EFECTS OF ORGANOCHLORINE COMPOUNDS AND HEAVY METALS ON MALE REPRODUCTIVE HEALTH

By

Julia Jennifer Wirth

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Epidemiology

2001

ABSTRACT

EFECTS OF ORGANOCHLORINE COMPOUNDS AND HEAVY METALS ON MALE REPRODUCTIVE HEALTH

By

Julia Jennifer Wirth

Ten to 17% of American couples currently seek medical help for infertility; half of these problems have a male cause usually of unknown etiology. Wildlife, experimental animal and human epidemiological studies indicate that environmental contaminants, especiallyorganochlorine compounds (OCs) such as polychlorinated biphenyls (PCBs), TCDD (dioxin) and DDT/DDE and heavy metals have adverse effects on male reproduction. To investigate the relationship between human male reproductive health problems, specifically semen quality and reproductive hormone levels (FSH, LH, testosterone and inhibin B), environmental contaminants and polymorphisms in genes involved in contaminant and sex steroid metabolism (P450), we propose to conduct a cohort study recruiting men based on exposure to Great Lakes sport-caught fish meals (none, 1 to 11 and greater than 12 meals) from infertility clinics in three areas of Michigan. Information on Great Lakes sport-caught fish consumption, as well as on other risk factors, and fertility will be obtained from a self-administered questionnaire. Secondary exposures (contaminants present in Great Lakes fish), including PCBs, dioxin, DDT and heavy metals, and P450 polymorphisms, will be measured from a blood sample. This study provides the opportunity to investigate the association between measures of male fertility, known reproductive toxicants found in fish, and gene-environment interactions affecting semen parameters and reproductive hormone levels.

To My Friends

Without whom this would not have been possible

ACKNOWLEDGEMENTS

I would like to thank my thesis advisor, Dr. Nigel Paneth, for his guidance, encouragement and faith that an idea could be transformed into a grant proposal, and for exemplifying a true scholar and mentor. Thank you for providing me the opportunities for growth and change.

Many thanks to Dr. Claudia Holzman and Dr. Larry Fischer, my committee members, for your their patience, advice and invaluable input.

Heart felt gratitude to the students and staff of the Department of Epidemiology.

Special thanks to Mary Fran Steele, Jan Pylar, Mel Kennicott, Julia Freije, Jeff Wirth, Andrew Mullard, and Teri Sexton for helping me to see the light.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

CI.....Confidence Interval

OCs.....Organochlorine Compounds

OR.....Odds Ratio

FFHP.....Fisheaters Family Health Project

FSH.....Follicle Stimulating Hormone

LH.....Luteinizing Hormone

PCBs.....Polychlorinated Biphenyls

TCDD.....2,3,7,8-Tetrachlorodibenzodioxin (dioxin)

TE.....Testesterone

A. SPECIFIC AIMS

To better understand the relationship between male infertility and environmental contaminants, we propose to conduct a retrospective cohort study recruiting men based on exposure from 3 infertility clinics in Michigan. Specifically, the aims of this proposal are to:

1. Recruit men attending infertility clinics in 3 areas of Michigan abutting Great Lakes, based on three levels of sport-caught Great Lakes fish consumption in the previous year:378 men who consumed 12 or more meals (most-exposed); 378 men who consumed less than 12 but at least one meal (moderately-exposed); and 378 those who consumed no meals (unexposed); matched within 5 years of age, within 2 months of enrolment and by clinic;
2. For subjects at all exposure levels:
   a. Obtain blood samples for analyses of reproductive hormones (TE, FSH, LH, inhibin B and estradiol), environmental contaminants (organochlorines, mercury and polymorphisms in genes involved in contaminant and steroid metabolism (cytochrome P450, glutathione S-transferase F{GST});
   b. Assess sperm number, motility, and morphology and semen quality;
   c. Administer a questionnaire on reproductive health, fish-eating habits, occupation and general demographics;
3. Collect semen samples from a subsample of men with high (upper quartile) and low (lower quartile) levels of PCB exposure, as determined in serum samples, to perform more specific and sensitive tests of sperm function;

4. Test the hypotheses that compared to unexposed subjects, moderately and most exposed subjects will have increased risks of altered sperm function parameters and increased risks of having abnormal levels of reproductive hormones.
5. Test the hypothesis that polymorphisms in cytochrome P450 and GST genes will interact with environmental contaminants (organochlorines, mercury) to produce an increased risk of alterations in sperm function and/or reproductive hormone levels in moderately and most exposed subjects.

B. BACKGROUND AND SIGNIFICANCE

1. Changes in male reproductive health

Reproductive problems are a concern for a growing number of American couples with between 10 and 17% seeking medical help. In 1987 about $1 billion dollars were spent on treatments for infertility (1). In about half of the cases, the problem is male and up to 80% of these cases have an unknown etiology (2, 3). Concern has focused on the reported changes in markers of male reproductive health, including decreasing sperm densities, increasing incidence of disorders of male reproductive development, including cryptochidism, hypospadias and testicular cancer, and decreasing sex ratios.

a. Changes in semen quality

Reports have been published both supporting and refuting a world-wide decline in sperm density over the last 50 years. A meta-analysis published in 1992 by Carlsen et al.(4), concluded that sperm densities have declined internationally by at least 50% in the past three decades. The study was criticized on methodological grounds (5) (6) and touched

off heated debate on the validity of the findings. The debate highlighted three controversial issues regarding the possible decline in male reproductive health: the conclusions of Carlsen et al.'s study in particular and the decline in semen quality in general; the association between semen quality and fertility; and the increase in congenital abnormalities of the male genital tract.

1) Carlsen et al.'s study and the decline in semen quality

a) Summary of Carlsen et al.'s study

In 1992 Carlsen et al.(4) in a meta-analysis of 61 studies, concluded that sperm density had declined significantly between 1940 and 1990. Using linear regression analysis of the data weighted for the number of men in the individual studies, they found that the mean sperm count decreased from 113 x 10^6/ml to 66 x 10^6/ml. The estimated regression coefficient was –0.934x10^6/ml per year (SE= 0.157, $p<0.0001$). The mean count rather than the median was used since the median could only be derived from 19 studies. Additionally, in 46 of the 61 studies in which the seminal volume was reported, linear regression showed a significant decrease during the same time period from 3.40 ml to 2.75 ml with an estimated linear regression coefficient of –0.0130 ml/year (SE= 0.0057, $p<0.027$). The meta-analysis did not include sperm motility or sperm morphology because their evaluation was considered to be rather subjective and values for them were not included in older publications.

The 61 studies included 14,947 men; 39 publications (8428 men) included men with proven fertility only, while 22 publications reported on men unselected with respect to fertility status. These latter men were considered to represent the "normal male

population" (their quotation marks). A separate analysis of mean sperm density including only studies with men with proven fertility had a regression coefficient of -1.062×10^6/ml per year (SE=0.185, $p<0.0001$). Information on age was provided in 42 of the studies and ranged from 17 to 64 years, with a mean of 30.8 years. A minimum period of sexual abstinence of three days was requested in 32 publications. The authors state, however, that the recorded data did not allow them to analyze the effects of the period of abstinence on semen parameters. While 21 countries from North and South America, Europe, Scandinavia, Africa, India and Hong Kong were represented, 28 of the reports came from the USA.

b) Criticism of Carlsen's et al. study and the decline in semen quality

Carlsen et al.'s report was criticized on methodological issues, including the non-comparability of the studies and the appropriateness of the methods used in their statistical analysis.

1)) Non-comparability of the studies

The criticism concerning the non-comparability of the studies focused on the performance of semen analyses for the individual studies in different laboratories, and the use different study populations.

a)) Use of different laboratories for semen analysis

The major concern is the possible introduction of variation when the semen analyses that are being compared are performed in different laboratories. As a consequence, spurious differences in semen parameters that arise due to different laboratory methods may be interpreted as true differences in sperm density over time and across studies.

A semen analysis involves few or many measures of sperm number, morphology and function as well as semen quality (pH, consistency, color). The parameters most frequently found in publications are the quantitative measures including sperm count (reported in millions), semen volume (ml), sperm density or concentration (millions per ml), and total sperm count per ejaculate (millions). Sperm counts should be presented as the median count, since the distribution is usually skewed and the median value is a better measure of central tendency. The sperm count itself is the parameter least susceptible to error (7). Sperm motility, morphology and viability are qualitative measures (8) (9), may be reported in different ways (10) and are thus more likely to involve technical error. Sperm count, sperm motility and sperm morphology are also subject to variation (11) (12). A within-subject variability of 50 to 75% of the total variability in sperm concentration, semen volume and motility measurements has been reported (13). Another study conducted as part of a longitudinal study of human semen characteristics of unexposed workers measured sperm motility variables Using computer- assisted sperm analysis (14). Motility analyses were conducted on monthly samples from 46 men for 9 months. The variability within a sample, between samples from the same individual (between monthly samples), and between individuals were calculated using a nested analysis of variance. For all sperm motility measurements, at least 90% of the variation was observed between microscope fields within a semen sample. For all sperm motility variables, variation between subjects was the smallest percentage, ranging from 1.3% to 4.0%. When sample means were used in the nested analysis of variation, at least 75% of the variation was observed between samples from the same individual.

Variation in the parameters of an individual's semen analysis is due mainly to measurement errors (15). Counting or statistical errors leading to lack of precision are associated with counting limited numbers of sperm. The exact number of sperm in a given volume follows a Poisson distribution where the variance is equal to the number counted, making the variation in count highly dependent on the number of sperm counted. Other random measurement errors include inadequate mixing of the semen sample, technician stress, poor technique and instrument variation (16). In fact, repeated analysis by the same technician using the same procedure will yield greater variation in a semen analysis than the counting error alone (15). In Carlsen et al.'s meta-analysis, errors of this sort would be expected to be randomly distributed amongst the studies and would affect the precision of the measurements, but not the validity.

Temporary life events can affect the semen analysis parameters. Febrile illnesses, infections, unusual stress, and seasons (17) (18) (reviewed in Clark (19) all can cause significant variations in sperm counts and thus affect the precision of the measured variables (13) (20). Increasing scrotal temperature from the optimum of 35°C can impair spermatogenesis (21) (reviewed in (22)). Different types of clothing, by their insulating effects, can raise scrotal temperature (23) and alter sperm function. A prospective randomized trial was conducted with healthy men of proven fertility between the ages of 25 and 50 years testing the effect of wearing tight-fitting versus loose-fitting underwear, 24 hours a day for 6 months, and sperm density and motility (24). After 6 months, the men switched protocols, wearing the other type of underwear for another 6 months.

Using a matched-pairs signed rank test, they found that sperm density was significantly reduced from 89.5 $x10^6$/ml to 46.0 $x10^6$/ml ($p<0.05$) and the concentration of motile sperm was decreased from 53.1$x10^6$/ml to 17.4$x10^6$/ml ($p<0.005$). The number of men participating, however, was low: 20 volunteers started the study and only nine completed it. Another study, also testing the effects of wearing tight versus loose-fitting underwear and using the same alternating protocol, but with even fewer men (n=2) also found a decline in sperm density and motility with duration of exposure (25). Several other studies have found similar results, although never with a sufficiently large study group (26) (27).

Seasonal changes have also been reported to affect sperm density, semen volume and motility by some (28) but not all (13) investigators. The seasonality of sperm counts may be more pronounced for studies taking place in areas with large yearly temperature variations (13). Information on these events was not obtained by Carlsen et al., but their variation would be expected to be randomly distributed amongst the subjects in the studies, especially if the recruitment phase of the study lasted at least a year to include all seasons. Thus, the validity should not be affected by these variables.

Duration of abstinence prior to semen collection is another life event that can affect sperm parameters (29). Values for the quantitative parameters have been reported to vary by threefold between 1 and 7 days of abstinence (16), with longer periods of sexual abstinence resulting in higher sperm counts and lower percentages of motility (30) (31). Sperm counts have been estimated to increase by 5-13 x 10^6 per ml per additional day of

abstin

again

Howe

contro

reduce

World

standa

length

standar

Not all

reductio

values fr

after one

Total spe

decreased

make it i

populatio

and total

from infer

to 4.4 days

evaluated

to be ferti

abstinence (32). If the duration of abstinence is not specified in a study, which is rare, it again should be randomly distributed among the subjects and only affect precision. However, if different studies recommended different durations and did not record and control for the differences in the analysis, it could affect the validity. In an effort to reduce variation in duration of abstinence as well as in other semen parameters, the World Health Organization (WHO), beginning in 1980, published a manual on standardized methods for evaluating semen analysis parameters, and recommended a length of abstinence between 2 and 7 days. Prior to that time there were no written standards.

Not all studies have found significant effects of length of abstinence, however. A reduction of only approximately 8% of control mean sperm concentration values (mean values from samples collected after abstinence of 3 to 5 days prior to entering the study) after one day of abstinence was found for 12 healthy men, aged 18 to 25 years (29). Total sperm count and semen volume, but not sperm motility or viability, were similarly decreased. The small sample size and restricted age range of the subjects in this study make it impossible to generalize the very small decrease in sperm density to the general population. Another study also found little association between duration of abstinence and total sperm count (33). A decrease in duration of abstinence was observed in males from infertile couples in Sweden from 7.5 days in 1956 and 1966 to 5.0 days in 1976 and to 4.4 days in 1986 (33). The total number of men evaluated was 785, with at least 141 evaluated per time period. Donors from 1956 were men whose partners were considered to be fertile, while those from 1986 donated semen prior to assignment of fertility status.

These investigators found a significant decease in total sperm count from 467 x 10^6 in 1956 to 305 x 10^6 in 1986 ($p<0.0001$), as well as a significant change in percentage of sperm with normal morphology (53% versus 37%, $p<0.0001$). When they restricted their analysis to men reporting 3 to 5 days of abstinence versus no restriction on the period of abstinence, the total sperm count recorded at each year (1956, 1966 and 1976) did not decrease significantly, and the difference between counts from 1956 and 1986 remained significant ($p<0.05$). The change in morphology, which is not affected by length of abstinence (17) (34) (29), was unchanged. While it is clear that sperm counts and semen volume vary according to duration of abstinence, it is unclear if the reduction is always significant. A period of abstinence from one to seven days may have little significant effect on sperm counts. However, if the period of abstinence is not specified in the study design and the actual length not recorded, it can be a confounder for sperm concentration.

The period of sexual abstinence was recorded by Carlsen et al., but was available for only 32 papers. For these 32 studies, the prescribed length of abstinence was at least three days. They did not, however, do a separate analysis of these studies. For the remaining studies, Carlsen et al. note that while the period of abstinence was not mentioned, andrologists have recommended a period of three days for the past 50 years. Additionally, to the best of their knowledge, Carlsen et al. state that no change in masturbation or coital frequencies had occurred since the 1930's, implying that whatever the periods of sexual abstinence for these studies were, they haven't changed over time and therefore should not confound their conclusions.

Twenty-seven of the studies in the meta-analysis were published before 1980 and would not have had access to the WHO standards on period of sexual abstinence. There is still no evidence that studies published after 1980 adhered to the WHO standards, or if they just recommended a specific period of abstinence or if they recorded the actual period. Carlsen et al. argue that the unwritten standard since the 1930's was three to five days. A quick inspection by myself of 10 publications included in the meta-analysis with semen analyses performed from 1941to 1993 revealed that all recommended at least three days, three to five days or three to ten days of abstinence, but none recorded the actual length of abstinence or controlled for it in their analyses. However, the semen analyses were performed in the same laboratory with the same standards over the indicated time span. It thus seems likely that the unwritten period of abstinence of three to at least five days was requested in many of the studies. Unless there is reason to think that the rate of compliance has changed over time, the length of abstinence has probably changed little over time and the various lengths should be randomly distributed within and between the studies.

Several studies have recorded changes in coital rates over time and, it is argued (16) these changes may have affected sperm densities. As mentioned above, Bendvold (33) reported a change in abstinence from 1965 to 1986 but found it had little effect on sperm counts or semen volume. A 21% age-standardized increase in marital coital rates in the US from 1965 to 1970 (7.0% in 1965 to 8.2% in 1970) was reported (35). The study compared responses of currently married women under age 45 years participating in the National Fertility Studies to a question on the frequency of intercourse in the last four weeks. The

author acknowledged a tendency to report stereotypical frequencies i.e. eight times per month. Additionally when the sample of 440 women were re-interviewed four to seven months later in 1965, 68% of the women gave responses with a range of plus or minus two days, which is greater than the reported difference in the frequencies from 1965 to 1970. It is also doubtful that the male partners of these women are representative of males in the general population or the population of men attending fertility clinics. James presented data from Trussell and Westoff (1980) and Abma (1993) showing a 22% increase in coital rates between 1965 and 1975 in married US couples and a subsequent decrease of 27% between 1975 and 1988 (36). The rise and fall in coital rates coincided with a rise and fall in the sex ratio, but no attempt was made to establish causality, so these two set of events remain a coincidence. Thus, the data supporting the effects of changing lengths of abstinence over time provided by these reports are weak or non existence, and these are the reports most frequently cited to illustrate the association between period of abstinence and changing sperm count values (16). Thus, the data on changing coital rates does not appear to support an effect on sperm density.

Another possible source of systematic error in sperm density measurements raised by Carlsen et al.'s critics is the differing counting techniques and instruments Used by different laboratories included in the meta-analysis. Changes in laboratory procedures, such as the type of counting chamber used and the technical training of the individual performing the analysis, can result in coefficients of variation between labs of 23-87% (11), although others have found no effect of different counting chambers or modifications to laboratory procedures (13). Laboratories may also differ in the analytic

n

b

ir

"r

fc

sa

fe

ra

th

dif

att

199

atte

thos

attri

morp

how

Carls

Carlso

metho

have l

ackno

methods used to evaluate sperm function (37) (38), which can also introduce systematic bias. For example, the determination of sperm morphology has changed since the introduction of "strict" criteria by Kruger et al. in 1986 (8). With this classification, a "normal" sperm must have a normal sperm head, neck, midpiece and tail. Any borderline form is considered abnormal. using this classification, the predictive value of semen samples containing at least 14% sperm with normal morphology for success in *in vitro* fertilization and pregnancy is good (81% *in vitro* fertilization rate and 22% pregnancy rate per embryo)(8). If comparisons are made between sperm morphology determined by the older criteria and the "strict" criteria of Kruger, differences due solely to the use of different criteria may be found. For example, a comparison of sperm morphology in men attending an infertility clinic in 1974-1984 with that of men attending the same clinic in 1991, revealed a decrease in the percent of morphologically normal sperm (20). The men attending the clinic in 1991 were expected to have a higher percent of normal sperm than those attending from 1974-1983 since their overall fertility was higher. The authors attributed the difference to the change in the criteria used to determine normal morphology. The possibility that environmental exposures contributed to the change, however, was not considered.

Carlsen et al., cite WHO as recommending the USe of different counting chambers (39). Carlsen et al. also excluded studies that USed computer assisted or flow cytometric methods for evaluating sperm counts as these methods were not available until 1980 and have lead to inaccurate counts for samples with low sperm densities (40). They acknowledge the apparent imprecision of sperm counting, but find no reason to believe

tha
her
not

As
diff
Mar
exar
well
sema
large
in 19
betw
range
sperm
heads
variat
the la
in Car
signifi
despite

that the test is subject to secular trends. In support of this, they mention that hematologists who have USed the same counting chambers for the past 50 years, have not reported a similar secular trend in blood cell counts (4).

As mentioned earlier, in an effort to reduce the variation in semen analyses performed in different laboratories, WHO publishes guidelines for evaluating semen. The WHO Manual for the Evaluation of Human Semen provides standards for collection and examination of human semen, sperm preparation techniques, quality control measures as well as references values for semen parameters. (The current WHO normal values for semen parameters are listed in Appendix A). Despite this attempt at standardization, a large multi-center trial on external quality control between eight laboratories in Germany in 1990 found large coefficients of variation for sperm count, motility and morphology between different laboratories (11). Coefficients of variation (CV) for sperm counts ranged from 23% to 73% for samples with high and low concentrations, respectively. For sperm morphology, the range of CVs for the different sperm parts was 25% for normal heads and 87% for abnormal midpieces, and the CV for motility was 21%. Some of the variation in sperm counts, which were made on formalin-fixed specimens and mailed to the laboratories, may have been due to the shipping conditions. Since most of the studies in Carlsen et al.'s meta-analysis used different laboratories, it is likely that there was significant variation in methodologies and in sperm density. This would seem to be true despite publication of the WHO guidelines.

b)) Use of different study populations

Another major criticism of the Carlsen et al.'s study was the inclusion of studies in the meta-analysis with populations too diverse to be compared so that no meaningful conclusion could be made. These population differences were fertility status and the geographical and racial composition of the participants in the different studies.

1)) Fertility status

The meta-analysis included data on men unselected with respect to fertility as well as on men with proven fertility. Their critics (6) (41) (42) (16) argue that men with proven fertility, which is defined as having fathered at least one child, and men with unknown fertility will differ with respect to semen parameters, including sperm count (10). The basis for this argument is that men who have fathered a child will have higher sperm counts than men unselected for fertility status, which presumably includes men who are infertile as well as fertile men. In fact, the men from the studies included in this group were sperm donors (43), men reporting for vasectomies (43) (18) and men coming in for pre-marital check-ups (43). In the study by Wang et al., 95% of the men were considered to be fertile. Carlsen et al. state that when they analyzed studies containing only fertile men, the regression coefficient for men sperm concentration was -8.52×10^6/ ml per year ($p<0.0001$), which is very close to the values for all of the studies. So there does not seem to be any bias regarding fertility status.

It is also possible, however, that as the knowledge and treatment of infertility problems has increased, men with lower sperm counts have become fathers. Thus these men would be included in the category of fertile men in the more recent studies, but would have been

considered in- or sub-fertile in the earlier studies and possibly not included in some studies. Consequently, the definition of normal sperm count has changed over time (6) Olsen 1994). In the earlier studies from the 1940's (44), a sperm concentration of 60 x 10^6/ml was considered normal, while currently a concentration of 20 x 10^6/ml is considered normal (15). If this change led to exclusion of men in the earlier studies with sperm counts less than 60 x 10^6/ml (that would be included in the later studies), this would artificially increase the mean of the earlier sperm counts. In support of this possibility Bromwich et al. (6) published a report criticizing Carlsen et al. on their statistical analysis. Bromwich et al. used various mathematical models of the probability distribution of mean sperm concentrations and then provided their own data from men attending an *in vitro* fertilization clinic to show that the distribution is heavily skewed towards lower counts. They argue that the data best fit a logarithmic distribution and that truncation of such a distribution at 60 x 10^6 sperm/ml alone would account for the drop in sperm count observed by Carlsen et al.

However, as pointed out by Keiding et al. (45), the studies from the 1940s (17) include many sperm counts lower than 60 x 10^6 sperm/ml, suggesting that the data was not truncated. Of interest, the sperm density distributions of these earlier studies (reviewed in (17) are actually skewed toward the higher concentrations.

2)) Comparison of studies with wide variation in geographic distribution

Another criticism of the populations compared in Carlsen et al.'s study involves the geographic distribution of the reviewed publications. The meta-analysis included publications from all over the world although almost half were from the USA. Inclusion of such diversity is sure to increase the variability of the counts due to genetic, life-style and environmental exposure differences of the populations as well as differences in the technical analysis of the sperm counts. On the other hand, there is no other way, given the data, to assess global changes.

To determine if geographical variations influenced Carlsen et al.'s findings, Fisch and Goluboff (46) re-analyzed the data using only those studies with at least 100 men. This gave them 20 publications, which still contained 91% of all the men in Carlsen et al.'s review. They noted that all 5 papers before 1970, which contained the high (100 x 10^6/ml or more) sperm counts, were from the USA, and 4 of the 5 were from New York. After 1970, when the counts were lower, only 3 out of 15 were from the USA and only one was from New York. Of the 7 papers reporting the lowest values, 5 originated from developing countries. Fisch and Goluboff concluded that, given the differences in methodologies and in patient selection, geographic variations in sperm count need to be considered when evaluating data from different locations. There is a problem with this interpretation. All but 2 (n= 100 men) of the 13 studies in Carlsen's review between 1938 and 1969 are from the USA (n=1680 men), so there is no other country with close to the same number of studies or men to serve as a comparison to the USA data. All but two of USA studies report counts above 100 x 10^6/ml. Thus there is no way to know if the high sperm counts reported by these early USA publications are due to the time frame of

semen collection or to the location. Of note, even though they represent only 50 men, the two studies not from the US reported counts of 103.2 x 10^6/ml (Peru, 50 men) and 87.5 x 10^6/ml (Denmark, 50 men) (in Carlsen et al. (4)), both of which are in the high range.

The issue of location has been addressed in several publications looking at data collected within countries. An early report by McLeod and Wang (7) compared studies from the USA from early times (1938 to 1951 in New York) and the "modern" era from 1970 to 1977 in New York, Iowa and Houston. Using the mean, median or the frequency distribution of sperm counts, they agreed that a depression of spermatogenesis had occurred since 1951. However, when they used their own data from 1951 on infertile men and compared it to data they collected from 1966 to 1977 on infertile populations, they found no decrease. They suggest that the use of the same experimental conditions in the same laboratory and essentially the same type of populations (infertile New Yorkers) eliminated the differences seen in the other studies. One hopes, however, that infertile New Yorkers are not representative of the USA males. On the other hand, Leto et al. (47) published a study on 275 semen donors from 1973 to 1980 from the Washington, DC area. They noted that the average sperm concentration, which was a mean of 12 replicates each, fell from 120 x 10^6/ml in 1973 to almost 90 x 10^6/ml in 1980.

Other investigators however have found an increase in sperm densities in studies within the USA. Fisch et al. (48) compared mean sperm counts, sperm motility and semen volume in three USA sperm banks (Roseville, MN, Los Angeles, CA and New York, NY), from 1970 to 1994. They reported that sperm counts differed significantly ($p<0.01$)

between locations with a high in New York of 131.5 x 10^6/ml (mean) and a low of 72.7 x 10^6/ml in California. Using linear regression of sperm concentrations for the total population, they found a significant increase from a mean of 77 x 10^6/ml in 1970 to 89 x 10^6/ml in 1994 ($p<0.04$). The also found an increase in sperm counts within the 3 centers, which was significant for New York and Minnesota, and in men with proven or unproven fertility status, both of which were significant. Finally in a multiple regression model with age and duration of abstinence as potential modifiers, sperm concentration still showed a significant increase ($p<0.03$). No data is presented on the distribution of the sperm counts in this paper; however, if it is skewed towards lower counts, as has been found in other studies (7) (6), the mean would be higher than the median and perhaps may have biased their data towards higher values. Nevertheless, this study is in agreement with McLeod and Wang (7) on the relatively high sperm counts from New York and adds support to the suggestion that the sperm densities from the early New York reports may have biased Carlsen et al's analysis by virtue of location rather than time. It also supports the notion of geographical variations in sperm densities in the USA (7).

Another study aimed at addressing the effect of geographical location on sperm counts was conducted in Seattle (49). Sperm counts from 510 healthy volunteers who served as untreated controls for an intervention study from 1972 to 1993 were analyzed. Since several semen samples were collected from each participant with a median number of 6 samples, the mean concentration was used in the analysis. Uniform methods of semen analysis including duration of abstinence were followed. No change was observed in the

g

2

te

re

U

ov

in

the

rev

bet

Rep

Eur

dete

men

and

ferti

exam

and t

the m

the 19

popul

10^6/ml

geometric mean during this time period. In 1999, Saidi et al. (50) published a review of 29 USA studies from 1938 to 1996, in which semen analyses were performed on 9,612 fertile or presumably fertile men. Mean sperm count was determined by geographic region. Mean sperm concentrations from New York were significantly higher than other USA cities (98.6 versus 71.6 x 10^6/ml, p=0.0006), and no significant change occurred over time for any city. However, an analysis performed without separating by location indicated a decline (p=0.047). Thus several studies from the USA on healthy men from the same geographic areas using standardized methods for semen analysis as well as a review found no change in sperm densities. Significant differences, however, were found between different locations.

Reports from Europe, on the other hand, have found divergent results. Two studies from Europe have also found a decrease in sperm count. In a study specifically designed to determine if fertility in Danish men has declined from 1952 to 1972, semen analyses of men from the Copenhagen area attending a single Sperm Analysis Laboratory in 1952 and in 1972 were compared (51). The men at both times were examined because of a fertility problem. In 1952, 6.2% of the men had azoospermia while 3.9% of those men examined in 1972 were azoospermic. The laboratory had one supervisor for the interval and thus provided consistent methodology and technical skill. Additionally, the ages of the men at both times were very close (3 year difference) but the social classification of the 1972 men was higher, and both classifications were higher than that of the general population. There was a significant decrease in the median sperm count from 73.4 x 10^6/ml in 1952 to 54.4 x 10^6/ml in 1972 ($p<0.01$). Although it is not clear in their

de
cal
cor
mo
clir
kno
rule
thei
the
volu
coho
(52).
that a
numb
The r
78 mi
at firs
associ

A stud
analys
criteria
time, al

description of the analysis, it seems likely the azoospermic men were included in this calculation. Additionally there was significant deterioration in the fertility class (a composite number reflecting semen volume, sperm count, perm mobility and sperm morphology) from 1.95 to 3.70 ($p<0.001$). Men having semen analyses performed in their clinic in 1972 did so as part of the routine work up before the fertility of the partner was known, whereas in the 1950's, the analysis was done only after a female problem was ruled out. This change would actually favor an increase in sperm density over time in their facility, whereas the opposite was found. This change may also suggest a change in the technical sophistication of infertility assessments. A study in Scotland of 577 volunteer donors for a program in gamete biology compared sperm counts by birth cohorts from 1951 to 1973, using a single laboratory with standardized methodology (52). This study was notable for using donors unselected as to fertility status. They found that a later year of birth was associated with a lower sperm concentration, a lower total number of sperm in the ejaculate and a lower number of motile sperm in the ejaculate. The median sperm concentration fell from 98 x 10^6/ml among donors born before 1959 to 78 mil/ml for those donors born after 1970 ($p<0.002$). The age of donors in the 4 cohorts at first donation decreased with year of birth, but was not found to be independently associated with sperm count in a multiple linear regression model.

