaths doubled since 1970 (Mizuno 2000), suggesting that males are more sensitive to gestational stress(es).

The trends in reproductive outcome presented earlier, although expressed at the individual level, went unrecognized until they were discovered by epidemiologists and statisticians using government data spanning several generations. Many disorders, or underlying causes, could very well have contributed to those statistics starting from pre-fertilization to birth in the affected individuals. In this article we use several approaches to demonstrate that there are enough data in the open literature at the individual level to suggest that pesticides are, in part, contributing to the population trends mentioned earlier.

Improvements in chemical analytical technology and non-invasive sampling protocols have made it easier to detect pesticides, their metabolites, and a diverse number of industrial chemicals at very low concentrations in human tissues. Monitoring has revealed that pesticides and other synthetic chemicals penetrate both maternal and paternal reproductive tissues and organs, thus providing a pathway for exposure to their offspring even before fertilization throughout gestation and lactation (see Table 1).

We open this article using DDT (*p,p′*-DDT (1,1-trichloro-bis(p-chlorophenyl) ethane) and its metabolite DDE (*p,p′*-DDE (1,1-dichloro-bis(p-chlorophenyl) ethylene) as a model for the mistake of having to depend on human epidemiological studies to prove that a chemical is, or is not, safe before regulatory action is taken.

Table 1. Human reproductive tissues in which pesticides have been detected.

Tissue	Citation(s)
Amniotic fluid	Bradman *et al.* (2003); Foster *et al.* (2000)
Blood serum	Brock *et al.* (1998)
Blood, maternal	Dorea *et al.* (2001); Saxena *et al.* (1983); Siddiqui *et al.* (2003); Simonetti *et al.* (2001)
Blood, umbilical cord	Dorea *et al.* (2001); Saxena *et al.* (1983); Siddiqui *et al.* (2003); Simonetti *et al.* (2001)
Breast milk	Dorea *et al.* (2001); Schinas *et al.* (2000); Polder *et al.* (2003)
Colostrum	Waliszewski *et al.* (2002)
Meconium	Whyatt and Barr (2001)
Ovarian follicular fluid	Jarrell *et al.* (1993)
Placenta	Dorea *et al.* (2001); Saxena *et al.* (1983); Siddiqui *et al.* (2003); Simonetti *et al.* (2001)
Semen	Arbuckle *et al.* (1999); Swan *et al.* (2003b)
Urine	Coronado *et al.* (2004); Curl *et al.* (2003)

Delaying for several generations by having to wait for population statistics to affirm (or not) the safety of a pesticide is not protective of human health. To do this we present the epidemiological data related to DDT/DDE exposure and other pesticides from the earliest life stages, starting with the pre-conception status of the parents (sperm and ovum), through to adulthood, revealing the continual vulnerability of individuals to pesticides. We then turn to laboratory evidence revealing that pesticides must be regulated based on more than one endpoint to determine their safety, and that additional testing for regulatory purposes must be done at ambient exposure concentrations. What we present challenges the U.S. Environmental Protection Agency's (USEPA's) outmoded approach that has almost completely missed the low-dose and endocrine system-mediated effects of pesticides. All confidence intervals (CI) are at 95%.

EVIDENCE OF EFFECTS

DDT/DDE

Probability of pregnancy

Preserved maternal serum samples collected between 1960 and 1963 were tested for *p*,*p*′-DDT and *p*,*p*′-DDE and the concentrations compared with time-to-pregnancy of their oldest daughters 28–33 years later (Cohn *et al.* 2003). For every 10 μg/L increase in *p*,*p*′-DDT pregnancy probability dropped by 32% (CI: 11–48). In contrast, for every 10 μg/L increase in *p*,*p*′-DDE the probability of pregnancy increased by 16% (CI: 6–27). The median concentration of *p*,*p*′-DDT in this study was 13.05 μg/L (range 3.04–48.4) and for *p*,*p*′-DDE, 48.9 μg/L (range 11.21–132.53 μg/L). The authors suspect that DDE's antiandrogen effect may mitigate any harmful DDT androgen damage in the ovary (see Kelce *et al.* 1995). The results also raise the question of whether *p*,*p*′-DDT's impact on the daughters' ability to conceive was also expressed in their mothers' generation and perhaps with more intensity? And whether the results reflect the effect of a shifting change in body burden in the ratio of *p*,*p*′-DDT to *p*,*p*′-DDE? This study exposed heretofore occult activity of DDT and DDE where their effects are manifested in the second generation—and not until adulthood—and with an ultimate effect at the population level in the third generation. These cryptic and confusing findings provide insight into the complexity and insidious nature of a pesticide that is not acutely toxic and has been considered safe by some (Attaran *et al.* 2000) for more than 60 years. This study points out the need for multigenerational testing of pesticides, especially those that are persistent and may have degradation products that have different health impacts than the parent compound. A recent calculation of the volatilization half life of total DDTs in Canadian agricultural soils is approximately 200 years (Kurt-Karakus *et al.* 2006). Because the DDTs are going to be around for a long time, if the DDT to DDE ratio in body tissue continues to decrease, the aforementioned study suggests that perhaps the health impacts might gradually shift, thus making cause and effect relationships even hazier. It certainly exacerbates the difficulty of regulating chemicals whose residues have differing and opposing health endpoints than the parent compound and which will be around for many generations.

Fetal loss

Nulliparous, non-smoking Chinese women ($n = 372$) working in the textile industry between 1996 and 1998 provided daily urine samples for chorionic gonadotropin in order to confirm conception (Venners *et al.* 2005). Preconception blood samples were taken to measure DDT and its isomers. Early pregnancy loss (128 occurring less than 6 weeks after the onset of the last menstrual period) and clinical spontaneous abortion (36 occurring after 6 weeks and no more than 20 weeks) were recorded out of 500 conceptions. Upon breaking the women into tertiles based on serum concentrations of total DDT, a significant monotonic relationship was discovered between increasing DDT levels and likelihood of early pregnancy loss. The odds for pregnancy loss were 1.17 (CI: 1.05–1.29) with each 10 ng/g increase in total serum DDT only for first conception. The odds for *p*,*p*′-DDT alone at a 1 ng/g increase were 1.21 (CI: 1.02–1.44) and *p*,*p*′-DDE at a 10 ng/g increase were 1.19 (CI:1.04–136). No association between DDT concentrations and spontaneous miscarriages were revealed. Serum total *p*,*p*′-DDE in this study accounted for 92% of the total DDT and together with *p*,*p*′-DDT they accounted for 98% total DDT. The range of total serum DDT found in this study was 5.52 ng/g to 113.3 ng/g. None of the women in this study worked on a farm. Instead they were processing material that could have been contaminated with residual DDT, because the ratio of DDE to DDT in their bodies suggests that the DDT had not been used recently. The findings in this study suggest that the odds for fetal loss are approximately 10 times greater in the presence of *p*,*p*′-DDT than *p*,*p*′-DDE. These results point out the need to treat each isomer as a separate stressor rather than basing conclusions on only the parent compound or one isomer. They point out what is possibly lost in an analysis when only total values are used, a lesson learned when early on only total dioxins and PCBs were used to make associations with health end points. Upon basing analyses on individual congeners (such as 2,3,7,8-TCDD, PCB 77, PCB 156, *etc.*), or isomeric groups (the highly chlorinated hexa-, septa-, and octo-PCBs), some associations became significant.

Length of gestation and birth weight

Using various data sets and cohorts that go back over the past 40 years, several investigative teams have discovered a relationship between maternal *p*,*p*′-DDE and preterm deliveries and low birth weight. For example, the odds increased from 1.0 to 1.5, 1.6, 2.5, and 3.1 (trend $p = 0.0001$) for pre-term births with each 10 μg/L *p*,*p*′-DDE increase for a subset of 44,000 children born between 1959 and 1966 (Longnecker *et al.* 2001). A similar trend in odds was found for small-for-gestational-age from 1.0 to 1.9, 1.7, 1.6, and 2.6 (trend $p = 0.04$) with *p*,*p*′-DDE. Births were considered pre-term if they occurred before the 37th week of pregnancy, and small-for-gestational age if they weighed 10% less than the norm for the study cohort. The median *p*,*p*′-DDE concentration for the general population in this study was 25 μg/L (range 3–178 μg/L).

Thirty years later, between 1990 and 1993, 20 women who experienced early deliveries were selected for comparison of birth outcome with 20 women who delivered at term (Berkowitz *et al.* 1996). There was a significant association between birth weight percentile groups and maternal serum *p*,*p*′-DDE concentration and early delivery ($p = 0.02$), and between preterm rupture of membranes and controls ($p = 0.04$).

