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Chapter 6

VALIDATION OF NEUROBEHAVIORAL STUDIES FOR EVALUATING THE PERINATAL EFFECTS OF SINGLE AND MIXTURE EXPOSURE TO PESTICIDES

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ABSTRACT

Laboratory studies have clearly revealed neurotoxic damage after exposure to specific pesticides and in some studies at concentrations equivalent to the environmental exposure. There are numerous opportunities during gestation where pesticides can alter the purpose of a cell, tissue, organ, or system function in the CNS. It is not uncommon for many classes of pesticides, such as insecticides, herbicides, and fungicides, to be used concomitantly. Additional work with environmentally-based mixtures is needed to test the hypothesis of dose-additivity or synergistic effects. Also, the maternal exposure to pesticides during the reproductive life may lead to animal developmental damage. Consequently, the evaluation of the possible effects due to this exposure is important to be evaluated during different developmental periods, especially those considered critical by the influence on nervous system. However, the quality and quantity of the data about the risk posed to humans by individual pesticides vary considerably and few are know about mixture pesticides effects. Consequently, sometimes it is difficult to quantify and compare neurodevelopmental impairment. The potential of pesticides mixtures to affect human toxicity or to pose a reproductive hazard to female rats exposed during critical periods of development needs more research attention. Facing this, the present study provides an overview of the theoretical discussion on potential neurodevelopmental

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effects of perinatal exposure to single or various chemical substances, considering different experimental situations and their limitations. Knowledge of data quality aids in the correct interpretation of the potential effects of a chemical pollutant exposure and opens perspectives of its use in public health. In this way, some criterions selected to the data evaluation and to guarantee the quality control are discussed, especially the dose-response effect and the variability obtained in a laboratorial test. Based on the findings from the data, a methodology was written with an overall decision rule for the acceptable behavior presented by the experimental units. It is also important to observe the possible influence of others factors interfering on results may cause experimental bias and establish a laboratorial historical control for test procedures. The criticism on the evaluation of them needs to be assessed in light of the end use of neurobehavioral results for improving the process in risk analysis to simple and mixture exposure.

INTRODUCTION

When two or more chemicals are applied simultaneously to a living system or unit, the combined effect may modify the individual toxic effect since they may have common cellular targets or metabolic pathways. Contaminated media at hazardous waste sites may contain hundreds of organic and inorganic compounds. Pesticide exposure can come from a variety of sources, including diet, drinking water, and both indoor and outdoor residential use. Despite the fact that almost all human chemicals exposure are mixtures, and that these mixed exposures occur in the context of numerous other risk modifiers, the current understanding of human health risks is based almost entirely on the evaluation of chemicals studied in isolation. Under realistic environmental conditions, concomitant or sequential exposure to pesticides mixtures dictates the necessity of exposure assessment, hazard identification, and risk assessment of them (Cory-Slechta, 2005). For example, exposure to the mixture of metals in water can carry for harmful effects in reproductive parameters of mice males (Jadhav et al., 2007). The acute and chronic health effects of individual chemicals may not accurately estimate the toxicological significances of mixtures. Methods are needed to investigate the interactions of complex mixtures (Donnelly et al., 2004).

Mixtures toxicology is proving to be different from single-chemical toxicology in several fundamental but barely recognized ways (Robinson and MacDonell, 2004). A complex chemical mixtures may consist of thousands of (often unidentified) components, each often at very low doses, but together constituting significant exposure levels and the exposure is nearly always via multiple routes, pathways. Other stressors such as noise, heat, infection, etc., may play a significant role in the overall environmental health response. The interactions are potentially many and varied: pharmacokinetic and pharmacodynamic interactions may occur at the same site, or at different sites via complex physiological processes (including defense mechanisms). Consequently, cumulative effects of different exposures/stresses over time need to be considered (altering the "baseline" susceptibility of the individual).

Attempts to deal with the problem of chemical mixtures have largely been restricted to classes of chemicals that are structurally related. While a within-class focus may be a logical starting point for evaluating the toxicity of chemical mixtures, it encompasses only a small

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fraction of the aggregate or overall exposure problem. The breadth of classes and types of chemicals to which humans are normally exposed are not structurally-related chemicals (Cory-Slechta, 2005).

