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Endocrine, immune, and behavioral effects of aldicarb (carbamate), atrazine (triazine) and nitrate (fertilizer) mixtures at groundwater concentrations

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This paper describes the results of 5 years of research on interactive effects of mixtures of aldicarb, atrazine, and nitrate on endocrine, immune, and nervous system function. The concentrations of chemicals used were the same order of magnitude as current maximum contaminant levels (MCLs) for all three compounds. Such levels occur in groundwater across the United States. Dosing was through voluntary consumption of drinking water. We used fractional and full factorial designs with center replicates to determine multifactor effects. We used chronic doses in experiments that varied in duration from 22 to 103 days. We tested for changes in thyroid hormone levels, ability to make antibodies to foreign proteins, and aggression in wild deer mice, *Peromyscus maniculatus*, and white outbred Swiss Webster mice, *Mus musculus*, ND4 strain. Endocrine, immune, and behavior changes occurred due to doses of mixtures, but rarely due to single compounds at the same concentrations. Immune assay data suggest the possibility of seasonal effects at low doses. We present a multiple-level model to help interpret the data in the context of human health and biological conservation concerns. We discuss six testing deficiencies of currently registered pesticides, and suggest areas of human health concerns if present trends in pesticide use continue.

Keywords: *aldicarb, atrazine, behavior, endocrine, groundwater mixtures, immune, nitrate.*

Introduction

Reproductive Effects

Evidence continues to accumulate that humans and domestic and wild animal species have suffered adverse health consequences from exposure to environmental chemicals that interact with the endocrine system (Colborn et al., 1993; Guillette and Crain, 1996). Endocrine disruption may be responsible for the data suggesting a decline in human sperm counts and production (Figure 1). These data are global in scope, as Figure 2 indicates. Geographic variation, especially urban *vs.* rural populations and possibly developed *vs.* developing countries, appear to be an important factor in sperm quality and quantity. A recent reanalysis of 61 studies on sperm density confirms that some intraregional differences are as large as the mean decline in sperm density between 1938 and 1990. The mean decline varies from 1.5% per year in the US (1938–1988) to more than 3% per year in Europe (1971–1990) (Swan et al., 1997). Moderate alcohol consumption may contribute to some of the variance in the data (Pajarinen and Karhunen, 1994; Pajarinen et al., 1996a,b). Nonwestern countries did not show this decline based on data from 1978–1989. Possible causes may include prenatal or early exposure to mixtures of polychlorinated biphenyls (PCBs) and dioxins and other agricultural and industrial chemicals (Colborn et al., 1993; Sauer et al., 1994; Gray et al., 1995).

Neurological Effects

Other developmental effects, such as altered learning and motor skills have also been linked with prenatal exposure to endocrine-disrupting chemicals. Brouwer et al. (1995) present evidence of polyhalogenated aromatic hydrocarbons affecting neurological function in animals and human infants. Jacobson and Jacobson (1996a,b)) and Lonky et al. (1996) present similar findings for children. A recent study in the Yaqui Valley, Mexico found "decreases in stamina, gross and fine eye–hand coordination, 30-minute memory, and the ability to draw a person" in children who lived in the agrarian region of the Valley compared with children in the foothills where pesticide use is avoided (Guillette et al., 1998). Attention deficit disorders and hyperactivity are common in chemically sensitive children, and laboratory

1. Abbreviations: ADHD, attention deficit hyperactivity disorder; ANOVA, analysis of variance; C.V., coefficient of variation; FTI, free thyroxine index; HGH, human growth hormone; MCL, maximum contaminant level; P450, cytochrome P450; PCB, polychlorinated biphenyl; PPAR, peroxisome proliferator-activated receptor; RTH, resistance to thyroid hormone; RXR, retinoid X receptor; SD, standard deviation; SE, standard error; SRBC, sheep red blood cells; T_3, triiodothyronine; T_4, thyroxine; UV, ultraviolet

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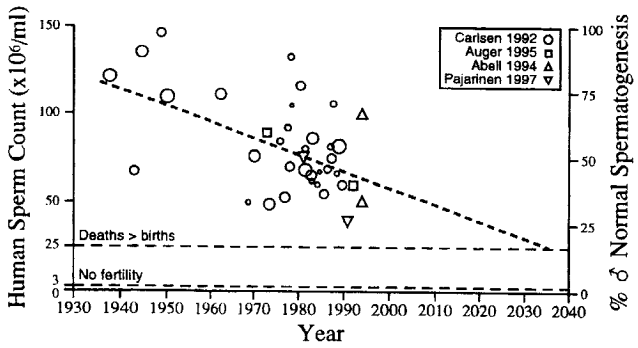
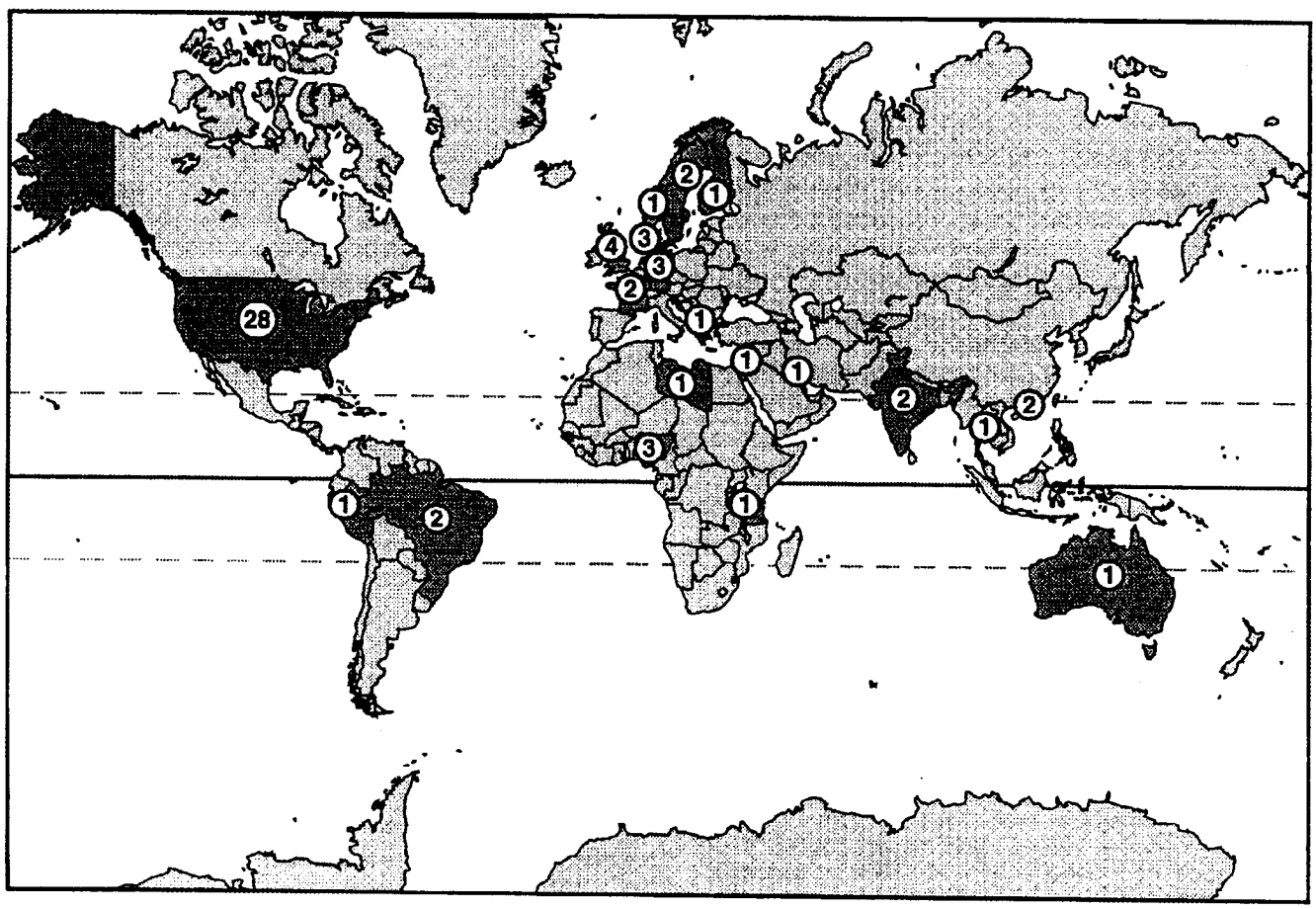


Figure 1. Data on human sperm counts from Carlsen et al. (1992), Abell et al. (1994) and Auger et al. (1995) and changes in normal spermatogenesis from Pajarinen et al. (1997). The area of the circles is proportional to the logarithm of the number of subjects in the study (Carlsen et al., 1992).

results are not consistent with a psychological origin of chemical sensitivity (Ziem and McTamney, 1997).

Diverse environmental chemicals may alter neurological development. For example, methoprene, a widely used mosquito control agent, closely resembles juvenile growth hormone and retinoic acid. Forms of retinoic acid are important for implantation of the embryo, neurological development and differentiation, organogenesis, craniofacial structures, and limb development (Mangelsdorf et al., 1994). Levels of retinoic acid that are too high or too low can lead to impaired brain and limb development (Maden et al., 1997). Temporal reductions in retinoic acid can produce specific neural crest, ocular, and nervous system defects (Dickman et al., 1997). Altered retinoid concentrations can also affect early heart development (Dickman and Smith, 1996; Smith et al., 1997, 1998).