In this more recent cohort, preterm birth was associated with *p,p′*-DDE (median 1.3 ng/ml (0.50–17.90) at a median level 18 times lower than in the general population in the Longnecker *et al.* (2001) study mentioned earlier. These results raise the question of whether the concentrations of DDT/DDE in the environment will ever reach a level where damage will be indiscernible. Keeping in mind the Cohn *et al.* (2003) study mentioned earlier, we add here that in all probability the women in this study were already exposed to *p,p′*-DDE in their mother's wombs a generation earlier, which may or may not have had some influence on the results in this study.

We present the following study even though statistically the odds were not significant because it provides insight into the problem of dealing with wider and wider exposure to pesticides and the difficulty of dealing with marginal results. In 1995 *p,p′*-DDE was marginally associated with preterm births among 100 Mexico City women with spontaneous preterm births when compared with 133 controls (Torres-Arreola *et al.* 2003). Maternal blood was collected within 24 hours after delivery and serum lipids tested for *p,p′*-DDE. An increased risk of 1.87 (CI: 0.95–3.68) for early delivery at an exposure level of 111.6–228.8 ng/g *p,p′*-DDE (the lowest tertile) was discovered. For those with DDE greater than 228.8 ng/g the odds were almost the same 1.67 (CI: 0.84–3.31). Results like these suggest that perhaps there is no safe level for exposure to DDT/DDE. For comparison purposes with the two previous studies, the *p,p′*-DDE concentrations in the lowest tertile in this article reported in blood lipid units would be equivalent to 0.92 μg/L–1.86 μg/L and for the top two tertiles, 1.86–17.56 μg/L.

The Longnecker *et al.* (2001) article mentioned earlier found a dose response effect on preterm births starting at 21 μg/L *p,p′*-DDE that is slightly higher than the highest concentration found in this Mexico City study. Granted, the earlier odds were not significant, but like most environmental situations dealing with contamination, there are always outliers that add considerable variation to the range of contamination and exposure—as in this study the range was 10 to 1923.41 ng/g and 10 to 1958.26 ng/g in the cases and controls, respectively. This study demonstrates the difficulty of finding a cohort for comparison purposes that has not been exposed. Even with a similarly exposed control population this study revealed a statistically weak association with preterm births and *p,p′*-DDE, and at the lowest exposure consistent with contemporary ambient background concentrations. The authors emphasize that individuals are still affected today although major uses of DDT for agricultural purpose were stopped in 1972 in the United States and are almost phased out in Mexico today. It is unlikely that outcomes such as these would be recognized by a woman and her doctor, and even if they were, their etiology would not be linked with pesticide exposure. Nonetheless, across a population, subtle impacts such as this add up and collectively pose a drain on public health resources.

The concentrations of two DDT metabolites in human breast milk in 4 northern Russian cities, when used as an indicator of prenatal exposure, revealed a "possible negative influence" on birth weight; *p,p′*-DDT ($p = 0.011$, $r^2 = 0.05$), *p,p′*-DDE ($p = 0.011$, $r^2 = 0.05$) (Polder *et al.* 2003). The mean concentration of total DDTs (750–1400 μg/kg milk fat) in the milk fat was equivalent to a daily intake of 4–7 μg/kg body weight for a 3–4 kg infant consuming 160 ml/kg body weight with 3% fat content. The World Health Organization's (WHO's) acceptable daily intake (ADI) for total is DDT 20 μg/kg body wt/day—four times higher than that found in the infants'

diets. The end point on which the ADI was determined is based on the lifetime exposure of a 70 kg adult male (Bouwman *et al.* 2006). As in previous studies, the concentrations at which these infants were affected are within ambient exposure levels. The physicians and the parents of the affected infants would probably not realize that their child is in the low birth-weight range let alone make a link with DDT exposure.

Placental blood and cord blood was collected at birth from Indian women who at 28 weeks were discovered by sonogram to be experiencing intrauterine growth retardation (IUGR) (Siddiqui *et al.* 2003). IUGR is defined as a birth weight 10% or more below normal. The blood levels of *p,p′*-DDE were negatively correlated with birth weight adjusted for gestational age ($p < 0.05$) with an odds ratio of 1.21 (CI: 1.03–1.42). The mean *p,p′*-DDE in this study was 8.79 μg/L and mean *p,p′*-DDT was 0.55 μg/L for mothers with IUGR. These authors hypothesize that because *p,p′*-DDE blocks progesterone receptors, this could contribute to shortening gestation periods and lowering birth weights.

Between 1997 and 1999, daily urine samples from a cohort of Southeast Asian immigrant women were assayed for DDT, DDE, and estrogen and progesterone metabolites (Windham *et al.* 2005). Mean DDT was 1.77 ng/L and median 0.79 ng/L. On a mean fat basis DDT was 287 ng/g and the median was 133 ng/g. Mean DDE was 20.8 ng/L and median was 13.1 ng/L. On a mean fat basis DDE was 3634 ng/g and the median was 2355 ng/g. Menstrual cycle length of women in the highest quartile was 4 days shorter compared with the lowest quartile. Higher concentrations of DDT or DDE were also associated with a reduction in progesterone metabolite levels and a decrease in the length of the luteal phase of the menstrual cycle. These authors suggested that the ovarian changes might signal DDT/DDE interference with the role of progesterone in cycling and maintaining pregnancy. This study went so far as to measure a hormone that is critical for the orchestration of pregnancy. The authors pointed out that the mean concentrations of DDT and DDE in these subjects is above that found in the U.S. population.

Birth defects and prenatal exposure

Information in a Ministry of Health of Mexico registry for a cohort of malaria control workers ($n = 2033$) who worked for a year or more applying DDT between 1956 and 1990 up to 2000 was used to evaluate the effects of occupational exposure to DDT on male reproductive outcome using the DDE in their body fat as a surrogate (Salazar-García *et al.* 2004). They fathered 9,187 children among whom 55 were born with birth defects based on the fathers' recall. Those defects recalled were classified as nervous system, osteomuscular, and obvious eye and ear anomalies. The odds for a congenital malformation increased to 3.37 (CI: 1.19–9.52) after the men started to apply DDT. The odds for those carrying the highest concentrations of DDE when broken out into quartiles jumped to 4.41 (CI: 1.41–13.84) in a nonlinear manner. We decided to add this recent study to this article because it is the first article we found that suggests the use of DDT might cause congenital malformations and that looked solely at the paternal contribution to the anomalies found. Again, like previous studies it uses only the concentrations of DDE in its statistical analysis and in this

case exposure was estimated by recall and selective partitioning of that information, not with chemical analysis.

A study by Longnecker *et al.* (2002) used archived maternal samples from women giving birth between 1959 and 1966 to look for cryptorchidism, hypospadias, and polythelia. The odds increased among the most highly exposed boys versus the least exposed boys but were not statistically significant. Keeping in mind the results of some of the studies mentioned earlier where there seemed to be no safe dose, Longnecker's results are not unusual. It is also important to keep in mind that hypospadias is an under-reported event for several reasons and even the most severe cases might be reluctantly reported by men.

Lactational effects

Using data collected between 1978 and 1982 Rogan *et al.* (1987) reported that the length of North Carolina mothers' ($n = 722$) ability to breast feed was inversely related to the amount of p,p'-DDE in their breast milk. From a median of 7.8 months, lactation dropped to 3.8 months as concentrations reached 10 to 12.5 ppm p,p'-DDE in breast milk fat. In a follow-up study, pregnant women expecting their first child in a Mexican agricultural town were recruited from September 1988 to October 1989 to provide breast milk samples at birth and to provide follow-up information when the child was weaned ($n = 229$) (Gladen and Rogan 1995). Only women giving birth to a single child were enrolled. As concentrations of p,p'-DDE increased to 12.5 ppm in breast milk fat, median lactation time decreased from 7.5 months to 4.0 months, similar to the results the authors found in their North Carolina study. These findings in the late 1980s received a great deal of attention among the scientific and academic community and provided the stimulus for several of the other studies described in this section.

Pubertal development

North Carolina children ($n = 594$) born between 1978 and 1982 whose records for prenatal and postnatal exposure to DDE and PCBs were available (placental blood, cord blood, maternal blood, and breast milk) had their height, weight, and stage of pubertal development measured at age 14 (Gladen *et al.* 2000). *In utero* exposure to DDE increased the height (6.3 cm) and weight (6.9 kg) of boys when adjusted for height, for those whose mothers' blood fat held more than 4 ppm p,p'-DDE. Girls were not affected similarly by p,p'-DDE and there was no effect on age of menarche. This was a follow-up study from the early 1960s work.