A study from Paris (53)(n=1351) using fertile semen donors and one laboratory for the analyses found a significant decrease in sperm concentration from 1973 to 1992. The criteria for entry into the study was not specified and the age of the men increased over time, although this did not completely explain the decrease. Another study performed in

Fr
ye
of
to
co
in
tha
loc
life
par
198
duri
was
acc
livi
the
(p<(
note
envi
den

In su
1950

France, in the Toulouse area, investigated whether sperm densities had changed in 16 years, using a time series of sperm donor's specimens from 1977 to 1992 at a sperm bank of a university hospital (54). Donors had proven fertility, were within ages 20 to 45, followed the recommended duration of abstinence of 3 to 5 days and thus were comparable to the men participating in the study of Auger et al. They found no difference in sperm counts. After discussing the similarities of the two studies, Bujan et al. suggest that the observed differences in sperm count between the two studies at two different locations may be due to environmental factors, including air quality, water supply and life style. In support of this notion, a study in London was conducted comparing semen parameters of partners of women who received gonadotropin therapy in 1978-1983 and in 1984-1989 (55). No change was observed in mean age, clinical or socioeconomic status during these two periods. A change in sperm density from 101 x 10^6/ml to 96 x 10^6/ml was observed. However, when they later re-analyzed the data by dividing the couples according to the source of their water supply, they found that the sperm density of men living outside the Thames Water Supply Area changed from 99 x 10^6 to 110 x 10^6, while the sperm density of men living inside the area changed from 105 x 10^6 to 76 x10^6 ($p<0.05$) (56). Significant changes in sperm motility (61.7% to 40.8%, $p<0.05$) were also noted, but only for the men living within the area. These results suggest that environmental factors, possibly present in the water, had adverse effects of sperm density.

In summary, some but not all studies showed that sperm densities have fallen between the 1950s and the 1970s. Whether the decline has continued is uncertain, especially in the

USA. Carlsen et al's review has a gap at this interface, and other models, which better fit the later data, show an increase (41). Despite the valid criticisms leveled against Carlsen et al's study regarding population non-comparability and lack of methodological consistency between studies, smaller studies that did not have these problems found similar results. There seems to be valid geographical differences in sperm densities, and, as suggested by some authors, this may be due to environmental conditions. Resolution of this issue awaits future epidemiological studies using uniform methods aimed at deciphering this relationship.

2)) Statistical methods

A major criticism of Carlsen's et al. data analysis was that they used inappropriate statistical methods to analyze data from the studies that were not evenly distributed over time (12 reports from 1930-1970, 48 from 1971 to 1990). Some critics have suggested that lack of earlier publications prevents any sort of valid statistical conclusion (5) (41). Carlsen et al. Used linear regression to describe the relationship between sperm density and year of publication. Subsequent re-analyses of their data have suggested that exponential, logarithmic or cyclic models better describe their data (57) (41). All of these models, however, show a decrease from the earliest times to the 1960's. Carlsen et al. in reply to these criticisms say they also tried the other models and found no better fit of their data (45). They conclude that regardless of the statistical model used, the data show a decline in sperm density over time.

Another re-analysis of the Carlsen et al data, which included more recent publications, found that the linear regression line was no longer useful in describing the data (58). They also said that with the inclusion of more recent data, they did not find a decrease in sperm densities since the 1960's. On the other hand, yet another re-analysis of the Carlsen et al. data (59) using multiple linear regression models controlling for period of abstinence, age, percent of subjects with proven fertility, specimen collection method, study goal and location, found that mean sperm densities differed significantly across countries with the highest densities found in Europe and lowest in non-Western countries ($p<0.02$). A decline in sperm density was seen in the USA from 1938 to 1988 (slope= -1.50; 95% CI l, -1.90 to –1.10) and in Europe from 1971 to 1990 (slope = -3.13; 95% CI, -4.96 to –1.30), but not in non-Western countries (slope = 1.56; 95% CI, -1.00 to 4.12). Results from the non-linear models were similar to the linear models. They also found significant regional differences often as large as the mean decline from 1938 to 1990. The same authors published another analysis including additional data from 47 English language studies published from 1934 to 1996 (60). With the additional studies their results were consistent with those of Carlsen et al. (slope = -0.94 vs -0.93) and their previous results, suggesting that the reported trends are not dependent on the particular studies included by Carlsen et al. and that the observed trends previously reported for 1938-1990 are also seen in data from 1934-1996. The authors did not include information on power analysis and did not provide the number of subjects in each study, so power could not be determined.

Thus, the different statistical models all support a decline in sperm densities from the earliest times to at least the 1960' or 1970's with regional differences playing an important role.

The largest uncertainty in the data from the meta-analysis is whether the earliest sperm densities, which predominately were from the USA and were high, are representative of world-wide sperm densities. Since more recent reports from the USA still indicate fairly high densities, especially those including data from New York (48) where the data in the oldest reports in the meta-analysis were from, the degree of the decrease reported by Carlsen et al. is uncertain. Many of the studies included in the meta-analysis (47, 61, 62) (33) and some of those published since then (52, 63) (64), have found significant decreases in sperm density. A commonly held view is that decreases in sperm densities have occurred over time, but are restricted to certain countries (46, 65), i.e. geographical areas. Thus while sperm densities have decreased in areas of France (53), Denmark (61) (64) and Scotland (52), they do not appear to have done so in Finland (66) (67). While an earlier report from the USA suggests a decline (47), the most recent reports indicate regional differences but no decline within those regions (49, 50). Several studies raise the possibility that environmental factors play a role in the lowering sperm densities over time (62) (54, 56).

2) Relationship between semen parameters and fertility

Semen quality has been used as a marker of male reproductive function in studies of occupational health for several decades (9). However, the association of semen

parameters with fertility status has not been directly investigated. In fact there has never been a population- based study on the possible association using a large, randomly selected group of men. Two groups of investigators have used prospective study designs to evaluate the association.

Bostofte and colleagues evaluated the association between measures of clinical fertility and sperm density, motility and morphology in a prospective study of 1077 men examined in The Sperm Analysis Laboratory in Copenhagen in 1950 and 1951(61). The men were contacted 20 years later and asked to complete a questionnaire and 72.9% responded. Fertility was defined as fathering a living child or as the number of living children. They found that decreasing fertility correlated with decreasing sperm count, increasing number of immobile sperm, poorer sperm motility and increasing number of morphologically abnormal sperm (68). These parameters were all interrelated with correlation coefficients from 0.36 to 0.67. Correlations between increasing number of abnormal spermatozoa or decreasing sperm motility and decreasing chances of having a live birth ($p<0.01$) and a longer time to pregnancy ($p<0.01$) were found (69) (51). Fertility was impaired when 60% or more of the sperm were morphologically abnormal. However men with poor motility still had a 32.7% chance of conceiving, so motility was not considered to be a predictor of fertility. Correlations between sperm count and number of living children and time to pregnancy were also observed ($p<0.01$).] The Cox proportional hazard regression model was employed on 765 men whose semen had been analyzed in 1950 and 1951, who had initially been diagnosed as infertile and who replied to a questionnaire 20 years later. Age at semen analysis, percentage normal

sperm, percentage mobile sperm and the degree of motility were all found to be significant independent predictors of individual pregnancy probability (70).

More recently Bonde et al.(71) studied the association between semen quality and the probability of conception in a single menstrual cycle (fecundability) in Danish couples with no previous reproductive experience. Couples (430), aged 20-35 years, one of which was a members of a Danish trades-union, were followed for 6 months or until a pregnancy occurred. Semen samples were collected at the start of the study and during the menstrual period of each menstrual cycle. Sampling after 3 days of abstinence was encouraged but not demanded and all analyses were carried out by two laboratories. Comparison of the variation in sperm counts of the same sample (n=28) by the two laboratories was not significant (p=0.82). They found that the probability of conception increased with increasing sperm concentration up to 40 x 10^6/ml. The odds ratios (ORs) for pregnancy with increasing sperm concentrations ranged from 0.30 (95% CI, 0.16-0.56) for counts of 0-9 x 10^6/ml to 0.96 (95% CI, 0.68-1.38) for counts of 40-79 x 10^6/ml. A threshold of about 40 x 10^6/ml above which there was no significant increase in the likelihood of pregnancy was obtained using a logistic regression model.

Sperm morphology was also a significant determinant of pregnancy. There was a linear increase in the likelihood of pregnancy with increasing proportions of sperm with normal morphology from 10% to 60%. A positive correlation with sperm concentration and proportion of normal sperm was found ($r=0.40$, $p<0.0001$), although both parameters were independently associated with likelihood of pregnancy. The likelihood of pregnancy

C

in

as

fer

pro

how

adh

cond

their

days

logis

In a s

used c

throug

were d

One se

by train

significantly decreased if the proportion of non-motile sperm was greater than 70%, although sperm motility was not a significant factor in a logistic regression model containing sperm count and morphology. In the analysis of menstrual cycle-specific semen samples, the likelihood of pregnancy increased from 8% in cycles with sperm counts below 10 x 10^6 to 25% in cycles with counts greater than 40 x 10^6. The adjusted OR for pregnancy in a menstrual cycle with sperm concentration increasing at 1x10^6/ml increments was 2.45 (95% CI, 1.53-3.89, p=0.0001). This study provides a much needed assessment of the association between semen quality parameters and a measure of fertility using a sample that is unbiased with regard to fertility potential, which has been a problem in many studies on sperm count. The selection of trade union members, however, may make it less representative of the general population. The lack of strict adherence to a 3 day period of sexual abstinence may make their measurement of sperm concentration less valid, and the authors mention that the mean sperm concentration in their study increased by 5.2 x 10^6 sperm/ml per day of sexual abstinence up to seven days. However, they mention that addition of duration of sexual abstinence in their logistic regression model did not change the likelihood of pregnancy.

In a study to evaluate infertility according to shifts in sperm counts, Bonde et al. (64) used data from 10 separate Danish occupational semen studies conducted from 1986 through 1995 to establish a baseline distribution of sperm counts. Although all the studies were designed to evaluate occupational exposures, all but two studies found no effect. One semen sample, collected by masturbation, was evaluated within 2 hours of collection by trained technicians. The median sperm count was 56 x 10^6/ml with a range of 0 to 504

a

w

th

le

m

ca

va

int

of c

In s

to p

Add

cont

cons

para

Furth

abnor

testicu

x 10^6/ml. Using this data and data from the study described above (Bonde et al), the authors constructed additive or multiplicative models to create alternative distributions with median sperm count values changed by 25-100%. The resulting models suggest that the relationship between sperm count shift and fecundability depends on the median levels of the sperm count and the type of shift. Variables interacting in a multiplicative model tend to have minor effects on fecundability while those with additive interactions can decrease fecundability by 30% and fertility by 70%. Thus, depending on the variables and their type of interaction, for example exposure to dioxin and DDT interacting additively, it is possible that changes in sperm counts may be an early warning of changes in fertility.

In summary, these studies have found an association between measures of fertility (time to pregnancy, live birth) and one or more semen parameters, including sperm counts. Additionally, a theoretical framework for the interactive effects of environmental contaminants on sperm counts has been developed, and stresses the importance of considering multiple exposures in designing studies to evaluated changes in semen parameters.

b. Changes in the incidence of abnormalities of the male genital tract

Further support for a decline in male reproductive health comes from studies on abnormalities of the male genital tract, including cryptorchidism and hyposapdias, and on testicular cancer.

Assessment of trends in the incidence of cryptorchidism and hyposapdias has been hampered by the lack of consistent measuring techniques and strict definitions of both conditions. An additional problem for diagnosing cryptorchidism is the age-dependent retractability of the testes leading to misclassification of both undescended and descended testes. It has also been reported that young boys with retractile testes that would have descended with time may have been diagnosed and operated on for undescended testes at an early age to prevent sterility (72). To compensate for this problem, a strict definition of cryptorchidism that eliminates the possibility of retraction should be Used, i.e. a testis that cannot be drawn at least 4 cc below the pubic crest at some time before 1 year of age (73). Additionally the evaluations for cryptochidism should be performed at birth, at 3 months and at one year. Problems in diagnosing hypospadias include the existence of forms considered minor (distal, glandular or coronal) and severe. Minor forms occur about 75% more frequently (74, 75). The rising trend for both these anomalies may be due to more frequent or earlier diagnosis of the minor forms over time or to an increasing tendency to report them to congenital anomaly registries (76).

a. Cryptorchidism

Three large prospective studies on the incidence of cryptorchidism, all adhering to the same standards, have been published and can be compared. The first study on 3500 infants born in a hospital in London in the 1950's used very accurate measurements of testes positions and a strict definition of a cryptorchid testis and has thus served as a reference for subsequent studies (73). This study found that at birth, 2.7% of full term male babies weighing 2500g or more had one or two undescended testes, while 21.0% of

infants weighing less that 2,500g had the same condition. At three months the incidence of cryptorchidism in boys with birth weights equal to or greater than 2500 g or less than 2500 g were 0.91% and 1.74%, respectively. Another study from England conducted in the 1980's on 7441 infants found the incidence to be 5.2% for infants less than 2500 g and 1.61% for those over 2500 g at 3 months (77), indicating an increase in incidence since Scorer's study.

A study from England in 1984 examined the Hospital Inpatient Enquiry data for England and Wales from 1962 to 1981 and found that the diagnosis of undescended testes had increased 2.3-fold (72). The cumulative discharge rate up to age 15 for undescended testes increased from 1.4% in 1952 to 2.9% for 1977, adjusted for double admissions. There are several problems with this data including a variable definition of cryptochidism by different physicians and the inclusion of subjects who were cryptorchid at birth but whose testes descended by one year of age.

Two more recent studies, one from England and one from New York, both using the same technique and definition as Scorer found contrasting results. The John Radcliffe Hospital Cryptorchidism Study Group (78) examined cryptorchidism at birth in 7441 boys, born from 1984 to 1988, whose mothers lived within the Oxfordshire Health Authority in England, and if present, at 3 months of age. At birth 22.8% of boys weighing less that 2500 g were cryptorchid while only 4.1% of boys weighing greater than or equal to 2500 g had the same condition. At 3 months, the values were 5.2% and 1.61%, respectively. These latter values represent a 197.4% and a 77.4% increase compared to

the values found by Scorer at 3 months. The study from New York examined 6935 consecutive male babies delivered at Mount Sinai Hospital in New York City from 1987 to 1990 (79). At birth 19.8% of babies less than 2500 g were cryptorchid while 2.2% of infants weighing greater than or equal to 2500 g were cryptorchid. At three months these values dropped to 1.94% and 0.91%, respectively. These values are close to the values reported by Scorer and suggest no change had occurred since Scorer's study. The New York cohort, however, was very diverse ethnically and racially and may have differed significantly from the British groups. Overall these data suggest an increasing trend in cryptochidism rates, although it is difficult to draw conclusions from these different populations.

2) Hypospadias

Accumulating evidence points to an international increase in the prevalence of hypospadias. Based on data from national registers, a steady increase in the incidence of hypospadias has been observed in England and Wales (80), Hungary (Czeizel 1985), Sweden (Kalen 1982; 1986), Norway (Bjerkedal et al 1975) and Denmark (Kallen 1986; WHO). The largest increases occurred from the 1970's to the late 1980's (WHO Clearinghouse for malformations, 1991). In England and Wales, the incidence of hypospadias increased from 7.3 per 10,000 births in 1964 to 16 per 10,000 births in the early 1980's and then decreased slightly to 11.7 per 10,000 in 1990. Data from Hungary followed a similar trend, rapidly increasing from 5.5 per 10,000 in 1964 to approximately 23.9 per 10,000 in the early 1980's where it has remained. Data from Denmark indicate an increase in incidence from 1970 to 1981 from approximately 7.5 to 12 per 10,000

(Kallen 1986). An additional increase occurred from 1982 to 1988, but may have been influenced by a change in the registration systems. Sweden also had an increase in the early 1970's: a 40% increase was noted from data collected in 1965 to 1968 compared to that collected in 1974 to 1982 (Kallen 1986). Finally in Norway, the prevalence of hypospadias at birth increased from 7-8 per 10,000 between 1967 and 1971 to 13 per 10,000 in 1973 (Bjerkedal) and to 20.7 per 10,000 in 1988 (WHO 1991).

In contrast, an increasing trend was not observed in Finland, Spain, New Zealand, Australia or Czechoslovakia (WHO 1991). A more recent study from Finland used their national hospital discharge registry birth defects data for boys born between 1970 and 1986 to find the number of boys treated for hypospadias by the age of 8 years (81). A prevalence of 28.1 cases per 10,000 live male births was found and it remained constant throughout the study period. The rate, however, was approximately 3-fold higher than previously reported, perhaps because of the variation in completeness of birth defects registry data used in the earlier studies. Thus in Europe and Scandinavia an increasing trend in the incidence of hypospadias was observed from the 1960's through the 1980's, but may have leveled off (82) (83).

In the USA the prevalence of hypospadias, determined from data from the Metropolitan Atlanta Congenital Defects Program (MACDP) for 1968 to 1993 and the nationwide Birth Defects Monitoring Program (BDMP) from 1970 to 1993, increased (75). In the MACDP database, the total hypospadias rate nearly doubled between 1968 and 1993. An annual rate of increase of 2.9% obtained by using linear regression and was significant

(p<
5.7
fron
from
rate
10.0

Two
surve
Cong
Syste
increa
discre
hypos
forms

The ev
for cry
indicat
worldv
is incre
countri

($p< 10^{-6}$). The overall increase occurred at a rate of 1.4% per year among Caucasians and 5.7% among non-Caucasians. The rate of severe hypospadias increased three-to five-fold from 1.1 per 10,000 live births in 1968 to between 2.7 and 5.5 per 10,000 births per year from 1990 to 1993 (p for trend $<10^{-6}$). Data from the BDMP database showed that the rate for hypospadias nearly doubled from 1970 to 1993, going from 20.2 to 39.7 per 10,000 (p for trend $< 10^{-6}$). All four regions of the US showed an increasing trend.

Two more recent studies arrived at conflicting conclusions. A study using two US surveillance systems also found an increase in prevalence (84). A study USing the Congenital Malformations Registry and State Wide Planning Research Cooperative System of New York State for the incidence and repair rates of hypospadias found no increase in incidence from 1983 to 1995 in New York State ($r=-0.225$, $p=0.4$)(85). The discrepancy between these two reports may in part be due to misclassification of hypospadias by Canning (84) since they did not differentiate between minor and severe forms.

3) Testicular cancer

The evidence supporting an increase in the incidence of testicular cancer is stronger than for cryptorchism and hypospadias at least in part because it is easier to diagnose. Studies indicate that the incidence of testicular cancer has increased for the last several decades worldwide (reviewed in (86)). Reports from cancer registries indicate that the incidence is increasing in England and Wales (87) (88), Scotland (89), the Nordic and Baltic countries (90) (91), Australia (92), New Zealand (93) (94), northern Europe (91), Canada

(95) and the USA (96, 97). A USA study using the Connecticut Tumor Registry for 1935 through 1979 found an epidemic increase over time in the risk of testicular cancer for men aged 15-44, with the most recent birth cohorts showing the greatest rate of increase (96). A subsequent study using the same database for 1935 to 1992, found that the incidence rate of testicular cancer (all histologies) has increased 3.5-fold for Caucasians during the past 60 years: 1.46 per 100,00 in 1935-1939 to 5.01 per 100,000 in 1990-1992 (97). The rates for seminomas and non-seminomas increased since the 1950's: the age-adjusted incidence rate for seminomas increased from 1.03 per 100,000 men in 1950-1954 to 2.60 per 100,000 in 1990-1992, while that for the nonseminomas increased from 1.14 to 2.41 for the same time period. For seminomas the largest increase occurred for men 20 to 44 years old, while the rate for nonseminoma was highest for men aged 15 to 34. The increases for men born after 1910 for both types of cancer were mainly explained by a strong birth-cohort effect.

A recent study from Canada also found an increase in testicular cancer incidence rates (95). The study from Canada involved 7296 incident cases of testicular cancer, divided again into seminomas and non-seminomas, which were recorded in the cancer registries in Ontario, Saskatchewan and British Columbia from 1970 and 1995. The age-adjusted rate for seminomas increased by 53% from 1.5 per 100,000 males in 1970-1971 to 2.3 per 100,000 in 1994-1995. The non-seminomas increased by 91% from 1.1 to 2.3 per 100,000 during the same time. Non-seminomas occurred in younger men, aged 15-19, with a four-fold increase. Both types of testicular cancer showed a birth cohort pattern. The high occurrence of this cancer in young men compared to men over 50 has been

interpreted to mean that exposure to risk factors early in life, possibly *in utero* are more likely to be important than exposures in adulthood (91).

4) Possible associations between sperm counts, cryptorchidism, hypospadias and testicular cancer

There are marked racial and geographic differences in the incidence of testicular cancer, some of which parallel the reported decreases in sperm count. For example Denmark has one of the highest incidences of testicular cancer (7.8 per 100,000) (90) (91) and has reported a decrease in sperm counts (28). On the other hand, Finland has a low incidence of testicular cancer (1.3 per 100,000) (91) (90) and has reported little change in sperm counts (98) (67). The etiology of testicular cancer is largely unknown, but cryptorchidism is the only established risk factor and the risk is reported to be increased for the descendend testis of monorchids (99). Additionally several prenatal risk factors are shared by both, including high levels of estrogen in the first trimester (100), premature birth (101), and hypospadia (78), suggesting that they may share etiological factors.

Based on these observations some investigators argue that changes in sperm counts should not be viewed as an isolated phenomenon, but as part of a larger decline in male reproductive health (102) (103) (86) (104) (105). They argue that the possible decline in sperm counts, the increased incidence in testicular cancer, as well as the increasing incidence of cryptorchidism and hypospadias result from adverse prenatal and postnatal effects on the testis. While it is obvious that the congenital anomalies of cryptorchidism and hypospadia have prenatal origins, it has been suggested that testicular cancer arises

from cells of carcinoma *in situ,* which are presumed to be malignant primordial gonocytes (106). The observations that lower sperm counts are associated with damage to the testis (107), testicular cancer (108) and a cohort effect (52), suggest a possible link with a perinatal exposure. It has been hypothesized that *in utero* exposure to high levels of estrogen or other hormonally active chemicals (86) or to low levels of testosterone (104) is responsible for the damage.

Studies on the effects of diethylstilbestrol (DES), a nonsteroidal estrogen administered to several million women between 1950 and 1970 to prevent miscarriage, both support and refute this hypothesis. In case-control studies, male offspring of DES-exposed mothers had an increased risk for hypospadias and cryptochidism [Gill , 1979 #837], although their fertility was not impaired (109). Several other case-control studies have found an association between DES exposure and testicular cancer, while others have not (reviewed in (110). Recently, a study reviewed and combined data from 4 prospective cohort studies on *in utero* DES exposure and testicular cancer (Mayo Clinic cohort, Dieckmann cohort, Women's Health Study cohort and Horne cohort) (110). The study population included 3613 men followed for 16 years. They concluded that testicular cancer rates among DES-exposed men may be elevated since the found a RR of 3.05 (95% CI, 0.65 to 22.0), which was not statistically significant. The increased testicular cancer risk was limited to men in the Mayo Clinic cohort, which suggests that this cohort may differ in some way from the others. The authors note that the men within the Mayo Clinic cohort were exposed to lower DES levels than men in the other three cohorts. Animal studies have found a negative effect of a low but not a high DES dose on prostate abnormalities (111).

A

ef

re

ho

de

rer

An

pla

estr

dev

hav

toxi

Rec

heal

prop

0.51

expe

Seve

USA

Gerr

Additionally the current study had only a 30% power to detect a statistically significant effect due to the low frequency of testicular cancer in the study population. Thus, this report lends marginal support to the hypothesis that increased levels of estrogenic hormones during fetal development have adverse effects on male reproductive development. Thus the role of endocrine disruption on male reproductive problems remains controversial (112) and awaits further investigation.

Another critical period in development is during adolescence when sex hormones again play an important role. It has been suggested that exposure to estrogen or chemicals with estrogenic or antiestrogenic properties during this time may adversely affect development and function of the testes. A variety of substances found in the environment have these properties as well as others that make them candidates for reproductive toxicants.

c. Changes in the sex ratio

Recently the stability of the sex ratio has been proposed as a marker of the reproductive health of a population (113). The sex ratio of a population, usually expressed as the male proportion (male births/total births), is usually stable over time and in most countries is 0.512 (114). A change in the sex ratio has also been viewed as a sensitive marker of exposure to toxic chemicals (115) and a sentinel event for changes in cancer risk (116). Several surveys have shown a decrease in the sex ratio in the last 20 to 40 years in the USA (117), Canada (118), Latin America (119), Denmark (120), Netherlands (121), Germany (122), England and Wales (123) and Japan (124). An increase in the sex ratio

wa

en

In

an

19

in t

bir

inc

for

fro

per

in fa

sex

Orga

conce

conti

diben

was noted in Italy from 1930 to 1989 and was speculated to be due to improvement in environmental conditions (125).

In the USA the sex ratio of live births, taken from birth certificates of live born infants and compiled by the US National Center for Health Statistics, declined from 0.513 in 1969 to 0.512 in 1995 for all live births (117). A change in the sex ratio of 0.001 results in the loss of one male birth per 1000 live births. The decline however was seen for white births (OR, 0.9935; 95% CI, 0.9919-0.9952) while the ratio for black births actually increased (OR, 1.0208; 95% CI, 1.0162-1.0254). Smaller regional changes were observed for white births. A greater decrease was reported in Canada where the sex ratio declined from 0.5147 in 1970 to 0.5125 in 1990 ($p<0.001$) (118), representing a loss of 2.2 males per 1000 live births. In these two countries there is no reason to suspect a reporting bias in favor of female births. Thus it appears that almost all reports indicate a decline in the sex ratio.

2. Exposure to OCs and mercury

a. OCs and mercury in the environment: natural history and trends

1) OCs

a) Natural history

Organochlorine compounds found in the Great Lakes have long been a public health concern. Those found at high enough concentrations historically and currently to be of continuing interest include polychlorinated biphenyls (PCBs), polychlorinated dibenzodioxins (PCDDs), specifically 2,3,7,8-; tetrachlorodibenzo-p-dioxin (TCDD;

diox

spec

majo

DDE

effec

chem

Fig

dioxin), polychlorinated dibenzofurans (PCDFs) and dichlorodiphenylchlorethanes, specifically 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (*p,p'*-DDT; DDT) and its major and persistent metabolite 1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene (*p,p'*-DDE). They have been found in Great Lake fish and have been shown to have adverse effects on reproduction in a variety of species. The diagram below illustrates their chemical structure.

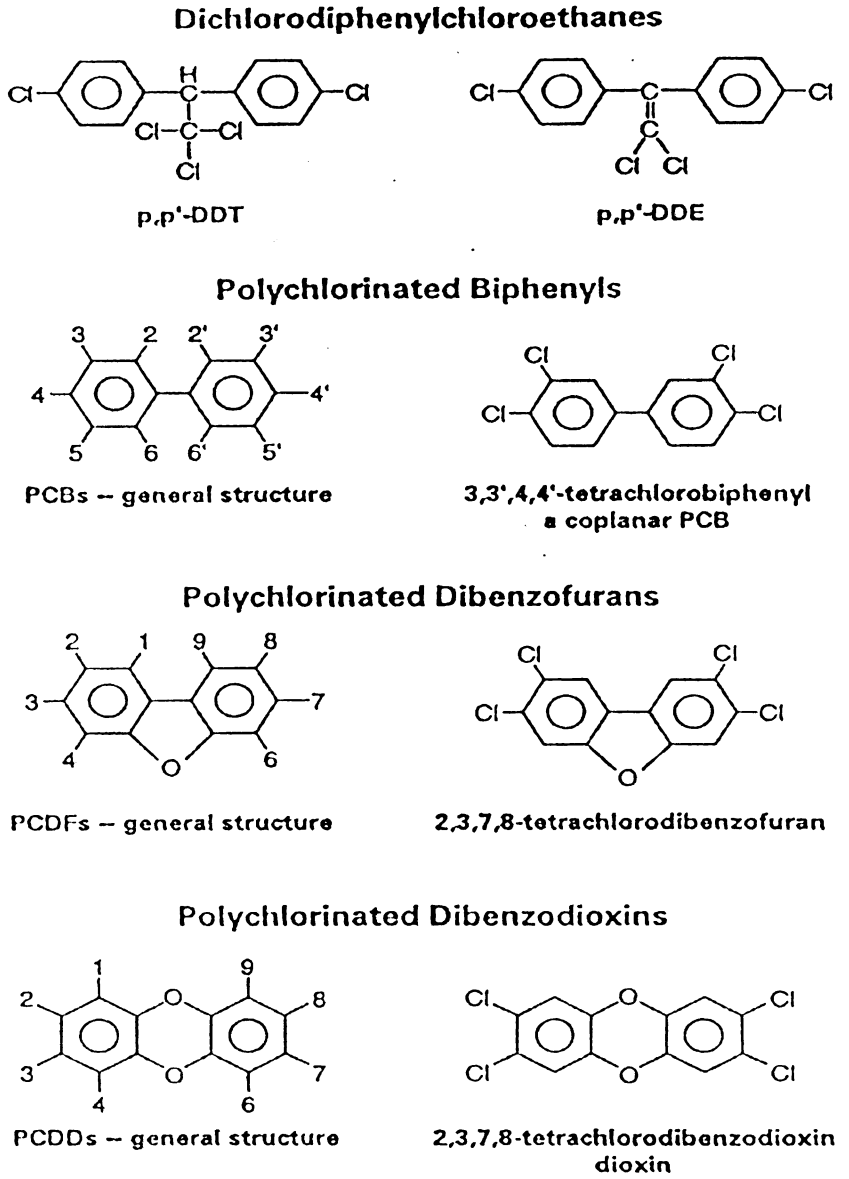

Figure 1. Structure of PCBs, dioxin, dibenzofurans

OC

OC

are

two

the

the

Grou

Their

rings,

(128)

adhe

organ

transf

manuf

OC can be broadly classified as pesticide and non-pesticide (126). The non-pesticide OC's, PCBs, dioxin and PCDFs, belong to the family of polyhalogenated aromatics and are man-made (127). PCBs were manufactured by the addition of chlorine atoms to the two biphenyl rings. The process yields variable levels of chlorination, producing a theoretical 209 congeners depending on the number and location of the chlorine atoms on the biphenyl rings (Figure 2).

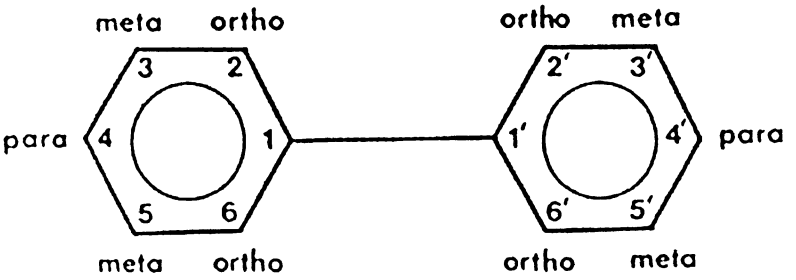

Figure 2. Positions of chlorines on PCBs

Groups of congeners with the same number of chlorines are referred to as homologs. Their stability depends on the number and position of the chlorine atoms on the biphenyl rings, with the higher chlorinated congeners possessing greater stability and persistence (128). Because of their chemical stability, PCBs were used as hydraulic fluids, adhesives, plasticizers in paint, heat transfer fluids, wax extenders, dedusting agents, organic dilutents, lubricants, flame retardants and as dielectric fluids in capacitors and transformers (127). It is estimated that approximately 1.4×10^9 pounds of PCBs were manufactured in the USA from 1930 to 1975. The commercially manufactured PCB

mixtures were marketed under trade names, such as Phenoclor, Kanechlor, Clophen, and, in the USA, Arochlor (127). Different Arochlor formulations were produced, containing mixtures of congeners and thus variable levels of chlorine ranging from 21% to 68%. Although they were first produced in the 1920's, significant production did not occur until the 1950's. Due to their detection in the environment production of PCBs was banned in the USA in the late 1970's. As a consequence of atmospheric transport, PCBs have been detected in rivers, lakes and ocean sediments and their biota. Through the bioaccumulation of PCBs in water and soil sediments, they bioconcentrate in the food chain, with the highest levels found in fatty tissues of carnivores such as the herring gulls and mink.