Retinoid receptors belong to a superfamily of ligand-dependent transcription factors that control fundamental aspects of development. There are receptors for steroid and thyroid hormones, vitamins A and D, and the invertebrate ecdysteroids (Evans, 1988; Green and Chambon, 1988; O'Malley, 1990; Mangelsdorf et al., 1994). The retinoid X receptor (RXR) serves as a heterodimer partner. These partnerships function as master regulators of several hormone receptor systems. The hormones, vitamins, and ecdysteroids can compete with a RXR to bind with another RXR to regulate gene expression (Mangelsdorf et al., 1994). One receptor that can form a heterodimeric partner with RXR is the peroxisome proliferator-activated receptor (PPAR). PPAR is activated by a variety of amphipathic



igure 2. Locations and number of studies of human sperm counts from the work of Carlsen et al. (1992).

carboxylates, including herbicides, industrial plasticizers, and hypolipidemic drugs like clofibric acid (Issemann and Green, 1990). Thus, a variety of environmental chemicals have the potential for influencing fundamental developmental effects through modulation of competitive RXR heterodimer formation.

Neurological development may be altered by thyroid hormone modulation as well, and pesticide mixtures have been shown to affect thyroid hormones (Boyd et al., 1990; Porter et al., 1993). In mammals, if a pregnant female is either hypothyroid or hyperthyroid, neurological development of the fetus may be jeopardized (Porterfield and Hendrich, 1993; Porterfield and Stein, 1994). Chiovato et al. (1993) point out that fetal and/or neonatal hypo- or hyperthyroidism produced by the transplacental passage of maternal thyroid autoantibodies can impair growth and neuropsychological development. Hyperthyroid individuals express multiple symptoms that are interconnected because of feedback. They may show hypersensitivity to stimuli; they may experience difficulty in concentration and learning; they may be overly irritable and quick to anger (Whybrow, 1991a). Altered thyroid levels also may change corticosteroid levels (Cotman et al., 1987). Altered corticosteroid levels may modify immune function, which may lead to increased infections (Wiegers and Reul, 1998). Infections may in turn stimulate the production of interleukins, which suppress certain central neurological functions (Cotman et al., 1987).

Immunological Effects

Retinoids and pesticides can affect the immune system. A recent summary of retinoid effects on the immune system (Ross and Hammerling, 1994) shows multiple effects on both B cells and T cells. These effects appear to be related to stimulation of the nonspecific arm of the immune system—through phagocytosis and perhaps by the modulation of cytokine production. A summary of pesticide effects on the immune system (Repetto and Baliga, 1996) highlights the variety of pesticides affecting immune-system properties. These include suppressing resistance to bacteria, viruses, and fungi. Weisglas-Kuperus et al. (1995) present evidence of immune parameter changes in Dutch infants due to prenatal and postnatal exposure to dioxins and PCBs in their mothers' blood and milk.

Effects on Defensive Enzyme Systems

It is often argued that naturally occurring defensive enzyme systems, such as cytochrome P450 (P450) enzymes, protect animals and humans from chemical stresses. Typically, these defensive P450 enzymes must be induced to detoxify foreign chemicals. However, there is a lag of hours to days before they reach 'full strength.' Moreover, many factors alter the ability to induce them, such as thyroid status (O'Leary et al., 1997) or exposure to retinoids and growth hormone (Westin et al., 1997). O'Leary et al. (1997) found that triiodothyronine (T_3)-activated transcription of the NADPH cytochrome P450 oxidoreductase gene is dependent on the thyroid hormonal status of the animal. Both transcriptional and posttranscriptional pathways are important in regulating the cellular P450 mRNA level. Retinoids and growth hormone are also essential for induction of another P450 enzyme, P450 2C7 (Westin et al., 1997). Retinoid-like molecules such as the mosquito control agent, methoprene, a juvenile growth hormone mimic, could conceivably alter defensive enzyme status. Another important defensive enzyme, glutathione peroxidase, is compromised by the ureic herbicide linuron. Linuron affects rats' defensive enzymes much more in the *commercial* formulation and in 'reagent grade' form than in the highly pure *technical* form (usually 95–100% active ingredient) at all doses tested (Scassellati-Sforzolini et al., 1994). This suggests that 'inert ingredients' may also have important impacts on defensive enzyme status.

Drug therapy also can lower the activity status of P450 enzymes (Murray, 1997). Some drugs known to have these effects include the first generation macrolide antibiotics, some antidepressants, antiepileptics, and tuberculostatic agents. Naturally occurring hormones at certain stages of development can also lower the activity status of P450 enzymes. Elevated growth hormone in young rats, especially pulses of growth hormone so characteristic of males, antagonizes phenobarbital induction of hepatic CYP2B1 and CYP2B2 P450 enzymes (Agrawal and Shapiro, 1996).

Possible pesticide induction of diabetes may occur through P450 induction mechanisms. The P450 mixed-function oxidase system is widely distributed in body tissues. It plays a key role in the metabolism of endogenous and exogenous compounds. Little attention has been paid to the expression of the system in the islets of Langerhans in the pancreas. The data of (Clarke et al., 1997) suggest that CYP1A-like protein expression within the pancreatic islets of Langerhans is inducible and may have a role in the alteration of pancreatic beta-cell secretory responsiveness.

Effects of Pesticide Mixtures

Neurological, endocrine, immune, and developmental effects may show up only when pesticides are tested in combination (Boyd et al., 1990; Porter et al., 1993). Garry et al. (1996) presents epidemiological evidence suggesting combinations of pesticides cause increased incidence of fetal abnormalities, including urogenital defects, in offspring of pesticide applicators and in the populace of an area of heavy pesticide use. Neurological effects in veterans who served in the US/Iraq war in the Persian Gulf have been associated with exposure to combinations of individually innocuous pesticides, insect repellents, and pyridostigmine bromide (chemical weapons protection)

(Haley and Kurt, 1997). Of particular significance in the collective work of Boyd et al. (1990), Porter et al. (1993), and our current study is that *thyroid hormone concentration change* was consistently a response due to mixtures, but *not* usually to individual chemicals. Even *transient* thyroid hormone change can have important *permanent* developmental, physiological, and behavioral consequences in amphibians and mammals if changes occur during key developmental windows (Porterfield and Hendry, 1998).

Effects of Multiple Stresses

Combinations of stresses may have more than just additive effects on animals in the wild (Parsons, 1989). In a series of recent studies, several species of anuran amphibians have shown increased susceptibility to a fungal infection when exposed to ultraviolet (UV) radiation (Blaustein et al., 1994a,b, 1995; Kiesecker and Blaustein, 1994). This susceptibility occurred even within the background range of UV-B. Pesticides that disrupt the endocrine or immune systems may confer the same types of system-weakening effects, causing animals to be susceptible to assaults that would not normally be a concern.

Multiple stresses can enhance biological effects of chemicals. Porter et al. (1984) studied growth rates of mouse offspring and the number of offspring that survived to weaning. They used five variable sequential fractional factorial experiments on outbred white mice and wild deer mice. Their experiments showed effects due to: food and water availability; presence of a commercial plant growth regulator, chlorocholine chloride, which is an immunosuppressant in mice; presence of a low level viral infection (Venezuelan equine encephalitis virus); and low-level PCB exposure. Those experiments also showed an important interaction between the chlorocholine chloride and water availability. In 'standard' toxicological testing with unlimited water, chlorocholine chloride *enhanced* growth of the young. In water stress conditions (80% of normal unlimited consumption), chlorocholine chloride *suppressed* growth. Stresses can also induce endocrine changes, especially corticosteroid changes. Corticosteroids are in feedback loops with thyroid hormone.

Purpose of Study

In summary, we might expect that some environmental chemicals, such as certain PCBs, dioxins, and pesticide/nitrate mixtures, will affect endocrine, immune, and neurological systems, as well as reproduction and development. These changes may alter learning abilities and aggression levels. Endocrine changes induced by environmental chemicals may also alter defensive enzyme status. These changes may affect individual survival, growth, and reproduction and finally cause changes in population levels and possibly community structure. The current study was undertaken to determine whether low concentrations of atrazine, aldicarb, and nitrate mixtures would affect the endocrine, immune, and/or nervous system function of mice by assessing their thyroid hormone levels, ability to make antibodies to foreign proteins, and behavior.

Materials and methods

In vitro systems lack the multiple connections and feedback mechanisms in whole organisms. With this in mind, we decided to use whole organisms, white outbred Swiss Webster mice and wild deer mice, to study a mixture of the most commonly occurring agricultural chemicals contaminating groundwater in the United States at levels detected in groundwater. We designed these experiments to test the effects of combinations of these chemicals.

The agrochemicals chosen were aldicarb (carbamate insecticide), atrazine (triazine herbicide), and nitrate (fertilizer). Aldicarb has previously shown immune effects at current groundwater concentrations (1 ppb) (Olson et al., 1987), and it appears that atrazine can upregulate aromatase, the enzyme that converts testosterone to estradiol (Crain et al., 1997). We chose nitrate because it co-occurs with virtually all pesticide applications, especially in the spring.