Observing the same population mentioned earlier of nulliparous, nonsmoking, Chinese women working in the textile industry between 1996–1998, researchers found an association between shortened menstrual cycles and serum total DDT (OR = 5.8, CI: 1.58 to 21.28) comparing those in the highest quartile of exposure with those in the lowest quartile (Ouyang *et al.* 2005). The youngest mean age at menarche was found in the highest quartile also. For each 10 ng/g increase in serum total DDT there was a reduction of 0.20 years at menarche. The mean total p,p'-DDT concentration in the lowest quartile was 1.8 (s.d.1.3) ng/g and the highest quartile 3.0 (s.d.1–8) ng/g; mean p,p'-DDE was 12.1 (s.d.3.4) ng/g and 53.3 (s.d.12.9) ng/g, respectively. The ratio of DDT to DDE in this study does not suggest that fresh DDT is

contributing to their body burdens. Because of improved technology the detection limits in these newer studies are now in the parts per trillion (ppt) range, 0.022 ng/g for p,p'-DDE and 0.031 ng/g for p,p'-DDT. These results suggest that perhaps even background levels of DDT/DDE have an effect on reproductive health.

Female offspring ($n = 151$) between the age of 20 and 50 whose mothers' serum concentrations of DDE were determined between 1973 and 1991 experienced earlier menarche ($p = 0.038$) (Vasiliu *et al.* 2004). The authors estimated that for each 15 μg/L of DDE increase in maternal serum the daughters' age of menarche was reduced by one year. This study adds to the concern about the long-term, multigenerational effects of DDT/DDE and other chemicals that can interfere with the endocrine system.

Reproductive effects measurable in adults

In 2000, healthy farmers, non-occupationally exposed to DDT ($n = 116$) between ages 18 and 40 living in an area of Mexico where DDT was sprayed in homes for malaria control until 1997 provided blood samples for p,p'-DDE and p,p'-DDT quantification along with two semen samples (de Jager *et al.* 2004, 2006). The statistical analyses were done using the concentrations of p,p'-DDE as the surrogate for DDT exposure. Men with diagnosed infertility and other health problems were excluded from the study. Study subjects represented the healthy segment of the population who had lived in this area for at least a year. p,p'-DDE was associated with abnormal sperm tail morphology ($p = 0.017$) and a weak association with impaired sperm motility ($p = 0.074$). With increasing concentrations of p,p'-DDE they found increasing poor sperm motility, abnormal sperm morphology, and inadequate sperm chromatin condensation using computer-assisted analysis. The authors pointed out that DDT, the parent compound, is an estrogen and p,p'-DDE is an antiandrogen, each capable of reducing testosterone production through a different mechanism. DDT could interfere via the hypothalamic-pituitary-axis by reducing testosterone production in the Leydig cells. DDE could interfere directly by blocking androgen receptors. In each case these could prevent the Sertoli cells from maintaining normal sperm production.

LABORATORY RESULTS

DDT/DDE

None of the aforementioned DDT/DDE studies revealed how DDT or DDE interfered with development that led to the health changes recognizable at the population level. The laboratory studies that follow reveal some mechanisms of action of DDT/DDE that add credence to some of the aforementioned findings among human populations.

Probability of pregnancy

A dose at the mean concentration of p,p'-DDE (0.33 μg/L) found in the cord blood of Arctic Alaskan women maintaining a traditional life style and a dose 10-fold higher were introduced into embryonic mouse fibroblasts and human fetal

fibroblasts in separate *in vitro* systems (Simonetti *et al.* 2001). At both doses used, cell death occurred in the mouse system whereas cell-cycling was arrested in the human system. Even though the doses used in this study were low, they were not low enough to determine the no effect level; yet what was used was well below what most studies used in the past.

Single-celled, preimplantation mouse embryos collected from oviducts and treated with *o*,*p*′-DDT (0.1 μg/ml) for 96 hrs experienced retarded development of the morula ($p < 0.001$) and increased apoptosis in blastomeres ($p = 0.05$), which attenuated embryo growth and increased programmed cell death prior to implantation (Greenlee *et al.* 2005). *o*,*p*'-DDT is the most powerful estrogen among the DDT isomers. These adverse effects did not impair implantation, alter litter rates, fetal weights, or sex ratio, or cause skeletal changes at birth. Consequently, they would never have been identified in a traditional toxicological study, and would also be missed in a human epidemiological study. Ideally, this study should have been extended beyond birth in the F1 generation (the stage at which this study stopped) in order to rule out damage that might be expressed in later life stages.

The findings of the two preceding studies bring us back to the Cohn *et al.* (2003) article where DDT and DDE had opposing effects on time to conception. It is probably much too simple to expect that one could ever determine the ratio of DDT to DDE that would result in a net no-effect level for this endpoint in the real world. We are presented two different mechanisms in the aforementioned studies that in each case could delay time to conception, but only the mechanisms of a single compound studied alone. The women in Cohn's study were exposed to vast mixtures of chemicals including pesticides, and no two women were exposed alike.

Which brings us to a laboratory study that used *p*,*p*′-DDE in the comet assay and found no effect on sperm (Hauser *et al.* 2003). Like the Cohn study, this article raises more questions about the role of *p*,*p*′-DDT and other metabolites on sperm integrity. The de Jager *et al.* (2006) article found sperm damage where the men were exposed to the other DDT metabolites (and many other chemicals). This suggests that damage to sperm integrity mentioned in other studies could be from *p*,*p*′-DDT and other DDT metabolites and not *p*,*p*′-DDE. This also points out the difficulty of comparing the significance of the results from study to study when one study used only DDT or DDE whereas others broke out the analysis by each DDT metabolite.

Fetal and pubertal development

In 1993, in what has now become a landmark article in the field of endocrine disruption, it was discovered that *p*,*p*′-DDE has little ability to bind to the estrogen receptor and is instead a potent inhibitor of androgen receptor (AR) binding (Kelce *et al.* 1995). This was a stunning discovery because from the time *p*,*p*′-DDE's parent compound DDT came on the market, *p*,*p*′-DDE had been considered an estrogen. It is only 10 times less potent than flutamide, a powerful antiandrogenic pharmaceutical. The dose required to inhibit androgen receptor (AR) binding and the subsequent inhibition of transcriptional activity in this cell culture assay was only 63.6 ppb, a little more than an order of magnitude higher than the amount found in human tissues today. Following this discovery the same team gavaged pregnant Long-Evans hooded rats with *p*,*p*′-DDE (100 mg/kg/d) from gestational day 14 to

18, the period of sexual differentiation in the rat, which would be equivalent to pregnancy week 7–8 in humans. Their male pups exhibited shortened anogenital distance (AGD) and retained nipples. In another assay, weanling rats 21 days old were dosed with 100 mg/kg/d *p,p′*-DDE until after puberty. Following this postnatal exposure, dosed animals reached puberty 5 days later than controls. Not only does *p,p′*-DDE affect development before birth, but it also has an effect on pubertal development if exposure occurs postnatally. The irony of these findings is that through its antiandrogen activity, *p,p′*-DDE demasculinizes and feminizes males, resulting in an estrogenic-like outcome, which standard testing protocols had not discovered.

It is important to note here that within the last three years medical researchers have begun to report on the activity of various isomers of DDT at the cellular level within the ovarian follicle. *p,p′*-DDE enhanced aromatase activity thus increasing estrogen production within the cell (Younglai *et al.* 2004). At 3 nmol/ml *p,p′*-DDE and *o,p′*-DDE increased calcium concentrations released from the smooth endoplasmic reticulum (Wu *et al.* 2006). At a low dose (4 ppb) *o,p′*-DDT exerted antiestrogenic activity and at higher doses estrogenic activity (Wójtowicz *et al.* 2004). Following up with assorted DDT isomers and doses, these researchers reported that with the exception of *o,p′*-DDT, they found elevated estradiol secretion with all isomers after a single low dose. However, repeated exposure led to "massive antiestrogenic activity." In a complementary study, it was revealed that DDT interferes with the ability of placental trophoblasts to support calcium transport to the embryo by interfering with the signaling pathways of estrogens (Derfoul *et al.* 2003). Results such as these suggest that there are extensive possible mechanisms driving the activity of DDT and explain in part the vague, but consistent, results of the human studies mentioned earlier.

EVIDENCE OF EFFECTS

Non-DDT/DDE

What follows is a review of the literature other than that devoted to DDT that also reveals a progression of disorders starting with delayed time to conception through to adulthood associated with pesticide exposure. (See Table 2 for statistics.) In many cases the studies that follow are dealing with pesticides that are not persistent and do not bioaccumulate, unlike DDT. Thus seasonal signatures of exposure can be matched with health problems. However, DDT along with other persistent industrial chemicals could be playing a role in the health effects reported in what follows.