Development and validation of mechanism-based models and predictive tools are essential for improving current risk assessment processes for mixtures. With mixtures, there is an added degree of complexity that requires explicit integration of multiple biochemical and physiological systems in order to fully understand many interaction mechanisms for even simple mixtures. As such mechanisms of biological processes can be embodied in inter-connected mathematical models; the scientists will develop a continually improving scientific infrastructure, which will allow one to generate better hypotheses based on plausible interaction mechanisms. Methods need to be developed to understand and integrate experimental data from the molecular to the whole organism level for understanding multiple data from mixed exposures. Understanding and prediction of precursors to adverse health effects will inevitably lead to identification of useful biomarkers of effect, and to earlier and more effective intervention strategies (Robinson and MacDonell, 2004). Therefore, it is recommendable for the modeler and toxicologist to include as much information as possible to limit uncertainties in the model. However, the models up to date have their limitations, as they cannot integrate every relevant biological mechanism, and mechanisms of activated toxicants might be quite different (Groten et al., 2004).

In managing risks to the environment and human health in modern economies, scientific evidence is a key knowledge input for decision-making (Allio et al., 2006). Many design elements impact data quality and therefore must be managed carefully for optimum outcome. These elements need to be managed in a consistent manner for optimum results (e.g., to reduce variability and facilitate interpretation of the results) (Crofton et al., 2004; Slikker Jr et al., 2005).

The most frequently studied situation in mixture toxicology is primarily to determine situations where the effects of combinations of chemicals are additive. Situations that differ from additive effects need a great deal of effort in creating statistical methods for assessing differences such as synergism or potentiation of the chemicals effects given individually. Better understanding of the patterns of exposure, the underlying variability within the human population, and the links between the animal toxicology data and human health effects will improve the evaluation of the risks to human health posed by pesticides mixtures (Mumtaz et al. 2004).

Some efforts were made to evaluate a complex mixture of pesticides. In this sense, a dietary study in rats with a combination of different groups of pesticides commonly found as residues in Denmark was made. The pesticides were alphacypermethrin, bromopropylate, carbendazim, chlorpyrifos and mancozeb. Some of these pesticides share common target organs in oral toxicity studies in rats including liver, kidney, testis, brain and nervous system. It was concluded that inhibition of acetylcholinesterase activity in plasma and brain by chlorpyrifos was not enhanced by coadministration of the other four pesticides. Effects were seen in liver, thyroid, thymus and blood in the combination groups. However, identification of the pesticide(s) responsible for these changes would require further studies of the individually pesticides as well as various combinations of the pesticides (Jacobsen et al., 2004).

Other study in relation to complex mixture aimed to analyze the alterations in the cortical and peripheral electrophysiological activity of rats acutely treated with combinations of

insecticides. Young adult male Wistar rats were treated with of insecticides dimethoate, propoxur, cypermethrin and amitraz, given alone or in triple or quadruple combinations. All treatments changed the cortical activity spectrum. The results indicate that simultaneous exposure by various pesticide agents deserves further investigation in, among others, neurotoxicological points of view (Lengyel et al., 2007).

Although standard reference texts, regulatory guidance documents, and journal articles contains useful theoretical concepts, clear definitions of most terminology, and well developed protocols for study design and statistical analysis, no general theoretical basis for the mechanisms and interactions of mixture toxicity could be discerned. There is also a poor understanding of the relationship between exposure-based and internal received dose metrics. Similarly, responses/adverse effects should be linked so that some understanding of how various effects are expressed at various levels of biological organization is also specified. Although the relative simplicity which might be achieved by focusing only an a single level of biological organization, such as the whole organism, has considerable attraction, the reality of both available data and toxicological concerns across the spectrum of the levels of biological organization dictates that multiple levels must be considered (McCarty and Borgert, 2006).