We measured body weight and spleen weight, and ran tests to assess an aspect of immune function (ability to make antibodies to a foreign protein), endocrine function [T_3, thyroxine (T_4), and free thyroxine levels], and behavior (aggression tests and exploratory holepoke tests).

All the experiments used a full factorial design with three chemicals, aldicarb, atrazine, and nitrate. In these designs, each chemical was tested at two levels, a low level (−), usually zero, and a high level (+), each of which have been detected in Wisconsin groundwater. The high levels are slightly above the maximum contaminant level (MCL) for each chemical. The MCL for aldicarb and atrazine is 3 μg/l (3 ppb), and the MCL for nitrate is 10 mg/l (10 ppm); the high-level doses were 10 ppb for aldicarb and atrazine and 28 ppm for nitrate. We exposed groups of mice to all possible combinations of the two levels and three chemicals. This requires 2^3 combinations, totaling eight groups of mice. We used six mice per group in these experiments. A sample design is shown in Table 1. In addition, we ran a center replicate group in which each chemical was administered at an intermediate level between the high and low dose. A positive control group using cyclophosphamide, a proven immunosuppressant, was also run in most experiments. Thus we distributed a total of 60 mice into 10 groups in any given experiment.

We purchased the chemicals from Chem Service, West Chester, PA. We mixed chemical solutions fresh each week, and changed the solutions in the animals' water

Table 1. A typical full factorial design used in the experiments[a].

Group	Chemical			Code		
	Aldicarb (Ald) (ppb)	Atrazine (Atr) (ppb)	Nitrate (N) (ppm)	Ald	Atz	N
A	0	0	0	−	−	−
B	10	0	0	+	−	−
C	0	10	0	−	+	−
D	10	10	0	+	+	−
E	0	0	28	−	−	+
F	10	0	28	+	−	+
G	0	10	28	−	+	+
H	10	10	28	+	+	+

[a]In addition to the eight groups shown, each chemical was usually administered at an intermediate level between the high and low dose (center replicate), and a positive control (cyclophosphamide) was usually run. Group numbers were randomly assigned for each experiment, and animals were randomly assigned to groups.

bottles twice each week. We used 5 ml of ethanol as a carrier to get the atrazine into solution. We diluted this ethanol by a factor of 10^6 in preparing the final solutions. We kept all solutions at 7–9°C before use in water bottles. We wrapped the storage bottles and the animal water bottles with aluminum foil to exclude light.

The experiments used male mice. Each mouse was housed singly in its own standard plastic mouse box (28 cm × 17 cm × 12 cm), and pine shavings were used as bedding. The outbred white mice for the experiment were strain ND4 purchased from Harlan Sprague–Dawley. According to our experience, these mice are more stable immunologically than the typical, less expensive strains used in toxicological studies. The deer mice, *Peromyscus maniculatus*, came from our outbred colony of locally derived Wisconsin deer mice. We kept the experimental mice in the Zoology Department's windowless animal rooms on a reversed light cycle. We reversed the light cycle so that the mice would be 'nocturnally' active during the day when we would observe their behavior. We acclimated the mice to the light cycle change and their new quarters for 1 week before dosing began. The length of dosing varied depending on the experiment from 22 to 103 days (see Table 2).

Behavioral Assays

In the aggression tests, treated resident male mice were exposed to intruder white mice males for 5 min. Each resident male was randomly assigned to one intruder male at the beginning of the experiment. Each resident male was exposed only to its assigned intruder male at two or more week intervals throughout the experiment. Experiments 1, 2, and 5 used deer mice as the resident male; the other experiments used white mice as the resident male. In all cases white mice were used as the intruder male.

Aggressive responses were observed under a red light. Aggression was scored as follows: 0, no encounter; 1, sniffing at the intruder; 1.5, a lunge and/or squeak by test mouse with no contact; 2, contact of any kind initiated by test mouse; 3, bite, but no blood drawn; and 4, bite with blood drawn. If more than one '4' occurred during the test, the mice were immediately separated. In most experiments we tested the animals at 2–4 week intervals during the experiment (see Table 2).

Holepoke assays were done to study possible changes in exploratory behavior. We reasoned that endocrine changes, especially thyroid changes, might alter exploratory behavior. In the holepoke assays, the treated mice were placed in a 40 cm × 40 cm × 40 cm box. The floor of the box was elevated 16 cm and had four 3-cm-diameter circular holes, one at the center of each quadrant. The mice were observed under a red light for two 5-min intervals, and the number of times each mouse poked its head down into a hole was recorded.

Table 2. The experiment descriptions for a 5-year series of experiments with mixtures of aldicarb, atrazine, and nitrate.

Experiment number	Season/year	Chemicals/experiment type	Species	Assay	Behavior test	Experiment dates	Lights on
1	Fall 1990	ald, atr, n full fact	*Peromyscus*	FTI PFC	aggression	11/2/90–1/22/91	1800
2	Spring 1991	ald, atr, n full fact	*Peromyscus*	FTI PFC	aggression	3/15/91–6/25/91	1900
3	Summer1991	ald, atr, n full fact	*Mus*	FTI PFC	aggression	7/26/91– 9/24/91	1900
4	Fall 1991	ald, atr, n full fact	*Mus*	FTI PFC	aggression	10/18/91– 1/28/92	1900
5	Spring 1992	ald, atr, n full fact	*Peromyscus*	FTI PFC	aggression	2/19/92– 3/24/92	2000
6	Spring 1992	ald, atr, n full fact	*Mus*	FTI PFC	holepoke	4/8/92– 4/30/92	2300
7	Summer 1992	ald, atr,[a] n full fact	*Mus*	FTI PFC	aggression	7/3/92– 9/9/92	2000
8	Fall 1992	ald, atr,[a] n full fact	*Mus*	FTI	holepoke	10/16/92–12/15/92	1100
11	Fall 1994	ald, atr, n full fact	*Mus*	T_4 PFC	aggression	9/9/94– 12/21/94	1800

[a]Atrazine concentrations = 100/1000.

Abbreviations: ald, aldicarb; atr, atrazine; n, nitrate; full fact, full factorial design; dose–res, dose response; *Mus*, *M. musculus*; *Peromyscus*, *P. maniculatus*; FTI, free thyroid index; PFC, plaque cell forming assay; T_4, the least active form of thyroid hormone.

Plaque-Forming Cell (PFC) Assay

Four days prior to the assay, the mice were injected intraperitoneally with 10% packed sheep red blood cells (SRBC) in a solution containing 0.85% NaCl and distilled water (2×10^8 SRBC). On the day of sacrifice, mice were weighed, then anesthetized with ether under a hood. Blood was removed from the postorbital sinus and placed on ice. The mice were killed by cervical dislocation, and the spleen was removed and weighed. After removal, spleens were placed in a petri dish containing 5 ml of phosphate buffered saline with 10% fetal bovine serum. A plunger from a syringe was used to break up the spleen against a small piece of stainless steel wire mesh. The suspension was then filtered through 30 gauge Nytex to remove fat, connective tissue, *etc.* The remaining suspension was centrifuged for 5 min at 2600 rpm. The supernatant was discarded and the pellet was resuspended in 2 ml of phosphate buffered saline with 10% fetal bovine serum. Ten microliters of the resuspended material was added to 990 μl of phosphate buffered saline with 10% fetal bovine serum. One drop of prepared SRBC and one drop of guinea pig complement was added to each test tube. This was mixed and then inserted with a pipette into a preprepared slide, using both halves of one slide per test tube. The slides were then sealed with a paraffin/Vaseline mixture and incubated for one hour at 37°C. After that time, the slides were removed from the incubator and plaques counted.

Thyroid Hormone Assay

The thyroid hormone assay used blood drawn from the mice at the start of the PFC assay. The blood was drawn into tubes treated with EDTA (ethylenediaminetetraacetate) and then centrifuged at 19,000 rpm ($600 \times g$) for 30 min at 4°C. We then separated the plasma from the pellet and stored the plasma at −70°C. We used 100 μl of serum for the assays. We determined T_4 concentration and T_3 uptake with the polarized fluorescence immune assay (Abbott Labs) and somatotropin concentrations with a Quantitope HGH (human growth hormone) (Kallestad Laboratories, Austin, TX). Validation was done by comparing in-house performance values with those stated by the manufacturer for items like inter- and intraassay variation, linearity, interferences, reference ranges, *etc.* Since we were looking for treatment differences, we did not determine antibody cross reactivity. Interassay variability had a coefficient of variation (C.V.) of 7.2% at a mean of 1.4 ng/ml HGH. Intraassay variability had a C.V. (SD/mean × 100) of 4.2% at a mean of 1.4 ng/ml HGH.

Results

We summarize the data in part by presenting bar graphs, where the experiments are denoted by fall/winter and by spring/summer (Figures 3–7). We also present a table of p values from the individual experiment ANOVA (analysis of variance) tables (Table 3). For each experiment, the degrees of freedom for each statistical analysis are presented at the bottom of the column. The denominator degrees of freedom differ due to occasional death of animals and loss of some samples.