Probability of pregnancy

When pesticides were applied more than 100 times a year, greenhouse workers experienced an increased risk in delays to conception (Petrelli and Figà-Talamanca 2001). Wives who mixed and applied herbicides for two years prior to attempting to conceive experienced a significantly elevated increased risk of infertility (Greenlee *et al.* 2003).

Table 2. Human epidemiological studies reporting reproductive and developmental effects associated with pesticide exposure and/or crop type.

Life stage	Pesticide	Conditions	Citation(s)
Probability of pregnancy	Pesticides	Greenhouse workers: OR = 2.4 (CI: 1.2–5.1)	Petrelli and Figà-Talamanca (2001)
	Herbicides	Wives mixing and applying: OR = 26.9 (CI: 1.9–384.8)	Greenlee *et al.* (2003)
Pre-term birth	Grain farming	Grain farmers: OR = 1.4 (CI: 1.0–1.9) with history of only term births, and OR = 11(CI: 7.7–15.9) with previous history of preterm birth	Kristensen *et al.* (1997c)
	Grain farming	Midpregnancy delivery (wk 21–24) 2.8/1000 grain farmers vs 1.8/1000 non-grain farmers (p.333), (2.9 and 1.9/1000 when restricted to main grain producing districts); OR = 1.8 (CI: 1.1–2.8) for post-harvest births (Sept–Dec); OR = 3.75 (CI: 1.72–8.20 for multiple-birth pregnancy; and OR = 2.42 (CI: 1.54–3.79) for poor harvest quality	Kristensen *et al.* (1997b)
	Herbicides	Preterm deliveries linked with paternal mixing and applying yard herbicides: OR = 2.1 (CI: 1.00–4.4); OR = 2.5 (CI: 1.1–5.8) with protective equipment; OR = 1.1 (CI: 0.03–4.5) without protective equipment	Savitz *et al.* (1997)
	Yard herbicides: Triazines	Preterm delivery OR = 3.2 (CI: 1.2–8.9)	Savitz *et al.* (1997)
	Yard herbicides: Atrazine	Preterm delivery OR = 4.9 (CI: 1.6–15)	Savitz *et al.* (1997)
	Yard herbicides: 2,4-DB	Preterm delivery OR = 3.5 (CI: 1.2–9.9)	Savitz *et al.* (1997)
	Atrazine, 2,4-D, Phenoxy herbicides, OPs and other insecticides	Preterm delivery OR > 2 combinations of activities with a variety of chemicals:	Savitz *et al.* (1997)
Fetal loss	Farming	Late-term abortion OR = 1.9 (CI: 1.6–2.3); with non-induced delivery OR = 2.18 (CI: 1.67–2.85) (farmers vs. non-farmers)	Kristensen *et al.* (1997b)

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Table 2. Human epidemiological studies reporting reproductive and developmental effects associated with pesticide exposure and/or crop type.

Life stage	Pesticide	Conditions	Citation(s)
Fetal loss cont.	Grain farming	Late-term abortion OR = 1.31 (CI: 1.11–1.55) grain farmers [vs. other farmers]	Kristensen *et al.* (1997b)
	Pesticides	Conceptions during heaviest use season (April to September) OR = 2.40 (CI: 1.53–3.77) due to congenital anomalies; OR = 1.72 (CI: 1.42–2.08) due to all other causes	Regidor *et al.* (2004)
	Pesticides (occupational exposure, agricultural counties)	First and second trimester exposure risk ↑ ($p < 0.05$)	Pastore *et al.* (1997)
	Agriculture	Women farm workers: OR = 1.4 (CI: 1.2–1.6, $p < 0.001$)	Vaughan *et al.* (1984)
	Halogenated hydrocarbon pesticides	First trimester (3rd–8th week): OR = 2.2 (CI: 1.3–3.9), application within same or adjoining square mile as residence	Bell *et al.* (2001b)
	Pyrethroids	First trimester (3rd–8th week): OR = 1.9 (CI: 1.1–3.2), applications within same or adjoining square mile as residence	Bell *et al.* (2001b)
	Phosphates	First trimester (3rd–8th week): OR = 3.0 (CI: 1.4–6.5), application within same square mile as residence	Bell *et al.* (2001b)
	Multiple exposure to 3 + pesticide classes	OR = 2.6 (CI: 1.3–5.3), applications within same or adjoining square mile as residence	Bell *et al.* (2001b)
	Phenoxyacetic acid herbicides[i]	First trimester: OR = 1.5 (CI: 1.1–2.1)	Arbuckle *et al.* (2001)
	Triazines	First trimester: OR = 1.4 (CI: 1.0–2.0)	Arbuckle *et al.* (2001)
	Any herbicides	First trimester: OR = 1.4 (CI: 1.1–1.9)	Arbuckle *et al.* (2001)
	Glyphosate	Late abortion: OR = 1.7 (CI: 1.0–2.9)	Arbuckle *et al.* (2001)
	Thiocarbamates	Late abortion: OR = 1.8 (CI: 1.1–3.0)	Arbuckle *et al.* (2001)
	Misc. pesticides	Late abortion: OR = 1.5 (CI: 1.0–2.4)	Arbuckle *et al.* (2001)
	Carbaryl	Women over 35: abortion risk ↑ 4-fold	Arbuckle *et al.* (2001)
	2,4-D and Carbaryl	Women over 35: abortion risk ↑ 27-fold	Arbuckle *et al.* (2001)
	Atrazine, 2,4-D, and Carbaryl	Exposure 3 mos. before and including conception month increases RR 20 to 40%	Arbuckle *et al.* (2001)
	Thiocarbamates	Miscarriage OR = 1.9 (CI: 1.1–3.3)	Savitz et al. (1997)

Fetal loss cont.	Carbaryl	Miscarriage OR = 1.9 (CI: 1.1–3.1) (with crop herbicide use), OR = 2.1 (CI: 1.1–4.1) (with crop insecticide or fungicide use)	Savitz et al. (1997)
	Pesticides	Women mixed, loaded and applied: OR = 1.81 (CI: 1.04–3.12)	Garry *et al.* (2002b)
	Organotins	OR = 1.55 (CI: 1.01–2.37)	Garry *et al.* (2002b)
	EBDC fungicides	OR = 1.77 (CI: 1.11–2.83)	Garry *et al.* (2002b)
	Fungicides, Insecticides, Herbicides combined	OR = 1.64 (CI: 1.01–2.67)	Garry et al. (2002b)
	Herbicides	More than 10% of spring conceptions miscarried among spouses applying only herbicides	Garry *et al.* (2002b)
	Sulfonylureas	OR = 2.11 (CI: 1.09–4.09) spring miscarriages ↑	Garry *et al.* (2002b)
	Imidazolinones	OR = 2.56 (CI: 1.11–5.87) spring miscarriages ↑	Garry *et al.* (2002b)
	Mixture 9100[ii]	OR = 2.94 (CI: 1.40–6.16) spring miscarriages ↑	Garry *et al.* (2002b)
	2,4-D LV4	↑MCF-7 cell proliferation	Lin and Garry (2000)
	2,4-D amine	↑MCF-7 cell proliferation	Lin and Garry (2000)
	Glyphosate	↑MCF-7 cell proliferation	Lin and Garry (2000)
	Roundup	↑MCF-7 cell proliferation	Lin and Garry (2000)
	X-77 (adjuvant)	↑MCF-7 cell proliferation	Lin and Garry (2000)
	Activate Plus (adjuvant)	↑MCF-7 cell proliferation	Lin and Garry (2000)
	Triphenyltin	Aneuploidy (cell cycle arrest) and apoptosis in MCF-7 cells	Lin and Garry (2000)
	Mancozeb or metabolite ETU	Apoptosis in MCF-7 cells	Lin and Garry (2000)
Impaired fetal development	Sugar beet production	Low birth weight ($p = 0.05$)	Xiang *et al.* (2000)
	Herbicides: Atrazine. Metolachlor, Cyanazine	Herbicide-contaminated groundwater. Communities' IUGR risk ↑, RR = 1.8 (CI: 1.3–2.7)	Munger *et al.* (1997)

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Table 2. Human epidemiological studies reporting reproductive and developmental effects associated with pesticide exposure and/or crop type.