Prior workshops, symposia, and roundtable discussions have discuss a variety of aspects of the developmental and reproductive toxicological studies of a single chemical exposure, including some appropriate endpoints. Therefore, the objective of this chapter is focus on the data quality and validation aspects of methods for evaluate perinatal neurobehavior toxicity due to single or mixture exposure to pesticides. Beyond that, there is a growing concern about the quality of the test results emitted by the laboratories which is promoting the implantation of quality systems and laboratory accreditation programs by the official organs.

In this way, due to the fact that the tests for reproductive and neurobehavioral toxicity parameters of evaluation sometimes are not specific for a determined toxicant, some of the criteria generally selected for the laboratory data evaluation and quality control are the dose-response effect and the observed variability of the response, as the studies of Castro et al. (2007) and Presibella et al. (2005). The tests planning and the data interpretation obtained in the accomplished behavioral evaluations should be done carefully, considering aspects related to experimental drawing and to the method used, the tests validation, to the control of intercurrences, the variability of the data, among others.

MATERNAL EXPOSURE AND PERINATAL EFFECTS

The female agricultural work in developmental countries produces around of 60 and 80% of the food. Beyond that, 70% of the infantile work is related to agricultural sector (Dinham and Sapna Malik, 2003). In this context, there is the possibility that children's neurobehavioral performance in preschool age, as sons of rural workers, can be affected by the exposure to the insecticides in small doses (Rohlman et al., 2005).

Poisoning of newborns has been associated with maternal exposure to pesticides at the place of residence or occupation. Multiple pesticides may be present at the same time in breast milk. The quantity of pesticide that is passed to the infant via breast milk is influenced by many variables (Weiss et al., 2004). In general, in the familiar agriculture at small

agricultural communities, all take part in the planting process, combat to pests and diseases and harvest. In this way, the pregnant women are also exposed to pesticides during the gestational period (Castro et al., 1999; Araújo et al., 2007).

Age related effects on susceptibility appear to depend on the chemical of concern, the effect that is observed, the dose that is received, its duration and the period of development during which exposure occurred (Scheuplein et al. 2002). It is also noteworthy that infants may be more susceptible to neurobehavioral effects, which may not be apparent until later in life (Moser et al. 2001, Scheuplein 2002, Costa et al. 2004, Ladics et al., 2005). The consequences of the exposure during are target of preoccupation because the young organisms are more susceptible to the pesticides effects due to differences in physiology of different phases of life (Cohen-Hubal et al., 2000). For example, developing animals are more susceptible to the acute toxicity of the organophosphorus (OP) chlorpyrifos in experimental tests that may result in neurobehavioral abnormalities (Richardson and Chambers 2005).

The identification of environmental agents that have adverse effects on reproductive health and animal development is particularly challenging. Prolonged exposure to environmental contaminants at apparently nontoxic doses might represent a major risk factor for the health of children (Ricceri et al., 2006). Protecting them from the developmental hazards of environmental agents requires a strategy capable of monitoring the patterns of toxic effects around weaning age, as well as in adulthood.

The central nervous system appears to be especially susceptible to toxic insults during development and there is evidence that functional changes can be induced at a lower exposure level than those resulting in toxicity in adults. Some developmental neurotoxicants are structural teratogens as well, but behavioral dysfunctions may be more serious than structural defects under certain circumstances (Hass, 2006).

Damage to a particular structure in the circuit or a connecting pathway in the developing nervous system, more susceptible than the adult one, may produce structural or functional changes that could result in behavioral changes (Costa et al., 2004). Furthermore, the vulnerability of the developing brain to toxic insults is dependent on exposure issues and on the stage of development of the potential target organ or system. For an accurate evaluation of the effects on development induced by xenobiotic exposure, the developing organism must be exposed to the product at times when the target organ under investigation is most likely to be affected, since also the maturity of enzyme systems can influence the effects of this product (Clewell et al., 2004). The evaluation of neurobehavioral performance can be a sensitive biomarker to assess the neurodevelopmental consequences of environmental exposures, as reported in relation to pesticide exposure (Dam et al., 2000).

Neurobehavioral evaluations are widely used to examine the potential neurotoxicity of pesticides and other chemicals (Ehman and Moser, 2006). There is a variety of methodologies that can be utilized to assess these processes, which depend on factors such as cross-species generality and parallels to human behavior (Hass, 2006).