The results for body weight at final assay show few significant effects. The aldicarb × nitrate interactions in experiments 5 and 8 show moderate evidence of significance ($p = 0.0402$ and 0.0525 respectively). The results for spleen weight at final assay show several significant effects. The effect of aldicarb in experiment 2 shows strong evidence of an effect ($p = 0.0277$), as does the aldicarb × atrazine interaction in experiment 8 ($p = 0.0198$). The aldicarb × nitrate interaction in experiment 5 shows moderate evidence of an effect ($p = 0.0479$). However, there are no apparent trends for lowered values across experiments.

The free thyroxine index (FTI) is an index of the unbound thyroxine in the blood that is free to bind with cell thyroxine receptors. It is calculated as FTI = (total $T_4 * T_3$ uptake %)/100. In experiment 2, FTI results show strong evidence of an effect for aldicarb ($p = 0.0053$) and moderate to strong evidence of an effect for the atrazine × nitrate interaction ($p = 0.0432$). The atrazine × aldicarb × nitrate interaction shows strong evidence of an effect in experiment 5 ($p = 0.0149$).

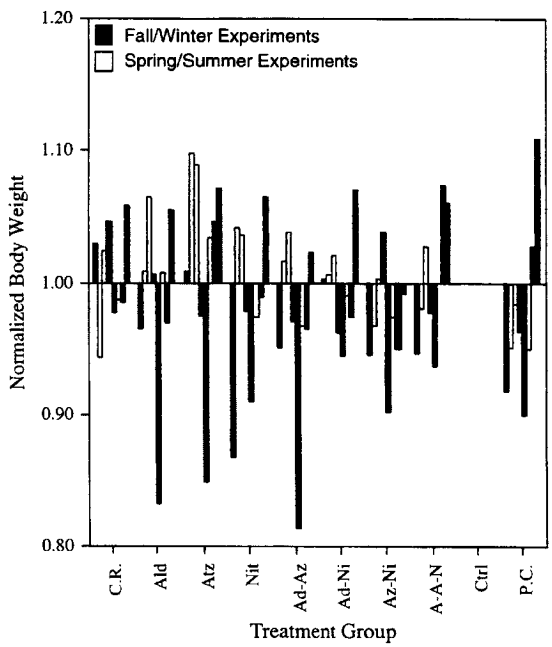


Figure 3. Final body mass (±1 SE) for each of the drinking water pesticide/fertilizer mixtures. Experiments in each group are shown in ascending order (C.R. = center replicate; Ctrl = control; P.C. = positive control). See Table 3.

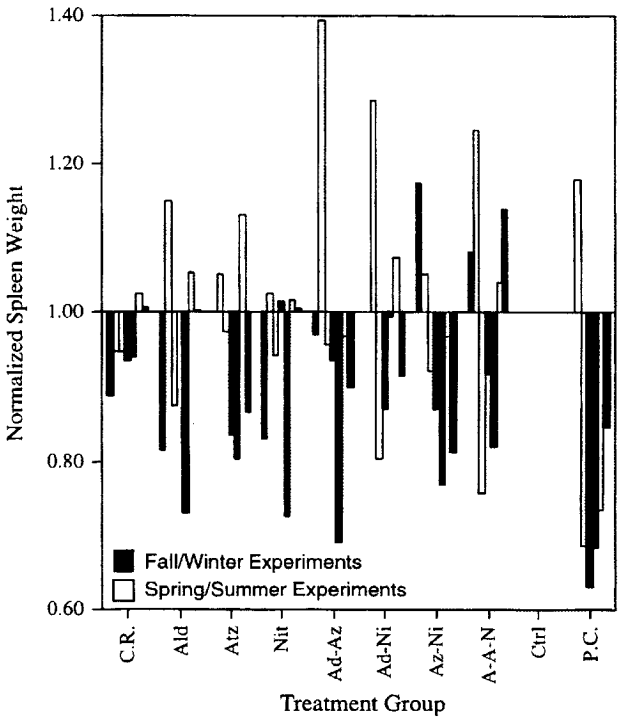


Figure 4. Mean spleen weight (±1 SE) for each of the drinking water pesticide/fertilizer mixtures. Fall and winter experiments are shown distinct from spring/summer experiments. See Table 1 for a description of the treatment groups. Experiments in each group are shown in ascending order (C.R. = center replicate; Ctrl = control; P.C. = positive control). See Table 3.

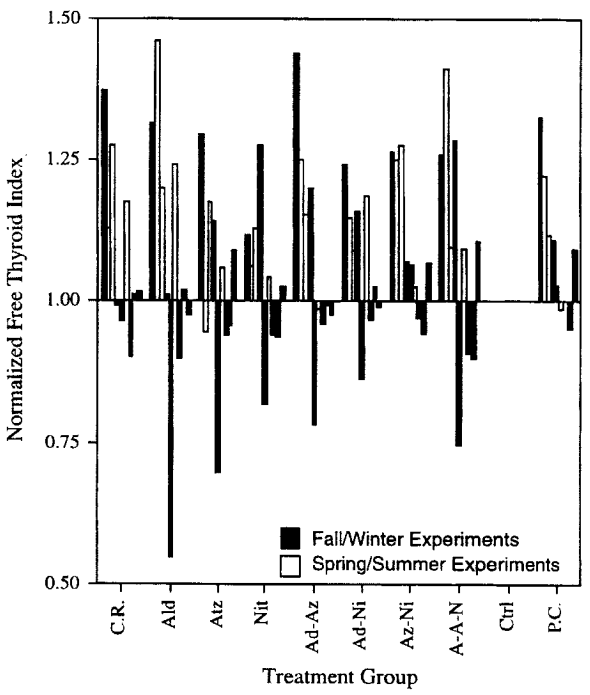


Figure 6. Free thyroxine index (±1 SE) for each of the drinking water pesticide/fertilizer mixtures. Free thyroxine index is an index of the unbound thyroxine in blood that is free to bind with cell thyroxine receptors. Experiments in each group are shown in ascending order (C.R. = center replicate; Ctrl = control; P.C. = positive control). See Table 3.

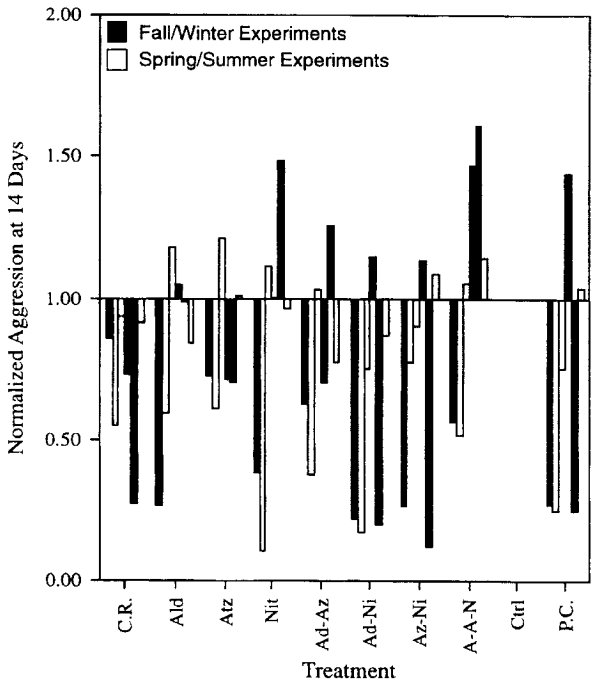


Figure 5. Aggression levels after 14 days exposure (±1 SE) for each of the drinking water pesticide/fertilizer mixtures. Experiments in each group are shown in ascending order (C.R. = center replicate; Ctrl = control; P.C. = positive control). See Table 3.

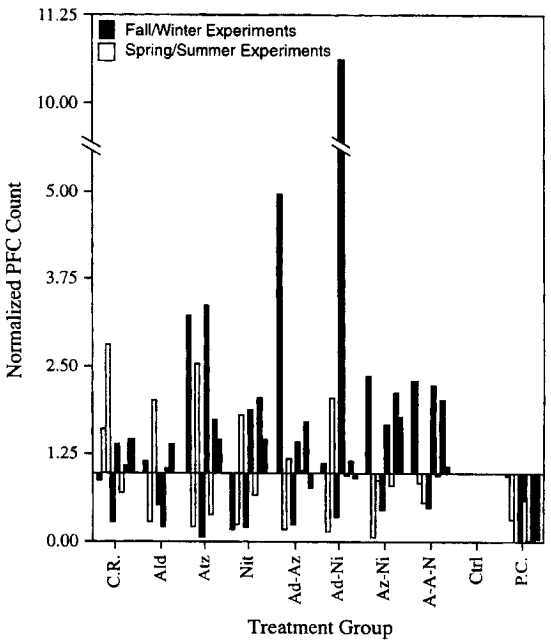


Figure 7. Plaque-forming cell index (±1 SE) for each of the drinking water pesticide/fertilizer mixtures. Plaque-forming cell index is used to assess the ability to make antibodies to a foreign protein, sheep red blood cells. Experiments in each group are shown in ascending order (C.R. = center replicate; Ctrl = control; P.C. = positive control). See Table 3.