Life stage	Pesticide	Conditions	Citation(s)
Impaired fetal development cont.	δ-HCH	Negative correlation between neonatal body weight and residues in cord blood ($r = -0.27$, $p < 0.05$) after adjusting for gestational age IUGR: OR = 1.61(CI: 1.01–2.54) residues in maternal blood; OR = 1.31(CI: 1.00–1.75) residues in cord blood;	Siddiqui *et al.* (2003)
	α-HCH	IUGR: OR = 1.22(CI: 1.02–1.460) residues in maternal blood	Siddiqui *et al.* (2003)
	γ-HCH (lindane)	IUGR: OR = 1.38(CI: 1.05–1.80) residues in maternal blood; OR = 1.14(CI: 1.00–1.31) residues in cord blood;	Siddiqui *et al.* (2003)
Altered sex ratio	Fungicides, Insecticides, Herbicides combined	Sex ratio 0.80 (21% fewer boys than girls) ($p = 0.02$); fathers applied	Garry *et al.* (2002b)
	Fungicides	Sex ratio (all children): 0.91 fathers applied; 1.13 fathers who did not ($p = 0.04$); Sex ratio of children with birth defects: 0.57 fathers applied; 1.80 fathers who did not ($p = 0.02$)	Garry *et al.* (2002a)
	Trichlorophenol factory Dioxin (2,3,7,8-TCDD)	Dioxin exposed population. Sex ratio: 0.40 ($z = 3.21$, $p < 0.001$). Fathers exposed: 0.38 ($z = 3.60$, $p < 0.001$). Mothers exposed: 0.51 (considered normal)	Ryan *et al.* (2002)
	Dioxin (2,3,7,8-TCDD)	When fathers' dioxin TEQ blood lipid >715 ppt sex ratio ↓ to 0.23	Ryan *et al.* (2002)
Birth defects related to the reproductive system and development	Pesticides Intensive greenhouse and other agriculture	Orchidopexy: OR = 2.32 (CI: 1.26–4.29, $p < 0.05$) in highest pesticide use areas	García-Rodríguez *et al.* (1996)

Birth defects related to the reproductive system and development cont.	Chlorophenoxy herbicides and fungicides—high use regions	Ratio of birth defects between boys and girls Male/female sex ratio for four birth anomaly categories (including urogenital tract) (major anomalies): 2.8 appliers; 1.5 general population ($p = 0.05$)	Garry *et al.* (1996)
	Chlorophenoxy herbicides and fungicides lower use regions	Ratio of birth defects between boys and girls Male/female sex ratio for four birth anomaly categories (including urogenital tract) (major anomalies): 2.1 appliers; 1.7 general population ($p = 0.05$)	Garry *et al.* (1996)
	Corn/soybeans region	Birth defect rate 26.8/1000 private appliers; 21.3/1000 general population.	Garry *et al.* (1996)
	Wheat, corn, soybeans, potatoes, sugar beets regions	Birth defect rate 30/1000 private appliers; 26.9/1000 general population.	Garry *et al.* (1996)
	Urban, forest (non-crop) regions	Birth defect rate 23.7/1000 private appliers; 18.3/1000 general population.	Garry *et al.* (1996)
	Grain production	Limb reduction: OR = 2.50 (CI: 1.06–5.90) pesticide purchase; April-June conception Hypospadias: OR = 1.51 (CI: 1.00–2.26) tractor spraying; April–June conception	Kristensen *et al.* (1997a)
	Orchards and greenhouses	Spina bifida: OR = 2.76 (CI: 1.07–7.13) tractor spraying; April–June conception Hydrocephaly: OR = 3.49 (CI: 1.34–9.09) tractor spraying; July–September conception Urinary system defects: OR = 2.94 (CI: 1.19–7.29) pesticide purchase; July-September conception	Kristensen *et al.* (1997a)
	Vegetable crops	Cryptorchidism: OR = 2.32 (CI: 1.34–4.01) pesticide purchase; April -June conception	Kristensen *et al.* (1997a)

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Human epidemiological studies reporting reproductive and developmental effects associated with pesticide exposure and/or crop type. *(Continued)*

Life stage	Pesticide	Conditions	Citation(s)
	Gardening mothers	Cyptorchidism: OR = 1.67 (CI: 1.14–2.47)	Weidner *et al.* (1998)
	Vegetarian mothers	Hypospadias: 2.2% of vegetarian mothers had a son with hypospadias vs 0.6% among omnivore mothers ($p = 0.001$), OR = 4.65 (CI: 1.97–10.98)	North and Golding (2000)
Adverse reproductive effects as a result of adult exposure	Alachlor	Semen quality ↓ Urinary concentrations ↑ OR = 30 (CI: 4.3–210) among highest Missouri group	Swan *et al.* (2003b)
	Diazinon (metabolite IMPY (2-isopropoxy-4-methyl-pyrimidinol))	Semen quality ↓ Urinary concentrations ↑ OR = 16.7 (CI: 2.8–98) among highest Missouri group	Swan *et al.* (2003b)
	Atrazine	Semen quality ↓ Urinary concentrations ↑ ($p = 0.012$) using those above detection limit in the Missouri group	Swan *et al.* (2003b)
	Herbicides	Appliers free testosterone ↑ post-season ($p = 0.032$); FSH ↑ during spray season ($p = 0.016$) and post-season ($p = 0.010$)	Garry *et al.* (1999)
	2,4-D LV4	Genotoxic *in vitro* (micronucleus frequency ↑; significant dose response)	Garry *et al.* (1999)
Adverse reproductive effects as a result of adult exposure cont.	Direct (adjuvant)	Genotoxic *in vitro* (micronucleus frequency ↑; significant dose response)	Garry *et al.* (1999)
	Nalco-trol (adjuvant)	Genotoxic *in vitro* (micronucleus frequency ↑; significant dose response)	Garry *et al.* (1999)
	X-77 (adjuvant)	Genotoxic *in vitro* (micronucleus frequency ↑; significant dose response)	Garry *et al.* (1999)
	Preference (adjuvant)	Genotoxic *in vitro* (micronucleus frequency ↑; significant dose response)	Garry *et al.* (1999)
	Carbaryl (metabolite 1N)	Sperm concentration <20 million/mL: OR = 4.2 (CI: 1.4–13.0) and OR = 4.2 (CI: 1.4–12.6) for medium and high metabolite tertiles, respectively	Meeker *et al.* (2004a)

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Carbaryl (metabolite 1N)	<50% Sperm motility: OR = 2.5(CI: 1.3–4.7) and OR = 2.4 (CI: 1.2–4.5) for medium and high metabolite tertiles, respectively	Meeker *et al.* (2004a)
Carbaryl (metabolite 1N)	DNA damage (↑% DNA in comet tail, regression coefficient = 4.13 (CI: 1.92–6.32, $p = 0.0003$))	Meeker *et al.* (2004b)
Chlorpyrifos (metabolite TCPY)	DNA damage (↑% DNA in comet tail, regression coefficient = 2.76 (CI: 0.89 –4.62, $p = 0.004$))	Meeker *et al.* (2004b)
Organically grown	Organic eaters' sperm concentration ↑43.1% (CI: 3.2–98.8%, $p = 0.033$)	Jensen *et al.* (1996)
Pesticides vs. organic	Traditional farmers: normal sperm heads ↓ 39.5% vs. 42.3% ($p = 0.01$) and normal sperm ↓ 2.5% vs. 3.4% ($p = 0.02$). Organic farmers testosterone sex hormone binding protein↑ ($p = 0.02$) and inhibin B↑ ($p = 0.05$)	Larsen *et al.* (1999)
EBDC degradate ETU	Correlation between thyroid nodule size and blood levels ($p = 0.001$)	Panganiban *et al.* (2004)
Wheat, sugar beet, potato region	↑prostate cancer mortality OR = 1.12 (CI: 1.00–1.26) and thyroid cancer OR = 2.95 (CI: 1.35–6.44)	Schreinemachers *et al.* (1999)

[i]For example, 2,4-D and dicamba.
[ii]Contains a chlorophenoxy, sulfonylurea, and benzothiodiazole compound.

Length of gestation and birth weight

Many of the studies that looked for an association between pesticides and gestational effects found not only links with occupational exposure but also with crop-type in a particular region. For example, the odds of pre-term births for grain farmers compared with non-grain farmers were greater (Kristensen *et al.* 1997b,c).

An association with low birth weight among infants born within a 300 to 500 meter zone of aerial application of pesticides for sugar beet production was discovered using geographic information system technology and regression analysis (Xiang *et al.* 2000). Intrauterine growth retardation (IUGR), defined as a birth weight that is 10% or more below normal, was discovered in a community dependent on a herbicide-contaminated ground water supply. Atrazine in the water was the strongest predictor of IUGR, followed by metolachlor, then cyanazine (Munger *et al.* 1997). IUGR, identified by sonogram at approximately the 28th week of gestation, revealed an association with placental and cord blood levels of hexachlorocyclohexane (HCH) isomers, and DDT and its metabolites as previously mentioned (Siddiqui *et al.* 2003).