PCDFs, which can also exist in various forms (approximately 135 congeners) again depending on the chlorination degree and pattern, are by-products of the PCB manufacturing process. Thus the commercial Arochlors actually contained a complex mixture of isomers and congeners of PCBs and PCDFs and were the major source for the introduction of these compounds into the environment. PCDDs (dioxins) are also by-products of industrial syntheses, such as in the production of 2,4,5-trichlorophenol and bleaching processes, and exits as 75 congeners again depending on the position and degree of chlorination. The most toxic and most widely studied PCDD is 2,3,7,8-tetrachlorodibenzo-p-dioxin, which is commonly referred to as "dioxin". It is the most toxic synthetic chemical known. It is important to emphasize that all human and environmental exposures to these compounds involve complex mixtures that interact additively, synergistically or antagonistically (129).

Di
ch
sec
rin
san
con
enz
chl
littl

The
maj
invo
cong
Once
as a
see s
activ
(TEF
comn
conge
halog

Dioxin and certain PCB congeners, although not structurally related, share a common chemical conformation. PCB congeners that lack chlorines in the *ortho* position (please see Figure 2), but have them in both *para* and at least 2 *meta* positions on the biphenyl ring, are referred to as coplanar since the two rings are hypothesized to rotate into the same plane giving the molecule a flat configuration. These congeners are generally considered to be the most toxicologically active based on their ability to induce CYP enzyme activity via the Ahr (129). Those congeners with a single *ortho*-substituted chlorine are considered to be less toxic while the lower chlorinated congeners are possess little or not activity (129).

The major, although not sole (130) (131), pathway thought to be responsible for the majority of dioxin's toxicity involves induction of cytochrome P450 (CYP) genes involved in activation of environmental toxicants. Both dioxin and the coplanar PCB congeners initiate this pathway by binding to the aryl hydrocarbon receptor (AhR) (132). Once these compounds bind to the AhR, the complex translocates to the nucleus and acts as a transcription factor activating a variety of genes, including P450 genes (133) (please see section B.4. a. 1) for more details). The toxicity of a compound that has dioxin-like activity, such as the coplanar PCBs, is often expressed as its toxic equivalency factor (TEF) with dioxin as the reference value (TEF=1). The TEF model for PCBs assumes a common mechanism of toxicity and additivity for the toxic effects of the individual congeners. The TEF value assigned to a specific PCB congener, as well as other halogenated aromatic compounds, is based on results of assays measuring binding to the

AhR, long term carcinogenicity, immunotoxicity, acute lethality, and enzyme induction, and often requires a subjective assessment of the results (134); (126). Additionally, both dioxin and the coplanar PCB congeners have weak anti-estrogenic activity (135) (136) (137).

The moderately chlorinated congeners, with 6 or 7 chlorines, have high TEF's, are the persistent in nature and are found most often in human tissues. If they are not chlorinated in the *ortho* positions, they are usually unable to bind to the AhR and lack the associated activities. These congeners have been reported to have neurotoxic, carcinogenic and endocrine disrupting activities (138). The lesser chlorinated congeners are readily taken up by organisms but also readily eliminated. Those more heavily chlorinated congeners (those having seven to ten chlorines) occur in low concentrations in the environment, remain tightly bound to soil and sediment and are thus less bioavailable (129).

The lower chlorinated coplanar and ortho-substitued PCB congeners also have biological activities. The lower chlorinated forms have lower TEF values and have weak estrogenic activity in various in vitro assays (135). The ortho-substituted non-coplanar PCBs congeners, which have low affinity for the AhR, have biological activities with potential toxicity (139). Some of these congeners, such as 2,2',6,6' tetrachlorbiphenyl with two chlorines in the ortho position, have been shown to have neurochemical effects and are weakly estrogenic. It is this group of congeners (140) (141) that appear to be partly responsible for the behavioral changes and learning deficits that have been observed in children exposed *in utero* to PCBs through fish consumption (142) (143). Investigations

of the effects of Archlor 1242 on release of reactive oxygen species from neutrophils (discussed in section 4. b.), has revealed that ortho, but not coplanar PCB congeners active several intracellular pathways required for super oxide anion (O_2^-) production (144). These PCB congeners are more prevalent in the environment than the dioxin-like congeners, possibly due to biotransformation of higher chlorinated and coplanar species (139).

Thus, it is becoming clear that analyzing associations between a health outcome and exposure to total PCBs may mask important effects mediated by specific congeners. The net effect of exposure to PCBs, through fish eating, for example, is thus likely to represent additive effects as well as opposing effects of the different congeners. As mentioned above, higher chlorinated PCBs have anti-estrogenic effects while the lower chlorinated congeners have estrogenic effects. It is thus important to use a technique for PCB analysis that will detect all possible congeners present in a specimen. Studies which have reported PCB exposures in terms of the specific congeners have found that different PCB congener profiles are associated with different exposures. For example, the profile of congeners observed in fisheaters (145) is quite different than the profile observed in ambiently exposed individuals (146). Occupational exposures yield a congener profile that differs from that of individuals exposed environmentally through diet (147). Similarly, the congener profile of fisheaters differs from that of firemen exposed to PCBs through smoke inhalation or from a mechanic working with hydraulic fluids (Mullard and Wirth, unpublished data).

DDT, a pesticide OC, was synthetically produced and widely used in agriculture and forestry from the 1940's to the 1960's. DDT preparations contain a mixture of two isomers, *p,p'*-DDT and *o,p'*-DDT. *P,p'*-DDE, the major break down product of *p,p'*-DDT, is the most frequently found form in human tissue due to its long half life.

Since it is likely that different OCs as well as different congeners of these OCs are found in the same locations and will bioaccumulate, most environmental exposures will involve a mixture of these compounds. Several studies have shown that OC can interact in vivo. Co-administration of dioxin and a non-dioxin-like PCB (congener 153) to rats resulted in a strong synergistic effect on porphyrin accumulation in the liver, an effect mediated by AhR-induced activation of CYP genes (CYP1A2) (148). Administration of dioxin alone or several concentrations of PCB 153 alone had no effect (1.4-3.0 μg/g liver). Dioxin plus PCB 153 at the lowest dose increased porphyrin levels to 300 μg/g liver and to 1223 ug/g liver at the highest level. DDT metabolites found in the heavily contaminated Lake Apopka in Florida were found to interact synergistically with isolated alligator oviduct estrogen receptors (aER) at concentrations measured in contaminated alligator eggs (149). P,p'-DDE and p,p'DDD, DDT metabolites, both bound to the alligator ER, and in combination decreased estradiol binding in a greater than additive manner (p,p'-DDD, 94% decrease; p,p'-DDE, 93% decrease; p,p'-DDD plus p,p'-DDE, 74% decrease; $p<0.05$ compared to no chemical control and individual metabolites). Similar results have been reported for other pesticides interacting to decrease estradiol binding to the human

ER and to increase human ER-mediated transcription of an estrogen-sensitive reporter gene in a synergistic manner (150, 151).

Administration of several PCB congeners to rats and mice increased hepatic TCDD receptor levels (Safe, 1986). Pretreatment of these animals with the PCB congeners followed by TCDD markedly increased hepatic AHH and EROD induction, activities regulated by the AhR.

OC can also interact antagonistically. Several halogenated aromatic compounds have been shown to antagonize the effects of TCDD (134). Aroclor 1254, a weak AhR agonist can partially antagonize several TCDD-mediated responses, including induction of AHH and EROD activities and immunological responses in vitro. A dibenzofuran congener, (1,3,6,8-TCDF) competitively antagonized binding of TCDD to the AhR in vitro (reviewed in (134)).

The observation that OC interact in both synergistically and antagonistically complicates risk assessment as well as attribution of causality to any single chemical or congener. Analysis of specific contaminants in an environmental exposure at least identifies the individual chemicals of concern, and combined with current knowledge of their effects, can offer a crude explanation for the observed effects.

b) Trends

Since PCB production ceased in 1977, reports indicate that the levels of PCBs found in the Great Lakes basin have generally declined (152) and will continue to decline although at a slower rate (153). According to the U.S. EPA, however, the concentration of PCBs in this "new equilibrium" is well above safe water quality standards (154). As recently as April, 2000, the EPA reported in a press release that PCBs still leak from Lake Michigan, and probably into other sites in the Great Lakes (155) providing new sources of potential human exposure. Such contaminant input from point sources, spills and direct run off from urban or rural areas will affect local concentrations and hence local exposures but will contribute little to the overall contaminant burden, which for PCBs in Lake Michigan is estimated to be 80,000 kg (156).

2) Mercury : natural history and trends in the environment

a) Natural history

Mercury is a toxic heavy metal that exists in trace amounts in the earth's crust. Various natural processes, including volcano eruptions, weathering of rocks and forest fires release inorganic mercury into the atmosphere where it is dispersed worldwide. Mercury, in dry or wet form, is deposited back to lakes, rivers or oceans.

Anthropogenic sources of mercury contribute significantly to the levels of mercury found in the environment. Elemental mercury is used in thermometers and dental amalgams and inorganic mercury is used in manufacturing processes and is usually present in hazardous waste sites. However, while exposure to these forms of mercury is associated with human health hazards, such as renal toxicity, these types of exposures tend to occur after

accidents. Otherwise exposure to these forms of mercury occur at low concentrations and is not a major public health concern (157). However, through a process of methylation, mediated by bacteria species found in soil, sediments, fresh water and salt water, mercury forms stable complexes with organic compounds. The newly formed methylmercury, which is the most toxic form of mercury (158), along with methyl mercury from pesticides and mildew control agents, enter the aquatic food chain (159). In water bodies, it can be taken up by fish and bioaccumulate in the aquatic food chain biomagnifying to tens of thousands to millions of times the concentration found in water (160) (157).

b) Trends

Since pre-industrial times, background concentrations of mercury in the atmosphere have increased two to five times (157) due to new human sources of mercury release. These new sources include burning of mercury-containing fossil fuels, release of mercury-containing by-products from manufacturing processes (chloralkali plants, incinerators) and the release of mercury from paints, broken fluorescent lamps, switches, thermometers, and mercury-containing batteries (158) (161). In 1996, the background concentration of mercury in the atmosphere over the oceans was estimated to be 1.6 nanograms per cubic meter (162) whereas in pre-industrial times, it was estimated to have been 0.5 ng/m3. Additionally the deposition of mercury onto the surface of the earth has also increased from approximately 3.7 μg/m2 in 1850 to 12.5 μg/m2 currently (160). Approximately 25% of the atmospheric mercury deposited over mid-continental North America has ended up in lakes. A modeling analysis conducted by the USEPA (160) predicted that the southern Great Lakes region will experience further high rates of

mercury deposition. Even small increases in atmospheric mercury has the potential to yield measurable increases in methymercury levels found in aquatic organisms (161).

b. OCs and mercury levels in fish

1) OCs

Due to their lipophilic nature, OC contaminants tend to accumulate in the fatty tissues of fish. Thus "fatty" fish, such as salmon and trout, tend to have higher contaminant levels than leaner fish such as perch and bluegills. Additionally the size of the fish, which in part is a function of its age, affects its contaminant burden.

The Michigan Department of Environmental Quality-Surface Water Quality Division (MDEQ-SWQD) monitors the fish levels of environmental contaminants commonly found in waters of the Great Lakes and inland water bodies. The monitored contaminants include PCBs, DDT and DDE, dioxin, other organochlorine pesticides and mercury (see Appendix B for a summary of chemicals quantified in fish). MDEQ-SWQD samples fish from 40 sites statewide and issues a report from which the Michigan Department of Community Health (MDCH) bases their fish consumption advisories for specific fish in specific locations. The number and species of fish monitored per site varies and the specific sites evaluated from year to year varies. The number of fish per species varies from 2 to10, with the majority over 5 fish. It is thus fair to say that the contaminant information for fish species and site per year are inconsistent, making it hard to generalize. When the permissible level of a particular contaminant is exceeded for a fish species (the "trigger level"), MDCH issues a fish consumption advisory. The "restrict

consumption" advisory is issued when more than 10% of a particular species of a given length exceeds the trigger levels, indicating that that there is a limit on the number of meals of that particular fish that can be eaten without possible health risks. Beside advisories for specific water bodies, MDCH also issues statewide advisories covering certain predator fish, such as northern pike, walleye, small mouth bass and large mouth bass, from inland lakes and reservoirs.

In their 1998 Michigan Fish Contaminant Monitoring Program Annual Report (163), MDEQ-SWQD found that contaminant concentrations were above the MDCH's trigger levels in all fish collected from 35 of 40 sites. (See Appendix B for a list of the trigger levels). For total PCBs, fish from 78% of the sites exceeded the women and children trigger level, while 11% of the sites exceeded the general population trigger level. At least one species from 19 of these 21 sites was under a fish a consumption advisory. For TCDD fish from 50% of the sites exceeded the MDCH trigger level and species from all of the sites were under a consumption advisory. Few sites reported fish with levels of DDT levels above the trigger level. An exception was all fish species from the Pine River, St. Louis impoundment, which were under a "no consumption" advisory based on levels of DDT and PBB. Total chlordane concentrations in fish from 3 sites exceeded the trigger level. Thus, even though, according to the state fishing advisories, overall levels of OC contaminants and mercury are decreasing in the Great Lakes, there is still cause for concern for Michigan residents consuming sport-caught fish.

2) Mercury

Methyl mercury is easily absorbed, rapidly accumulates in most aquatic organisms and attains it highest concentrations in older, predator fish at the top of the food chain (164). Unlike the OCs, mercury accumulates in muscle tissue and is thus found at significant levels in many more fish species. About 90% of the mercury found in fish is methylmercury (165). US freshwater fish frequently have mercury concentrations exceeding 1.0 ppm (157). Pike, bass and walleye from contaminated waters tend to have the highest levels (166).

According to the 1998 Michigan Fish Contaminant Monitoring Program Annual Report (167), mercury concentrations exceeded the "restrict" consumption trigger level of 0.5ppm in fish from 54% of the sites monitored and the "no consumption" trigger level in fish from 4% of the sites. Seventy-five percent of inland lakes and or reservoirs sampled in 1997 had one or more fish with mercury concentrations greater than 0.5 ppm.

c. OC and mercury levels in humans

1) OCs

Human populations resident in the Great Lakes region are at higher risk of exposure to OCs as a result of the historic contamination of much of the Great Lakes basin with these synthetic compounds. OC resist degradation, are lipophilic and tend to bioaccumulate. Most humans have low ambient levels of some or all of these OCs.

According to a Canadian Government Report, 80-90% of human exposure to PCBs and related environmental contaminants in the Great Lakes region comes mainly from sport-

caught Great Lakes fish (168, 169). Great Lakes fish consumers, such as anglers and fishing boat captains, have increased body burdens of PCBs and DDE compared to non-fish consumers (170-173), although the levels have declined (145, 174). The number of exposed people is not insignificant: approximately 2 million Michigan residents and 334,000 nonresidents fish in Michigan each year (175). Both men and women consume some Great Lakes fish during most of their reproductive years with men consuming more than women. A variety of species are caught and consumed, often with little regard for the fishing advisories (176).

PCB levels detected in human serum or plasma from non-fisheaters and fisheaters in the Great Lakes region have declined in concert with the levels detected in the water bodies (177). Median PCB levels decreased from 21.4 ppb in fisheaters and 6.6 ppb in non-fisheaters in from Lake Michigan in 1973-1774 (178) to 5.3 ppb in high fish consumers and 2.7 ppb in low fish consumers from Montreal in 1994 (Kosatsky et al. 1996, as reported in Kearney et al. (179)). In a time trend analysis of levels of Arochlor 1260 in the serum of a cohort of Michigan anglers, He et al (177) found that the median PCB levels in male fisheaters changed little from 17.0 ppb in 1973-1974 to 22.9 ppb in 1979-1982 to 21.1 ppb in 1989-1993. For non-fisheaters, however, the levels decreased from 10.0 ppb to 8.1 ppb to 6.8 ppb in the same time period.

Of the 209 theoretically possible PCB congeners, only a portion are considered to be environmentally relevant. Many have never been reported in environmental samples, are not toxic or have low bioavailability (129). In a recent report 89 individual congeners

were detected in serum from Michigan fish consumers (145); (Wirth, unpublished data). However, a subset of these congeners consisting of 25 congeners accounted for over 90% of the total PCBs found in the serum of Michigan fish consumers (145).

Using data from 59 reports, McFarland and Clark (129) divided the PCB congeners into four groups based on potential for toxicity (inferred from their ability to induce the CYP enzyme activities), frequency of occurrence in the environment, and relative abundance in animal tissues. Based on this scheme congeners 77, 105, 118,126, 128, 138, 156, 169 and 170 were in the group with the highest toxic potential. All but congener 77 are members of the penta-, hexa, and hepta-chlorobiphenyl isomer groups. The lesser chlorinated congeners are readily taken up by organisms but are also readily eliminated. Those more heavily chlorinated congeners (those having seven to ten chlorines) occur in low concentrations in the environment, remain tightly bound to soil and sediment and are thus less bioavailable. In the recent re-analysis of PCB congeners found in serum from members of the original Michigan fisheaters cohort (first assessed in 1979-1982), Humphrey et al.(145) focused their analysis on 25 congeners that represented over 90% of the total PCBs in the subjects' serum. This list included only three (118, 138 and 170) from McFarland and Clark's list of the most potentially toxic congeners. Three congeners (126,138 and 156) from McFarland and Clark's list were not found in any subject's serum sample.

Overall, PCB levels in humans seem to be declining with median levels in the general US population ranging from 2 to 9 ppb (180). However, selected populations such as anglers are still at risk through consumption of contaminated sport-fish from the Great Lakes.

2) Mercury

The major source of methyl mercury for humans is fish and shellfish (158) (181, 182) from mercury that has deposited into the Great Lakes and into many of the freshwater inland lakes. When ingested by humans, nearly 99% of methyl mercury is absorbed, spreads throughout the body, and is excreted slowly over time (183). The highest levels are found in the kidney, liver and brain after a few days of exposure. The average person excretes methylmercury with a half life of approximately 68 days (Institute of Medicine, 1991).

Levels of methylmercury are usually measured in hair, blood, or urine. Little methylmercury is found in urine, but can usually be detected in hair and blood. The differential between hair and blood levels varies in different studies (184) (185), but can be as high as 250 times higher in hair than blood. Concentrations of <20 ppb in blood and <6ppm in hair are considered to be in the normal acceptable range for adults (186). Chronic exposure to methylmercury at 0.7 to1.1 μg/kg/day has been estimated to correspond to hair mercury levels ranging from 10 to 70 μg/g (ppm) and blood level of 44 μg/L (ppm) (183) (in (157)). The upper part of this range may lead to toxic effects in adults.

Health risks of methymercury exposure have been found to occur at varying concentrations. Exposure of pregnant women to 10 to 20 ppm, as measured in maternal hair, were associated with motor retardation and CNS toxicity in the child, while levels for 60 ppm to 500 ppm were associated with paresthesia, ataxia and deafness in adults (184). Consumption of fish containing 1 ppm, if consumed in sufficient quantity, could potentially cause levels of methylmercury that have been associated with such adverse health effects. For example, a hair mercury level of 1 ppm, which can be achieved by daily consumption of about 0.4oz of fish containing 0.5ppm methylmercury, would produce a dose of 0.1 μg/kg/day, which is the USEPA's reference dose for causing an adverse health effect. Reference standards, however vary between regulatory organizations and range between 0.05-0.07 μg/kg/day to 0.5 μg/kg/day. Additionally, an adverse health effect would also depend on the particular effect and on individual variables. WHO has set hair levels of mercury in hair at 6 ppm as the acceptable level and reports that the estimated average intake of methylmercury is 2.41 μg/day, coming almost exclusively from fish consumption (186).

3. Effects of OCs on male reproductive outcomes

a. Observations on wildlife and their exposures

Wildlife studies provided the first support for adverse effects of OC exposure on male reproductive parameters. (187-192). Many animal studies (fish, birds, reptiles, mammals) have found associations between OC compounds and reproduction (187). Juvenile male alligator living in Lake Apopka, a Florida lake contaminated with waste from agricultural activities, a sewage treatment facility and an extensive spill containing dicofol, DDT and

sulfuric acid in 1980, exhibited abnormal gonadal morphology, plasma sex steroid concentrations and greatly reduced plasma testosterone concentrations compared to alligators from a control lake (Lake Woodruff) (193). Further work indicated that unstimulated testes from Lake Apopka males synthesized significantly more estradiol than testes from Lake Woodruff males (5.3 pg/mg tissue/hr versUS 1.1 pg/mg tissue/hr) (194) while testosterone syntheses was comparable. These results suggest that xenobiotic chemicals including DDT, modified gonadal steroidogenic activity and hepatic metabolism of steroids.

The population of Florida panthers has been decimated in part through exposure to a variety of environmental contaminants found in raccoons, a major source of food. Necropsies of dead panthers showed potentially toxic concentrations of mercury (110 ppm in liver), as well as p,p'DDE, PCBs and other pesticides (195). Additionally, sperm abnormalities of the Florida panther were the highest reported for any feline and included low ejaculate volume, low sperm concentrations ($3\text{-}15 \times 10^6$ sperm/ml), poor motility, and a very high proportion of abnormal sperm (92.2%) (196). Eleven of 17-free-ranging panthers and two captive males were cryptorchid (197).

A variety of reproductive abnormalities have been found in wildlife in the Great lakes region. The increased incidence of teratogenesis and embryonic mortality of colonial fish-eating water birds from the Great Lakes is at least in part due to exposure to PCBs (188). Adult bald eagles that have migrated to the Great Lakes shoreline exhibit reproductive impairment after feeding on fish and other

foods from the lakes for 2 or more years (reviewed in Guillette et al (198).

More powerful evidence comes from laboratory studies in which various animals were fed fish from the Great Lakes contaminated with PCBs as well as other chemicals. Ingestion of levels of PCBs found in Great Lakes coho salmon was associated with reproductive failure in mink (199). Similarly, mink fed Great Lakes carp, sucker, perch scraps, or whitefish had decreased reproductivity (200). In particular, those fed carp failed to reproduce at all. It has been suggested that PCBs diminish reproductive performance in terms of decreased fertility and litter sizes in most animal species tested under laboratory conditions (201).

b. Effects on spermatogenesis

Early studies focused on treatment of adult experimental animals with PCBs, usually as Arochlor mixtures, and TCDD, found increases in testes weights (202) (203), reductions in sperm counts (204), decreased fertility (Khera and Ruddick, 1973) and altered plasma androgen levels (205) (206). However, the doses required to achieve these effects were often toxic and far above levels ever seen in human exposures. For example, a single dose of PCB congener 77, which has a high TEQ, of 18mg/kg (ppm) resulted in permanently reduced daily sperm production and a significant increase of abnormal sperm (207). For comparison, the median serum level of men exposed to PCBs through fish consumption in the 1970's was 21.4 ppb (208). Similarly, for TCDD, the ED^{50} (the dose that will cause an effect in 50% of the animals), for androgenic deficiency is 15,000 ng/kg (205), while the average background body burden for humans is 8-13 ng/kg (209).

Subsequently, investigators shifted focus to more sensitive periods in mammalian development to determine if exposure to contaminants in the range of human exposures during these periods would have an effect on reproductive outcomes. Animals exposed in *utero* and perinatally, when organogenesis and further organ development, respectively, occurs, required significantly lower does of OC to produce adverse reproductive effects (reviewed in (210). Exposure of experimental animals ***in utero*** appears to be the period of development most sensitive to the harmful effects of OC (142).

More recent data indicate that for both experimental animals and humans, puberty is also a time when lower exposures can lead to adverse reproductive effects (211). During puberty, levels of gonadotroping-releasing hormone (GnR) produced by the hypothalamus increases, which leads to increased levels of gonadal sex hormones and secondary sexual development (212). In males secretion of FSH leads to the development of seminiferous tubules and an increase in testis size. LH release leads to testosterone secretion by the Leydig cells while FSH secretion leads to production of inhibin secretion by Sertoli cells, thus establishing the regulatory networks in the hypothalmus-anterior pituitary-gonadal axis. Exposure to hormone disrupting chemicals, such as PCBs and TCDD, during this time may have adverse effects on spermatogenesis. Since these chemical are lipophilic and have long half-lives, the effects could persist into adulthood (211).

Since the focus of this proposal is exposures occurring from adolescence through adulthood, this section will review studies covering these life periods.

1) Effects of exposure to PCBs on experimental animals and humans

a) Experimental animals

Studies on experimental animals exposed to PCBs have added increased support for the notion that exposure to PCBs explains some of the effects of OC exposure on wildlife. PCB treatment of adult rats with a single subcutaneous dose of 18mg/kg or 60 mg/kg body weight of 3,3',4,4'- tetrachlorobiphenyl (congener 77 which has a TEF value of 10^{-3} (132) resulted in a reduction of daily sperm production from the control value of $31x10^6$ sperm to $22x10^6$ and $20x10^6$ sperm, respectively, for the two PCB groups at 8 weeks. The sperm number per caudal epididymus was significantly reduced one week after treatment from the control level of 222 $x10^6$ sperm to 135 x 10^6 and 142 x 10^6 for the PCB treatment groups. Significant increases in the percentage of abnormal sperm were observed in both treatment groups compared to the control group, with changes in the tails being the most frequently observed abnormality (207).

In another study, female mice were fed diets containing 30 ppm daily of PCB congener 77 for two weeks before pairing to untreated males. Females received the same daily dose throughout mating, gestation and lactation. F1 males were given the same diet postnatally (213). At three weeks of age, PCB-exposed male mice had significantly increased testes weight compared to unexposed controls (43.2 mg vs. 32.9 mg,

respectively, ($p<0.05$). The percentage of eggs fertilized *in vitro* by sperm from similarly PCB-treated mice at 19 weeks of age was significantly reduced to 46.7% compared to 57.9% fertilized by control mice ($p<0.05$).

In a third study, the effects of exposure of male rat pups to Arochlor 1254 during lactation on their adult reproductive functions were investigated (202) (214). During lactation in rats, development is continuing and transfer of PCBs is greater than during gestation (215). Male pups were exposed to PCBs in the milk of mothers given Arochlor 1254 on days 1,3,5,7, and 9 day of lactation, at doses of 8mg/kg, 32mg/kg and 64mg/kg (214). Beginning on 130 days of age, the PCB-exposed males were mated to unexposed females. The number of embryo implants, the number of embryos and the implants per corpora lutea were all significantly reduced in females mated to males exposed to 32mg/kg and 64mg/kg Arochlor compared to females mated to unexposed males ($p<0.001$). In a follow-up study by the same authors using a similar protocol and Arochlor doses, a significant decrease was observed in the number and percent of normal fertilized eggs and eggs at the two-to four blastocyst stages in females mated to Arochlor-exposed males compared to females mated to unexposed males (216). No reduction in caudal sperm number, sperm production, epididymal sperm morphology and either FSH or testosterone levels was observed. While there are significant differences in the protocols used by these three groups (PCB congener(s) type, dose, and route), they indicate that exposure of animals early in life to PCB congener mixes or specific PCB congeners can lead to adverse effects on fertility and sperm function during adulthood.

b) Effects of PCBs on humans

Effects on human sperm parameters have been harder to document, in part because of the difficulty of obtaining semen samples. A study compared the levels of 74 PCB congeners in 33 fertile men, 50 subfertile men (20-60 x 10^6 sperm/ml), 50 men with idiopathic oligospermia (<20 x 10^6 sperm/ml) and 25 men post vasectomy (N=170). A significant correlation was noted between sperm motility and the levels of congeners 123, 138, and 167 in samples with sperm counts below 20 x 10^6 in regression analysis (217). The mean total PCB level in seminal fluid was 5.8 ppb and the maximum levels of the three congener were 2.3 ppb (congener 123), 1.1 ppb (congener 138) and 1.1 ppb (congener 167). In a more recent pilot case-control study investigating the relationship between OC and human sperm parameters, Hauser *et al.* (218) showed that men with decreased sperm counts (<20 x 10^6/ ml), decreased sperm motility (<50%) and decreased percentage of morphologically normal sperm (<40%) had higher levels of PCB congeners 118, 138 and 153 than controls with normal semen parameters. Due to their small sample size (29 men), these results can be no more than suggestive. Recently, a modest association in men with high levels of Lake Michigan sport-caught fish consumption and risk of conception delay (OR=2.8 CI, 1.0-8.0) has been found (219). Another study conducted with sport fish consumers from Lake Ontario, however, did not find such an association (220). Both studies obtained the analyzed data cross-sectionally and both had low participation rates for the over all study: 29% (219) and 39% (220). Each study used a different method to calculate the number of fish meals consumed, neither of which was precise, so it is difficult to compare the levels of exposure. Additionally, differences in fish species, location, and contaminant types and concentrations between the two studies

may contribute to the differing results. A study of transformer repair workers did not find an association between level of PCB exposure and sperm counts (221). However, the levels of individual congeners was not determined and PCBs were not analyzed as a continuous variable, which may have decreased their ability to detect smaller differences. Other studies have also detected PCBs in human semen with mean values for total PCBs of 5.8 ng/g (217), 7.0 ng/g (222) and 11.7 ng/g (223), but did not correlate these levels with sperm parameters.

A follow up study of individuals exposed to PCBs and dibenzofurans in the large scale poisoning in Taiwan in 1979 through ingestion of contaminated rice oil (Yu-Cheng) found an effect on male children exposed *in utero* (224). All prenatally exposed men 16 years or older and 48 healthy volunteers of comparable age and no history of chemical exposures were recruited. A significant difference in the percentage of morphologically abnormal sperm (37.5% in 12 exposed men and 25.9% in 23 unexposed men, $p<0.0001$) was found. A significant reduction in the percentage of total motile sperm (35.1% for exposed men, 57.1% for unexposed men, $p<0.0058$) was reported. Additionally, a reduction in the percentage of hamster oocytes penetrated (65.8, exposed and 73.5, unexposed ($p<0.017$) was found. No differences in semen volume or sperm count were found.