Table 3. Summary of P values obtained from ANOVA analyses of experimental data.

	Experiment 1	Experiment 2	Experiment 3	Experiment 4	Experiment 5	Experiment 6	Experiment 7	Experiment 8	Experiment 11
Final body mass									
Ald	–	–	–	–	–	–	nd	–	–
Atz	–	–	–	–	0.1557	–	nd	–	–
N	–	–	–	–	0.139	–	nd	–	–
Ald–Atz	–	–	–	–	–	–	nd	–	–
Ald–N	0.098	–	–	–	0.0402 *	0.1713	nd	0.0525	–
Atz–N	–	0.1848	–	0.1312	–	–	nd	–	–
Ald–Atz–N	–	–	0.1008	–	–	–	nd	–	0.149
Degrees of freedom	1, 29	1, 39	1, 37	1, 35	1, 39	1, 39	nd	1, 38	1, 40
Final spleen weight									
Ald	–	0.0277 *	0.1592	–	–	–	nd	–	nd
Atz	0.1062	–	–	0.1046	–	–	nd	0.1746	nd
N	–	–	–	–	–	–	nd	–	nd
Ald–Atz	–	–	–	0.1514	–	–	nd	0.0198 *	nd
Ald–N	–	–	–	–	0.0479 *	0.1617	nd	0.1682	nd
Atz–N	–	–	–	–	–	–	nd	0.1117	nd
Ald–Atr–N	–	–	–	–	–	0.1696	nd	–	nd
Degrees of freedom	1, 30	1, 39	1, 37	1, 35	1, 39	1, 39	nd	1, 38	nd
FTI									
Ald	–	0.0053 * *	–	0.0777	0.1779	–	0.194	–	
Atz	–	–	–	–	–	–	–	–	0.1842
N	–	–	–	–	0.1942	–	–	–	–
Ald–Atz	–	–	0.1611	–	–	0.1654	–	0.1582	–
Ald–N	–	0.1418	0.1289	–	–	–	–	–	–
Atz–N	–	0.0432 *	–	–	–	–	–	–	–
Ald–Atz–N	–	–	–	–	0.0149 *	–	0.0614	–	–
Degrees of freedom	1, 26	1, 37	1, 37	1, 33	1, 37	1, 39	1, 35	1, 38	1, 35
Day 14 aggression score									
Ald	–	–	–	–	–	nd	–	nd	nd
Atz	–	–	–	–	–	nd	–	nd	nd
N	0.1662	–	0.0579	0.008 * *	–	nd	–	nd	nd
Ald–Atz	0.1948	–	–	–	0.0347 *	nd	–	nd	nd
Ald–N	–	–	–	–	–	nd	–	nd	nd
Atz–N	–	0.0547	–	0.0247 *	–	nd	0.1963	nd	nd
Ald–Atz–N	–	–	0.0077 * *	–	0.1549	nd	–	nd	nd
Degrees of freedom	1, 33	1, 40	1, 40	1, 40	1, 40	nd	1, 40	nd	nd
PFC assay									
Ald	–	–	–	–	–	0.0827	nd	0.0179 *	0.1222
Atz	0.0348 *	0.1937	–	0.1275	–	–	nd	–	–
N	–	–	–	–	0.0541	–	nd	–	–
Ald–Atz	–	0.0026 * *	0.0775	–	0.0832	–	nd	0.0362 *	–
Ald–N	–	0.0039 * *	–	–	0.0280 *	–	nd	–	–
Atz–N	–	0.0044 * *	0.0587	0.0045 * *	0.0258 *	–	nd	0.1158	–
Ald–Atr–N	–	–	–	0.1691	0.1858	–	nd	–	–
Degrees of freedom	1, 34	1, 39	1, 37	1, 35	1, 39	1, 39	nd	1, 38	1, 40

Dash is $p > 0.2$. *Significant, $p < 0.05$; **Significant, $p < 0.01$.
Abbreviations: Ald, aldicarb; Atz, atrazine; N, nitrate; FTI, free thyroxine index; PFC, plaque forming cell; nd, no data.

We chose to present data for the aggression tests that were conducted after 14 days of chemical exposure, because we have the largest body of data for this time. The 14-day aggression data showed strong evidence of an

effect for nitrate in experiment 4 ($p = 0.008$) and aldicarb × atrazine × nitrate in experiment 3 ($p = 0.0077$). Aldicarb × atrazine in experiment 5 ($p = 0.0347$) and atrazine × nitrate in experiment 4 ($p = 0.0247$) showed moderate to strong evidence of an effect. None of the significant effects show consistent recurrence across experiments, which varied in season and duration.

Holepoke experiments to test for changes in exploratory behavior did not show any significant effects and are not shown.

The PFC values show the most significant effects. The effect of aldicarb was significant in experiment 8 ($p = 0.0179$). The effect of atrazine was significant in experiment 1 ($p = 0.0348$). The aldicarb × atrazine interaction was significant in experiment 2 ($p = 0.0026$) and experiment 8 ($p = 0.0362$). The aldicarb × nitrate interaction was significant in experiment 2 ($p = 0.0039$) and experiment 5 ($p = 0.0280$). The atrazine × nitrate interaction was significant in experiments 2 ($p = 0.0044$), 4 ($p = 0.0045$), and 5 ($p = 0.0258$). This interaction was particularly striking, being significant in three experiments and also nearly significant in experiments 3 and 8. The aldicarb × atrazine interaction was also noteworthy, being significant in two experiments and nearly significant in two other experiments (3 and 5).

Tables 4 and 5 show the results of a Type III sum of squares analysis of the 2^3 factorial experiments on the ability to make antibodies to foreign proteins (PFC assay). Table 4, where the data are sorted by season, shows no significant effects for either aldicarb or atrazine alone. However, there are significant effects for combinations of the two chemicals, and for combinations of either chemical with nitrate, in both winter and spring. Table 5, where the data are sorted by duration, shows, with one exception, no significant effects for individual pesticides. However, multiple significant effects show up for all two-factor mixtures

Table 4. *P* values of effects on PFCs by season using type III sum of squares: an analysis of effects of single factor, two factor and three factor interactions of chemical effects on ability to produce antibodies to a foreign protein.

	Winter	Spring	Summer	Fall
Model[a]	0.0092**	0.0033**	0.2038	0.5508
Aldicarb	0.2111	0.8585	0.8340	0.4562
Atrazine	0.3374	0.1893	0.2986	0.8563
Nitrate	0.0541*	0.3473	0.3858	0.0664*
Aldicarb–Atrazine	0.0832*	0.0068**	0.0774*	0.5545
Aldicarb–Nitrate	0.0280**	0.0301**	0.8568	0.8312
Atrazine–Nitrate	0.0258**	0.0044**	0.0587*	0.2126
Aldicarb–Atrazine–Nitrate	0.1858	0.4041	0.2760	0.9095

*Significant at 0.10.
**Significant at 0.05.
[a]Model of overall seasonal effects.

Table 5. *P* values of effects on PFCs by duration using type III sum of squares: an analysis of effects of single factor, two factor and three factor interactions of chemical effects on ability to produce antibodies to a foreign protein.

	22 days	34 days	64 days	103 days
Model[a]	0.4229	0.0092**	0.131	0.0001**
Aldicarb	0.0827*	0.2111	0.5181	0.3432
Atrazine	0.4354	0.3374	0.6724	0.0557
Nitrate	0.9262	0.0541*	0.0591*	0.1847
Aldicarb–Atrazine	0.5274	0.0832*	0.0404**	0.0018**
Aldicarb–Nitrate	0.7507	0.0280**	0.8224	0.0025**
Atrazine–Nitrate	0.2038	0.0258**	0.2347	0.0001**
Aldicarb–Atrazine–Nitrate	0.2400	0.1858	0.1924	0.1755

*Significant at 0.10.
**Significant at 0.05.
[a]Model of overall duration effects.

in both the 34-day and 103-day duration data. Again, there is evidence that nitrate with a pesticide affects the ability to make antibodies to a foreign protein.

Discussion

Single Compounds versus Mixtures

One notable result in this 5-year study is that the trend of our results for *individual* pesticides agrees with results of standard toxicological testing protocols used for pesticide product registration. Namely, at current groundwater concentrations there is little or no biological effect. There were exceptions to this, however, namely the effect of aldicarb on T_4 and PFC and the effect of atrazine on PFC. A second notable result in this study is that effects appear frequently for *mixtures*, and the level of statistical confidence often increases in comparison to the effects of individual pesticides. Of special significance are the effects that appear when nitrates co-occur with individual pesticides (Table 3). We saw effects on aggression scores at 14 days with mixtures of atrazine/nitrate and aldicarb/atrazine/nitrate. We saw effects on body mass with mixtures of aldicarb/nitrate, atrazine/nitrate, and aldicarb/atrazine/nitrate. We saw effects on free thyroid index and on plaque forming (antibody production) with mixtures of aldicarb/nitrate, atrazine/nitrate, and aldicarb/atrazine/nitrate. Aldicarb/nitrate also affected final spleen weight.