Fetal loss

Fetal loss was found among grain farmers compared with non-grain farmers especially during mid-pregnancy after grain harvest and in years of poor harvest (Kristensen *et al.* 1997b). When conception took place during the heaviest pesticide-use season (between April and September) the wives of farming fathers experienced an elevated risk of fetal loss (Regidor *et al.* 2004). Women occupationally exposed to pesticides for one month in the first trimester had an increased risk of losing their babies with the risk increasing if exposure was throughout the first trimester (Pastore *et al.* 1997). The odds of fetal loss were significant during the second trimester for women living within one square mile of halogenated pesticide application, and as the number of chemicals increased in the mixtures of pesticides applied, the risk increased (Bell *et al.* 2001a,b). Women engaged in farming compared with women working in other occupations had an excess risk for fetal deaths, although it was also discovered that 10 other occupational groups posed higher risks (Vaughan *et al.* 1984).

More specifically, increased risk for early spontaneous abortion has been linked with phenoxyacetic acid herbicides (like 2,4-D and dicamba) and triazines; late abortions increased with preconception exposure to glyphosate and thiocarbamates, and older women had the greatest risk, as much as 4-fold greater, when exposed to miscellaneous pesticides and carbaryl, and as much as 27-fold greater when exposed to both carbaryl and 2,4-D (Arbuckle *et al.* 2001). Exposure to glyphosate, 2,4-D, carbaryl, and atrazine during the 3 months before and including the month of conception poses a 20% to 40% relative increase in risk for spontaneous abortion (Arbuckle *et al.* 2001). Reported farm use of thiocarbamates, carbaryl, and assorted other pesticides combined with yard herbicide activities produced odds ratios for fetal loss of 4.9 for atrazine and 3.5 for 2,4-DB (Savitz *et al.* 1997).

For women who personally mixed, loaded, and applied pesticides their odds for fetal loss per pregnancy ran as high as 30% more when compared with those who did not engage in these activities (21%) (Garry *et al.* 2002b). Fetal loss per pregnancy was 15% in those who handled pesticides, versus 8.9% in those who did not. Those who

used organotins, triazoles, and ethylenebisdithiocarbamates (EBDC)s had increased risks, with the highest risk from fungicide use. More than 10% of spring conceptions miscarried among spouses who applied only herbicides. Breaking out the pesticides by class, the sulfonylureas, imidazolinones, and Mixture 9100 significantly increased the odds for spring miscarriages (Garry *et al.* 2002b).

Sex ratio

Among farm families using fungicides, insecticides, and herbicides, fewer boys were born than girls versus families using only herbicides (Garry *et al.* 2002b). The sex ratio of the children of fathers who applied fungicides was 0.91 and was 1.13 for the fathers who did not use fungicides (Garry *et al.* 2002a).

The sex ratio of children born to workers in a pesticide factory producing trichlorophenol and the herbicide, 2,4,5-T, was low compared with worldwide figures and study controls (Ryan *et al.* 2002). When the children in this study were broken into paternal and maternal groups, the ratio of boys born from exposed fathers was even lower. The ratio was normal when only the mothers were occupationally exposed. For men whose blood lipid dioxin (2,3,7,8-TCDD) equivalents (TEQ) levels were higher than 715 ppt, the sex ratio was 0.23. This is in line with other work that found dioxin-like effects on sex ratio, at again elevated levels of exposure, are transferred by the male partner (Mocarelli *et al.* 2000).

Birth defects related to the reproductive system and development

In a statewide study, farm children were born with more defects than any other group of children. More boys were born with birth anomalies than girls in agricultural areas with spring conceptions producing the most birth defects (Garry *et al.* 1996). The ratio of boys compared to girls for any anomaly was almost double in areas where phenoxy herbicides and fungicides were applied compared with the general population. Even in areas where lower amounts of chlorophenoxy herbicides and fungicides were applied, boys were at greater risk for all birth anomalies if their fathers applied pesticides than for the general population. And even among the general population the risks for boys were higher where chlorophenoxy herbicides and fungicides were used suggesting that drift is a factor. Crop type also had an influence on the birth defect rate with the combined wheat, sugar beet, and potato growing region experiencing the highest rate among farmers' children. The most frequent birth defects reported were circulatory/respiratory, urogenital, and musculoskeletal/integumental. Chlorophenoxy herbicides and fungicides were related to these as well as to nervous system anomalies (Garry *et al.* 1996). Many chlorophenoxy herbicides (e.g. 2,4-D, 2,3,5,-T) contain dioxin (Schecter *et al.* 1997) impurities and another common contaminant, hexachlorobenzene (HCB), which must be taken into consideration when looking at these results (Huwe *et al.* 2003). See Simanainen *et al.* (2004) for effects of dioxin after gestational and lactational exposure on the male reproductive system.

Birth defects were associated with the incidence rates of surgery for cryptorchidism based on both year of surgery and year of birth in areas where crops are grown under plastic in greenhouse-like conditions (García-Rodríguez *et al.* 1996). High risks for spina bifida and hydrocephaly were found for children of farmers

who worked in orchards or greenhouses (Kristensen *et al.* 1997a). The risks for limb reductions were elevated in grain farming regions. And tractor spraying in grain regions was moderately associated with hypospadias. Based on the amount of pesticides purchased and in regions of vegetable farming, cryptorchidism odds were more than two. Prevalence of these birth defects was greatest for boys conceived from April to June and for hypospadias, where there was grain farming. Ironically, the authors suggested that the odds for these two birth defects were probably not the result of exposure to pesticides because the pesticides in use were not estrogenic (Kristensen *et al.* 1997a). Since they published this article, it was discovered that some pesticides are anti-androgens and by blocking male development can induce estrogen-like outcomes (see Kelce *et al.* 1995). This relatively recent evidence about anti-androgens alters the possible explanation and significance of the results in this study.

Sons of women working as gardeners have an increased risk for cryptorchidism (Weidner *et al.* 1998). Boys born of vegetarian mothers had an increased risk of hypospadias, and if the mother's diet was supplemented with iron, the risk increased significantly (North and Golding 2000). Percentage wise, more vegetarian mothers had sons with hypospadias than omnivore mothers. However, sons of mothers who ate only organic produce had no hypospadias, where 1.07 cases were expected, raising the question of whether pesticides were involved. In this study, mothers who drank soy milk and ate soy products delivered a larger proportion of boys with hypospadias, although the increase was not significant. The dietary influence of pesticides as well as phytoestrogens on gestational outcome must be taken into consideration in this case (Doerge *et al.* 2006; Jefferson *et al.* 2005).

Reproductive effects as a result of adult exposure

The effects discussed thus far were the result of prenatal or early postnatal exposure to compounds that interfered with development. Effects reported in adults that could be the result of adult exposure are now discussed, although in the more recent studies, fetal or early life stage exposure as an underlying cause cannot be ruled out.

The semen of partners of 512 women recruited through prenatal clinics in four U.S. cities was evaluated at a central laboratory for volume, sperm concentration, and sperm motility. The hemocytometer mean/median sperm counts were as follows: Columbia, MO 58.7/53.5, New York City 102.9/88.5, Minneapolis, MN 98.6/81.8, and Los Angeles, CA 80.8/64.8 million sperm/ml (Swan *et al.* 2003a). Total million mobile sperm per ejaculate was 113, 196, 201, and 162, respectively. The fact that the Missouri samples were collected in a highly rural area led the investigators to quantify urinary pesticide metabolite concentrations in a subset of the Missouri and Minnesota populations, and to compare pesticide concentrations with semen quality (Swan *et al.* 2003b). The men in the two cities were broken into two groups based on semen quality parameters: low semen quality (cases) and normal (controls). Alachlor, atrazine, and a metabolite of diazinon were significantly elevated in Missouri men. The odds ratio for poor sperm quality was extremely high in Missouri cases with high levels of alachlor and diazinon metabolites in their urine. The odds

were lower, but still significant, for men with atrazine concentrations above the detection limit.