In summary, while experimental animal studies indicate adverse effects of PCB on male reproduction and on sperm parameters, data from human studies are sparse. It is

noteworthy, however, that those studies in which levels of individual PCB congeners were measured found significant reductions in sperm function.

2) Effects of OC pesticides on semen quality

1) Dibromochloropropane (DBCP)

OC pesticide exposure has also been shown to affect spermatogenesis in several studies. Similar effects of DBCP on spermatogeneis were reported for production workers in pesticide factories using DBCP as an ingredient in California (225) (226) and Israel (227-229) in 1976 and 1977, respectively. Direct quantitation of DBCP exposure levels were not made in either study, so length of time at their job was used as a proxy for exposure. In investigations of both exposures, fertility problems were reported only by men coming into direct contact with DBCP during its production. In both studies, semen analysis of DBCP exposed men revealed decreasing numbers of sperm with increasing years of exposure.

The California study began with the 39 people working in the Agricultural Chemical Division of the pesticide plant, but after excluding women, none of whom complained of fertility problems, men with vasectomies or men with intermediate sperm counts (10-30 x 10^6/ml), ended up with 11 men with normal (> 40 x 10^6 sperm/ml; group A) and 11 men with low (<1 x 10^6 per/ml; Group B) sperm counts. Nine volunteers consented to a testicular biopsy. Significant differences between Groups A and B ($p<0.01$) were found for years exposed (A, 8.0 y vs B, 0.08 y), sperm count (A, 0.2 x 10^6/ml vs B, 93 x 10^6/ml), FSH (A,11.3 vs B, 2.6 mi.U/ml), and LH (A, 28.4 vs B, 14.0 mi.U/ml) but not with

testosterone (459 vs B, 463 ng/dl). Two men in Group B who were not azoospermic (0 x 10^6/ml sperm) had sperm with decreased motility and increased morphological abnormalities. Biopsies of men in Group B revealed loss of spermatognia. A high level of DBCP exposure in the current year was significantly associated with depressed sperm counts ($p<0.01$)(230). The level of exposure to DBCP in early 1977 was measured at 0.4 ppm USing personal air monitoring devices.

The Israeli study found that longer, continuous exposures to DBCP by production workers was associated with a decrease in sperm count and, if long enough, to oligospermia (greater than 0 but less than 20 x 10^6 sperm/ml), azoospermia and sterility (231). Testicular biopsies on an azoospermic man showed severe atrophy of the germinal epithelium with no evidence of active spermatogenesis (107). Follow up of the 30 exposed men at five years (231) found that some of the oligospermic and azoospermic men had recovered and fathered children. Azoospermic men who did not recover had FSH levels that were almost three times higher than in men who recovered (approximately 9 mIU/ml versus 25 mIU/ml, respectively). LH levels were initially comparable in both groups of azoospermic men, but by four years the levels increased and stayed elevated in men who did not recover (approximately 10 mIU/ml versus 19 mIU/ml, respectively). Testosterone levels remained normal in all men at all times.

A study conducted in 1977 of California pesticide workers exposed to DBCP included a total of 96 men from 5 counties who came in for examination (230). Ten men reported clinical infertility, and two had sperm counts less than 1 x 10^6 sperm/ml. An association

between mean sperm count and current year exposure (61.7 x 10^6 sperm/ml for zero days to 21.9 x 10^6 sperm/ml for greater than two months) was significant ($p<0.01$), while the trend for past years exposure to DBCP (61.7 x 10^6 sperm/ml for 0 years to 30.2 x 10^6 sperm/ml for eight or more years) was marginally non significant for trend ($p<0.054$). A significant association between FSH levels and days exposed in current year, but not LH levels, was significant ($p<0.5$). They also suggested that the effects of DBCP appeared to be reversible, since lower sperm counts were associated with current rather than past years exposure. Thus DBCP, a halogenated hydrocarbon, has been shown to have toxic effects on the spermatogonia, which depending on the exposure, can lead to long-term sterility. While DBCP may seem like an extreme example, it should be kept in mind that over 1000 chemicals have been identified in the waters of the Great Lakes and only a fraction of them have been tested for any type of adverse health-related effects, much less for their ability to interact with each other.

b) Other pesticides

Occupational exposure to pesticides has been reported to affect time to pregnancy, a measure of fecundability. A correlation between pesticide spraying season and time to pregnancy was found for fruit growers in the Netherlands exposed to pesticides (de cock et al. 1994). On the other hand, Savitz and associates assessing the potential reproductive hazards associated with farming in the Ontario Farm Family Study, were not able to find significant associations between farm chemicals and time to pregnancy (232) or an altered sex ratio (233). Similarly, no association was found between farmers and agricultural workers in France and Denmark and time to pregnancy (234). Differences in study populations and chemicals examined may explain these contrasting results.

Pesticides have been shown to disrupt endocrine function (235). P,p'DDE (dichlorodiphenyldichloroethene), the major and persistent metabolite of DDT, was shown to be a potent androgen receptor antagonist capable of inhibiting the effects of androgens (236). Effective doses were in the range of those found in humans in areas contaminated with DDT. Of interest, the biologically active metabolite of methoxychlor (the methylated isomer of DDT and a xenoestrogen), was shown to significantly reduce testosterone production by Leydig cells in vitro (237). This effect was mediated via a decrease in the activity of CYP11A1, a P450 enzyme involved in steroidogenesis. O,p'DDT (an isomer of DDT) as well as p,p'DDE, and p,p'DDT have been found in human semen (238).

3) Effects of dioxin

Much of the evidence supporting a role for dioxins and male fertility has come from experiments with animals. Dioxins can affect male fertility in experimental animals if administered to adult animals although their strongest effects seem to occur after *in utero* exposure. In post pubertal experimental animals, dioxin, at doses causing overt toxicity, causes decreases in the weight of the testis and the accessory sex organs, decrease in spermatogenesis, abnormal testicular morphology, inhibition of testicular steroidogenesis, reduction in plasma androgen concentrations and adverse reproductive performance (239-244). If administered during gestation at a time when the hypothalamic/pituitary/testis axis is developing, a single dose of dioxin adversely affects the male offspring by decreasing serum androgen concentrations and impairing androgen-dependent perinatal development (245), by caUSing demasculinization and feminization, by impairing sexual

differentiation of the central nervoUS system (246), and by decreasing spermatogenesis (247). Maternal and lactational exposure of rats to dioxin increased the frequency of cryptorchidism in the male offspring, caUSed dose-dependent reductions in male offspring of testicular weight (247) and sperm counts (248). Sperm from male rats exposed to dioxin *in utero* had decreased motility, decreased in vitro fertilizing capacity (213) and permanently altered sperm-transit through the epididymUS (210, 249). Dioxins thUS appear to have clear cut effects on spermatogenesis and male sexual development in animals but at doses much higher than seen in humans. However, if the exposure occurs during sensitive developmental times, such as during puberty, they may potentially affect spermatogenesis at doses seen in human studies (211). A target of these toxic effects is the Leydig cell, whose number and steroidogenic enzyme activity are reduced after TCDD treatment of adult rats (205).

Male workers exposed to high levels of dioxin where found to have statistically significant, elevated levels of LH and FSH, and decreased levels of testosterone (250). A study on the risk of infertility and delayed conception and workplace exposures found that fishermen had a significantly increased risk of sperm abnormalities ($p<0.05$) although the specific exposure was not determined (251). Dioxins have been detected in human semen (252).

In summary, the evidence suggests that PCBs, pesticides and dioxin can adversely affect various aspects of male reproduction in animals and in humans if the dose is high enough or if the exposure occurs during a susceptible time during development. From the FFHP

data we know that 80-90 % of male participants reported eating sport-caught Great Lakes fish during their teens and twenties, possibly times when the effects of these contaminants may be harmful to processes involved in spermatogenesis and regulation and function of reproductive hormones.

b. On the sex ratio

The male proportion, commonly referred to as the sex ratio, is number of live males births divided by the total number of live births. For most western countries this value is usually 0.512 to 0.515 and remains stable (114) (115). The ratio is determined early in gestation when sexual differentiation takes place. Prenatal sexual development is a complex process requiring balance and timing of exposure to androgens and estrogens (reviewed in (253) (115)). Initially all embryos appear to be female and will remain phenotypically female without androgenic stimulation. Between 6 and 9 weeks of gestation, Sertoli cells of the testis or follicular cells of the ovary become active. At this point the embryo has a unisex pair of gonads and two sets of ducts, referred to as the wolffian and mullerian ducts. In genetic males, the gonads differentiate into testes and produce testosterone, which further drives masculine development. Testosterone stimulates the differentiation of the wolffian ducts into the ductus deferens and the descent of the testes into the scrotal sac. Mullerian inhibitory hormone, produced by the Sertoli cells, directs the regression of the mullerian ducts. In genetic females, the wolffian ducts disappear in the absence of androgens and the mullerian ducts develop into the oviducts. Normal differentiation of the testes depends on the undisrupted functioning of

the Sertoli cells at this critical stage. Thus, exogenous agents capable of changing androgen or estrogen levels (endocrine disruption) or damaging to the cells of the testes at this time could affect the phenotypic determination of sex and subsequent development.

It has been hypothesized by James that mammalian parental hormone levels at the time of conception affect the sex ratios of the young at birth (James 1996). Thus the sex ratio of offspring is increased by high levels of testosterone and estrogen in the parents at the time of conception, and decreased by high levels of gonadotrophin and progesterone. Exposure to environmental contaminants with the potential to modulate gonadotrophin levels has been linked to changes in the sex ratio through studies of accidental and occupational exposures.

1) Effects of PCBs and PCDFs on the sex ratio

Two mass food poisonings of contaminated rice oil, called Yusho in Japan (254) and Yucheng in Taiwan (255), resulted in widespread exposures to PCBs and PCDFs. In Taiwan, about 2000 individuals were affected from 1978 to 1979 and the estimated amount of PCBs ingested was 0.7 to 1.84 g (255). Serum PCB concentrations from 613 individuals ranged from 3 to 1,156 ppb. Recently, the sex ratio of 137 live births in Taiwan from 1978 to 1985 by 74 women who had registered with the health department as being affected was determined (256). Sixty-nine girls and 68 boys were born giving a ratio of 0.496. The authors state that the number of girls born was not excessive, but do not provide the sex ratio of a comparison group, such as children born to unexposed mothers. For comparison, the sex ratio for Japan for 1978-79 was 0.514 (124). In this

study, the eligibility criteria for the women included having at least one child alive at the time of the interview in 1985, which could be up to 8 years after the exposure. The reason for this criterion is unclear, since if anything, it would bias the ratio in favor of more girls since boys die at a faster rate than girls at all life stages. Additionally, there is no information on the exposure levels of the 74 women. This could bias the results in either direction since these volunteers could either be more health conscious and perhaps healthier or they could be the most seriously affected. No consideration was given to the exposure status of the fathers, which again could have increased, if exposed, or decreased, if unexposed, the effects on the sex ratio. Thus the effect of PCB exposure on the sex ratio cannot clearly be ascertained from this report. No reports on the sex ratio of children of the Yusho patients could be found to evaluate the effects of PCBs and PCDF's on the sex ratio for that population.

Experimental animal studies provide some additional evidence for the effect of PCBs on the sex ratio, although the exposures were *in utero*. In a study on the effects of Arochlor 1254 on the sex ratio of rhesus monkeys, 80 menstruating rhesus monkeys were untreated or treated with from 5 to 80ug Arochlor 1254/kg/day for 25 months. A total of 36 impregnations occurred (257). Of these, the sex could be determined on 26 live births, stillbirths and abortions. The sex ratio for the offspring of the untreated monkeys was 0.500 and for the treated, 0.4375. Of the abortions and stillbirths in the treated group whose sex could be determined, all were male. The authors conclude that there was some suggestion that Arochlor 1254 may adversely affect the viability of the male rhesUS fetUS. In another study, pregnant female rats were dosed with 6 mg/kg of 3,3',4,4'

tetrachlorobiphenyl (PCB 77) daily by gavage from day 6 to day 18 of gestation (258). PCB 77 reduced the sex ratio before and after birth from 1.00 (control) to 0.72 and 0.69, respectively. While both sexes were affected, males were preferentially killed. Significant mortality occurred late in gestation, on days 19 and 20, suggesting that the congener acted directly on the fetUS rather than by affecting maternal physiology.

b) Effects of dioxin exposure on the sex ratio

Studies on a human dioxin exposure resulting from an explosion in a herbicide plant in 1976 in Seveso, Italy, assessed the effects on the sex ratio of children born to exposed parents (259). Seventy-four children born in the high dioxin exposure zone (A-zone) from 1977 to 1984 had a sex ratio of 0.351 (26 males vs 48 females), compared to the expected ratio of 0.514 (X^2 test, $p<0.001$)(260). In families in which both parents were in the A-zone at the time of the explosion and also had high serum dioxin levels (104-2340 ppt in 1976), no males were born from 1977 to 1984. In a more extensive analysis of the sex ratio of children born to exposed parents in whom serum dioxin levels were determined, it was found that exposure of both parents to greater than 15 ppt dioxin decreased the sex ratio to 0.442 ($p=0.03$, compared to the expected ratio of 0.514)(211). Only the father's exposure was shown to significantly decrease the sex ratio and the ratio decreased with increasing dioxin concentrations (X^2 for trend, $p=0.008$). Fathers exposed before or during puberty (younger than 19 years old) had a lower sex ratio than those exposed later in life (0.382 versus 0.469, respectively), suggesting that the time before and during puberty may be a very sensitive period for dioxin toxicity in men. Additionally, fathers who had greater than 15 ppt serum dioxin levels and who were

exposed during puberty continued to father significantly more girls than boys more than 15 years after the 1976 exposure, suggesting that these effects of dioxin may be long-lived, if not permanent.

Thus, exposure of males to environmental chemicals during critical periods of their development can affect aspects of their fertility involved in the determination of the sex ratio. It has in fact been suggested that the timing of such exposures may be more critical than the total dose rate in determining a broad range of outcomes (261).

Servicemen in the Vietnam War were also exposed to dioxin as a result of extensive aerial spraying with the herbicide Agent Orange. An ingredient of Agent Orange, 2,4,5-T, was contaminated with dioxins at a mean concentration of 2 ppm (262). Exposure assessment was based on service location as determined from self-administered questionnaires and service records or spraying locations (263). Exposure to Agent Orange was not associated with any of the reproductive outcomes measured including difficulty in conception, time to conception or the sex ratio of the offspring. Since the risks of exposure to Agent Orange were unforeseen, semen quality was not assessed at any time following exposure. The study design was ecological and as such individual exposure levels were not determined. Additionally it is likely that assessment of location by self-report and by official records may not accurately reflect the actual war-time situation. It thus difficult to determine the exposure parameters and correctly assess the effects of Agent Orange on specific individuals and their reproductive health from this study.

3) Effects of OC pesticides on the sex ratio

a) Dibromochloropropane (DBCP)

OC pesticide exposure has also been shown to affect the sex ratio in several studies. The study investigating the effects of DBCP exposure in pesticide workers in Israel described above also found alterations in the sex ratio. Follow up of the 30 exposed men in the Israeli study at five years (231) found that some of the oligospermic and azoospermic men had recovered and fathered children. Of the 13 children born to exposed fathers, 11 were girls, yielding a sex ratio of 0.154 ($p=0.011$)(228, 229). The sex ratio of men who were normospermic (greater than 20 x 10^6 sperm/ml) was 0.80 (5 live births) compared to 0.53 for the pre- or unexposed men ($p>0.05$). The sex ratio of men who remained oligospermic was 0.25 while that of men who remained azoospermic was 0.00 (4 live born females) ($p< 0.015$ for oligospermic and azoospermic men).

The Israeli group of men were evaluated again at 8 and 17 years post exposure. At 8 years, 15 formerly azoospermic men and 7 formerly oligospermic were followed up and their sex ratio compared with the sex ratio of men formerly unexposed and men with pre-exposure live births. The sex ratio of the pre-exposed and unexposed men (a total of 51 live births) was 0.529. The sex ratio for pregnancies occurring during the exposure (17 live births), 0.352; and for pregnancies occurring during the recovery period (19 live births), 0.210 (107). At the 17 year follow-up of 9 formerly azoospermic and 6 formerly oligospermic men, no further recoveries had occurred. Of the 41 live births of the 7 recovered men, 17 were male, yielding a sex ratio of 0.414 (264). Thus the effects DBCP

exposure on the sex ratio was severe and long lasting, much like that seen with the dioxin exposure in Seveso.

Another study did not find that exposure to DBCP affected the sex ratio. They found that exposure to DBCP in drinking waters did not adversely affect birth outcomes, including the sex ratio (265), even at the highest level of contamination (1-3 ppb in the drinking water). This was an ecological study, however, with exposure being determined by census tract residence rather than individual body burdens. As such, a direct association between DBCP exposure and the sex ratio cannot be made.

2)) Clordecone (kepone)

Clordecone, a chlorinated hydrocarbon insecticide, was involved in one of the most costly chemical disasters in the United States (266). The disaster occurred in a small, single-product manufacturing factory, named Life Sciences Products Company, which made the insecticide Kepone for Allied Chemical Corporation in Hopewell, Virginia. In 1974 and 1975, 76 of 133 people working at the plant developed a clinical illness associated with production work with kepone. Kepone blood levels in workers with the illness were 2.53 ppm and those without the illness were 0.060 ppm ($p<0.001$) (267). Thirteen patients (17.1%) had oligospermia with abnormal and nonmotile forms predominating (268).

Taken together, these results suggest a link between damage to the germinal epithelium by DBCP, decreased sperm counts and decreased sex ratio. These results suggest a

continuum of deleterious effects on the spermatogonium by increasing levels of exposure to environmental toxicants like DBCP and dioxin. On a population level, the first effect may be a decrease in the sex ratio followed by falling sperm counts and ultimately by increases in sterility and/or testicular cancer. Movement from the first effect to the subsequent ones may involve cumulative exposures of related chemicals, which may interact to amply their effects as well as exposure to very high doses of a single chemical, which rarely occurs. For example the effects of both DBCP and dioxin in Seveso (260) appear to be reversible up to a certain level of exposure, after which the higher doses may cause permanent damage to the germinal epithelium.

d. Comparison of animal and human OC doses

DeVito et al. (209) compared estimated body burdens of dioxin-like chemicals reported in studies on humans and experimentally animals associated with the same health outcome. In humans, body burdens were estimated from lipid-adjusted serum concentrations of dioxin-like chemicals, including dibenzo-p-dioxins, dibenzofurans and PCBs using the TEF method. The value obtained thus reflected the body burden of chemicals with dioxin-like properties and was expressed as the TCDD equivalent factor (TEF). In the general human population, average background concentrations were estimated as 58 ng TCDD equivalents (TEF)/kg serum lipid, which corresponds to a body burden of 13 ng TEQ/kg body weight. Human populations with known exposures to dioxins have body burdens of 96-7,000 ng TEQ/kg body weight, or about 8 to 538 times

the average exposure level. Background levels of TCDD were 1 and 4 ng/kg in rats and mice, respectively.

For effects clearly associated with dioxin exposure, such as chloracne and induction of CYP1A1, a gene induced by TCDD-like chemicals that regulates hormone and contaminant metabolism (see section 4. for more details), humans and animals responded at similar body burdens. Development of chloracne in humans occurred at body burdens of 96-3,000 ng/kg while the animal dose was 1,000ng/kg in monkeys and 13,900 ng/kg (4,000 ng/kg, 3 days/week/2 weeks) in mice. The lower human exposure value, 96 ng/kg, was found in an individual with the lowest reported adipose dioxin concentration for any human with chloracne. This individual was exposed to a mixture of chlorinated dibenzodioxins and chlorinated dibenzofurans. The higher value, 3,000 ng/kg, represents the average body burden of TEQs in individuals from Yusho with chloracne.

Induction of CYP1A1 in human placenta occurred at 2,130 ng/kg while induction in rat liver required 2,582 ng/kg (125 ng/kg/day, 5 days/week/13 weeks). The human exposure level was obtained in placentas from mothers highly exposed during the Yu-Cheng incident. These mothers gave birth to babies with lower birth weights compared to babies from unexposed mothers and had altered levels of placental CYP1A1 and placental epidermal growth factor receptors. Induction of CYP1A1 activity was also measured in German chemical plant workers exposed to TCDD (Masten et al 1997). Elevated levels of CYP1A1 enzyme activity were found in 34 workers diagnosed with chloracne with TEQ values ranging from 22.7 to 914.7 ppt.

In contrast, the body burdens associated with the less clearly defined carcinogenic effects of TCDD ranged from 944 ng/kg in mice to 137,000 ng/kg in hamsters. In epidemiological studies that reported an association between TCDD exposure and cancer, body burdens were estimated between 109 and 7,000 ng/kg at the time of highest human exposure. For example, a report on cancer incidence from 1977 to 1986 in individuals exposed to TCDD in Seveso, Italy, in 1976, found significant increases in cancers of the hematopoietic system in men and hepatobilliary system in women (Bertazzi et al 1993), although overall cancer rates were not increased. The increases occurred in residents of Zone B, whose blood concentrations of TCDD were estimated at 74 to 526 ppt (ng/kg). No increases in cancer incidence were found in the 724 residents of Zone A, perhaps because, at the time of this publication, there were only 14 reported cancer cases. Thus, the TCDD levels associated with cancer development in this group are approximately 5 to 40 times the background level for humans, but are much lower than the dose required to cause cancer in rodents.

In contrast to the better established effects of dioxin-like chemicals that occur at relatively high exposure levels, several other effects occurring at variable doses have been reported in experimental animals. Decreased sperm counts in rats occurred at a TCDD body burden of 64 ng/kg (64 ng/kg, maternal dose) (247), while testis abnormalities in rats and mice occurred at 12,500 (rats)(205) and 100,000 ng/kg in mice (240). Sperm from rats exposed during lactation to mothers given a total of 150,000ng/kg Arochlor 1252 during lactation (32μg/kg on days 1,3,5,7 and 9) had

significantly decreased linear motility ($p<0.5$) (216). In humans, significantly decreased sperm motility occurred with seminal fluid PBC congener concentrations of 1.1 to 2.3 ng/g (ppb) (217).

Little can be concluded on dose equivalences from these experiments on reproductive outcomes since the actual amounts of PCBs to which the males rodents are exposed is not known.

In conclusion, it appears that for outcomes causally associated with TCDD exposure, the levels required in experimental animals and humans are similar, and higher than background levels for both species. For cancer, the levels required to see an effect in humans is appreciably lower than in animals, but still higher than the levels seen in the general population. For reproductive comes, the data is much more limited and insufficient to draw any conclusions.

4. Mechanisms of OCs effects on male reproductive paramters

a. Role of genetic polymorphisms

1) In genes encoding cytochrome P450 enzymes

Enzymes coded by cytochrome P450 genes, also referred to as CYP genes, play critical roles in the activation and detoxification of a wide variety of environmental toxicants and in the metabolism of sex hormones, including hydroxylation of estradiol and testosterone. Some of these genes have stable genetic variants, or polymorphisms, that encode enzymes with no, increased or decreased activity. Some of the genes are inducible

by a variety of chemicals including environmental contaminants. Substrates for the enzymes also include a wide variety of environmental contaminants, drugs, and steroid hormones, and the substrates are often the same as the inducers.

Dioxin and the coplanar PCB congeners induce transcription of CYP genes involved in activation of environmental contaminants to chemical forms able to damage a variety of human systems (269, 270). CYP1A1 and CYP1B1 catalyze the formation of mutagenic intermediates from several polycyclic aromatic hydrocarbons, some of which are potent mammary gland carcinogens in rodents (271, 272). Other CYP genes induced by dioxin-like compounds are involved in the metabolism of estrogen and testosterone (273). The basic mechanism is described in the following figure (Fig 3).

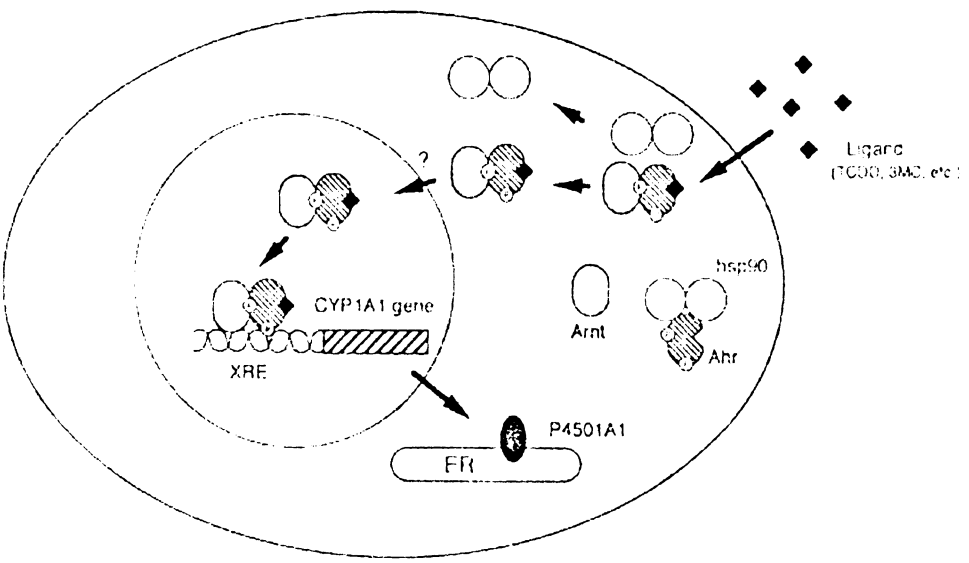

Figure 3. Model for dioxin activation of genes via AhR binding .
Dioxin (L) binds to AhR/hsp90 complex, releasing hsp90 and creating a binding site for the AhR nuclear transporter (Arnt). This complex translocates to the nucleus and binds to xenobiotic receptor elements (XRE) in the DNA and initiates transcription of the CYP1A1 gene. The CYP1A1 RNA is transported to the endoplasmic reticulum (ER) where the CYP1A1 message is translated into the CYP1A1 enzymes (from Kawajiri and Hayashi (274).

The toxicity of dioxin and the coplanar PCBs is positively correlated with their ability to bind to the aryl hydrocarbon receptor (AhR) (133) and induce transcription of several CYP genes, including CYP1A1, CYP1A2 and CYP1B1 (269) (273). In a study on the effects of exposure to dioxin-containing OC herbicides and pesticides, the transcriptional levels of CYP1A1 in peripheral blood lymphocytes from German chemical workers were shown to increase with increasing dioxin exposure levels (275). The serum levels of dioxin in exposed workers with chloracne (n=16), a skin disorder associated with high dose dioxin exposure, was 32.1 ppt compared to 11.5 ppt in exposed workers without chloracne (n=15). The corresponding levels of CYP1A1 activity, expressed as EROD (ethoxyresorufin O-deethylase) activity, were 2.81 and 2.32 pmol/min/mg, respectively. CYP1B1 activity is also induced by dioxin (276) and other OC compounds (272).

Both human CYP1A1 and CYP1B1 encode enzymes that are also 17β-estradiol (E2) hydroxylases; CYP1A1 is expressed primarily extra-hepatically and CYP1B1 is expressed primarily in the breast (reviewed in (277). CYP1A1 catalyzes the hydroxylation of E2 at the C-2, -6a and -15a positions while CYP1B1 is more active at the C-4 position (278). The 4-hydroxylated metabolite is elevated in human malignant, but not normal breast tissue (279) and is carcinogenic in animals (280).

Several polymorphic CYP genes that are induced by PCBs have been shown to be associated with increased risks of cancers of the reproductive tract possibly by altering reproductive hormone metabolism or increasing reproductive toxicant levels.

Four polymorphisms have been identified in the human CYP1A1 gene (reviewed in (281), one of which has been associated with postmenopausal breast cancer and PCB exposure (282) and 10-15% of white Americans have an allele with this substitution (283). The mutation does not appear to alter enzymatic activity (284), but the activity seems to be more inducible than the wild type gene (285). In a case control study of 154 women with postmenopausal breast cancer, women with the CYP1A1*2B polymorphism (substitution of valine for the wild type isoleucine) in at least one chromosome who were also exposed to high levels of PCBs [3.73-19.04 ng/g (ppb) of serum] had a significantly increased risk for postmenopausal breast cancer (OR, 2.96; 95% CI, 1.18-7.45) (282). In this case-control study there is a possibility that the cachexia associated with cancer resulted in weight loss and mobilization of PCBs from fat stores to the blood, artefactually increasing serum PCB levels. This issue was not addressed by the authors. Additionally, the possible interaction between this CYP1A1 polymorphism and cigarette smoking, previously shown to significantly increase breast cancer risk (286) (287), was not investigated due to the small sample size. Thus while the results of this study are suggestive of an interaction between a genetic variant involved in toxicant and ES metabolism and a hormone-dependent cancer, the association needs to be evaluated with the proper controls (women without cancer but with cachexia and the same polymorphism) or by using a prospective study design. The study does highlight, however the possible interaction between a PCB-inducible gene and alterations in sex hormone metabolism.

In an earlier report in which potential interactions with OC exposure were not investigated, the same polymorphism was associated weakly and nonsignificantly with postmenopausal breast cancer risk (286). In light smokers, however, the presence of at least one allele with the polymorphism was significantly associated with increased breast cancer risk (OR, 5.22; CI,1.16-23.56) although the numbers were small (N=29). No effect was seen in heavy smokers (20 or more pack-years), perhaps due to the reported anti-estrogenic effects of smoking (288). Similar results were reported by Ishibe et al.(287) who found that women who had commenced smoking before the age of 18 and had the CYP1A1 2* genotype had an increased risk of breast cancer (RR, 3.61; 95% CI, 1.11-11.17). The authors interpreted their results to suggest that exposure to the carcinogenic polyaromatic hydrocarbons found in cigarette smoke induced expression of the mutant CYP1A1 allele, which codes for an enzyme with an increased capacity for metabolizing estrogen to a metabolite (16-hydroxy estrogen) that is an estrogen agonist (289, 290). Alternatively, since CYP1A1 is also involved in activation of genotoxic substances with the ability to produce reactive oxygen intermediates capable of damaging DNA (291) (292), increased inducibility of the mutant gene or production of an enzyme with increased activity could lead to increased DNA damage.

CYP3A4 is another gene involved in xenobiotic metabolism and in the hydroxylation of testosterone to a form that is eliminated. The gene has been shown to be induced by PCBs in monkeys (293). Men with a genetic polymorphism in CYP3A4, CYP3A4-V, were found to have a significantly increased risk of prostate cancer (294). Significant trends for all men regardless of family history (p=0.0003) or men with no family history

(p=0.0008) for any stage of tumor diagnosis or increasing severity of tumor stage at diagnosis and the polymorphism compared to men without the polymorphism were found. The polymorphism was found in the 5'regulatory element of the gene, possibly resulting in an alteration in transcription and a consequent decrease in enzyme levels. The investigators did not look for associations between genotype and environmental exposure. These results suggest that the polymorphism may increase prostate cancer risk via increasing testosterone levels.