It is not clear yet how or why nitrate in combination with a pesticide may be more active. Possibilities include reactions in acidic environments outside or inside the organism that create new potent molecules, and cellular effects due to strong ionic charges on pesticides and nitrates that collectively impact ion communication inside cells. Ammonium nitrate fertilizer has been shown to affect amphibian tadpoles (Hecnar, 1995), but no mixtures

were used. To our knowledge, fertilizer–pesticide interactions are not a part of any official federal toxicological testing protocol. This is an important shortcoming that needs further investigation, because such mixtures are ubiquitous in aquatic environments, soils, and the atmosphere.

The data presented here and prior data (Boyd et al., 1990; Porter et al., 1993) suggest that some mixtures can have effects that individual chemicals do not have. It is not known at present whether this is due to direct effects of individual chemicals on biological processes or indirect effects due to new chemicals created by reactions between the individual chemicals either outside or inside the organism. Whatever the cause, the data clearly indicate that even when doses are given in drinking water, without any of the agents in soil or air that might facilitate reactions between chemicals, more biological responses occur in the presence of *mixtures* of common groundwater contaminants than if the contaminants occur singly.

We know little at this point about the biological effects of common pesticide/fertilizer mixtures that occur due to current crop rotation practices in different regions of the country. Current testing procedures do not take into account mixtures that result from serial applications of chemicals in one field or those that result from parallel applications of different pesticide/fertilizer combinations in neighboring fields where different crops are grown.

Duration vs. Seasonal Effects

When viewed as a whole, the most striking results of the experiments are the differences from run to run. Significant single chemical effects and significant interactions occur in some experiments, but not in others. A number of unreplicated factors in the experiments could account for these differences. These include species differences (*Peromyscus vs. Mus*), exposure time differences, and seasonal differences. Data analysis suggests that seasonal differences may be important.

When these experiments were originally conceived, we were focused on duration of exposure effects. No thought was given to the possibility of seasonal effects, especially at low concentrations (Table 4). In retrospect, it was precisely at low concentrations where we should have expected seasonal effects. Disruption of the subtle *seasonal* endogenous endocrine variations by low concentrations of pesticide mixtures in drinking water or in the air should have been expected. It is important to remember that laboratory animals are not the same endocrinologically throughout the year. This seasonal endocrine variation possibility confounded the duration experiments (Table 5), and it will require further work to fully unravel what currently appears to be duration and/or seasonal effects of exposure to pesticide mixtures.

Human Developmental Implications of Thyroid Hormone Disruption

The induction of thyroid hormone changes by the low-level mixtures we used in this study raises two concerns. One concern is for postnatal individuals. In hyperthyroidism, *elevated thyroid* levels have been associated with increased irritability and quickness to anger (Whybrow, 1991a). Elevated irritability can affect the ability to concentrate and to learn (Boyd et al., 1990). In hypothyroidism, *depressed thyroid* levels have been associated with lower motivation to learn or to work at full capacity (Whybrow, 1991b). Thyroid hormones are also critical for fetal brain development (Porterfield and Hendrich, 1993; Porterfield and Stein, 1994; Hendrich and Porterfield, 1996), which could determine an individual's disposition in adulthood.

Thyroid hormone levels also affect corticosteroid levels (Cotman et al., 1987), which implies altered aggression levels and immune properties. Other possible consequences, including altered pituitary secretions from the brain, have not yet been explored in the context of mixture exposures.

Pesticide Exposure and Thyroid Hormone Changes

Thyroid hormone concentrations can rise or fall depending on the pesticide mixture and possibly duration or season of exposure. In an earlier study, metribuzin, an asymmetric triazine, *elevated* thyroxine levels (Porter et al., 1993). Atrazine, however, has been shown to cause dose dependent decreases of serum T_3 concentrations at all doses used (0.57–0.21 nmol/l) (Kornilovskaya et al., 1996). Thiram, a carbamate, can cause decreased lipoprotein lipase activity, which corresponds to the profiles of plasma lipoproteins typical of thyroid hypofunction in rats (Sadurska and Boguszewski, 1993). The herbicide nitrofen (2,4-dichlorophenyl *p*-nitrophenyl ether) 'greatly resembles thyroid hormone,' a well-known growth factor in lung development (Bos et al., 1994). Nitrofen interferes with lung development in rats and can induce diaphragmatic hernia. It seems to function by decreasing the binding of T_3 to the alpha 1 and beta 1 form of the thyroid hormone receptor in a noncompetitive way (Brandsma et al., 1994). Amitrole, a widely used herbicide, can produce thyroid and liver tumors in rodents. Twelve days of exposure to amitrole in drinking water at 200 mg/kg induced a steady reduction in plasma levels of T_3 and T_4. The mechanism of cancer induction appears to be hormone imbalance, rather than genotoxic (Mattioli et al., 1994). Fenvalerate, a pyrethroid insecticide, can decrease serum T_3 and T_4 and impact hepatic lipid peroxidation (Maiti et al., 1995; Maiti and Kar, 1997). A new acetanilide-type herbicide undergoing regulatory testing, FOE 5043, appears to reduce circulating levels of T_4 through an increase in the biotransformation

and excretion of thyroid hormone in the liver (Christensen et al., 1996). Positive trends for thyroid, testicular, mammary gland, and lymph node tumors were observed with folpet, an agricultural fungicide, in CR, CD, Wistar, or F344 strains of rats (Quest et al., 1993). In this same study, other fungicides caused an increased incidence of tumors in the kidney (captan and captafol), uterus (captan), mammary gland and liver (captafol) in the same strains of rats.

Hormone Disruption, Immune Status, and Learning Disabilities in Children

Valentine et al. (1997) explored the relationship of thyroid function in a population of children with attention deficit hyperactivity disorder (ADHD). Their results did not indicate thyroid hormone abnormalities. However, Weiss et al. (1997) point out that 48% to 73% of children with resistance to thyroid hormone (RTH) have ADHD. Treatment with liothyronine helped about half the children with ADHD and RTH. RTH appears to be due to mutations in the thyroid hormone receptor beta gene. These mutants mediate the clinical phenotype by interfering with transcription of thyroid hormone-regulating genes *via* a dominant negative effect. Wong et al. (1997) used transgenic mice to model RTH. Their study used a human mutant thyroid hormone beta 1 receptor. Transgenic mice with the mutant thyroid hormone beta 1 receptor showed 1.5-fold higher mean serum total of L-thyroxine levels than controls (nontransgenic mice), weight reduction, and hyperactivity. Thus the transgenic mice had phenotypic features consistent with the commonly observed clinical features of RTH. Interestingly, they found no significant differences in thyroid stimulating hormone levels between transgenic mice and controls. Attention deficit disorder may be divided into two distinct subgroups, one with and one without hyperactivity. Hauser et al. (1997) present data supporting the hypothesis that thyroid hormones may provide a physiological basis for the dichotomy between symptoms of inattention and symptoms of hyperactivity in ADHD.

Ziem and McTamney (1997) report that attention deficits and hyperactivity are commonly seen in chemically sensitive children. Neuroimaging studies suggest that the relevant regulatory circuits in ADHD include the prefrontal cortex and the basal ganglia, which are modulated by dopaminergic innervation from the midbrain and stimulant medications (Castellanos, 1997). Dysfunction of D2 dopamine receptors leads to aberrant substance-seeking behaviors (ethanol, drugs, tobacco, and food) and other related behaviors (pathological gambling, Tourette's disorder, attention-deficit/hyperactivity disorder) and schizoid/avoidant behavior (Blum et al., 1997). Dopaminergic neurons with melanin can be attacked by two herbicides, paraquat and diquat, which break down to MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), and induce Parkinson-like symptoms (Lindquist et al., 1988).

ADHD is complex in that there appear to be genetic and environmental factors involved (Hechtman, 1997). Multiple endocrine interactions, especially norepinephrine, epinephrine, and dopamine, are involved in modulating attention and impulse control (Pliszka et al., 1997). Some of these hormones are also involved in stress responses, which modulate immune system function.

Immune status of mothers appears to affect learning in children. McAllister et al. (1997) present evidence that maternal immunoreactivity, as represented by women with systemic lupus erythematosus, may present a special risk factor for subsequent learning difficulties in their children, particularly males.

Interpretation of the Results Using a Model of Interacting Biological Systems

Figure 8 illustrates our current conceptual model of interactive effects of environmental chemicals across multiple levels of biological organization. The left half of the figure illustrates healthy systems, and the right side illustrates consequences of some pesticide impacts. Pesticides may undermine endocrine, immune, and nervous system functions and cellular/molecular processes that depend on mass, energy, and nutrients. The availability of mass, energy, and nutrients for cellular/molecular processes depends on proper functioning of the gut and the nervous system that controls gut function. The nervous, endocrine, and immune systems are intimately interconnected and affect each other *via* extensive feedback mechanisms (Cotman et al., 1987). At the *individual* level of organization, behavior, growth, and reproduction (including developmental processes) depend on cellular/molecular and organ system functions. Individual processes support *population* level processes of birth rates and death rates and social structure. Population level processes support *community* level properties. These properties may include relative species abundance, immigration, and emigration rates. Thus, pesticide effects on processes at the organ system level and the cellular/molecular level may affect the entire superstructure by undermining or altering functions at suborganismal levels of organization.