Another lab-based study group collected blood from applicators using herbicides only, applicators using herbicides and insecticides, applicators using herbicides and fumigants, and organic farmers (Garry *et al.* 1999). Blood was tested before, during, and after the season of pesticide application for several hormones, cholesterol, triglycerides, and HDL levels. Herbicide applicators' free testosterone was significantly elevated in the post-season sprayers and follicle stimulating hormone (FSH) levels were much higher during the spray season and after spraying, compared with the organic farmers (Garry *et al.* 1999). Fumigants were associated with increased triglycerides and lowered HDL, which appeared to be an irreversible, year-round condition. The authors pointed out that fumigant applicators are generally commercially licensed and apply herbicides in the spring and insecticides during the summer and, consequently, are exposed to a broader mix of pesticides than the other two applicator groups. The results of an *in vitro* micronuclei assay using human lymphocytes provided by the organic farmers revealed a significant dose response for the herbicide formulation, 2,4-D LV4, and four surfactant adjuvants: (1) Direct (polyvinyl polymers with other inert ingredients), (2) Nalco-trol (polyvinyl polymer and other inert ingredients), (3) X-77 (alkyl polyoxyethylene, free fatty acids, glycol and isopropanol mixture), and (4) Preference (proprietary with less than 0.002% ethylene oxide and 0.001% dioxane). The adjuvants selected for testing came from a list provided by the subjects in the study who applied the pesticides. The authors were not able to determine if the adjuvant effects were additive or independent of the pesticides and suggested that more investigations are needed with formulations to determine their safety.

After finding the non-persistent pesticides, chlorpyrifos (CPF) >90%, carbaryl >75%, and naphthalene metabolites in the urine of males nationwide (CDC 2003; Duggan *et al.* 2003) researchers discovered increased urinary concentrations of 1-naphthol (1N), a metabolite of carbaryl and naphthalene, were associated with increased damage to human sperm measured as percent of DNA in the tail, length of tail, and an integrated value that accounts for the distance and intensity of fragmented sperm using the comet assay (Meeker *et al.* 2004a,b). Significant increases in the concentration of 3,5,6-trichloro-2-pyridinol (TCPY), a CPF metabolite, were associated with increased tail percent of DNA but with decreased fragmentation and distance of the sperm. Subjects' blood testosterone concentrations decreased significantly as urine concentrations of TCPY and 1N increased when the cohort was broken into quartiles based on exposure levels and quartiles compared. The range of exposure found in these men was nearly identical to that found in the CDC (2003) study (Meeker *et al.* 2006). The technology used in this study is just one among many now being employed to examine seminal tissues. It is sensitive enough to reveal covert damage that would never be detected using traditional toxicology. Unfortunately, these are not among the standard protocol required to determine product safety.

We close this section with two studies that attempted to answer the questions of whether organic diets or organic farming are better than conventional diets or farming. Men who ate only organic food had higher sperm concentrations than those eating conventional diets (Jensen *et al.* 1996). The prevalence of previous

genital disorders was three times less among those who ate organic food compared with controls. In contrast, cryptorchidism was reported more often among those on organic diets, raising a red flag once again about the possible influence of phytoestrogens in the diet. The percentage of men in each group with less than 20 million sperm per ml was the same. In a second study there was no difference in sperm concentration between organic farmers and traditional farmers (64 million sperm/ml versus 58 million sperm/ml, respectively) (Larsen *et al.* 1999). However, upon closer examination, traditional farmers had fewer normal sperm heads, fewer normal spermatozoa, and lower levels of testosterone sex hormone binding globulin and inhibin B than organic farmers. This study again reinforces the need to utilize the latest technology when determining the integrity of semen and not just count the sperm.

Adult impacts on complementary systems essential for reproductive success

The following studies are presented as examples of damage to complementary systems that play critical roles in the development and function of the reproductive system and impair fertility. The concentrations of ethylenethiourea (ETU), a breakdown product of EBDC fungicides, were an order of magnitude lower in the blood of banana plantation workers than in those who worked on banana plantations where EBDCs were applied (Panganiban *et al.* 2004). Only EBDC applicators developed nodular thyroid tumors and, when the nodules exceeded 4 cm, a dose response association was found with blood EBDC. A comparison among four statewide, major, crop-based regions in the United States revealed an increased mortality ratio for prostate cancer and thyroid cancer in men where wheat, sugar beets, and potatoes were grown (Schreinemachers *et al.* 1999). Both the prostate gland and thyroid gland play a critical role in development and reproductive success (Jahnke *et al.* 2004).

LABORATORY RESULTS

Non-DDT/DDE Effects

Even though the odds in several of the human epidemiological studies in the previous section were high, it is still impossible to make a direct link between a specific chemical and a specific health endpoint. Just as an epidemiological study cannot predict with certainly that a chemical caused an effect, neither can a laboratory study predict that a chemical will cause a similar effect in a human. Nonetheless, when evidence from the laboratory continues to complement the signals from the human population, this should be of concern on the part of public health authorities. What follows takes a closer look at the range of possible mechanisms behind adverse reproductive health effects from *in vitro* studies to transgenerational evidence.

Delayed implantation, interrupted development, and fetal loss

Several laboratory studies have discovered biological explanations for how a specific pesticide can cause delayed implantation and/or fetal loss. For example, the commonly used dithiocarbamate fungicide, thiram, was administered to adult female

rats either on the day before mating or on the day of mating (Stoker *et al.* 1996). In previous studies, these researchers demonstrated that a single dose of thiram, or the insecticide chlordimeform, delayed ovulation for 24 hours by interfering with the luteinizing hormone (LH) surge of the ova, which initiates ovulation (Cooper *et al.* 1994). Similar to their prior results, significantly fewer females became impregnated than controls if the rats were exposed to thiram 24 hours before mating ($p < 0.01$). By the 7th day of gestation, more fetal pups had been resorbed among the thiram-treated mothers than among controls, although the number of implantations was the same. By the 11th day, or mid-gestation, researchers reported that fetal head length, crown-rump length, and somite numbers were reduced, compromising the embryos that were still alive. By the 20th day, the number of viable embryos was significantly reduced causing a significant reduction in litter size ($p < 0.01$). These studies demonstrated that just a single dose of a selected pesticide at a critical stage in the estrus cycle can delay conception and can contribute significantly to fetal loss. The lowest dose of thiram that delayed ovulation was 25 mg/kg i.p. (Stoker *et al.* 1993). The authors noted that other dithiocarbamate pesticides such as metam sodium, disulfiram, and carbon disulfide delay the LH surge similarly in the same chain of events as thiram.

A total of 16 agricultural chemicals, including herbicides, fungicides, and adjuvants were tested in the MCF 7 breast cancer cell assay for estrogen-induced proliferation, an assay used to compare the estrogenicity, or cell-proliferative response, of a compound to that of endogenous estrogen 17β-estradiol. They were also tested for cell viability, morphology, ploidy, and apoptosis (Lin and Garry 2000). The adjuvants X-77 (0.01 μg/ml) and Activate Plus (0.1 μg/ml) caused significant proliferation. 2,4-D caused proliferation at 1 μg/ml, reagent grade glyphosate at 2.28 μg/ml, and formulated Roundup (containing glyphosate as the active ingredient) at 10 μg/ml, which compared well with positive controls. Using taxol as a positive control, triphenyltin caused aneuploidy at 0.41 μg/ml and apoptosis at 4.1 μg/ml. Mancozeb at 50 μg/ml and its metabolite, ethylenethiourea (ETU) at 1,000 μg/ml, also caused apoptosis (Lin and Garry 2000). Aneuploidy has been associated with stillbirths, birth defects, and mental retardation (Hunt *et al.* 2003).

Lindane (γ-HCH) was administered to artificially inseminated rabbits (1 mg/kg/day) starting the 8th day after insemination for two weeks, and then every other day to the end of lactation (Fausto *et al.* 2001). Both control and treated offspring were weaned at day 35, and, at day 173 (puberty), males were randomly chosen from each group ($n = 10$) for mating with untreated females. Lindane had no effect on growth performance, libido, and reproductive parameters such as mating time, litter size, and pup survival. The only differences observed were higher semen volume in the lindane-treated animals and changes in sperm tail morphology. The differences between the lindane-treated group and the controls were limited to increases in the percent of cytoplasmic droplets with missing mitochondria (10.3% and 5.3%, respectively) and coiled tails (4.3% and 1.3%, respectively). In the case of de Jager *et al.* (2004), there was an inverse relationship between sperm motility ($p = 0.015$) and the concentration of p,p'-DDE in blood plasma of the subjects; the latter was positively correlated with sperm tail defects ($p = 0.016$).