In summary, these studies have shown that polymorphic forms of genes involved in sex hormone metabolism that can be regulated by PCBs and possibly by dioxin, increase the risk for two hormone-dependent cancers. As discussed below, endocrine disruption by environmental contaminants is a major hypothesis for the increasing incidence of hormone-related cancers as well as increasing male fertility problems.

Some investigators have suggested that male reproductive problems may be linked to endocrine hormone-disrupting chemicals (86, 105, 235, 295, 296) and OCs have been shown to disrupt normal endocrine functions (105, 235, 296). The time of exposure to these chemicals is critical with in utero exposure and exposure before and during puberty being the most sensitive (297). PCBs can express a mixture of estrogenic, non-estrogenic and anti-estrogenic effects in *in vitro* systems (135), depending on the congener involved, with less chlorinated compounds expressing more estrogenic activity (298). Arochlors suppressed expression of male-specific CYP genes involved in testosterone metabolism in rats (299). Dioxins have antiestrogenic effects in rats (136), and can cause partial

demasculinization and feminization of sex behavior in male rats exposed *in utero* (300). Additionally, a mixture of PCBs and β-estradiol were found to have a synergistic effect on sex reversal of turtles (301), suggesting that detrimental interactions may occur between hormones metabolized by CYP enzymes and environmental contaminants.

2) In genes encoding glutathione *S*-transferase (GST) enzymes

GSTs are enzymes that catalyze the conjugation of glutathione to electrophilic xenobiotics in order to inactivate them and facilitate their excretion from the body. They thus play an important role in detoxifying potentially toxic compounds, including pesticides and environmental pollutants (reviewed in (281)). However, they have also been shown to bioactivate some xenobiotics, including the mutagen 1,2-bromoethane (302). Because of their role in detoxification of genotoxic compounds, most research has focused on their role in oncogenesis.

Polymorphisms in several GST genes have been identified and characterized. A polymorphism in GST Mu (*GSTM1-1*) was identified and found to result in a null genotype due to deletion of the gene (303). The mutation is most frequently (304), although not universally (305), associated with an increased risk of lung cancer. It was also found to slightly increase the risk of postmenopausal breast cancer in the youngest women (age less than 58 years old) (OR, 2.44 CI, 0.89-6.64) (286). The frequency of the GSTM1-1 genotypes in white Americans is 52% (306). A second GST gene, GST theta (*GSTT1-1*), which is involved in detoxification of low-molecular weight halogenated compounds, including several pesticides, was also found to have a null genotype present

in 38% of white Americans (307). Like the GSTM1 null mutation, the GSTT1 null genotype may be associated with different types of cancer due to a reduced capacity to inactivate toxic compounds. Two recently identified polymorphisms in GSTPi (*GSTP1-1*) have been identified and one, the *GSTP1b* allele, is reported to have decreased activity for compounds such as benzo[a]pyrene diol epoxide and acrolein, carcinogens found in cigarette smoke (reviewed in (308)). In a study of 155 healthy volunteers from the Edinburgh area, a total of 6.5% of the individuals were homozygous for the low activity allele (309). In the same study, the authors also reported on a survey of different types of cancer that found that subjects with testicular cancer had a prevalence of 18.7% for this allele vs. 6.5 % for random controls (OR 3.3; CI (1.5-7.7), while a significant decrease in the GSTP1a allele was observed in prostate cancer cases 27.8% vs. 51% for controls (OR 0.4: CI 0.02-3.3).

Two studies have investigated the association of polymorphisms in GST genes and reproductive health. The first study examined the relationship between the GST Mu null genotype, spermatogenic disorders and alcohol consumption (310). The study involved 271 autopsies of moderate and heavy drinkers, as determined by interviews with relatives. Spermatogenetic abnormalities were determined by cytological analysis of biopsied testicular tissue and the GST genotype from cardiac tissue. Heavy drinkers with the null genotype were less likely to have cytological abnormalities (morphology of the somniferous epithelium, presence and number of all developmental stages of spermatozoa) in testicular sections than moderate drinkers. The ORs for disordered testicular cytology, partial spermatogenic arrest or complete spermatogenic arrest in

heavy drinkers with the wild type genotype compared to the variant genotype were 2.0, (95% CI, 1.0-4.0), 2.0 (95% CI, 0.9-4.2), and 2.0 (CI,0.9-4.1.) These results suggest that the null genotype was protective against alcohol-induced testicular damage, a surprising finding since GSTs are usually associated with detoxification pathways. There are several problems with the study including small study size (50 moderate drinkers and 212 heavy drinkers), lack of a non-drinking reference group and the inclusion of men with co-morbidities, such as "other diseases", drug overdoses and other health problems associated with heavy alcohol consumption. Additionally the effects of polymorphisms in CYP 2E1, a gene strongly induced by alcohol, involved in activation of procarcinogens and consequently associated with ethanol-induced hepatotoxicity (reviewed in (311)) were not investigated. Thus it is difficult to determine the role of the GSTM1 polymorphism in the testicular damage they report.

A second study examined the relationship between polymorphisms in three phase II detoxification genes, N-acetyltransferase 2 (NAT2), GSTM1 and GSTT1, and endometriosis (312). Endometriosis is a multifactorial disease with significantly elevated frequency in industrial areas, possible genetic components and possible associations with environmental contaminants, including OCs and dioxin (reviewed in (313)). This study found significant increases in the proportions of patients with minimal and mild endometriosis and the GSTM1 null genotype compared to controls (75.0% and 79.0% versus 45.8%, $p<0.0001$), suggesting that women with the null genotype are at increased risk for endometriosis. A non- significant increase in the proportion of GSTT1 null genotypes among the patients was also found. Analogous to the modifying role of

CYP1A1in the development of postmenopausal breast cancer in PCB exposed women (282), it seems plausible that a reduced capacity to detoxify reproductive toxicants in subjects with null mutations in GST genes could play a role in male as well as female reproductive problems.

In summary, the findings of these reports are suggestive of possible roles of genetic polymorphisms in genes involved in sex hormone and toxicant metabolism and reproductive problems in males and females. Clearly more research is needed to better define these associations.

b. Role of direct toxicity and reactive oxygen species (ROS)

Several researchers have demonstrated a significant relationship between defective sperm function and oxidative stress (314-318), arising primarily from excessive exposure to ROS. ROS are produced by leucocytes found in semen, usually as a consequence of inflammation, or by the sperm themselves during capacitation (319). Evidence for production of ROS by capacitating spermatozoa was obtained in *in vitro* experiments using chemiluminescence probes to detect the superoxide anion. Using a Cypridina luciferin analog, MCLA, as probe, significant SOD-inhibitable chemiluminescence was associated with the capacitation of spermatozoa incubated in media supplemented with fetal cord serum ultrafiltrate. Chemiluminescence was 1270 mV/10 s (with 8×10^6 cells/ml), and corresponded to levels of sperm hyperactivation (12 %) and capacitation (17%) that were significantly different from those of control spermatozoa (4.9 % and 6

%, respectively). The level of capacitation-associated chemiluminescence was directly related to sperm concentration up to 30 x 10^6 cells/ml.

Production of ROS by sperm is associated with serious defects in the semen profile, including severe oligospermia (less than 1 x10^6 sperm/ml) (320). Support for the role of ROS produced by the sperm themselves in infertility comes from a study on the semen quality of oligospermic men. Cells isolated from the ejaculates of a high proportion of patients exhibiting oligozoospermia are characterized by generation rates of reactive oxygen species that considerably exceed those obtained for the normal fertile population (Aiken 1992). Semen samples from a cohort of oligozoospermic patients and a group of fertile controls were fractionated to generate three cell populations of differing density. For each fraction, both the steady-state and the induced (using a phorbol ester) chemiluminescent signals were significantly ($p< 0.001$) greater for the oligozoospermic samples than for the fertile controls. In the fertile donors, leucocytes comprised the major source of reactive oxygen species, especially in the low-density fractions; in oligozoospermic patients, however, spermatozoa were identified as a second major source of reactive oxygen species. An intense phorbol-ester-induced chemiluminescent signal generated by purified oligozoospermic spermatozoa, free of leucocyte contamination, was 167 times greater than the median signal generated by the corresponding fraction from the fertile controls ($p< 0.001$). These results emphasize the importance of spermatozoa as a major source of reactive oxygen species in oligozoospermia.

Ortho-substituted but not meta- or para- substituted (i.e. non-coplanar) PCBs have been shown to activate the oxygen burst in neutophils (321). A PCB congener (3,3',4,4'–tetrachlorobiphenyl) that binds the AhR had no effect on *in vitro* measures of neutrophil activation and ROS production while a congener that does not bind (2,2',4,4' – tetrachlorobiphenyl), does (322). Thus the well documented presence of PCBs in seminal fluid (see above) may affect ROS production by leucocytes present in semen.

Lower chlorinated PCB congeners (1-3 chlorines), although shorter lived than the higher chlorinated congeners, have been found to persist in environmental samples, biota and in humans tissues (127) (323, 324), most likely as a result of the dechlorination of the higher chlorinated congeners. *In vitro* experiments have shown that the lower chlorinated congeners are hydroxlated in reactions catalyzed by CYP1A and 2B (127) (325) (326) and can then be metabolically activated to electrophilic semiquinones and quinones (McLean et al 1996a). These metabolites can then form PCB- DNA adducts (327) with the production of superoxide radicals (291). These mechanisms are thought to provide a basis for the observation that PCB mixtures are complete carcinogens in rodent models (reviewed in (269). Additionally, production of ROS, including superoxide radicals, have been shown to damage sperm by inducing lipid peroxidation in the sperm plasma membrane leading to loss of motility (328), chromatin cross-linking (329), DNA strand breaks (330) and DNA base oxidation (331).

5. Effects of mercury on male reproduction

Studies on experimental animals have shown that administration of methyl mercury to rodents alters spermatogenesis (332) and results in accumulation of mercury in Sertoli cells and interstitial tissues of the testes (333). Mercury can induce lipid peroxidation and ROS generation in rat tissues (334), and liver extracts from rats administered mercury have reduced CYP2E1 activity (335), possibly as a result of ROS generated during mercury intoxication.

Several epidemiological studies have found associations of mercury levels in hair or blood and impaired male fertility. A study on the relationship between Hong Kong male subfertility and fish consumption found that subfertile males had approximately 40% more mercury in their hair than did fertile males (336) after adjusting for age.

C. PRELIMINARY STUDIES

1. Previous results from the Fisheaters Family Health Project (FFHP)

The FFHP is an ongoing study funded since 1992 by the CDC that investigates the association, in licensed Michigan anglers, between exposure to PCBs via Great Lakes sport-caught fish consumption and male and female reproductive outcomes.

a. Association between fish consumption and PCB levels

The number of sport-caught fish meals consumed in the previous 12 months is a statistically significant predictor of serum PCB levels for male participants of the FFHP. A linear model with log10 transformed total PCBs modeled on the dichotomized number of fish meals in past 12 months (0=less than 12 meals, 1=greater than or equal to 12 meals) was significant (Prob > F = 0.04) and explained 5% of the total variation in serum

PCB concentration. A second linear model including age was also significant (Prob > F =0.02) and explained 28% of the total variation in serum PCB concentration. Adding age adds so much to the explanation of variance partially because it is confounded with long term past fish consumption exposures. In the proposed study, we will use the same categories of fish consumption exposure.

b. PCB congener analysis

PCB congener analyses of 142 male and female participants in the FFHP indicate that of the 209 possible congeners, 36 were found in the serum of at least one FFHP participant. Congeners 138/163 (two congeners which co-eluted), 153, 179/190, 180, 187, 194 were present in at least 30% of the participants, while congeners 138/163 153, 180, and 194 were present in at least 60% of the subjects. These congeners are typical of the congener profile associated with Great Lakes fish consumption and are consistent with the other Great Lakes fisheater studies (145, 337). Most of the detected congeners have TEF values between 10^{-8} to 10^{-10} and are not likely to exhibit dioxin-like toxicity (129, 136, 338). Three congeners, 105, 118 and 156, which were detected in 6, 27 and 13% respectively, of the samples, have TEF values between 10^{-3} and 10^{-4}, which is within the range at which dioxin-like effects are likely to occur (339, 340).

c. Association between PCB levels and reproductive hormone levels

Preliminary analysis of the FFHP data indicates an inverse association between serum total PCB levels and LH (luteinizing hormone) levels ($r=-0.27$, $p<0.05$) in males. Total PCB values are calculated as the sum of the detected values without substitution for

missing values. The levels were not adjusted for lipids. The sample number is small (N=62), so caution must be used in interpretation of these results.

2. Pilot study: Michigan Fisheaters Study

As part of the FFHP, we tested the feasibility of conducting an exposure-control study on male fertility problems and exposure to PCBs via Great Lakes sport-caught fish consumption. We did this in collaboration with Dr. Michael Stahler, the Scientific Director of the IVF Laboratory, Beaumont Center for Reproductive Endocrinology in Royal Oak, MI. We collaborated with CDC investigators at the Wisconsin Division of Public Health, Bureau of Environmental Health (Claire Falk, Henry Anderson, Marty Kanarek) who are conducting a similar pilot study, in the development of the questionnaire. We developed the specific protocols and instruments, including a questionnaire modified from the main male questionnaire from the FFHP, for the study and determined the most efficient way to recruit participants. We found that having a receptionist briefly mention the study to clinic clients and then call a staff physician to talk to the potential participant about the study did not work well. The receptionist was often uncomfortable or too busy to present the study to the clients, and the physician, often seeing other clients or supervising laboratory work, was not readily accessible. Similar results were found by our Wisconsin colleagues and by Dr. Hauser (personal communications). Due to lack of funds we were not able to hire our own recruiter, as Dr. Hauser did. This pilot study nevertheless provides the framework for the proposed study, as described below.

3. Research Team

Julia Wirth, PhD MS Principal Investigator. Dr. Wirth was the Project Director of the FFHP from July 1, 1997 to September 30, 2000. She is currently the Scientific Coordinator for a large international study on breast cancer in women of Polish ancestry She has a PhD in microbiology and substantial experience, grant support and publications in molecular biology, virology, immunology and parasitology. She was a graduate student in the Department of Epidemiology at MSU from 1996 to 2001, finishing in her masters degree in May, 2001.

Nigel Paneth, MD, MPH Co-Investigator Dr. Paneth is Chairman of the Department of Epidemiology, and was the Principal Investigator of the FFHP from July 1, 1997 to October, 1999. He is a pediatrician and perinatal epidemiologist and has been involved with this project since its inception. He was the recipient of a Great Lakes Foundation planning grant to develop collaborative research on reproductive effects of Great Lakes contaminants. Dr. Paneth has extensive research experience in reproductive and perinatal epidemiology, including the epidemiology of congenital malformations and perinatal illnesses and the effects of adverse prenatal and perinatal exposures on child development. Due to his experience in large scale epidemiolgic field studies and analysis of large data sets, he will be directly involved in data analysis and interpretation of the results.

Andrew Mullard, BS Project Manager. Mr. Mullard was associated with the FFHP from 1996 until January, 2001. For the last two years he was a graduate assistant and was instrumental in developing the questionnaires for the main study and the male

reproductive health helped develop the recruiting and tracking protocols and questionnaire databases. He is currently employed by the Wisconsin Division of Public Health, Bureau of Environmental Health as Project Director for their ATSDR-funded study on fish consumption and reproductive health outcomes. Mr. Mullard will be responsible for the daily conduct of the project, coordinating the efforts of team members, aiding in manuscript and grant proposal preparation, supervision of student employees, and organizing and chairing staff meetings.

Jenny Wang, MS Data Analyst. Ms Wang was previously associated with the FFFP as data analyst and is familiar with the databases and statistical analyses used in that study. Ms Wang will design and set up the databases for the participant questionnaire information, hormone analysis, semen analysis and genetic analysis results. She will then design systems for integrating information from the databases to produce forms and reports. She will clean the data and perform the statistical analysis.

Michael Diamond, MD Consultant. Dr. Diamond is the Kamran S. Moghissi Professor of Obstetrics and Gynecology and the Director of Reproductive Endocrinology and Infertility at the Detroit Medical Center, Wayne State University, Detroit, MI. He has extensive experience and substantial publications in many aspects of fertility. He will serve as advisor on the collection and analysis of data from the semen analyses. He will help oversee the analysis of the semen samples collected from participants at his clinic and aid in the analysis and interpretation of the results. He will oversee the conduct of semen analysis for the specific function tests for the PCB subsample study. He will also lend his expertise on issues regarding male infertility and assessment of outcomes.

Harold Humphrey, PhD, Consultant. Dr. Humphrey is a public health scientist recently retired from the Michigan Department of Community Health but retaining strong ties with them and active research interests on the effects of PCB exposure and Great Lakes fish consumption. He has over 25 years of experience in developing and directing studies examining the human health implications of exposure to environmental chemical contaminants. He directed the federally funded projects, which established the Great Lakes fisheater cohort database. He has been a consultant for the FFHP for the last three years and will provide advise on measuring OC and heavy metal exposures for this proposal.

Vasantha Padmanabhan, PhD, Consultant. Dr. Padmanabhan is the Senior Research Scientist and Director of Pediatric Endocrine Research in the Department of Pediatrics, University of Michigan, and a Senior Research Scientist in the Reproductive Sciences Program, also at the University of Michigan. She has extensive experience and publications in the areas of the physiology of reproductive hormone regulation and in the analysis of human of reproductive endocrine hormones, including inhibin B. Her laboratory is one of the few that has the capability of measuring specific inhibin subunits using monoclonal antibody-based assays (ELIZA). She will advise on issues and interpretation of results of the inhibin B analyses and its relationship to the results of the other hormones and the semen analyses.

D. RESEARCH DESIGN AND METHODS

1. Overall design

We propose to conduct a cohort study in which we will recruit participants based on exposure to consumption of Great Lakes sport-caught fish. The source population will be male partners of couples attending infertility clinics in 3 areas of Michigan. For the study population, we will recruit men based on exposure to consumption to sport-caught Great Lakes fish. Men who consumed no meals of sport-caught Great Lakes fish in the last year will be considered unexposed, men who consume 1-11 meals will be considered moderately exposed and men who consume 12 or more in the last year will be considered most exposed. We will thus stratify fish consumption into three levels, insuring that we will have both a highly exposed (12 or more meals), moderately exposed (between 1 and 11 meals) and an unexposed (no meals) groups. This strategy, providing a wide range of exposures, will allow to detect any differences between the fish consumption groups if they exist. Since we will recruit men from infertility clinics, we also ensure that we will have the outcome of interest, semen parameters, which include sperm density, sperm motility and sperm morphology. To investigate possible gene-environment interactions, we will examine DNA isolated from whole blood samples for polymorphisms in genes that are involved in contaminant and steroid metabolism. Since the strongest evidence for an effect of a specific OC on human semen quality comes from the reported adverse effects of PCBs (217, 218), we will further investigate this relationship with the proposed cohort. To do this, we will conduct a substudy of men with serum PCB levels in the upper and lower quartiles of the PCB distribution after we have collected information on individual serum PCB levels from all the participants. From these men, we will request a semen sample, which we will use to perform more specific and sensitive tests of sperm

function, including the hypoosomotic swelling test, the acrosin release assay and the zona-binding assay.

a. Study areas

This proposal will focus on three areas of Michigan, each of which abuts on one of Michigan's Great Lakes: Allegan, Muskegon and Ottawa counties (Lake Michigan), Midland, Saginaw and Bay counties (Lake Huron) and St. Claire, Macomb, Wayne and Monroe counties (Lake Erie)(see the Appendix for a map of the locations). These counties cover the Great Lakes "areas of concern", so designated because their waters contain high levels of persistent and toxic pollutants (176, 341). These areas have distinct as well as overlapping contaminant profiles reflective of the particular industries along the lakeshore and tributaries and thus provide a spectrum of Great Lakes contaminants. Since these areas have been the focus of recruitment for the FFHP, we have questionnaire data, including information on fishing habits and fish consumption, male reproductive hormone data and PCB levels on 73 men from these areas, which has aided US in designing the proposed study. The following clinics representing theses areas have agreed to participate in our proposed study (see the Appendix for their letters of collaboration).

1) *Beaumont Center for Reproductive Endocrinology*, In vitro Fertilization Laboratories, Royal Oak, MI. Scientific Director: Michael Stahler;
2) *Division of Reproductive Endocrinology and Infertility*, the Detroit Medical Center: Director: Michael Diamond, MD
3) *Michigan Reproductive and IVF Center, P.C.*, Grand Rapids, MI. Louise Plante, PhD, Director ART Laboratory.

4) *To be determined*, Midland, Saginaw or Bay county

b. Recruitment

Men who come into the clinics for a routine appointment will be approached by a study recruiter. Those men indicating an interest in participating in the study will be given a short questionnaire to assess their eligibility and to determine their level of Great Lakes sport-caught fish in the last year: none, 1-11 meals or 12 or more. The eligibility criteria include age 18-50 years, not taking hormonal therapy, does not have diabetes, thyroid or adrenal disorder, does not have genetic disorder related to fertility, does not have testicular cancer and has not had bilateral orchiectomy. Eligible me will be given a consent form to sign in which they agree to complete the questionnaire, donate approximately 20 ml of blood to be used to measure reproductive hormones and genetic polymorphisms in genes involved in hormone and contaminant metabolism and give us permission to read their semen analysis report.

We will first recruit an eligible man who has eaten 12 or more meals a year, since, based on our previous experience, men in this category constitute the lowest percentage of the general population and mostly likely of the clinic clientele. Once we recruit the highly exposed man, an eligible moderately exposed man and an unexposed man will be recruited. Highly exposed men will be matched to moderately and unexposed men on clinic, age (within 5 years) and time of entry into the study (within 2 months).

This recruitment strategy has two important advantages compared to other studies on fish consumption and reproductive outcomes. First, by recruiting men from an infertility

clinic we will have the semen analysis results, which are sensitive indicators of male reproductive health (248), and, based on our experience in the FFHP, are often hard to obtain. We will also have a range of outcomes since the fertility problem has been reported to be female in a third to a half of the couples (2) (3). Secondly, by recruiting based on exposure, we ensure that we have some highly exposed men, a population that is often missing in fisheater studies and may partly explain their inability to find an association with adverse reproductive outcomes.

We used the following information to estimate that we will be able to recruit about 126 exposed men a year.

Table 1. Estimated recruitment of most exposed men by clinic

Clinic	No. Samples/year	No. Assuming 80% Participation	No. Exposed Assuming 20% Anglers	No. Eating >12 fish meals/year Assuming 32%
Diamond (Detroit)	420	336	67	22
Plante (Grand Rapids)	1530	1224	245	78
Stahler (Royal Oak)	500	400	80	26
TOTALS	2380	1960	392	126

The participation rate is based on the study by Hauser (218), which had rates between 70-90%, depending on the type of individual doing the recruiting (investigator, nurse) and time of year the recruiting took place (R. Hauser, personal communication). We will use a nurse researcher and begin recruiting in the late summer, early fall, 2002, which, based on Dr. Hauser's experience, should give us a rate of about 70-80%. Recruiting will be

year round in part to randomize any seasonal variations in sperm count that may occur (18, 67). Based on FFHP cohort A data, we assume an average of 20% of men in Michigan will obtain yearly fishing licenses. Most anglers in our experience consume between 5 and 20 fish meals a year. Based on the FFHP cohort A data, 32% of the anglers consume 12 or more sport-caught fish meals in the last 12 months. We thus should be able to recruit about 120 exposed and 120 unexposed participants a year for a total of 376 exposed and unexposed pairs. These calculations were made without including a possible clinic in the Lake Huron area, which, if included, would increase the number of exposed subjects recruited by approximately 50 per year.

c. Substudy

Thirty men in the upper and 30 men in the lower quartiles of the PCB distribution from all three clinics will be selected and asked to donate a semen sample on which to perform additional sperm function tests. This study will allow us to assess the relationship between PCB levels and more specific and sensitive measures of sperm function. The additional sperm function tests will be the: hypoosmotic swelling test, the acrosin assay, and the zona binding assay. The additional tests have been shown to correlate positively and significantly with the outcome of in vitro fertilization and sperm fertilizing potential (342, 343).

d. Questionnaires: development and modification

The questionnaire from the Pilot Study will be used. Prior to administering it to study participants, it will be field tested for clarity, ease of use and ease and accuracy of data

entry on 20 men attending the clinics. The men will also be asked to give their comments either in writing at the end of the questionnaire or to the study recruiter. Any consistent comments or recommendations will be used to modify the questionnaire prior to starting the study.

To assess non-participant bias, we will administer a short questionnaire on fish eating habits and reproductive health to men who decline to participate. During the three years of recruiting, approximately 150 randomly selected weeks will be selected during which all men declining participation will be asked to fill out this questionnaire at the clinics. We estimate that we will have about 350 completed questionnaires.

e. Training of study recruiters

It has been our experience with the Pilot Study, the experience of our collaborators in Wisconsin and Dr. Hauser's experience that having an enthusiastic, trained study representative dedicated to recruiting participants at the clinic is absolutely critical to enrolling sufficient participants and thus to the study's success. We will thus hire a part time nurse at each clinic whose major responsibility will be to recruit participants. This person will be present in the clinic from approximately 7 am to 1 pm, which is when the majority of men come into the clinic (Michael Diamond, personal communication). This person will be trained by us at MSU regarding the rational of the study, its importance, how it will be conducted, the requirements and benefits of participation and the purpose and meaning of the tests and questionnaire. We will stress that the recruiters' enthusiasm for the study is critical. This person will also draw the blood sample, oversee data

collection, tracking and reimbursement, and communicate weekly with the Project Manager at MSU.

2. Data Collection

a. Measurement of consumption of Great Lakes fish

Information on consumption of Great Lakes sport caught fish will be obtained from the questionnaire, which was modified from the FFHP male questionnaire. Men who fish are asked to identify the water body in which the fish were caught, the fish species caught, how the fish were prepared, and the amount consumed. This information is critical in refining the effects of fish consumption on human health outcomes. Both the species and location of the fish consumed have been shown to be important predictors of total PCB burdens. We anticipate using this information in models to estimate PCB exposure. Additional questions about general health, including recent infections with fever, reproductive health, occupation, medications, smoking habits, and alcohol and caffeine consumption are also asked. We will also ask about the frequency and duration of hot tub and Jacuzzi use. We have prepared an identical "core" section of our questionnaire to be administered both by ourselves and by our Wisconsin colleagues, that includes questions about fish consumption, medical conditions, risk factors, lifestyle habits, occupation and environmental exposures and other demographic variables.

b. Other risk factors for infertility

We will exclude men with congenital disorders such as cryptorchidism, even after repaired by orchipexy, since it is associated with infertility and impaired testicular

function (344-346). If men with specific genetic disorders that affect sperm function, such as Kallman's syndrome, are identified in the questionnaire, we will exclude them. Unless varciocele or anti-sperm antibodies contribute to the final diagnosis, we will not exclude men with these conditions.

Clinically apparent infections of the testes, epididymis, or the prostate can lead to reduced sperm count or motility (reviewed in (19). The physical examination the men undergo as part of the fertility work-up will identify men with these conditions. To identify past infections or recent subclinical infections that might affect spermatogenesis, we ask in our questionnaire if the men have ever had specific diseases and conditions that are associated with impaired sperm function. We also ask if the men have had any viral or bacterial infections or flu that caused a fever in the last 3 months prior to semen collection. Thus, we will identify men with these possible risk factors but will not exclude them.

Smoking has clearly been associated with a variety of sperm abnormalities in several studies (342, 347-349). In our questionnaire we ask about smoking, both the type of tobacco used and the quantity, and will control for it in the analysis. Chronic alcohol abuse can lead to testicular atrophy (350), while little consistent effect on sperm function has been found for caffeine consumption (351). We ask about consumption of both in the questionnaire and will control for them if necessary.

c. Laboratory tests

1) Reproductive hormones

To obtain as accurate and as sensitive a measure of spermatogenesis as possible, we will assay the serum samples for inhibin B and estradiol, as well as for FSH, LH and testosterone (TE). Serum inhibin B level correlated with sperm concentration in several studies (352-354) and is considered to be the best available endocrine marker of spermatogenesis in subfertile men (355). The two-site ELISA assay for inhibin B (355, 356) uses a monoclonal antibody able to specifically detect and distinguish between the two bioactive forms of inhibin, A and B. The assay will be performed in the laboratory of Dr. Vasantha Padmanabhan at the University of Michigan who has extensive experience using it in large scale studies (please see the Appendix for her letter of collaboration).

Spermatogenesis is controlled by the hypothalamic-pituitary-testicular axis (reviewed in de Kretser (357). Gonadotropin-releasing hormone (GnR) stimulates the pituitary gland to release FSH and LH. FSH in the male, in combination with TE, is required for the initiation and maintenance of spermatogenesis. The primary target of FSH is the Sertoli cell, which provides the nutrients needed by the stem cells in the somniferous tubules to differentiate into mature motile sperm. Sertoli cells also secret inhibin B, which inhibits FSH secretion by the anterior pituitary cells. LH, also produced by the anterior pituitary, regulates the function of Leydig cells in the interstitial tissues of the testis. Leydig cells produce TE and may also utilize it. TE inhibits LH secretion by the anterior pituitary and may also negatively hormone secretion by the hypothalamus. Estradiol in adult men is required for synthesis of sex hormone binding globulin, which controls the level of available TE in the circulation, and helps regulate gonadotropin secretion (358). FSH,

LH, TE and estradiol will be measured from serum samples, using routine laboratory methods.

Secretion of FSH, LH, and TE is subject to biological rhythms, but there is no consensus as to their effects on serum hormone levels. TE levels have been reported to decrease during the day (359), although the variation diminishes with age (360). The secretory patterns of FSH and LH are pulsatile, but no clear pattern has been found (361, 362). Another study found that differences in FSH and inhibin B levels could not be explained by sampling time (353). The participating clinics report that most men come in between early morning and early afternoon, which should help reduce any possible variation due to sampling time. Additionally, we will record the time of day the serum samples are drawn and assess its effect on hormone levels in the analysis. To ensure comparability between the clinics, random serum samples from participants will be aliquoted and assayed in each lab. Kappa statistics will be calculated to evaluate interobserver agreement.

2) Semen analysis

Although the clinical value of the traditional semen parameters in the diagnosis of male fertility has been debated recently (363), several studies have found that the measured parameters are predictive of pregnancy outcome (364) (363) as well as for the likelihood of successful in vitro fertilization (reviewed in (365)). Routine semen analyses measures sperm number, sperm motility, sperm morphology as well as semen characteristics, such as liquification, volume, viscosity, pH and number of white blood

cells. Semen analysis is routinely performed in all the participating clinic laboratories as part of the fertility work-up. All of the clinics follow the procedures recommended by the World Health Organization (15) and have rigorous internal quality control measures (periodic analyses of the same sample by the technicians and multiple analyses of the same sample by the same technician).