For example, there are multiple ramifications for changes in fitness due to thyroid hormone disruption. Some of these are described in Porter et al. (1994). Changes in thyroid-hormone-controlled metabolic rate affect expenditure and acquisition of mass and energy. Changes in immune status/health status affect infection frequency and duration and survival probability. Infection/disease modulates expenditure and acquisition of mass and energy for growth and reproduction potential. Survivorship, growth, and reproduction are key variables affecting population dynamics and community structure. Thus, every level of biological organization is impacted by exposure to biologically active environmental chemicals.

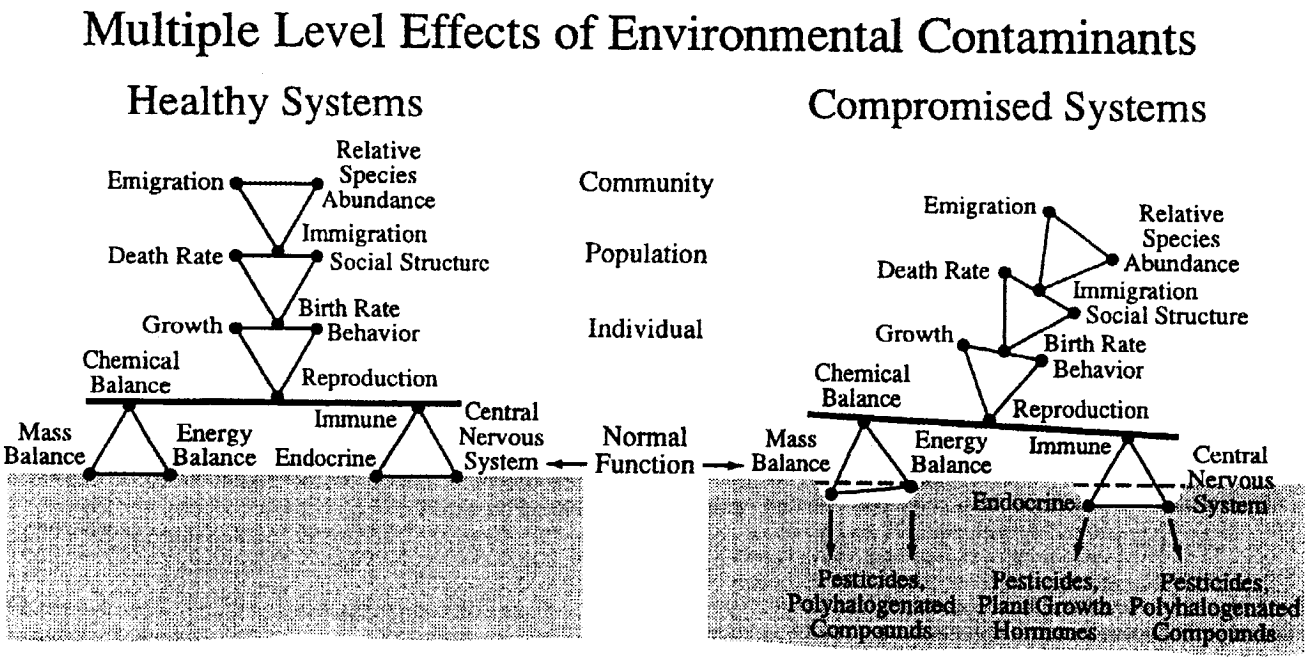


Figure 8. Multiple level effects of environmental contaminants on biological systems. The working hypothesis is that environmental contaminants compromise cellular/molecular interconnected processes that depend on mass, energy and nutrients and that compromises to the interacting organ system functions of the endocrine, immune and nervous system serve to undermine functions at higher levels of organization that depend on lower level functions. At the individual level of organization, reproduction (which includes development), growth and appropriate behavior depend critically on the interactive functions of the endocrine, immune and nervous systems, as well as cellular/molecular processes. Population level variables of birth and death rates and social structure depend on interacting individual parameters. Community level parameters like relative species abundance depend on population variables.

Shortcomings in Current Toxicity Testing Protocols

We have identified six significant shortcomings in the toxicological testing protocols currently used to register pesticides. We refer to these shortcomings by the acronym DREAMS for public awareness (see Figure 9).

Dosing deficient refers to the fact that current testing protocols do not require chemicals to be tested at low dose pulse exposures. Pulse doses of low levels of pesticides at critical times when developmental windows are open and body defenses are unable to respond may lead to permanent developmental changes in a fetus. For example, P450 enzymes or other toxicologically defensive enzymes may take from one to five days to reach maximum levels after stimulus for induction, and their efficiency varies widely within and between races of humans (Rowland and Tozer, 1989). Moreover, endocrine status, drug therapy, retinoids, and sex dependent patterns of hormonal release can slow their speed of induction. Mammalian fetal developmental patterns, especially those influenced by thyroid hormones (*e.g.*, brain development), may be altered before the parent's defensive enzymes can be induced. The same could be true of developing eggs of amphibians, reptiles, or birds. Pulse doses of pesticides in frog tadpoles and spotted salamander larvae have not shown increases in sensitivity (Berrill et al., 1995), but the experiments did not include the critical first 48 h of development when endocrine modification of gene expression is the most likely to have long-term developmental effects. It is important to remember that the embryo has almost no defensive systems against chemicals and no feedback systems to modulate chemical concentrations early in its development (Vom Saal et al., 1997).

DREAMS for public awareness

Dosing deficient – pulse doses at low concentrations not considered

Routes restricted – single exposure routes used; need oral, cutaneous, respiratory routes

Endpoints excluded – cancer and mutation endpoints used; need immune, endocrine, nervous system, and development endpoints

Additions absent – manufacturing contaminants, toxic waste contaminants deliberately added ("reworking"), and "inert" ingredients (organic soaps, surfactants, etc.) missing in "reagent grade" tests

Mixtures missing – little or no testing for commonly occurring mixtures

Stresses suppressed – nutrition, disease, climate stress not in tests

Figure 9. The acronym for six important deficiencies in current testing protocols for registered pesticides, DREAMS, illustrates how far laboratory toxicology tests are from dealing with a variety of relevant real world variables. These variables can profoundly change risk assessments of chemical exposures.

Routes restricted refers to standard toxicological tests that only evaluate one route of exposure at a time, rather than examine all possible routes of exposure (oral, cutaneous, and respiratory). Furthermore, exposure levels are typically evaluated without the organic soaps and surfactants ('inert ingredients') in commercial preparations that can alter the properties of pesticides and influence the route of exposure.

Endpoints excluded refers to the fact that toxicological tests have typically focused on cancer and mutation endpoints and have not considered other critical endpoints, such as endocrine and immune system effects.

Additions absent refers to the fact that most toxicological testing is done with highly pure forms of pesticidal active ingredients rather than with the commercial formulations, which include additives, that are actually released into the environment. There are three types of chemical additions that are missing from most toxicological testing. The *first* of these is permitted contaminants of manufacturing processes. Even though the concentrations may be 'low' from a chemist's perspective, fetuses can be affected by hormones in the parts per quadrillion concentration (Nagel et al., 1997; Vom Saal et al., 1997). Fetuses may also not respond at high doses, but only at lower physiological range doses (Vom Saal et al., 1997). The *second* of these additions is the deliberate addition of toxic waste from chemical reactor cleaning processes to subsequent products manufactured (Lea, 1991). This usage of one product to dispose of toxic wastes from prior products may pose an important health hazard. No testing is done for these random additions. Nothing is known about chemical reactions in the packaged mixtures created prior to use by the consumer. The *third* of these additions is the 'inert' ingredients (organic soaps, surfactants, *etc.*) that hasten pesticide penetration through the cuticle and the stomata of leaves. The analogous surfaces in animals are the skin and the respiratory system.

Studies using commercial formulations containing surfactants and organic soaps, rather than only the active ingredients, are showing greater biological activity. The ureic herbicide linuron (3-(3,4-dichlorophenyl)-1-methoxy-1-methylurea) affects rats' defensive enzyme glutathione peroxidase much more in the commercial formulation at all doses tested (Scassellati-Sforzolini et al., 1994). Some commercial formulations of glyphosate are much more biologically active in amphibian tadpoles than the active ingredient alone (Mann and Alexander, 1997). Surfactants appear to be the most active agents. They appear to interfere with the natural surfactants in amphibian larval skins that control ion and respiratory gas exchange (Shoemaker et al., 1995).

Mixtures missing refers to the substantial lack of knowledge about exposure effects from chemical mixtures. It is clearly impossible to examine all possible mixtures experimentally. However, common mixtures generated in specific areas due to crop rotation practices and tillage practices could be examined. Mixtures can be created by both serial applications on a given field and by parallel applications on crops in adjacent fields. Either process could result in mixtures that contaminate groundwater or occur in runoff or in the atmosphere. Analogous urban mixtures can be identified due to lawn pesticide spraying and other practices.