The following laboratory study provided some insight into the complex mechanisms of action that lead to expression of reproductive impairment long after

exposure. One-day-old female CD-1 mice were treated for 14 days with the widely used DDT substitute, methoxychlor (MXC) (0.1, 0.5, 1.0 mg in oil i.p.), to determine its effect on their ability to mate, ovulate, and conceive upon reaching maturity (Swartz and Eroschenko 1998). The results were compared with mice that were treated at the same age with 17β-estradiol (10 μg i.p. for 14 days) as positive controls. At maturity only one estradiol-treated mouse mated whereas all but one of the MXC treated mice mated. Even though the MXC mice mated, pregnancy percentage was significantly reduced ($p < 0.05$) in the 1.0 mg MXC-treated mice and the mean number of mice per litter was reduced. The number of resorptions per litter significantly increased ($p < 0.05$) in the 0.5 mg MXC-treated mice. The ovaries in the MXC mice had less follicular tissue and more interstitial tissue, fewer corpora lutea than normal, and increased hypertrophied thecal cells. All of these tissues are under control of the hypothalamic-pituitary-ovarian axis, confirming that early life-stage exposure to MXC can indirectly impair fertility in adulthood. If MXC had acted like the estrogenic hormone, 17β-estradiol, the mice would not have mated similarly to the estrogen-treated controls. MXC is a complex, chameleon-like pesticide that can act as an alpha (ERα) agonist and a beta (ERβ antagonist and also act an antiandrogen depending on the tissue present (Gaido *et al.* 1999). Prenatal exposure to MXC at 20 μg/kg/day (maternal dose) resulted in increased adult prostate weight in mice, a result achieved at a dose only 100-fold lower than the powerful estrogen, diethylstilbestrol (DES) (Welshons *et al.* 1999). This dose is below the RFD for MXC. Discoveries like this reaffirm the need to incorporate the results of assays like those reported earlier into decisions about the safety of pesticides and other chemicals.

The chameleon effects of HCB should also be mentioned here. This banned pesticide and often-detected contaminant in chlorinated pesticides at low prenatal doses has an agonistic effect on prostate weight and an antagonistic effect at high doses reducing prostate weight in rat offspring (Ralph *et al.* 2003). Similar effects were discovered using *in vitro* transcriptional assays with prostate tissue. Unpredictable results, such as these, reveal the futility of trying to determine the safe level of chemicals that have different effects at different stages of development.

A mixture of 2,4-D (0.01 mg/kg/d), mecoprop (0.004 mg/kg/d), dicamba (0.0009 mg/kg/d), and their inert ingredients when administered in drinking water at four different dose levels to pregnant mice (GD 5–15) reduced litter size and implantations at the very low ($p < 0.05$) and lowest ($p < 0.01$) doses (Cavieres *et al.* 2002) but not at higher doses. The lowest dose (given earlier) was environmentally relevant. The authors (p. 1085) pointed out that "... 2,4-D and dicamba reportedly do not produce reproductive toxicity (Stevens and Breckenridge 2001)," but in combination in their study they did. Their non-monotonic dose-response curve, which is a often seen in hormonally driven systems, points out the necessity for testing at ambient exposure levels and using mixtures.

Several researchers explored the transgenerational effects of lindane in both sheep (Beard *et al.* 1999) and mink (Beard and Rawlings 1998) and reported the same lack of observable effects on mating behavior, litter size, and so on as the aforementioned study, using doses of 1 mg/kg/day throughout pregnancy and lactation. However, pregnancy rate was reduced in the second-generation female sheep exposed throughout gestation and lactation (Beard *et al.* 1999). In the case of the second-generation mink, a smaller percentage of mated animals whelped and they

had a 60% reduction in litter size. Third generation male mink had smaller testes (Beard and Rawlings 1998).

The final study in this section provides an explanation for the aforementioned results and at the same time reveals worrisome evidence that population-wide, male-driven infertility is entirely possible and irreversible. Gestating rats exposed to vinclozolin, an antiandrogenic fungicide, and MCX separately gave birth to sons whose sons and their grandsons (F4) suffered sperm damage, passed down through the generations via the male germ cell line (Anway *et al.* 2005). More than 90% of the males in each generation were affected. This endocrine disruption, transgenerational effect was induced between gestation days 8 to 15, expressed as increased sperm cell apoptosis, reduced sperm forward motility, and reduced sperm number when compared with controls ($p < 0.001$). Mutation was ruled out, using DNA methylation analysis. Instead, they found that the epigenetic programming that controls methylation and demethylation was altered. These studies were done with high doses of both pesticides (100 and 200 mg/kg per day for a week) and must be repeated at more realistic doses and over longer periods of time. The implications of this study are far reaching and reveal the imminent need to incorporate transgenerational considerations in standard risk assessment.

DISCUSSION

Over the past decade direct empirical data have shown that pesticides are found in humans at all life stages from fertilization to death. Yet, most people are unaware that they are exposed to pesticides, and if told that they were carrying a particular pesticide in their body tissue, they would have no idea where or when they were exposed. In addition, extensive data are now available demonstrating that pesticide residues are found on more than 70% of conventionally grown fresh produce, whether produced domestically or imported (Benbrook 2004). There is also adequate evidence that food exposure contributes to one's measurable pesticide burden (Brock *et al.* 1998; Lu *et al.* 2005). On-farm and off-farm exposure studies have demonstrated that the blood, serum, and urine of those living in agricultural areas often reflect exposures to the specific pesticide(s) used in the surrounding areas, and those in closest proximity to intensive applications carry and excrete the highest concentrations. Unfortunately, tracking how a pesticide gets into the body and what percent of body burden at a given stage of life is from ingestion, inhalation, or dermal sorption is not an exact science.

Recent knowledge gained about the multigenerational effects of pesticides should change how data on pesticide safety are reviewed in the future. Data on pesticide residues in food, drinking water, or the home environment should no longer be written off as irrelevant even when the reported low levels of exposures are an order of magnitude or more below USEPA's current "level of concern." The outmoded "Maximum Tolerated Dose" or the U.S. Agency for Toxic Substances and Disease Registry's (ATSDR's) Minimum Risk Levels (MRLs) are based on crude, traditional toxicological protocols and endpoints that have almost completely missed low-dose, endocrine system-mediated effects. To move forward, transgenerational tests should be conducted on all environmental chemicals as thoroughly as those used to test

pharmaceuticals, for which the U.S. Food and Drug Administration (FDA) already has some tests in place.

Advances in knowledge at the genetic, molecular, cellular, tissue, organ, and system levels can provide supportive evidence to back up human studies that can only infer or make correlations between pesticide exposure and a disorder or undesirable change in function. The overwhelming complexity that exists because of the vast mixtures of pesticides and industrial chemicals in use, the many exposure pathways, the multiple mechanisms of action of each active ingredient, the timing and life stage of the tissue exposed, the long-term delayed expression of many exposures, and the amazing lack of knowledge about the intricacies of human development on the part of the biomedical, scientific community all contribute to the inability to make specific causal links. Consequently, inferences drawn from the existing body of work remain as the only choice in crafting answers to basic questions about exposure and health outcomes, questions that are on the minds of many people, scientists, regulators, and public health professionals.

The delayed confirmation that gestational exposure to DDT at ambient levels can lead to preterm birth, low birth weight, reduced breast milk production, and shortened lactation is of extreme importance and must be factored into decisions concerning future uses of DDT. It suggests that many families in the past were unknowingly impacted healthwise and economically. It is accepted today that low birth weight is associated with increased early mortality and life-long morbidity (Hediger *et al.* 2002). Also, it is the second cause of neonatal deaths in the United States (CDC 2005). Public health authorities must take these findings into consideration when determining how to deal with impending increases in insect vector borne diseases as the result of global warming in regions where poverty and malnutrition already put millions of babies at risk.

As we look back on the case of DDT, many of the human studies mentioned earlier were not *initiated* and completed until decades (a generation or more) after the data were collected, and DDT's production and use were already restricted. We have shown that the health effects of a pesticide can be easily overlooked because they are invisible and not life threatening—but in the long term could have significant health, social, and economic impacts at the individual and population levels.

The lesson learned from DDT and the other studies cited earlier is that developmental, transgenerational testing is critical to protect public health and future generations from widely dispersed chemicals. Certainly we cannot wait for prospective studies that could resolve the uncertainties. See Longnecker (2005) for a discussion on this. Until risk analysis is broadened and takes into consideration the effects of chemicals on future generations, the DDT mistake will be repeated over and over again. DDT did not cause obvious birth defects, it did not cause cancer, and it did not irritate the eyes or skin. And as many people are able to recall, DDT seemed perfectly harmless and a blessing as they ran behind trucks spraying DDT in order to cool off on sultry, summer days. DDT passed all the traditional toxicological tests that governments required to determine its safety, and so do practically all the pesticides on the market today. And, like DDT, none are being challenged at the regulatory level for their endocrine disrupting and/or transgenerational effects. Yet, the aforementioned studies, which look only at the reproductive effects of DDT, signal that something is wrong. It is apparent that although there are adequate scientific data

available to make sound public health decisions about certain pesticides, neither the political will nor the correct vehicle are available to translate that knowledge into policy to protect human health.

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