To ensure comparability of the laboratory analyses, prior to recruitment test samples from volunteers will be analyzed at each laboratory. A portion of the semen will be washed and resuspended in buffered 1% formalin (11). Several dilutions will be made and aliquoted into 3 sets of cryovials. The vials will be transported by courier to each of the three labs for analysis of sperm density. For analysis of morphology, slides will be prepared from the samples and sent to the laboratories. For motility analysis, the semen samples will be aliquoted in to 3 cryovials and frozen at -180^0C. The vials will be transported by the same courier as for the semen samples for sperm counting to the three laboratories (11). Interobserver agreement between the different laboratories will be assessed using the Kappa statistic. Additionally, the Detroit Medical Center has the capability of videotaping semen samples. The actual semen material can then be viewed at the other laboratories and sperm count and motility can be analyzed. We will also test the feasibility of this procedure for external quality control. After the study has begun, random samples will be collected at each clinic and then analyzed by all 3 clinics. Interobserver agreement will be assessed using the Kappa statistic. All laboratory personnel in the clinics will be blinded as to the identity and level of fish consumption of

the participants. We will also have access to information on the final diagnosis and if the problem is male or female when it becomes available.

3) Additional sperm function tests

Ideally for the substudy, sperm function tests would be chosen to test each of the critical reactions a sperm must undergo to successfully fertilize an ovum. Twelve such steps have been described (343). While a plethora of such tests are available (reviewed in (343) (366), many of them are not accurate or reproducible, or are too expensive for large scale studies such as this one (365). For this study, three tests were chosen to reflect specific critical reactions that correlate with *in vitro* fertilization potential and sperm fertilizing capacity (342) (365) that can be measured accurately and reproducibly. The additional tests will be performed under the supervision of Dr. Diamond at the Detroit Medical Center.

The hypoosomotic swelling test is a test of sperm survival and membrane integrity and will be assessed by a dye exclusion test. This test has been reported to be useful in assessing male fertility (367). Before sperm can penetrate the ovum, it must undergo several physiological changes in the female genital tract. The first is capacitation, a series of biochemical and functional changes involving removal and redistribution the membrane constituents of the sperm surface. Following this, the acrosome, a membrane enclosed structure filled with enzymes at the sperm head, reacts resulting in the dissolution of the outer acrosome membrane and the release of the acrosomal contents, one of which is the lytic enzyme acrosin. An additional consequence of capacitation is a

change in the sperm tail beat pattern to one of hyperactivity. After capacitation and the acrosome reaction, the sperm are able to attach to receptors on the zona pellucida of the ovum and penetrate it. We will use two tests to evaluate steps in these processes. To evaluate the acrosome reaction, we will use a test to detect released acrosin (368). To evaluate sperm binding to the zona pellucida, we will use the zona-binding assay, possibly with recombinant zona proteins (365).

4) Genetic analysis

We are collecting a whole blood sample from which to isolate genomic DNA. In collaboration with Dr. Michael Shi at Parke-Davis Pharmaceutical Research in Ann Arbor, MI, we will look for polymorphisms in 11 genes associated with contaminant, steroid and/or drug metabolism (see the Appendix for his letter of collaboration). The genes with polymorphisms that are relevant to this proposal (please see Section C.4.c.) are CYP1A1, CYP3A4, GST Mu and GST Theta. In addition, Parke-Davis is interested in determining the gene frequency in our study population of polymorphisms in CYP2D6 (debrisoquine hydroxylase), NQO1 (NAD(P)H (quinone oxidoreductase), SULTI (STP1)(phenol sulfotransfease), CYP2C9 (tolbutamide methylhydroxylation), and CYP2C19 (S-mephenytoin 4'-hydroxylation), N-acetyltransferase (NAT2) because these genes are also involved in metabolism of drugs. DNA will be isolated using standard protocols and the sequences of interest amplified using polymerase chain reaction technology with the appropriate primers. A portion of each isolated DNA sample will be frozen and stored for future analysis.

5) Contaminant analysis

a) PCBs

Serum samples will be analyzed for 208 PCB congeners, DDT, DDE and several other pesticides for which the laboratory routinely screens. The analyses will be performed at Michigan Department of Community Health (MDCH), Lansing, MI, which has performed identical analyses for the FFHP, as well as for most other Fisheater studies conducted in Michigan (145, 208). For PCB analysis, the laboratory uses capillary column gas chromatography to identify the specific PCB congeners, DDT and DDE. The detection sensitivity of this method varies according to congener, ranging from 0.03 to 1.30 ppb. Aliquots of the remaining sera will be stored frozen and will be available for future determinations of other pesticides and industrial contaminants. The MDCH laboratory adheres to strict internal and external QA/QC practices and has over twenty years of experience in developing and conducting chemical analyses on human specimens. The analyses will be performed in a comparable manner to those conducted on previous and contemporary cohorts to provide full comparability to previous results and permit longitudinal as well as geographic comparisons. We are aware that serum PCB levels may require correction for serum lipid level(369). Although serum PCB levels do appear to correlate well with adipose tissue PCB levels (370), enough blood will also be drawn for serum lipid analysis in order to correct for lipid levels as required.

b) Dioxin

Dioxin analysis will be performed by Xenobiotic Detection Systems, Inc (XDS)(please see the Appendix for their letter of collaboration). XDS uses a patented chemical activated luciferase (CALUX) gene expression assay to screen for persistent bioaccumulating toxins, including dioxins and PCBs. The assay is based on toxicant

binding to the aryl hydrocabon receptor (AhR), which has been attached to the firefly luciferase gene and thus controls its expression. A second step then separates PCBs, dioxins and dibenzofurans. The assay routinely measures dioxins in low parts per trillion. A study measuring dioxin concentrations found a positive correlation (r=0.81) between this method and the gas chromatography/mass spectrometry (GC/MS) method (371). The advantages of this system compared to GC/MS are its low cost and high sensitivity. Samples that are positive for dioxins using this method will be saved for further analysis using GS/MS, if warranted.

c) Mercury

To detect mercury, whole blood samples will be analyzed using a modification of the cold vapor atomic adsorption spectrophotometry method. The MDCH lab routinely analyzes samples for mercury using this method. The laboratory will also perform an analysis for lead at no extra cost. The results of this analysis will be recorded and stored in a database, but not evaluated as part of this study. While lead exposure has been consistently associated with male reproductive problems, including decreased sperm concentrations and total sperm counts, poor sperm motility, and abnormal sperm morphology (372) (373), there are many sources of exposure beside fish consumption.

3. Data analysis and interpretation

a. Data analysis

The aims of this study are both descriptive and comparative. Information on several exposure variables and a large number of outcome variables, including covariates, will be

collected on men attending infertility clinics in four areas of Michigan. Exposure to consumption of sport-caught Great Lakes fish is the primary exposure. Secondary exposures are the contaminants found in these fish: PCBs, DDE, dioxin, and mercury. The primary outcome is sperm density, which is one of the group of outcome variables obtained from the semen analysis. Other semen variables include semen volume, sperm motility, and sperm morphology. The second group of outcome variables include the male reproductive hormones FSH, LH, testosterone and inhibin B. The outcome variables will first be inspected as continuous variables and then may be categorized as appropriate.

The descriptive statistics will be used to characterize the cohort and will include distributions of age, BMI, area of recruitment, fish consumption level, and contaminant levels, including total and congener-specific levels PCB level, and reproductive hormone levels. Distribution of the genetic polymorphisms will also be determined.

For the univariate analyses of high and low fish consumers matched on age and recruitment area, all variables will be categorical. Comparisons of the pairs with the outcomes will be made using rate ratios derived from 2 x 2 contingency tables. For analysis of the variable as continuous, t-tests and correlational methods will be used. If the variable is not normally distributed, which is likely for sperm densities (4) (6) and PCB values (145), the data will be log-transformed. If the log-transformed values are not normally distributed, non-parametric tests, such as the Wilcoxon rank sum test and rank correlations methods will be used. Since PCB levels usually have a wide distribution,

PCB exposure levels may be divided into quantiles. While the analytic procedure used by MDCH can theoretically detect 209 PCB congeners, only 33 peaks, representing 37 congeners (four peaks were co-eluting pairs) were detected in the serum from FFHP cohort A participants. However, since multiple comparisons can generate significant relationships by chance alone, multiple-comparison procedures will be used if appropriate. For statistical analysis, PCB congeners may be grouped into various categories including: chlorination clusters (374), isoforms, co-planar and ortho-substituted congeners, and the most prevalence congeners. Logistic regression models will be used to ascertain the contribution of these congener groups to any detected adverse health effects.

Exposure variables found to be significantly associated with an outcome will be incorporated into a multivariate regression models, logistic for categorical variables and linear for continuous variables. Included in these models will be various covariates, including BMI, infections, and occupation. A model using species and location as predictors of ingested dose will also be tested (375). The final model will include the significant exposure variables as well as potential confounders.

Interaction between fish consumption and the secondary exposure variables and genetic polymorphisms will tested using statistical models designed to examine interactions.

b. Sample size/power calculation

The sample size was calculated for two independent means for differences in sperm density. Alpha was set at 0.05, power at 0.80, the true group mean under the null hypothesis at 100.8 x 10^6 (million) sperm/ml, the smallest group 2 mean at 80.64, the maximum group 2 mean at 88.0 million/ml, and the standard deviation was calculated at 72.0 million/ml (48). The sperm count values were taken from a report on semen analyses on US men who banked sperm prior to vasectomy in the years 1970 to 1994. The specific values used in the above calculations were from 662 men that donated their samples at Cryogeneic Laboratories, Inc., Roseville, MN.

Table 2. Sample size requirements

SAMPLE SIZE REQUIREMENTS FOR COMPARING TWO MEANS where Alpha=.05, Sides=2, STD=72.0, Power=.80, Ratio N2/N1=2, Group 1 true mean=100.8(million/ml)

Group 1 Mean (million/ml)	Group 2 Mean (million/ml)	N1	N2
100.8	80.64	151	302
100.8	81.14	159	318
100.8	81.64	167	334
100.8	82.14	176	352
100.8	82.64	186	372
100.8	83.14	197	394
100.8	83.64	208	416
100.8	84.14	221	442
100.8	84.64	235	470
100.8	85.14	250	500
100.8	85.64	267	534
100.8	86.14	285	570
100.8	86.64	306	612
100.8	87.14	328	656
100.8	87.64	354	708
100.8	88.14	382	764
100.8	88.64	414	828
100.8	89.14	450	900
100.8	89.64	491	982

Power is calculated with the same values for the variables as above. With a sample size of 378 most-exposed men, 378 moderately exposed men and 378 unexposed men (N2=752) and we have 80% power to detect a difference between means of 11.2 million sperm/ml.

Table 3. Power estimates

POWER ESTIMATES FOR TESTING HYPOTHESIS WHERE
Alpha=.05, Sides=1, Pooled SD=72.0, N1=378,
N2=756, Group 1 true mean=100.8(million/ml)

Group 1 Mean (million/ml)	Group 2 Mean (million/ml)	POWER
100.8	80.64	0.99740
100.8	81.14	0.99636
100.8	81.64	0.99497
100.8	82.14	0.99313
100.8	82.64	0.99071
100.8	83.14	0.98757
100.8	83.64	0.98356
100.8	84.14	0.97849
100.8	84.64	0.97215
100.8	85.14	0.96433
100.8	85.64	0.95479
100.8	86.14	0.94330
100.8	86.64	0.92962
100.8	87.14	0.91354
100.8	87.64	0.89486
100.8	88.14	0.87342
100.8	88.64	0.84911
100.8	89.14	0.82187
100.8	89.64	0.79173

c. Project timetable

Prior to beginning recruiting, we will conduct the external quality control study. When we are satisfied that minimal differences exist between the laboratories (kappa above 0.75) will begin recruiting subjects. Since the instruments and protocols have already been developed, excluding the substudy, we will begin recruiting within 3 months of receiving funding. During that time, we will also seek approval from the participating

institutions' internal review boards (IRBs), select and train the half time personnel responsible for recruiting at each clinic, set up databases, develop computer linkages for data entry and pilot test the questionnaire. Based on the calculations above (please see section D.2.b.), we estimate it will take approximately 3 years to recruit the 800 subjects. During that time, questionnaire data will be collected, cleaned and entered into the databases. Contaminant, hormone and genetic analyses will be performed and the results entered into the appropriate databases. Data analysis will begin when this is complete and continue through the end of the funding period.

6. Strengths and weaknesses

a. Strengths

This study has several strengths that set it apart from other studies examining the relationship between environmental contaminants and male reproductive health. First, our study subjects are men who attend infertility clinics, and thus a higher percentage are likely to have fertility problems than in an unselected population sample. It is estimated that approximately one third to one half of couples' infertility problems will be male (3) (2). We will have access to their semen analysis results as well as the final diagnosis. We will also perform sperm function tests in a subsample of men exposed to high and low levels of PCBs in order to detect more subtle changes in sperm functions as a consequence of PCB exposure. Second, we are recruiting subjects based on exposure. This will ensure that we have a range of exposures, including highly exposed subjects, thus increasing our chances of detecting adverse effects if they exist. Third, we will look

for polymorphisms in the subjects' genes involved in contaminant and sex steroid metabolism that are known to alter toxicant metabolism and ultimately concentration.

b. Weaknesses

Sampling from an infertility clinic creates a risk for selection bias. Not all couples with infertility problems seek medical help (376). If factors related to the decision to seek medical help are also related to our exposure, Great Lakes fish consumption, this may result in etiologically irrelevant differences in exposure between infertile care-seeking cases and fertile controls who did not seek medical help. However, this is unlikely since there is no a priori reason to associate care-seeking behavior and fish consumption. Selection patterns might differ according to the type of procedures the couples select. To minimize the potential for selection bias, we will recruit more and less exposed men from the same group of couples coming in for consultation. Ideally we would chose couples having their first consultation only, but based upon the experience of another similar ongoing study by Dr. Hauser (218), this approach would eliminate a significant number of potential participants. It is also possible that men attending infertility clinics may represent a more susceptible population, which would potentially influence the relationship between exposure and outcome. However, studies comparing sperm counts from infertility clinics and from semen donors have not found great differences (4).

We recognize that there is significant day to day variability in semen parameters. This misclassification bias should be nondifferential and reduces power because it reduces our ability to discriminate between men with normal and abnormal semen parameters, but

only biases our results toward the null. Ideally we would collect more than one sample but due to the large sample size (756 men) this would make the study prohibitively expense. We have some limited data from the FFHP on men who donated a semen sample that was found to be abnormal and were subsequently asked to donate a second one. In 4 of 5 cases, even though there were variations in the values, the abnormal values remained within the abnormal range.

E. HUMAN SUBJECTS

Participants in the proposed study will be adult (18-50 year old) males attending infertility clinics in Michigan. All potential participants will receive an informed consent statement for their consideration and signature before any additional information or samples are obtained. Participants will be asked to complete a questionnaire on reproductive history, fish consumption, occupation and other factors potentially confounding the relationship between contaminant exposure and semen parameters. Men will be asked to have their blood drawn on one occasion and the risks to participants are minimal. The phlebotomy will be performed by technicians employed by the clinics and will be in compliance with Michigan licensing laws. A subsample of men will be asked to donate a semen sample for this study for which there are minimal risks. All data will be maintained under confidential conditions as mandated by Michigan State University. Participants will be tracked using the clinic study identifiers unrelated to personal information. Personal identifiers will be stored locked and separate from the study data. All data from the original forms will be coded and entered into a computer file, and the coded computer file will be used for all data analyses. Participants will receive several

incentives to increase participation. First, they will receive the results of their own tests for contaminants, reproductive hormones, semen quality and genetic analysis. Second, they will receive a cash incentive of $50 after completion of the phlebotomy and the questionnaire. All test results will be made available to the study participants' physicians upon request.

F. REERENCES

1. NORA. Fertility and pregnancy abnormalities: NIOSH National Occupational Research Agenda (NORA), 1999.

2. Skakkebaek NE, Giwercman A, de Kretser D. Pathogenesis and management of male infertility [see comments]. Lancet 1994;343:1473-9.

3. De Kretser DM, Baker HW. Infertility in men: recent advances and continuing controversies. J Clin Endocrinol Metab 1999;84:3443-50.

4. Carlsen E, Giwercman A, Keiding N, Skakkebaek NE. Evidence for decreasing quality of semen during past 50 years [see comments]. Bmj 1992;305:609-13.

5. Brake A, Krause W. Decreasing quality of semen. Bmj 1992;305:1498.

6. Bromwich P, Cohen J, Stewart I, Walker A. Decline in sperm counts: an artefact of changed reference range of "normal"? [see comments]. Bmj 1994;309:19-22.

7. MacLeod J, Wang Y. Male fertility potential in terms of semen quality: a review of the past, a study of the present. Fertil Steril 1979;31:103-16.

8. Kruger TF, Menkveld R, Stander FS, et al. Sperm morphologic features as a prognostic factor in in vitro fertilization. Fertil Steril 1986;46:1118-23.

9. Bonde JP, Giwercman A, Ernst E. Identifying environmental risk to male reproductive function by occupational sperm studies: logistics and design options. Occup Environ Med 1996;53:511-9.

10. Sheriff DS. Analysis of semen in a constantly changing social context of medicine. Arch Androl 1995;34:125-32.

11. Neuwinger J, Behre HM, Nieschlag E. External quality control in the andrology laboratory: an experimental multicenter trial. Fertil Steril 1990;54:308-14.

12. Matson PL. External quality assessment for semen analysis and sperm antibody detection: results of a pilot scheme. Hum Reprod 1995;10:620-5.

13. Mallidis C, Howard EJ, Baker HW. Variation of semen quality in normal men. Int J Androl 1991;14:99-107.

14. Schrader SM, Turner TW, Simon SD. Longitudinal study of semen quality of unexposed workers. Sperm motility characteristics. J Androl 1991;12:126-31.

15. Organization WHO. WHO laboratory manual for the examination of human semen and sperm-cervical mucus interaction. Cambridge, MA: World Health Organization, 1999.

16. Mees MM, Cuijpers CEJ, Piersma AH. Is sperm quality actually declining? A literature survey. Bilthoven, the Netherlands: National Institute of Public health and Environment, 1997.

17. MacLeod J, Gold RZ. The male factor in fertility and infertility. II. Spermatozoon counts in 1000 men of known fertility and in 1000 cases of infertile marriage. J Urol 1951;66:436-49.

18. Tjoa W. Circannual rhythm in human sperm count revealed by serially independent sampling. Fertil Steril 1982;38:454-9.

19. Clark R. Male infertility. In Principles and practice of endocrinology and metablism, second edition, ed: K.L. Becker, J.B.Lippincott Co., Philadelphia; pp.1102-1110. 1995.

20. Tielemans E, Heederik D, Burdorf A, Loomis D, Habbema DF. Intraindividual variability and redundancy of semen parameters. Epidemiology 1997;8:99-103.

21. Rock J, Robinson D. Effect of induced intrascrotal hyperthermia on testicular function in man. Am J Obstet Gynecol 1965;93:793-801.

22. Mieusset R, Bujan L. Testicular heating and its possible contributions to male infertility: a review. Int J Androl 1995;18:169-84.

23. Brindley GS. Deep scrotal temperature and the effect on it of clothing, air temperature, activity, posture and paraplegia. Br J Urol 1982;54:49-55.

24. Tiemessen CH, Evers JL, Bots RS. Tight-fitting underwear and sperm quality. Lancet 1996;347:1844-5.

25. Sanger WG, Friman PC. Fit of underwear and male spermatogenesis: a pilot investigation. Reprod Toxicol 1990;4:229-32.

26. Bujan L, Mieusset R. [Male contraception by hyperthermia]. Contracept Fertil Sex 1995;23:611-4.

27. Lynch R, Lewis-Jones DI, Machin DG, Desmond AD. Improved seminal characteristics in infertile men after a conservative treatment regimen based on the avoidance of testicular hyperthermia. Fertil Steril 1986;46:476-9.

28. Gyllenborg J, Skakkebaek NE, Nielsen NC, Keiding N, Giwercman A. Secular and seasonal changes in semen quality among young Danish men: a statistical analysis of semen samples from 1927 donor candidates during 1977-1995. Int J Androl 1999;22:28-36.

29. Levin RM, Latimore J, Wein AJ, Van Arsdalen KN. Correlation of sperm count with frequency of ejaculation. Fertil Steril 1986;45:732-4.

30. Mortimer D. The male factor in infertility. Part I. semen analysis. Curr Probl Obstet Gynecol Fertil 1985;8:3-87.

31. Pellestor F, Girardet A, Andreo B. Effect of long abstinence periods on human sperm quality. Int J Fertil Menopausal Stud 1994;39:278-82.

32. Schwartz D, Mayaux MJ, Guihard-Moscato ML, et al. Study of sperm morphologic characteristics in a group of 833 fertile men. Andrologia 1984;16:423-8.

33. Bendvold E, Gottlieb C, Bygdeman M, Eneroth P. Depressed semen quality in Swedish men from barren couples: a study over three decades. Arch Androl 1991;26:189-94.

34. Nelson CM, Bunge RG. Semen analysis: evidence for changing parameters of male fertility potential. Fertil Steril 1974;25:503-7.

35. Westoff CF. Coital frequency and contraception. Fam Plann Perspect 1974;6:136-41.

36. James WH. What stabilizes the sex ratio? Ann Hum Genet 1995;59:243-9.

37. James WH. Secular trend in reported sperm counts. Andrologia 1980;12:381-8.

38. Seracchioli R, Porcu E, Flamigni C. The diagnosis of male infertility by semen quality. Sperm morphology is not the only criterion of male infertility. Hum Reprod 1995;10:1039-41.

39. Organization WHO. WHO laboratory manual for the examination of human semen and sperm-cervical mucus interaction. Geneva: WHO, 1987.

40. Chan SY, Wang C, Chan ST, et al. Predictive value of sperm morphology and movement characteristics in the outcome of in vitro fertilization of human oocytes. J In Vitro Fert Embryo Transf 1989;6:142-8.

41. Olsen GW, Bodner KM, Ramlow JM, Ross CE, Lipshultz LI. Have sperm counts been reduced 50 percent in 50 years? A statistical model revisited [see comments]. Fertil Steril 1995;63:887-93.

42. Lerchl A, Nieschlag E. Decreasing sperm counts? A critical review. Exp Clin Endocrinol Diabetes 1996;104:301-7.

43. Wang C, Chan SY, Leung A, et al. Cross-sectional study of semen parameters in a large group of normal Chinese men. Int J Androl 1985;8:257-74.

44. MacLeod J aHL. Characteristics and variations in semen specimens in 100 normal young men. J Urol 1945;54:474-82.

45. Keiding N, Giwercman A, Carlsen E, Skakkebaek NE. Falling sperm quality [letter; comment]. Bmj 1994;309:131.

46. Fisch H, Goluboff ET. Geographic variations in sperm counts: a potential cause of bias in studies of semen quality. Fertil Steril 1996;65:1044-6.

47. Leto S, Frensilli FJ. Changing parameters of donor semen. Fertil Steril 1981;36:766-70.

48. Fisch H, Goluboff ET, Olson JH, Feldshuh J, Broder SJ, Barad DH. Semen analyses in 1,283 men from the United States over a 25-year period: no decline in quality [see comments]. Fertil Steril 1996;65:1009-14.

49. Paulsen CA, Berman NG, Wang C. Data from men in greater Seattle area reveals no downward trend in semen quality: further evidence that deterioration of semen quality is not geographically uniform [see comments]. Fertil Steril 1996;65:1015-20.

50. Saidi JA, Chang DT, Goluboff ET, Bagiella E, Olsen G, Fisch H. Declining sperm counts in the United States? A critical review. J Urol 1999;161:460-2.

51. Bostofte E, Serup J, Rebbe H. Relation between spermatozoa motility and pregnancies obtained during a twenty-year follow-up period spermatozoa motility and fertility. Andrologia 1983;15:682-6.

52. Irvine S, Cawood E, Richardson D, MacDonald E, Aitken J. Evidence of deteriorating semen quality in the United Kingdom: birth cohort study in 577 men in Scotland over 11 years [see comments]. Bmj 1996;312:467-71.

53. Auger J, Kunstmann JM, Czyglik F, Jouannet P. Decline in semen quality among fertile men in Paris during the past 20 years [see comments]. N Engl J Med 1995;332:281-5.

54. Bujan L MA, Pontonnier F and Mieusset R. Time series analysis of sperm concentration in fertile men in Toulouse, France between 1997 and 1992. British Medical Journal 1996;312:471-2.

55. Ginsburg J, Hardiman P. Ovulation induction with human menopausal gonadotropins--a changing scene. Gynecol Endocrinol 1991;5:57-78.

56. Ginsburg J, Okolo S, Prelevic G, Hardiman P. Residence in the London area and sperm density. Lancet 1994;343:230.

57. Farrow S. Falling sperm quality: fact or fiction? Bmj 1994;309:1-2.

58. Bahadur G, Ling KL, Katz M. Statistical modelling reveals demography and time are the main contributing factors in global sperm count changes between 1938 and 1996. Hum Reprod 1996;11:2635-9.

59. Swan SH, Elkin EP, Fenster L. Have sperm densities declined? A reanalysis of global trend data. Environ Health Perspect 1997;105:1228-32.

60. Swan SH, Elkin EP, Fenster L. The question of declining sperm density revisited: an analysis of 101 studies published 1934-1996. Environ Health Perspect 2000;108:961-6.

61. Bostofte E, Serup J, Rebbe H. Has the fertility of Danish men declined through the years in terms of semen quality? A comparison of semen qualities between 1952 and 1972. Int J Fertil 1983;28:91-5.

62. Osser S, Liedholm P, Ranstam J. Depressed semen quality: a study over two decades. Arch Androl 1984;12:113-6.

63. Menchini-Fabris F, Rossi P, Palego P, Simi S, Turchi P. Declining sperm counts in Italy during the past 20 years. Andrologia 1996;28:304.

64. Bonde JP, Kold Jensen T, Brixen Larsen S, et al. Year of birth and sperm count in 10 Danish occupational studies. Scand J Work Environ Health 1998;24:407-13.

65. Fisch H, Ikeguchi EF, Goluboff ET. Worldwide variations in sperm counts. Urology 1996;48:909-11.

66. Bendvold E. Semen quality in Norwegian men over a 20-year period. Int J Fertil 1989;34:401-4.

67. Vierula M, Niemi M, Keiski A, Saaranen M, Saarikoski S, Suominen J. High and unchanged sperm counts of Finnish men. Int J Androl 1996;19:11-7.

68. Bostofte E, Serup J, Rebbe H. Interrelations among the characteristics of human semen, and a new system for classification of male infertility. Fertil Steril 1984;41:95-102.

69. Bostofte E, Serup J, Rebbe H. Relation between morphologically abnormal spermatozoa and pregnancies obtained during a twenty-year follow-up period. Int J Androl 1982;5:379-86.

70. Bostofte E. Prognostic parameters in predicting pregnancy. A twenty-year follow-up study comprising semen analysis in 765 men of infertile couples evaluated by the Cox regression model. Acta Obstet Gynecol Scand 1987;66:617-24.

71. Bonde JP, Ernst E, Jensen TK, et al. Relation between semen quality and fertility: a population-based study of 430 first-pregnancy planners [see comments]. Lancet 1998;352:1172-7.

72. Chilvers C PM, Forman D, Fogelman K, Wadsworth MEJ. Apparent doubling of frequency of undescended testis in England and Wales in 1962-81. Lancet 1984;8398:330-332.

73. Scorer C. The descent of the testis. Arch Dis Childh 1964;39:605-609.

74. Calzolari E CM, Roncarati E, et al. Aetiologic factors in hypospadias. J Med Genet 1986;23:333-337.

75. Paulozzi LJ, Erickson JD, Jackson RJ. Hypospadias trends in two US surveillance systems. Pediatrics 1997;100:831-4.

76. Dolk H. Rise in prevalence of hypospadias. Lancet 1998;351:770.

77. Ansell PE BV, Bull D et al,. Cryptorchidism: a prospective study of 7500 consecutive male births, 1984-8. Arch Dis Child 1992;67:892-899.

78. Group JRHCS. Cryptorchidism: a prospectivestudy of 7500 consecutive male births, 1984-8. Arch Dis Child 1992;67:892-899.

79. Berkowitz GS, Lapinski RH, Dolgin SE, Gazella JG, Bodian CA, Holzman IR. Prevalence and natural history of cryptorchidism. Pediatrics 1993;92:44-9.

80. Matlai P, Beral V. Trends in congenital malformations of external genitalia. Lancet 1985;1:108.

81. Aho M, Koivisto AM, Tammela TL, Auvinen A. Is the incidence of hypospadias increasing? Analysis of Finnish hospital discharge data 1970-1994. Environ Health Perspect 2000;108:463-5.

82. Systems ICFBDM. Annual Reports 1980-1993. Rome: International Center for Birth Defects, 1982-95.

83. Group EW. 15 years of surveillance of congenital anomalies in Europe 1980-1994. Brussels: Scientific Institute of Public Health-Louis Pasteur, 1997.

84. Canning DA. Hypospadias trends in two US surveillance systems. Rise in prevalence of hypospadias. J Urol 1999;161:366.

85. Choi J, Cooper KL, Hensle TW, Fisch H. Incidence and surgical repair rates of hypospadias in New York state. Urology 2001;57:151-3.

86. Toppari J, Larsen JC, Christiansen P, et al. Male reproductive health and environmental xenoestrogens [see comments]. Environ Health Perspect 1996;104 Suppl 4:741-803.

87. Nethersell AB, Drake LK, Sikora K. The increasing incidence of testicular cancer in East Anglia. Br J Cancer 1984;50:377-80.

88. Pike MC, Chilver CED, Bolerow LG. Classification of testicular cancer in incidence and mortality statistics. Br J Cancer 1987;56:83-85.

89. Boyle P, Kaye SB, Robertson AG. Changes in testicular cancer in Scotland. Eur J Cancer Clin Oncol 1987;23:827-30.

90. Hakulinen T, Andersen A, Malker B, Pukkala E, Schou G, Tulinius H. Trends in cancer incidence in the Nordic countries. A collaborative study of the five Nordic Cancer Registries. Acta Pathol Microbiol Immunol Scand Suppl 1986;288:1-151.

91. Adami HO, Bergstrom R, Mohner M, et al. Testicular cancer in nine northern European countries. Int J Cancer 1994;59:33-8.

92. Stone J, Sandeman TF, Ironside P, Cruickshank DG and Matthews JP. Time trends in accuracy of classification of testicular tumurs, with clinicalf and epidemiological implications. Br J Cancer 1992;66:396-401.