The literature contains few studies of toxic mixtures. The work of Yang and colleagues is an exception to this. In a series of studies using a mixture of 25 groundwater contaminants (Chapin et al., 1989; Germolec et al., 1989; Yang et al., 1989; Yang, 1992, 1994a,b; Hong et al., 1992, 1993; Kligerman et al., 1993; Heindel et al., 1994) they found relatively few biological effects. They observed alterations in hematopoietic responses, altered immune parameters that suggested immune suppression, increases in sister chromatid exchange, and enhanced myelotoxicity due to irradiation, but they did not find any treatment related effects on fertility or any measures of reproduction changes in parental or first generation offspring. The vehicles used to dissolve the lipid soluble compounds in the drinking water in their study were different from ours. This may have affected absorption and/or excretion of the test chemicals. The strain of mice used was also different than the more immunologically stable strain we used. There may also have been reactions between the large mix of chemicals in the gastrointestinal tract of the animals, which may have altered their reactivity.

Stresses suppressed refers to the fact that laboratory animals generally receive excellent care and live in an environment where climate, nutritional, and disease stresses are absent. We already know that when these stresses are present, toxic responses to registered chemicals show up that do not appear under current standard testing procedures (Porter et al., 1984).

Concerns about Possible Future Human Health Impacts

Figure 10 illustrates some of what we know about exposure during pregnancy, including the potential to interfere with development of the nervous, endocrine, and immune systems. These disruptions may come about through interactions at the genome level with retinoids, powerful developmental morphogens, and through impacts on physiological processes after birth. The question marks represent some of the next logical steps in research needed to assess whether/how pesticide/fertilizer mixtures may impact animal/human development and function before and after birth.

Koopman-Esseboom et al. (1994) present evidence of effects of dioxins and PCBs on thyroid hormone status of pregnant women and their infants. Might pesticides and/or

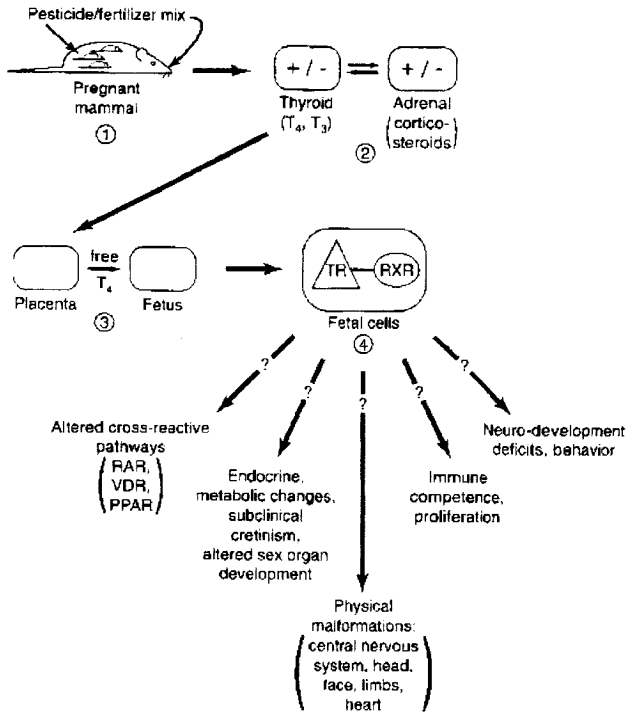


Figure 10. Schematic of the possible sequence of events resulting from chemical exposure to pregnant mammals and potential consequences for development and function of fetal nervous, immune, and endocrine systems. Here the timing of dosing may be critical. Chemical exposures to pregnant mammals may cause developmental impacts if those initial exposures occur during developmental windows that determine brain formation, sex organ development, and other events. Stress response to toxic exposure alters maternal corticosteriod and thyroid levels, which interact with each other. Thyroid hormone changes in the mother are communicated to developing fetuses across the placenta. Thyroid hormone bound to its receptors (TR) in the cells of the developing fetus compete with other receptor molecules bound to fat-soluble hormones to form heterodimers with retinoid X receptor molecules (RXR). These heterodimers activate promoter regions in the genome that initiate critical gene expressions affecting developmental programs of the fetus. Some of the other molecules that can form heterodimers with RXR include the retinoic acid receptor (RAR), the vitamin D receptor (VDR), and the peroxisome proliferator activated receptor (PPAR). RXR homodimers or RXR heterodimers formed with RAR, TR,VDR, or PPAR positively regulate gene expression through hormone receptor elements composed of tandem repeat sequences spaced by 1, 2, 3, 4, or 5 nucleotides. These heterodimer combinations represent complexes with distinct binding site preferences and represent transcription factors with multiple activation states (Mangelsdorf et al., 1994). Of special interest from a toxicological perspective is that when hormone receptor elements are palindromes, they tend to be promiscuous. For example, human osteocalcin response elements also bind VDR (Schule et al., 1990), and *Xenopus* vitellogenin A2 response elements also bind the estrogen receptor (Klein-Hitpass et al., 1986) or the thyroid hormone receptor (Glass et al., 1987). Some possible consequences of altered developmental pathways that need to be explored are also illustrated with question marks.

other environmental chemicals be associated with the developmental effects associated with the surge in learning disabilities, attention deficit disorders, and orthopedic problems exhibited by children in the United States (Childcount, 1997)?

It would appear from what we know that the following questions should be addressed (see Table 6 for a summary of research areas needing more attention). Are children's aggression levels and learning disabilities rising? Are they different in rural *vs.* urban children? Has there been an increase in juvenile onset diabetes, an autoimmune disease? Have arthritic conditions associated with autoimmune factors increased? Are there higher frequencies of joint replacements of hips and knees in at risk groups? Is chemical hypersensitivity increasing? Are infections becoming more frequent and of longer duration? Is there more frequent development of new diseases? Are the frequencies of hyper- and hypothyroidism in children, adults, and/or domestic animals on the rise?

Chronic induction of P450 defensive enzymes due to chemical exposure may alter Islets of Langerhans function in adults. Are frequencies of adult onset diabetes increasing? Are diabetes rates higher in environments of intense chemical industry activity? Will cancer rates in children and young adults continue to rise? Will more birth deformities, including undescended testes in male newborns, continue to rise? Will instances of abnormal neonatal brain development increase?

These specific neurological, endocrine, immune, and developmental questions can be addressed with epidemiological studies of high exposure groups, such as farmers, pesticide applicators, children of individuals who use lawn

Table 6. Possible human health concerns of exposure to environmental chemicals with areas of research needing more attention.

Increased frequencies of system dysfunction?
1. Nervous system:
a. Altered aggression levels?
b. More learning disabilities?
2. Immune system:
a. Increased juvenile onset diabetes?
b. Increased frequency of arthritic conditions/ joint replacements, especially hip and knee?
c. Increased chemical hypersensitivities?
d. More frequent and longer infections?
e. Faster development of new diseases?
3. Endocrine system:
a. Increased hyperthyroidism?
b. Increased hypothyroidism?
c. Increased adult onset diabetes (chronic P450 induction)?
4. Developmental disorders:
a. Continued decline in human sperm counts?
b. Continued higher cancer rates in children and young adults?
c. Increased rates of birth deformities, especially orthopedic problems?
d. More neonatal undescended testes (precancerous)?
e. Higher frequencies of brain development problems?

care products regularly, or children of individuals who use pesticides regularly in the home. Herbicide exposure has been associated with the development of neurological diseases in mature adults. A recent Canadian epidemiological study indicates that exposure to herbicides increases the probability of contracting Parkinson's disease by a factor of five (Semchuk et al., 1992). Lindquist et al. (1988) described a possible mechanism for similar effects of paraquat and diquat. Other at risk groups could include individuals living near industries, such as plastic, pesticide, or carpet/fabric production facilities.

Conclusions

Mixtures of three common groundwater contaminants in the United States, aldicarb, atrazine, and nitrate, are capable of altering immune, endocrine, and nervous system parameters in outbred white mice and deer mice at concentrations of the same order of magnitude as current MCLs. We measured aggressive behavior, thyroxine hormone levels, and ability to make antibodies against a foreign protein. Alterations in these endpoints occurred at concentrations currently in groundwater and are confined primarily to mixtures, namely nitrate plus a single pesticide. Our data indicate that season and/or duration of exposure affect the immune system response. Mice appear to be most sensitive in winter, then spring, summer, and fall in that order. This may be due to endogenous seasonal low-level fluctuations of hormone concentrations that may be altered by low concentrations of pesticides and fertilizers in combination. The effects of pesticide mixtures become more apparent when a series of experiments over time are viewed as a whole.

Thyroid disruption in humans has multiple consequences. Some of these include effects on brain development, level of irritability, sensitivity to stimuli, potential interference with ability or motivation to learn, altered immune status, altered ability to induce defensive enzymes for protection against xenobiotics, and impacts on growth potential. In amphibians thyroid disruption may alter gene expression, affecting gut function, sexual development, reproductive function, and muscle function. Thyroid disruption can also affect amphibian metamorphosis, including tail resorption, remodeling of the head, size at metamorphosis, and survivorship.

Testing protocols for pesticides currently registered for use are deficient in six testing arenas (DREAMS: *D*osing, *R*outes, *E*ndpoints, *A*dditions, *M*ixtures, *S*tresses) and do not adequately assess the potential for biological effects under real world exposure scenarios. Specific human health concerns should be closely monitored if current trends in pesticide use continue.

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