93. Pearce N, Sheppard RA, Howard JK, Fraser J, Lilley BM. Time trends and occupational differences in cancer of the testis in New Zealand. Cancer 1987;59:1677-82.

94. Wilkinson T, Colls BM and Schluter PJ. Increased incidence of germ-cell testicular cancer in New Zealand Maoris. Br J Cancer 1992;65:769-771.

95. Liu S, Semenciw R, Waters C, Wen SW, Mery LS, Mao Y. Clues to the aetiological heterogeneity of testicular seminomas and non- seminomas: time trends and age-period-cohort effects. Int J Epidemiol 2000;29:826-31.

96. Brown LM, Pottern LM, Hoover RN, Devesa SS, Aselton P, Flannery JT. Testicular cancer in the United States: trends in incidence and mortality. Int J Epidemiol 1986;15:164-70.

97. Zheng T, Holford TR, Ma Z, Ward BA, Flannery J, Boyle P. Continuing increase in incidence of germ-cell testis cancer in young adults: experience from Connecticut, USA, 1935-1992. Int J Cancer 1996;65:723-9.

98. Suominen J, Vierula M. Semen quality of Finnish men. Bmj 1993;306:1579.

99. Henderson BE, Benton B, Jing J, Yu MC, Pike MC. Risk factors for cancer of the testis in young men. Int J Cancer 1979;23:598-602.

100. Cosgrove MD, Benton B, Henderson BE. Male genitourinary abnormalities and maternal diethylstilbestrol. U Urol 1977;117:220-222.

101. Depue RH. Cryptorchidism, and epidemiologic study with emphasis on the relationship to central nervous system dysfunction. Teratology 1988;37:301-5.

102. Giwercman A, Carlsen E, Keiding N, Skakkebaek NE. Evidence for increasing incidence of abnormalities of the human testis: a review. Environ Health Perspect 1993;101 Suppl 2:65-71.

103. Carlsen E, Giwercman A, Skakkebaek NE. Declining sperm counts and increasing incidence of testicular cancer and other gonadal disorders: is there a connection? Ir Med J 1993;86:85-6.

104. James WH. Offspring sex ratio as a potential monitor of reproductive disorders in communities near hazardous chemical sites [letter; comment] [see comments]. Reprod Toxicol 1997;11:893-5.

105. Giwercman A, Bonde JP. Declining male fertility and environmental factors. Endocrinol Metab Clin North Am 1998;27:807-30, viii.

106. Skakkebaek NE, Berthelsen JG, Giwercman A, Muller J. Carcinoma-in-situ of the testis: possible origin from gonocytes and precursor of all types of germ cell tumours except spermatocytoma. Int J Androl 1987;10:19-28.

107. Potashnik G, Yanai-Inbar I. Dibromochloropropane (DBCP): an 8-year reevaluation of testicular function and reproductive performance. Fertil Steril 1987;47:317-23.

108. Jacobsen R, Bostofte E, Engholm G, et al. Risk of testicular cancer in men with abnormal semen characteristics: cohort study. Bmj 2000;321:789-92.

109. Wilcox AJ, Baird DD, Weinberg CR, Hornsby PP, Herbst AL. Fertility in men exposed prenatally to diethylstilbestrol. N Engl J Med 1995;332:1411-6.

110. Strohsnitter WC, Noller KL, Hoover RN, et al. Cancer risk in men exposed in utero to diethylstilbestrol. J Natl Cancer Inst 2001;93:545-51.

111. vom Saal FS, Timms BG, Montano MM, et al. Prostate enlargement in mice due to fetal exposure to low doses of estradiol or diethylstilbestrol and opposite effects at high doses. Proc Natl Acad Sci U S A 1997;94:2056-61.

112. Safe SH. Endocrine disruptors and human health--is there a problem? An update. Environ Health Perspect 2000;108:487-93.

113. Weijin Z, Olsen J. Offspring sex ratio as an indicator of reproductive hazards [letter; comment] [see comments]. Occup Environ Med 1996;53:503-4.

114. Lloyd OL, Fodor JG, Lloyd MM, Chockalingam A. The geography of the community sex ratios for young children in Newfoundland. Sci Total Environ 1991;106:165-74.

115. Davis DL, Gottlieb MB, Stampnitzky JR. Reduced ratio of male to female births in several industrial countries: a sentinel health indicator? [see comments]. Jama 1998;279:1018-23.

116. Goldsmith JR. Dibromochloropropane: epidemiological findings and current questions. Ann N Y Acad Sci 1997;837:300-6.

117. Marcus M, Kiely J, Xu F, McGeehin M, Jackson R, Sinks T. Changing sex ratio in the United States, 1969-1995 [see comments]. Fertil Steril 1998;70:270-3.

118. Allan BB, Brant R, Seidel JE, Jarrell JF. Declining sex ratios in Canada [see comments] [published erratum appears in Can Med Assoc J 1997 Feb 1;156(3):348]. Cmaj 1997;156:37-41.

119. Feitosa MF, Krieger H. Demography of the human sex ratio in some Latin American countries, 1967-1986. Hum Biol 1992;64:523-30.

120. Moller H. Change in male:female ratio among newborn infants in Denmark [letter; comment] [see comments]. Lancet 1996;348:828-9.

121. van der Pal-de Bruin KM, Verloove-Vanhorick SP, Roeleveld N. Change in male:female ratio among newborn babies in Netherlands [letter; comment] [see comments]. Lancet 1997;349:62.

122. Bromen K, Jockel KH. Change in male proportion among newborn infants [letter; comment]. Lancet 1997;349:804-5.

123. Dickinson HO, Parker L. Decline in sex ratios at birth, England and Wales, 1973-90 [letter; comment]. J Epidemiol Community Health 1997;51:103.

124. Ohmi H, Hirooka K, Mochizuki Y. Reduced ratio of male to female births in Japan [letter]. Int J Epidemiol 1999;28:597.

125. Ulizzi L, Zonta LA. Factors affecting the sex ratio in humans: multivariate analysis of the Italian population. Hum Biol 1995;67:59-67.

126. Longnecker MP, Rogan WJ, Lucier G. The human health effects of DDT (dichlorodiphenyltrichloroethane) and PCBS (polychlorinated biphenyls) and an overview of organochlorines in public health. Annual Review Of Public Health 1997;18:211-44.

127. Safe S. Polychlorinated biphenyls (PCBs) and polybrominated biphenyls (PBBs): biochemistry, toxicology, and mechanism of action. Crit Rev Toxicol 1984;13:319-95.

128. Giesy JP, Kannan K. Dioxin-like and non-dioxin-like toxic effects of polychlorinated biphenyls (PCBs): implications for risk assessment. Crit Rev Toxicol 1998;28:511-69.

129. McFarland VA, Clarke JU. Environmental occurrence, abundance, and potential toxicity of polychlorinated biphenyl congeners: considerations for a congener-specific analysis. Environ Health Perspect 1989;81:225-39.

130. Smith S, Schecter A, Papke O, Do T, Coulibaly D, Brandt-Rauf P. Quantitation of the extracellular domain of epidermal growth factor receptor in the plasma of dioxin-exposed individuals. Am J Ind Med 1998;34:1-5.

131. Tian Y, Ke S, Denison MS, Rabson AB, Gallo MA. Ah receptor and NF-kappa B interactions, a potential mechanism for dioxin toxicity. Journal Of Biological Chemistry 1999;274:510-5.

132. Kafafi SA, Afeefy HY, Ali AH, Said HK, Kafafi AG. Binding of polychlorinated biphenyls to the aryl hydrocarbon receptor. Environ Health Perspect 1993;101:422-8.

133. Landers JP, Bunce NJ. The Ah receptor and the mechanism of dioxin toxicity. Biochemical Journal 1991;276 (Pt 2):273-87.

134. Safe S. Polychlorinated biphenyls (PCBs), dibenzo-p-dioxins (PCDDs), dibenzofurans (PCDFs), and related compounds: environmental and mechanistic considerations which support the development of toxic equivalency factors (TEFs). Crit Rev Toxicol 1990;21:51-88.

135. Bitman J, Cecil HC. Estrogenic activity of DDT analogs and polychlorinated biphenyls. J Agric Food Chem 1970;18:1108-12.

136. Safe S, Astroff B, Harris M, et al. 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) and related compounds as antioestrogens: characterization and mechanism of action. Pharmacol Toxicol 1991;69:400-9.

137. Buchanan DL, Sato T, Peterson RE, Cooke PS. Antiestrogenic effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin in mouse uterus: critical role of the aryl hydrocarbon receptor in stromal tissue. Toxicol Sci 2000;57:302-11.

138. Seegal RF. Epidemiological and laboratory evidence of PCB-induced neurotoxicity. Crit Rev Toxicol 1996;26:709-37.

139. Fischer LJ, Seegal RF, Ganey PE, Pessah IN, Kodavanti PR. Symposium overview: toxicity of non-coplanar PCBs. Toxicol Sci 1998;41:49-61.

140. Schantz SL, Moshtaghian J, Ness DK. Spatial learning deficits in adult rats exposed to ortho-substituted PCB congeners during gestation and lactation. Fundam Appl Toxicol 1995;26:117-26.

141. Schantz SL, Seo BW, Wong PW, Pessah IN. Long-term effects of developmental exposure to 2,2',3,5',6- pentachlorobiphenyl (PCB 95) on locomotor activity, spatial learning and memory and brain ryanodine binding. Neurotoxicology 1997;18:457-67.

142. Jacobson JL, Jacobson SW. Intellectual impairment in children exposed to polychlorinated biphenyls in utero. N Engl J Med 1996;335:783-9.

143. Schantz SL, Gardiner JC, Gasior DM, Sweeney AM, Humphrey HE, McCaffrey RJ. Motor function in aging Great Lakes fisheaters. Environ Res 1999;80:S46-S56.

144. Tithof PK, Watts S, Ganey PE. Protein tyrosine kinase involvement in the production of superoxide anion by neutrophils exposed to Aroclor 1242, a mixture of polychlorinated biphenyls. Biochem Pharmacol 1997;53:1833-42.

145. Humphrey HE, Gardiner JC, Pandya JR, et al. PCB congener profile in the serum of humans consuming great lakes fish [In Process Citation]. Environ Health Perspect 2000;108:167-72.

146. DeVoto E, Fiore BJ, Millikan R, et al. Correlations among human blood levels of specific PCB congeners and implications for epidemiologic studies. Am J Ind Med 1997;32:606-13.

147. Korrick SA, Altshul L. High breast milk levels of polychlorinated biphenyls (PCBs) among four women living adjacent to a PCB-contaminated waste site. Environ Health Perspect 1998;106:513-8.

148. Van Birgelen A, DeVito MJ and Birnbaum LS. Toxic equivalency factors derived from cytochrome P450 induction in mice are predictive for cytochrome P450 induction after subchronic exposure to mixtures of PCDDs, PCDFs, and PCBs in female B6C3F1 mice and Sprague Dawley rats. Organohalogen Compounds 1996;29:251-256.

149. Vonier PM, Crain DA, McLachlan JA, Guillette LJ, Arnold SF. Interaction of environmental chemicals with the estrogen and progesterone receptors from the oviduct of the American alligator. Environ Health Perspect 1996;104:1318-22.

150. Arnold SF, Klotz DM, Collins BM, Vonier PM, Guillette LJ, McLachlan JA. Synergistic activation of estrogen receptor with combinations of environmental chemicals. Science 1996;272:1489-92.

151. Arnold SF, Bergeron JM, Tran DQ, et al. Synergistic responses of steroidal estrogens in vitro (yeast) and in vivo (turtles). Biochem Biophys Res Commun 1997;235:336-42.

152. Stow CA, Carpenter SR, Eby LA, Amrhein JF, Hesselberg RJ. Evidence that PCBs are approaching stable concentrations in Lake Michigan fishes. Ecological Applications 1995;5:248-260.

153. Fensterheim RJ. Documenting temporal trends of polychlorinated biphenyls in the environment. Regul. Toxicol. Pharmacol 1993;18:181-201.

154. Agency UEP. Fed Regist 1993;58:20806-09.

155. Webber T. Study says banned toxin is entering Lake Michigan. Lansing State Journal. Lansing, MI, 2000.

156. Wong C ea. Accumulation and preliminary inventory of PCBs and organochlorine pesticides in Lake Ontario sediments. First international conference on contaminated sediments:historical records, environmental impact and remediation. Milwaukee, WI, 1993.

157. Provinces NSECP. Mercury Study. A framework for action, 1998.

158. Harris RaH, C. Mercury-measuring and managing the risk. Environment 1978;20:25-36.

159. Wilkinson S, Golding, KH and Robinson, PK. Mercury accumulation and volatilization in immobilzed agal cell systems. Biotechnol Lett 1989;11:861-864.

160. EPA. The USEPA Mercury Study: Report to Congress. Volume I: Executive summary. 1997.

161. Fitzgerald WF, Clarkson TW. Mercury and monomethylmercury: present and future concerns. Environ Health Perspect 1991;96:159-66.

162. EPA. Mercury Study: Report to Congress. Volume III: An assessment of exposure from anthropogenic mercury emmissions in the US: USEPA, 1996.

163. Michigan. Michigan fish contaminant monitoring program. Lansing: State of Michigan, 1998:8-9.

164. Stevens JaB, BE. Occurrence of heavy metals in the blue shark, Prionace glauca, and selected pelagic fish in the north-east Atlantic Ocean. Mar Biol 1974;26:287-293.

165. Galal-Gorchev H. Dietary intake, levels in food and estimated intake of lead, cadmium, and mercury. Food Addit Contam 1993;10:115-28.

166. Organization WHO. Mercury. Geneva, Switzerland: WHO, 1989:115.

167. Day R. Michigan Fish Contaminant Monitoring Program 1998 Annual Report: Michigan Department of Environmental Quality-Surface Water Quality Division, 1998.

168. Zabik ME, Zabik MJ, Booren AM, et al. Pesticides and total polychlorinated biphenyls residues in raw and cooked walleye and white bass harvested from the Great Lakes. Bull Environ Contam Toxicol 1995;54:396-402.

169. DeVault DS, Hesselberg R, Rodgers PW, Feist TJ. Contaminant Trends in Lake Trout and Walleye from the Laurentian Great Lakes. J. Great lakes Res. 1996;22:884-895.

170. Kreiss K. Studies on populations exposed to polychlorinated biphenyls. Environ Health Perspect 1985;60:193-9.

171. Hanrahan LP, Falk C, Anderson HA, Draheim L, Kanarek MS, Olson J. Serum PCB and DDE levels of frequent Great Lakes sport fish consumers-a first look. The Great Lakes Consortium. Environ Res 1999;80:S26-S37.

172. Hanrahan LP FC, Anderson HA, Draheim L, Steeprot D, Olson J, Fiore B, Kanarek M, and the Great Lakes Consortium. Serum PCB Levels and Great Lakes Sport Fish Consumption. Health Conference '97 Great Lakes and St. Lawrence. Montreal Quebec, Canada, 1997.

173. Falk C, Hanrahan L, Anderson HA, et al. Body burden levels of dioxin, furans, and PCBs among frequent consumers of Great Lakes sport fish. The Great Lakes Consortium. Environ Res 1999;80:S19-S25.

174. Hovinga ME SM, and Humphrey HEB. Historical changes in serum PCB and DDT levels in an environmentally-exposed cohort. Arch. Environ. Contam. Toxicol. 1992;22:362-366.

175. Resources MDNR. Resources and conservation: MI DNR, 2000.

176. Johnson BL HH, De Rosa T. Key Environmental Human Health Issues in the Great Lakes and St. Lawrence River Basins. Environ. Research Section A 1999;80:S2-S12.

177. He J-P, Humphrey HEB, Paneth N, Courval JM. Time trends in sport-caught Great Lakes fish consumption and serum polychlorinated biphenyl levels among Michigan anglers, 1973-1999. Environ Sci Technol 2001;35:435-440.

178. Humphrey HE, Budd ML. Michigan's fisheater cohorts: a prospective history of exposure. Toxicol Ind Health 1996;12:499-505.

179. Kearney JP, Cole DC, Ferron LA, Weber JP. Blood PCB, p,p'-DDE, and mirex levels in Great Lakes fish and waterfowl consumers in two Ontario communities. Environ Res 1999;80:S138-S149.

180. Robinson PE, Mack GA, Remmers J, Levy R, Mohadjer L. Trends of PCB, hexachlorobenzene, and beta-benzene hexachloride levels in the adipose tissue of the U.S. population. Environ Res 1990;53:175-92.

181. Airey D. Total mercury concentrations in human hair from 13 countries in relation to fish consumption and location. Sci Total Environ 1983;31:157-80.

182. Airey D. Mercury in human hair due to environment and diet: a review. Environ Health Perspect 1983;52:303-16.

183. Stern AH. Re-evaluation of the reference dose for methylmercury and assessment of current exposure levels. Risk Anal 1993;13:355-64.

184. Clarkson T. An outbreak of mercury poisoning due to consumption of contaminated grain. Fed Proc 1975;34:2395-2399.

185. Gerstenberger SL, Tavris DR, Hansen LK, Pratt-Shelley J, Dellinger JA. Concentrations of blood and hair mercury and serum PCBs in an Ojibwa population that consumes Great Lakes region fish. J Toxicol Clin Toxicol 1997;35:377-86.

186. WHO. Methylmercury. Geneva, Switzerland: World Health Organization, 1990.

187. Peterson RE, Theobald HM, Kimmel GL. Developmental and reproductive toxicity of dioxins and related compounds: cross-species comparisons. Crit Rev Toxicol 1993;23:283-335.

188. Tillet DE AG, and Giesy JP. Planar chlorinated hydrocarbons (PCH's) in colonial fish-eating waterbirds eggs from the Great Lakes. Govt. reports Announcements and Index (GRS&I) 1991.

189. TR. C. Toxicants in the Aquaeous Ecosystem: John Wiley & Sons Ltd., 1979.

190. Mac MaS, TR. Investigations into the effects of PCB congeners on reproduction in lake trout from the Great Lakes. Chemosphere 1992;25:189-192.

191. Jennings M, Percival HF, and Woodward, AR. Evaluation of alligator hatchlings and egg removal from three Florida lakes. Proc Ann Conf Southeast Assoc Fish Wildl Agencies 1988;42:283-294.

192. Guillette LJ, Jr., Pickford DB, Crain DA, Rooney AA, Percival HF. Reduction in penis size and plasma testosterone concentrations in juvenile alligators living in a contaminated environment. Gen Comp Endocrinol 1996;101:32-42.

193. Guillette LJ, Gross TS, Masson GR, Matter JM, Percival HF, Woodward AR. Developmental abnormalities of the gonad and abnormal sex hormone concentrations in juvenile alligators from contaminated and control lakes in Florida. Environ Health Perspect 1994;102:680-8.

194. Guillette LJ, Gross TS, Gross DA, Rooney AA, Percival HF. Gonadal steroidogenesis in vitro from juvenile alligators obtained from contaminated or control lakes. Environ Health Perspect 1995;103 Suppl 4:31-6.

195. Roelke ME SD, Facemire CF and Sundlof SF. Mercury contamination in the free-ranging endangered Florida panter (Felis concolor coryi). Am Assoc Zoo Vet Annu Proc 1990;1991:277-283.

196. Roelke ME MJaOBS. The consequences of demographhic reduction and genetic depletion in the endangered Florida panther. Curr Biol 1993;3:340-350.

197. Facemire CF, Gross TS, Guillette LJ, Jr. Reproductive impairment in the Florida panther: nature or nurture? Environ Health Perspect 1995;103 Suppl 4:79-86.

198. Guillette LJ, Crain DA, Rooney AA, Pickford DB. Organization versus activation: the role of endocrine-disrupting contaminants (EDCs) during embryonic development in wildlife. Environ Health Perspect 1995;103 Suppl 7:157-64.

199. Aulerich RJ, Ringer RK. Current status of PCB toxicity to mink, and effect on their reproduction. Arch Environ Contam Toxicol 1977;6:279-92.

200. Hornshaw TC, Aulerich RJ, Johnson HE. Feeding Great Lakes fish to mink: effects on mink and accumulation and elimination of PCBS by mink. J Toxicol Environ Health 1983;11:933-46.

201. Allen JR, Barsotti DA, Lambrecht LK, Van Miller JP. Reproductive effects of halogenated aromatic hydrocarbons on nonhuman primates. Ann N Y Acad Sci 1979;320:419-25.

202. Sager DB. Effect of postnatal exposure to polychlorinated biphenyls on adult male reproductive function. Environ Res 1983;31:76-94.

203. Johansson B. Lack of effects of polychlorinated biphenyls on testosterone synthesis in mice. Pharmacol Toxicol 1987;61:220-3.

204. Sanders OT, Kirkpatrick RL, Scanlon PE. Polychlorinated biphenyls and nutritional restriction: their effects and interactions on endocrine and reproductive characteristics of male white mice. Toxicol Appl Pharmacol 1977;40:91-8.

205. Moore RW, Potter CL, Theobald HM, Robinson JA, Peterson RE. Androgenic deficiency in male rats treated with 2,3,7,8- tetrachlorodibenzo-p-dioxin. Toxicol Appl Pharmacol 1985;79:99-111.

206. Moore RW, Jefcoate CR, Peterson RE. 2,3,7,8-Tetrachlorodibenzo-p-dioxin inhibits steroidogenesis in the rat testis by inhibiting the mobilization of cholesterol to cytochrome P450scc. Toxicol Appl Pharmacol 1991;109:85-97.

207. Faqi AS, Dalsenter PR, Mathar W, Heinrich-Hirsch B, Chahoud I. Reproductive toxicity and tissue concentrations of 3,3',4,4'- tetrachlorobiphenyl (PCB 77) in male adult rats. Hum Exp Toxicol 1998;17:151-6.

208. Humphrey HEB. Chemical contaminants in the Great Lakes: The human health aspect. In: M E, ed. Toxic Contaminants and Ecosystem Health: A Great Lakes Focus: Wiley, New York., 1988:153-165.

209. DeVito MJ, Birnbaum LS, Farland WH, Gasiewicz TA. Comparisons of estimated human body burdens of dioxinlike chemicals and TCDD body burdens in experimentally exposed animals. Environ Health Perspect 1995;103:820-31.

210. Roman E, and Peterson, RE. Developmental male reproductive toxicology of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and PCBs. In: Korach, KS, ed. Reproductive and developmental toxicology, New York: Marcel Dekker, 1998: 593-624. 1998.

211. Mocarelli P GP, Ferrari E, et al. Paternal concentrations of dioxin and sex ratio of offspring. Lancet 2000;355:1858-1863.

212. Styne D. Puberty. In: GJ GFaS, ed. Basic and Clinical Endocrinology. Stamford, CT: Appleton & Lange, 1997:521-547.

213. Huang A, Powell D, Chou K. Pre- and postnatal exposure to 3,3',4,4'-tetrachlorobiphenyl: I. Effects on breeding ability and sperm fertilizing ability in male mice. Arch Environ Contam Toxicol 1998;34:204-8.

214. Sager DB, Shih-Schroeder W, Girard D. Effect of early postnatal exposure to polychlorinated biphenyls (PCBs) on fertility in male rats. Bull Environ Contam Toxicol 1987;38:946-53.

215. Shain W, Overmann SR, Wilson LR, Kostas J, Bush B. A congener analysis of polychlorinated biphenyls accumulating in rat pups after perinatal exposure. Arch Environ Contam Toxicol 1986;15:687-707.

216. Sager D, Girar, D and Nelson, D. Early postnatal exposure to PCBs: sperm function in rats. Environ Toxicol Chem 1991;10:737-746.

217. Bush B, Bennett AH, Snow JT. Polychlorobiphenyl congeners, p,p'-DDE, and sperm function in humans. Arch Environ Contam Toxicol 1986;15:333-41.

218. Hauser R, Altshul L, Schiff I, et al. Semen quality and polychlorinated biphenyls. Recent Advances in the Environmental Toxicology and Health Effects of EPCs (PCB Workshop) 2000;2000.

219. Courval J DJ, Stein A, Tay E, He J, Humphrey H, Paneth N. Sport-Caught Fish Consumption and Conception Delay in Liscensed Michigan Anglers. Environmental Research Section A 1999;80:S183-s188.

220. Buck GM, Mendola P, Vena JE, et al. Paternal Lake Ontario fish consumption and risk of conception delay, New York State Angler Cohort. Environmental Research 1999;80:S13-S18.

221. Emmett EA, Maroni M, Jefferys J, Schmith J, Levin BK, Alvares A. Studies of transformer repair workers exposed to PCBs: II. Results of clinical laboratory investigations. Am J Ind Med 1988;14:47-62.

222. Dougherty R, Whitaker, MJ, Tang, SY et al. Sperm density and toxic substances: a potential key to environmental health hazards. Environ Health Chem 1980;13:268.

223. Schlebusch H, Wagner U, van der Ven H, al-Hasani S, Diedrich K, Krebs D. Polychlorinated biphenyls: the occurrence of the main congeners in follicular and sperm fluids. J Clin Chem Clin Biochem 1989;27:663-7.

224. Guo YL, Hsu PC, Hsu CC, Lambert GH. Semen quality after prenatal exposure to polychlorinated biphenyls and dibenzofurans. Lancet 2000;356:1240-1.

225. Whorton D, Krauss RM, Marshall S, Milby TH. Infertility in male pesticide workers. Lancet 1977;2:1259-61.

226. Whorton D, Milby TH, Krauss RM, Stubbs HA. Testicular function in DBCP exposed pesticide workers. J Occup Med 1979;21:161-6.

227. Potashnik G, Ben-Aderet N, Israeli R, Yanai-Inbar I, Sober I. Suppressive effect of 1,2-dibromo-3-chloropropane on human spermatogenesis. Fertil Steril 1978;30:444-7.

228. Potashnik G, Goldsmith J, Insler V. Dibromochloropropane-induced reduction of the sex-ratio in man. Andrologia 1984;16:213-8.

229. Goldsmith JR, Potashnik G, Israeli R. Reproductive outcomes in families of DBCP-exposed men. Arch Environ Health 1984;39:85-9.

230. Glass R, Lyness, RN, Mengle, DC, et al. Sperm count depression in pesticide applicators exposed to dibromochloropropane. Am J Epidemiology 1979;109:346-351.

231. Potashnik G. A four-year reassessment of workers with dibromochloropropane-induced testicular dysfunction. Andrologia 1983;15:164-70.

232. Curtis KM SD, Weinberg CR and Arbuckle TE. The effect of pesticide exposure on time to pregnancy. Epidemiology 1999;10:112-117.

233. Savitz DA, Arbuckle T, Kaczor D, Curtis KM. Male pesticide exposure and pregnancy outcome. Am J Epidemiol 1997;146:1025-36.

234. Thonneau P, Abell A, Larsen SB, et al. Effects of pesticide exposure on time to pregnancy: results of a multicenter study in France and Denmark. ASCLEPIOS Study Group. Am J Epidemiol 1999;150:157-63.

235. Sharpe RM, Skakkebaek NE. Are oestrogens involved in falling sperm counts and disorders of the male reproductive tract? [see comments]. Lancet 1993;341:1392-5.

236. Kelce WR, Monosson E, Gray LE, Jr. An environmental antiandrogen. Recent Prog Horm Res 1995;50:449-53.

237. Akingbemi BT, Ge RS, Klinefelter GR, Gunsalus GL, Hardy MP. A metabolite of methoxychlor, 2,2-bis(p-hydroxyphenyl)-1,1, 1- trichloroethane, reduces testosterone biosynthesis in rat leydig cells through suppression of steady-state messenger ribonucleic acid levels of the cholesterol side-chain cleavage enzyme. Biol Reprod 2000;62:571-8.

238. Waliszewski SM, Szymczynski GA. Determination of selected chlorinated pesticides, bound and free, in human semen. Arch Environ Contam Toxicol 1983;12:577-80.

239. Kociba RJ, Keeler PA, Park CN, Gehring PJ. 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD): results of a 13-week oral toxicity study in rats. Toxicol Appl Pharmacol 1976;35:553-74.

240. McConnell EE, Moore JA, Haseman JK, Harris MW. The comparative toxicity of chlorinated dibenzo-p-dioxins in mice and guinea pigs. Toxicol Appl Pharmacol 1978;44:335-56.

241. Moore JA HM, AP. Tissue distribution of [14C]tetrachlorodibenzo-p-dioxin in pregnant and neonatal rats. Toxicol Appl Pharmacol 1976;37:146-147.

242. Mebus CA, Reddy VR, Piper WN. Depression of rat testicular 17-hydroxylase and 17,20-lyase after administration of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Biochem Pharmacol 1987;36:727-31.

243. Chahoud I, Krowke R, Schimmel A, Merker HJ, Neubert D. Reproductive toxicity and pharmacokinetics of 2,3,7,8- tetrachlorodibenzo-p-dioxin. 1. Effects of high doses on the fertility of male rats. Arch Toxicol 1989;63:432-9.

244. Kleeman JM, Moore RW, Peterson RE. Inhibition of testicular steroidogenesis in 2,3,7,8-tetrachlorodibenzo- p-dioxin-treated rats: evidence that the key lesion

occurs prior to or during pregnenolone formation. Toxicol Appl Pharmacol 1990;106:112-25.

245. Mably TA, Moore RW, Peterson RE. In utero and lactational exposure of male rats to 2,3,7,8- tetrachlorodibenzo-p-dioxin. 1. Effects on androgenic status. Toxicol Appl Pharmacol 1992;114:97-107.

246. Mably TA, Moore RW, Goy RW, Peterson RE. In utero and lactational exposure of male rats to 2,3,7,8- tetrachlorodibenzo-p-dioxin. 2. Effects on sexual behavior and the regulation of luteinizing hormone secretion in adulthood. Toxicol Appl Pharmacol 1992;114:108-17.

247. Mably TA, Bjerke DL, Moore RW, Gendron-Fitzpatrick A, Peterson RE. In utero and lactational exposure of male rats to 2,3,7,8- tetrachlorodibenzo-p-dioxin. 3. Effects on spermatogenesis and reproductive capability. Toxicol Appl Pharmacol 1992;114:118-26.

248. Faqi AS, Dalsenter PR, Merker HJ, Chahoud I. Reproductive toxicity and tissue concentrations of low doses of 2,3,7,8- tetrachlorodibenzo-p-dioxin in male offspring rats exposed throughout pregnancy and lactation. Toxicol Appl Pharmacol 1998;150:383-92.

249. Gray LE, Ostby JS, Kelce WR. A dose-response analysis of the reproductive effects of a single gestational dose of 2,3,7,8-tetrachlorodibenzo-p-dioxin in male Long Evans Hooded rat offspring. Toxicol Appl Pharmacol 1997;146:11-20.

250. Egeland GM, Sweeney MH, Fingerhut MA, Wille KK, Schnorr TM, Halperin WE. Total serum testosterone and gonadotropins in workers exposed to dioxin [see comments]. Am J Epidemiol 1994;139:272-81.

251. Rachootin P, Olsen J. The risk of infertility and delayed conception associated with exposures in the Danish workplace. J Occup Med 1983;25:394-402.

252. Schecter A, McGee H, Stanley JS, Boggess K, Brandt-Rauf P. Dioxins and dioxin-like chemicals in blood and semen of American Vietnam veterans from the state of Michigan [published erratum appears in Am J Ind Med 1997 Mar;31(3):370-1]. Am J Ind Med 1996;30:647-54.

253. De Kretser D. Morphology and physiology of the testis. In: Becker K, ed. Principles and practice of endocrinology and metabolism. Philadelphia, PA: J.B. Lippincott company, 1995:1032-1041.

254. Kuratsune M YT, Matsuzaka J and Yamaguchi A. Epidemilogical study on Yusho, a poisoning caused by ingestion of rice oil contaminated with commercial brand of polychlorinated biphenyls. Environ Hlth Perspec 1972;1:119-128.

255. Hsu ST, Ma CI, Hsu SK, Wu SS, Hsu NH, Yeh CC. Discovery and epidemiology of PCB poisoning in Taiwan. Prog Clin Biol Res 1984;137:71-9.

256. Rogan WJ, Gladen BC, Guo YL, Hsu CC. Sex ratio after exposure to dioxin-like chemicals in Taiwan. Lancet 1999;353:206-7.

257. Arnold DL, Bryce F, McGuire PF, et al. Toxicological consequences of aroclor 1254 ingestion by female rhesus (Macaca mulatta) monkeys. Part 2. Reproduction and infant findings [see comments]. Food Chem Toxicol 1995;33:457-74.

258. Simmons DL, Valentine DM, Bradshaw WS. Different patterns of developmental toxicity in the rat following prenatal administration of structurally diverse chemicals. J Toxicol Environ Health 1984;14:121-36.

259. Mocarelli P, Needham LL, Marocchi A, et al. Serum concentrations of 2,3,7,8-tetrachlorodibenzo-p-dioxin and test results from selected residents of Seveso, Italy. J Toxicol Environ Health 1991;32:357-66.

260. Mocarelli P, Brambilla P, Gerthoux PM, Patterson DG, Jr., Needham LL. Change in sex ratio with exposure to dioxin [letter]. Lancet 1996;348:409.

261. Nagel SC vSFTCea. Relative binding affinity-serum modified access (RBA-SMA) assay predicts the relative in vivo bioactivity of the xenoestrogens bisphenol A and octyphenol. Environ Health Perspect 1997;105:70-77.

262. Young AL CJ, Thalken CE and Trembly JW. The toxicology, environmental fate and human risk of herbicide orange and its associated dioxin. Brooks Air Force Base, TX: Medical Division: USAF Occupational and Environmental Health Laboratory (OEHL), 1978.

263. Stellman SD, Stellman JM, Sommer JF, Jr. Combat and herbicide exposures in Vietnam among a sample of American Legionnaires. Environ Res 1988;47:112-28.

264. Potashnik G, Porath A. Dibromochloropropane (DBCP): a 17-year reassessment of testicular function and reproductive performance. J Occup Environ Med 1995;37:1287-92.

265. Whorton MD, Wong O, Morgan RW, Gordon N. An epidemiologic investigation of birth outcomes in relation to dibromochloropropane contamination in drinking water in Fresno County, California, USA. Int Arch Occup Environ Health 1989;61:403-7.

266. Reich MR, Spong JK. Kepone: a chemical disaster in Hopewell, Virginia. Int J Health Serv 1983;13:227-46.

267. Cannon SB, Veazey JM, Jr., Jackson RS, et al. Epidemic kepone poisoning in chemical workers. Am J Epidemiol 1978;107:529-37.

268. Taylor JR, Selhorst JB, Houff SA, Martinez AJ. Chlordecone intoxication in man. I. Clinical observations. Neurology 1978;28:626-30.

269. Silberhorn EM, Glauert HP, Robertson LW. Carcinogenicity of polyhalogenated biphenyls: PCBs and PBBs. Crit Rev Toxicol 1990;20:440-96.

270. Parkinson A, Safe SH, Robertson LW, et al. Immunochemical quantitation of cytochrome P-450 isozymes and epoxide hydrolase in liver microsomes from polychlorinated or polybrominated biphenyl-treated rats. A study of structure-activity relationships. J Biol Chem 1983;258:5967-76.

271. Shimada T, Yun CH, Yamazaki H, Gautier JC, Beaune PH, Guengerich FP. Characterization of human lung microsomal cytochrome P-450 1A1 and its role in the oxidation of chemical carcinogens. Mol Pharmacol 1992;41:856-64.

272. Shimada T, Hayes CL, Yamazaki H, et al. Activation of chemically diverse procarcinogens by human cytochrome P- 450 1B1. Cancer Res 1996;56:2979-84.

273. Pang S, Cao, JQ, Katz, BH, et al. Inductive and inhibitory effects of non-ortho-substituted polychlorinated biphenyls on estrogen metabolism and human cytochromes P450 1A1 and 1B1. Biochem Pharmacol 1999;58:29-38.

274. Kawajiri KaHS-I. The CYP1 Family. In: C I, ed. Cytochromes P450 Metabolic and Toxicological Aspects: CRC Press, Inc, 1996:77-97.

275. Masten SA GJ, Miller CR et al. Population-based studies of dioxin responsiveness: individual variation in CYP1A1 levels and relationship to dioxin body burden. Organohalogen Compounds 1998;38:13-16.

276. Spink DC, Hayes CL, Young NR, et al. The effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin on estrogen metabolism in MCF-7 breast cancer cells: evidence for induction of a novel 17 beta-estradiol 4-hydroxylase. J Steroid Biochem Mol Biol 1994;51:251-8.

277. Zhu BT, Conney AH. Functional role of estrogen metabolism in target cells: review and perspectives. Carcinogenesis 1998;19:1-27.

278. Sutter TR, Tang YM, Hayes CL, et al. Complete cDNA sequence of a human dioxin-inducible mRNA identifies a new gene subfamily of cytochrome P450 that maps to chromosome 2. J Biol Chem 1994;269:13092-9.

279. Liehr JG, Ricci MJ. 4-Hydroxylation of estrogens as marker of human mammary tumors. Proc Natl Acad Sci U S A 1996;93:3294-6.

280. Han X, Liehr JG. 8-Hydroxylation of guanine bases in kidney and liver DNA of hamsters treated with estradiol: role of free radicals in estrogen-induced carcinogenesis. Cancer Res 1994;54:5515-7.

281. Wormhoudt LW CJaNP. Genetic polymorphisms of human N-acetyltransferase, cytochrome P450, glutathione-S-transferase, and epoxide hydrolase enzymes: relevance to xenobiotic metabolism and toxicity. Crit Rev Toxicol 1999;29:59-124.

282. Moysich KB, Shields PG, Freudenheim JL, et al. Polychlorinated biphenyls, cytochrome P4501A1 polymorphism, and postmenopausal breast cancer risk. Cancer Epidemiol Biomarkers Prev 1999;8:41-4.

283. Shields PG, Caporaso NE, Falk RT, et al. Lung cancer, race, and a CYP1A1 genetic polymorphism. Cancer Epidemiol Biomarkers Prev 1993;2:481-5.

284. Zhang ZY, Fasco MJ, Huang L, Guengerich FP, Kaminsky LS. Characterization of purified human recombinant cytochrome P4501A1- Ile462 and -Val462: assessment of a role for the rare allele in carcinogenesis. Cancer Res 1996;56:3926-33.

285. Cosma G, Crofts F, Taioli E, Toniolo P, Garte S. Relationship between genotype and function of the human CYP1A1 gene. J Toxicol Environ Health 1993;40:309-16.

286. Ambrosone CB, Freudenheim JL, Graham S, et al. Cytochrome P4501A1 and glutathione S-transferase (M1) genetic polymorphisms and postmenopausal breast cancer risk. Cancer Res 1995;55:3483-5.

287. Ishibe N, Hankinson SE, Colditz GA, et al. Cigarette smoking, cytochrome P450 1A1 polymorphisms, and breast cancer risk in the Nurses' Health Study. Cancer Res 1998;58:667-71.

288. Baron JA. Smoking and estrogen-related disease. Am J Epidemiol 1984;119:9-22.

289. Taioli E, Trachman J, Chen X, Toniolo P, Garte SJ. A CYP1A1 restriction fragment length polymorphism is associated with breast cancer in African-American women. Cancer Res 1995;55:3757-8.

290. Jellinck PH, Forkert PG, Riddick DS, Okey AB, Michnovicz JJ, Bradlow HL. Ah receptor binding properties of indole carbinols and induction of hepatic estradiol hydroxylation. Biochem Pharmacol 1993;45:1129-36.

291. Oakley GG, Robertson LW, Gupta RC. Analysis of polychlorinated biphenyl-DNA adducts by 32P-postlabeling. Carcinogenesis 1996;17:109-14.

292. Amaro AR, Oakley GG, Bauer U, Spielmann HP, Robertson LW. Metabolic activation of PCBs to quinones: reactivity toward nitrogen and sulfur nucleophiles and influence of superoxide dismutase. Chem Res Toxicol 1996;9:623-9.

293. Dalet-Beluche I, Boulenc X, Fabre G, Maurel P, Bonfils C. Purification of two cytochrome P450 isozymes related to CYP2A and CYP3A gene families from monkey (baboon, Papio papio) liver microsomes. Cross reactivity with human forms. Eur J Biochem 1992;204:641-8.

294. Rebbeck TR, Jaffe JM, Walker AH, Wein AJ, Malkowicz SB. Modification of clinical presentation of prostate tumors by a novel genetic variant in CYP3A4 [published erratum appears in J Natl Cancer Inst 1999 Jun 16;91(12):1082] [see comments]. J Natl Cancer Inst 1998;90:1225-9.

295. Golden RJ, Noller KL, Titus-Ernstoff L, et al. Environmental endocrine modulators and human health: an assessment of the biological evidence. Crit Rev Toxicol 1998;28:109-227.

296. Danzo BJ. Environmental xenobiotics may disrupt normal endocrine function by interfering with the binding of physiological ligands to steroid receptors and binding proteins. Environ Health Perspect 1997;105:294-301.

297. Roman BL, Sommer RJ, Shinomiya K, Peterson RE. In utero and lactational exposure of the male rat to 2,3,7,8- tetrachlorodibenzo-p-dioxin: impaired prostate growth and development without inhibited androgen production. Toxicol Appl Pharmacol 1995;134:241-50.

298. Krishnan V, Safe S. Polychlorinated biphenyls (PCBs), dibenzo-p-dioxins (PCDDs), and dibenzofurans (PCDFs) as antiestrogens in MCF-7 human breast cancer cells: quantitative structure-activity relationships. Toxicol Appl Pharmacol 1993;120:55-61.

299. Yeowell HN, Waxman DJ, LeBlanc GA, Linko P, Goldstein JA. Suppression of male-specific cytochrome P450 2c and its mRNA by 3,4,5,3',4',5'-hexachlorobiphenyl in rat liver is not causally related to changes in serum testosterone. Arch Biochem Biophys 1989;271:508-14.

300. Bjerke DL, Brown TJ, MacLusky NJ, Hochberg RB, Peterson RE. Partial demasculinization and feminization of sex behavior in male rats by in utero and lactational exposure to 2,3,7,8-tetrachlorodibenzo-p- dioxin is not associated with alterations in estrogen receptor binding or volumes of sexually differentiated brain nuclei. Toxicol Appl Pharmacol 1994;127:258-67.

301. Bergeron JM, Crews D, McLachlan JA. PCBs as environmental estrogens: turtle sex determination as a biomarker of environmental contamination. Environ Health Perspect 1994;102:780-1.

302. Commandeur JN, Stijntjes GJ, Vermeulen NP. Enzymes and transport systems involved in the formation and disposition of glutathione S-conjugates. Role in bioactivation and detoxication mechanisms of xenobiotics. Pharmacol Rev 1995;47:271-330.

303. Seidegard J, Vorachek WR, Pero RW, Pearson WR. Hereditary differences in the expression of the human glutathione transferase active on trans-stilbene oxide are due to a gene deletion. Proc Natl Acad Sci U S A 1988;85:7293-7.

304. McWilliams JE, Sanderson BJ, Harris EL, Richert-Boe KE, Henner WD. Glutathione S-transferase M1 (GSTM1) deficiency and lung cancer risk. Cancer Epidemiol Biomarkers Prev 1995;4:589-94.

305. Nazar-Stewart V, Vaughan TL, Burt RD, Chen C, Berwick M, Swanson GM. Glutathione S-transferase M1 and susceptibility to nasopharyngeal carcinoma. Cancer Epidemiol Biomarkers Prev 1999;8:547-51.

306. London SJ DA, Cooper J et al. Polymorphism of glutathione S-transferase M1 and lung cancer risk among African-Americans and Caucasians in Los Angeles county, California. J Natl Cancer Institute 1995;87:1246-1253.

307. Pemble S, Schroeder KR, Spencer SR, et al. Human glutathione S-transferase theta (GSTT1): cDNA cloning and the characterization of a genetic polymorphism. Biochem J 1994;300:271-6.

308. Hayes JD, Pulford DJ. The glutathione S-transferase supergene family: regulation of GST and the contribution of the isoenzymes to cancer chemoprotection and drug resistance. Crit Rev Biochem Mol Biol 1995;30:445-600.

309. Harries LW, Stubbins MJ, Forman D, Howard GC, Wolf CR. Identification of genetic polymorphisms at the glutathione S- transferase Pi locus and association with susceptibility to bladder, testicular and prostate cancer. Carcinogenesis 1997;18:641-4.

310. Pajarinen J, Savolainen V, Perola M, Penttila A, Karhunen PJ. Glutathione S-transferase-M1 'null' genotype and alcohol-induced disorders of human spermatogenesis. Int J Androl 1996;19:155-63.

311. Ronis MJJ LKaI-SM. The CYP2E subfamily. In: DV ICaP, ed. Cytochromes P450. Metabolic and toxicologic aspects. Boca Raton: CRC Press, 1996:211-239.

312. Baranova H, Canis M, Ivaschenko T, et al. Possible involvement of arylamine N-acetyltransferase 2, glutathione S- transferases M1 and T1 genes in the development of endometriosis. Mol Hum Reprod 1999;5:636-41.

313. Osteen KG, Sierra-Rivera E. Does disruption of immune and endocrine systems by environmental toxins contribute to development of endometriosis? Semin Reprod Endocrinol 1997;15:301-8.

314. Aitken RJ, Clarkson JS. Cellular basis of defective sperm function and its association with the genesis of reactive oxygen species by human spermatozoa. J Reprod Fertil 1987;81:459-69.

315. Sharma RK, Agarwal A. Role of reactive oxygen species in male infertility. Urology 1996;48:835-50.

316. Aitken RJ. Pathophysiology of human spermatozoa. Curr Opin Obstet Gynecol 1994;6:128-35.

317. Twigg J, Fulton N, Gomez E, Irvine DS, Aitken RJ. Analysis of the impact of intracellular reactive oxygen species generation on the structural and functional integrity of human spermatozoa: lipid peroxidation, DNA fragmentation and effectiveness of antioxidants. Hum Reprod 1998;13:1429-36.

318. Twigg JP, Irvine DS, Aitken RJ. Oxidative damage to DNA in human spermatozoa does not preclude pronucleus formation at intracytoplasmic sperm injection. Hum Reprod 1998;13:1864-71.

319. de Lamirande E, Gagnon C. Capacitation-associated production of superoxide anion by human spermatozoa. Free Radic Biol Med 1995;18:487-95.

320. Aitken RJ, Buckingham DW, West KM. Reactive oxygen species and human spermatozoa: analysis of the cellular mechanisms involved in luminol- and lucigenin-dependent chemiluminescence. J Cell Physiol 1992;151:466-77.

321. Voie OA, Wiik P, Fonnum F. Ortho-substituted polychlorinated biphenyls activate respiratory burst measured as luminol-amplified chemoluminescence in human granulocytes. Toxicol Appl Pharmacol 1998;150:369-75.

322. Ganey PE, Sirois JE, Denison M, Robinson JP, Roth RA. Neutrophil function after exposure to polychlorinated biphenyls in vitro. Environ Health Perspect 1993;101:430-4.

323. Jacobson JL, Humphrey HE, Jacobson SW, Schantz SL, Mullin MD, Welch R. Determinants of polychlorinated biphenyls (PCBs), polybrominated biphenyls (PBBs), and dichlorodiphenyl trichloroethane (DDT) levels in the sera of young children. Am J Public Health 1989;79:1401-4.

324. Falck F, Jr., Ricci A, Jr., Wolff MS, Godbold J, Deckers P. Pesticides and polychlorinated biphenyl residues in human breast lipids and their relation to breast cancer. Arch Environ Health 1992;47:143-6.

325. Kaminsky LS, Kennedy MW, Adams SM, Guengerich FP. Metabolism of dichlorobiphenyls by highly purified isozymes of rat liver cytochrome P-450. Biochemistry 1981;20:7379-84.

326. Kennedy MW CN, Dymerski PP et al. Metabolism of monochlorobiphenyls by hepatic microsomal cytochrome P-450. Biochem Pharmacol 1980;29:727-736.

327. McLean MR, Robertson LW, Gupta RC. Detection of PCB adducts by the 32P-postlabeling technique. Chem Res Toxicol 1996;9:165-71.

328. Jones R, Mann T, Sherins R. Peroxidative breakdown of phospholipids in human spermatozoa, spermicidal properties of fatty acid peroxides, and protective action of seminal plasma. Fertil Steril 1979;31:531-7.

329. Nackerdien Z, Rao G, Cacciuttolo MA, Gajewski E, Dizdaroglu M. Chemical nature of DNA-protein cross-links produced in mammalian chromatin by hydrogen peroxide in the presence of iron or copper ions. Biochemistry 1991;30:4873-9.

330. Martins EA, Meneghini R. Cellular DNA damage by hydrogen peroxide is attenuated by hypotonicity. Biochem J 1994;299:137-40.

331. Oluski R, Nackerdien, Z, and Dizdaroglu, M. DNA protein cross linking between thymine and tyrosine in chromatin of gamma irradiated and hydrogen peroxide treated cultured human cells. Arch Biochem Biophys 1992;297:139-143.

332. Lee IP, Dixon RL. Effects of mercury on spermatogenesis studied by velocity sedimentation cell separation and serial mating. J Pharmacol Exp Ther 1975;194:171-81.

333. Earnst E, and Lauritsen, JG. Effects of organic and inorganic mercury on human sperm motility. Pharmacol Toxicol 1990;69:440-444.

334. Stacey NH, Kappus H. Cellular toxicity and lipid peroxidation in response to mercury. Toxicol Appl Pharmacol 1982;63:29-35.

335. Alexidis AN, Rekka EA, Kourounakis PN. Influence of mercury and cadmium intoxication on hepatic microsomal CYP2E and CYP3A subfamilies. Res Commun Mol Pathol Pharmacol 1994;85:67-72.

336. Dickman MD, Leung KM. Mercury and organochlorine exposure from fish consumption in Hong Kong. Chemosphere 1998;37:991-1015.

337. Sonzogni W. Maack L GT, Degenhardt D, Anderson H, and Fiore B. Polychlorinated biphenyl congeners in blood of Wisconsin sport fish consumers. Arch. Environ. Contam. Toxicol 1991;20:56-60.

338. Birnbaum LS. The role of structure in the disposition of halogenated aromatic xenobiotics. Environ Health Perspect 1985;61:11-20.

339. Kafafi SA, Said HK, Mahmoud MI, Afeefy HY. The electronic and thermodynamic aspects of Ah receptor binding. A new structure-activity model: I. The polychlorinated dibenzo-p-dioxins. Carcinogenesis 1992;13:1599-605.

340. Kafafi SA, Afeefy HY, Said HK, Kafafi AG. Relationship between aryl hydrocarbon receptor binding, induction of aryl hydrocarbon hydroxylase and 7-ethoxyresorufin O-deethylase enzymes, and toxic activities of aromatic xenobiotics in animals. A new model. Chem Res Toxicol 1993;6:328-34.

341. Canada NHW. Toxic chemicals in the Great Lakes and associated effects. Environment Canada, Department of Fisheries and Oceans, Ottawa 1991;2.

342. Sofikitis N, Miyagawa I, Dimitriadis D, Zavos P, Sikka S, Hellstrom W. Effects of smoking on testicular function, semen quality and sperm fertilizing capacity. J Urol 1995;154:1030-4.

343. Muller CH. Rationale, interpretation, validation, and uses of sperm function tests. J Androl 2000;21:10-30.

344. Lipshultz LI, Caminos-Torres R, Greenspan CS, Snyder PJ. Testicular function after orchiopexy for unilaterally undescended testis. N Engl J Med 1976;295:15-8.

345. Lipshultz LI. Cryptorchidism in the subfertile male. Fertil Steril 1976;27:609-20.

346. Alpert PF, Klein RS. Spermatogenesis in the unilateral cryptorchid testis after orchiopexy. J Urol 1983;129:301-2.

347. Close CE, Roberts PL, Berger RE. Cigarettes, alcohol and marijuana are related to pyospermia in infertile men. J Urol 1990;144:900-3.

348. Stillman RJ, Rosenberg MJ, Sachs BP. Smoking and reproduction. Fertil Steril 1986;46:545-66.

349. Vine MF, Margolin BH, Morrison HI, Hulka BS. Cigarette smoking and sperm density: a meta-analysis. Fertil Steril 1994;61:35-43.

350. Van Thiel DH, Lester R, Sherins RJ. Hypogonadism in alcoholic liver disease: evidence for a double defect. Gastroenterology 1974;67:1188-99.

351. Wilcox A, Weinberg C, Baird D. Caffeinated beverages and decreased fertility. Lancet 1988;2:1453-6.

352. Anawalt BD, Bebb RA, Matsumoto AM, et al. Serum inhibin B levels reflect Sertoli cell function in normal men and men with testicular dysfunction. J Clin Endocrinol Metab 1996;81:3341-5.

353. Jensen TK, Andersson AM, Hjollund NH, et al. Inhibin B as a serum marker of spermatogenesis: correlation to differences in sperm concentration and follicle-stimulating hormone levels. A study of 349 Danish men. J Clin Endocrinol Metab 1997;82:4059-63.

354. Klingmuller D, Haidl G. Inhibin B in men with normal and disturbed spermatogenesis. Hum Reprod 1997;12:2376-8.

355. Pierik FH, Vreeburg JT, Stijnen T, De Jong FH, Weber RF. Serum inhibin B as a marker of spermatogenesis. J Clin Endocrinol Metab 1998;83:3110-4.

356. Illingworth PJ, Groome NP, Byrd W, et al. Inhibin-B: a likely candidate for the physiologically important form of inhibin in men [see comments]. J Clin Endocrinol Metab 1996;81:1321-5.

357. De Kretser DM. Morphology and physiology of the testis. In: KL B, ed. Principles and practice of endocrinology and metabolism. Philadelphia: JB Lippincott, 1995:1032 -1041.

358. Pollard I. The testis and control of spermataogenesis. A guide to reproduction. Cambridge: Cambridge University Press, 1994:74-93.

359. Winters SJ. Diurnal rhythm of testosterone and luteinizing hormone in hypogonadal men. J Androl 1991;12:185-90.

360. Bremner WJ, Vitiello MV, Prinz PN. Loss of circadian rhythmicity in blood testosterone levels with aging in normal men. J Clin Endocrinol Metab 1983;56:1278-81.

361. Krieger DT, Ossowski R, Fogel M, Allen W. Lack of circaidan periodicity of human serum FSH and LH levels. J Clin Endocrinol Metab 1972;35:619-23.

362. Veldhuis JD, Iranmanesh A, Johnson ML, Lizarralde G. Twenty-four-hour rhythms in plasma concentrations of adenohypophyseal hormones are generated by distinct amplitude and/or frequency modulation of underlying pituitary secretory bursts. J Clin Endocrinol Metab 1990;71:1616-23.

363. Barratt CL, Naeeni M, Clements S, Cooke ID. Clinical value of sperm morphology for in-vivo fertility: comparison between World Health Organization criteria of 1987 and 1992. Hum Reprod 1995;10:587-93.

364. Bostofte E, Bagger P, Michael A, Stakemann G. Fertility prognosis for infertile men: results of follow-up study of semen analysis in infertile men from two different populations evaluated by the Cox regression model. Fertil Steril 1990;54:1100-6.

365. Barratt CL, St John JC. Diagnostic tools in male infertility. Hum Reprod 1998;13 Suppl 1:51-61.

366. Overstreet JW. Clinical approach to male reproductive problems. Occup Med 1994;9:387-404.

367. Zanaveld LJD JR. Modern assessment of semen for diagnostic purposes. Semin Reprod Endrocrinol 1988;6:324-337.

368. Tummon IS, Yuzpe AA, Daniel SA, Deutsch A. Total acrosin activity correlates with fertility potential after fertilization in vitro. Fertil Steril 1991;56:933-8.

369. Phillips DL, Pirkle JL, Burse VW, Bernert JT, Jr., Henderson LO, Needham LL. Chlorinated hydrocarbon levels in human serum: effects of fasting and feeding. Arch Environ Contam Toxicol 1989;18:495-500.

370. Patterson DG, Jr., Needham LL, Pirkle JL, et al. Correlation between serum and adipose tissue levels of 2,3,7,8- tetrachlorodibenzo-p-dioxin in 50 persons from Missouri. Arch Environ Contam Toxicol 1988;17:139-43.

371. Aarts J. Application of the chemical activated luciferase expression (CALUX) bioassay for quantification of dioxin-like compounds in small samples of human milk and bloodplasma. Organohalogen Compounds 1996;27:285-289.

372. Lancranjan I, Popescu, HI, Klepsch, I et al. Reproductive ability of workmen occupationally exposed to lead. Arch Environ Health 1975;30:396-401.

373. Thomas JA, Brogan WCd. Some actions of lead on the sperm and on the male reproductive system. Am J Ind Med 1983;4:127-34.

374. Stewart P, Darvill T, Lonky E, Reihman J, Pagano J, Bush B. Assessment of prenatal exposure to PCBs from maternal consumption of Great Lakes fish: an analysis of PCB pattern and concentration. Environmental Research 1999;80:S87-S96.

375. Mullard AK, W and Wirth, JJ. Human exposure to polychlorinated biphenyls in Michigan sport caught fish. 2001 (submitted).

376. Olsen J, Juul S, Basso O. Measuring time to pregnancy. Methodological issues to consider. Hum Reprod 1998;13:1751-3.

APPENDIX A

WHO GUIDELINES FOR SEMEN ANALYSIS PARAMETERS

Reference values	
Volume	2.0 ml or more
pH	7.2 or more
Sperm concentration	20×10^6 spermatozoa/ml or more
Total sperm number	40×10^6 spermatozoa per ejaculate or more
Motility	50% or more motile (grades a + b) or 25% or more with progressive motility (grade a) within 60 minutes of ejaculation
Morphology	•
Vitality	75% or more live, i.e., excluding dye
White blood cells	Fewer than 1×10^6/ml
Immunobead test	Fewer than 50% motile spermatozoa with beads bound
MAR test	Fewer than 50% motile spermatozoa with adherent particles

• Multicentre population-based studies utilizing the methods of morphology assessment in this manual are now in progress.

Data from assisted reproductive technology programmes suggest that, as sperm morphology falls below 15% normal forms using the methods and definitions described in this manual, the fertilization rate in vitro decreases.

APPENDIX B

SUMMARY OF CHEMICALS QUANTIFIED IN FISH TISSUE

TRIGGER LEVELS USED BY MDCH TO ESTABLISH CONSUMPTION WARNINGS

Summary of chemicals quantified in edible-portion fish tissue samples.

Chemical*	# of Sites Monitored	# of Sites Quantified	Concentration Range (ppm)	Location and Species with Maximum Concentration
a-Chlordane	27	21	K 0.003 - 0.344	Lake Michigan-Lake Sturgeon
g-Chlordane	27	18	K 0.003 - 0.114	Lake Michigan-Lake Sturgeon
cis-Nonachlor	27	19	K 0.003 - 0.597	Lake Michigan-Lake Sturgeon
trans-Nonachlor	27	25	K 0.003 - 0.608	Lake Michigan-Lake Sturgeon
Oxychlordane	27	20	K 0.003 - 0.047	Lake Michigan-Lake Sturgeon
DDD	27	24	K 0.005 - 45.1	Pine River-Carp
DDE	27	27	K 0.003 - 24.0	Lake Michigan-Lake Sturgeon
DDT	27	21	K 0.005 - 2.93	Pine River-Carp
Dieldrin	27	18	K 0.005 - 0.156	Lake Michigan-Lake Whitefish
Heptachlor Epoxide	27	14	K 0.003 - 0.063	Lake Michigan-Lake Whitefish
Hexachlorobenzene	27	18	K 0.001 - 0.011	Pine River-Carp
Mercury	26	26	0.02 - 5.74	Deer Lake-Northern Pike
Octachlorostyrene	21	13	K 0.001 - 0.012	Au Sable River-Carp
PBB	21	2	K 0.001 - 0.800	Pine River-Smallmouth Bass
Total PCB	27	23	K 0.025 - 20.4	Lake Michigan-Lake Sturgeon
Apparent Toxaphene	27	8	K 0.050 - J 2.8	Lake Michigan-Lake Sturgeon
Dioxin TEQ	18	17	0.0 - 47.1	Lake Huron-Lake Whitefish

K = indicates unquantified at the level shown

J = estimated value, value may not be precise

* Aldrin, Heptachlor, Lindane (g-BHC), Mirex, PBB, Pentachlorostyrene, Heptachlorostyrene, Hexachlorostyrene, and Terphenyl were not quantified at any of the sites monitored.

Trigger levels used by the Michigan Department of Community Health to establish fish consumption advisories.

Chemical	MDCH Trigger Level	
Total Chlordane	0.3 ppm	(= mg/kg)
Total DDT	5.0 ppm	
Dieldrin	0.3 ppm	
Dioxin Toxic Equivalents#	10.0 ppt	(= ng/kg)
Heptachlor (+Heptachlor Epoxide)	0.3 ppm	
Mercury		
Restrict Consumption	0.5 ppm	
No Consumption	1.5 ppm	
Mirex	0.1 ppm	
Total PCB		
General Population	2.0 ppm	
Women of Child Bearing Age and Children Under 15 Years		
1 Meal Per Week	0.05 ppm	
1 Meal Per Month	0.2 ppm	
6 Meals Per Year	1.0 ppm	
No Consumption	1.9 ppm	
Toxaphene	5.0 ppm	

The MDCH advisory trigger level for dioxin applies to total 2,3,7,8-TCDD toxic equivalent concentrations.