In the literature, numerous agrochemicals are described as able to change the endocrine system, since the differentiation and development of the reproductive system are dependent of the hormones action. So, chemicals with potential to affect the endocrine system can interfere in the hormones production or action compromising the sexual identity, fertility or behavior (Castro et al., 2007; Castro, 2000; Vinggaard et al., 2005). Endocrine disruptors are agents and chemical substances that promote alterations in the endocrine system. Many of them are

persistent in the environment, can be found at the rivers sediment and are easily transported at the long distances by the atmosphere (Waissmann, 2002).

Endocrine active compounds are a significant public health concern since these compounds interfere with normal function of pathways responsible for both reproduction and development. Several modes of action could contribute to the same outcome, including aromatase inhibition, anti-estrogenicity, testosterone biosynthesis disruption, and anti-androgens that alter upregulation of aromatase in the target regions within the brain. More complex biological responses will generally represent combinations of several physiological processes integrated through multiple biological pathways (Andersen and Dennison, 2004).

The developing nervous system is extremely sensitive to the organizational actions of gonadal steroids, actions that are manifested as permanent changes in both functional and structural characteristics of the brain. It is known that any manipulation of the hormonal milieu during the perinatal critical period has significant consequences on brain organization for those regions controlling reproductive behaviors and also on the modulation of behaviors not directly linked to reproduction, such as cognition, memory, and stress response (Calamandrei et al., 2006).

Neurobehavioral outcomes are important endpoints for assessing potential human health risks of environmental contaminants since endogenous estrogens, synthesized from androgens by the enzyme aromatase, are involved in organizing both the brain and sexually differentiated behavior during development and activating sexual behavior in adult rodents (Laessig et al., 2007). Behavior represents an integrated response of the nervous system that can reveal functional changes important to the overall fitness and survival of the organism exposed to sigle or mixtures pesticides.

Deltamethrin, methiocarb, prochloraz, simazine, and tribenuron-methyl are all commonly dissimilarly acting pesticides used for agricultural and horticultural purposes. Their mixture effects were analyzed for antiandrogenic effects in vitro and in vivo. The pesticides were found to act additively in vitro. In vivo, the organ weight changes indicated that the pesticides had an accumulating effect that was not observed for the individual pesticides (Birkhøj et al., 2004). Also, it was observed that the potential of organophosphorous insecticides used in combination with pyrethroid insecticides have enhanced human toxicity needs more research attention (Perry et al., 2007).

Data Quality and Critical Evaluation of Experimental Data

In order to accomplish a good experimental planning, some points before the beginning of the study should be observed, as: a) the problem should be defined carefully based on previous evidences (association cause-effect consistent); b) the study goal should be clear; c) the data collection should be rigorously planned with emphasis in criteria's choice of the experimental and statistical methods aiming at reproducibility of the obtained data; d) the possibility of adequate human and physical infrastructure and; e) the adequacy of the data interpretation in view of the experimental delineation.

The principal purpose of a study is an important consideration for test selection and study design; namely, the term validation is used in many ways and includes animal-to-human

predictive validity and construct validity. Validation can be local (specific for that laboratory) or global (generalizable and reproducible across laboratories). The validation process should include data to show that the equipment works as it should (hardware and software), that the equipment tests the behavioral domain of interest, and that the results are robust, reproducible, and relevant to the behavior (or effect) of interest. The validation should include evaluation of appropriate installation as well as defining the range of use of equipment. Also, it should be accomplished the facilities environmental controls (Slikker Jr. et al., 2005).

The discussion of experimental subject variables that can confound treatment effect includes species, strain, and developmental age. Differences in developmental age in breeding studies with postnatal components were seen as a common source of variability that could confound interpretation of effects, e.g., developmental delays.

One of the oldest principles in biomedical research is to guarantee comparability and reproducibility of results within and between laboratories. This is especially true for testing for regulatory purposes. Among others, experimental conditions are believed to affect experimental results (Verwer et al., 2007, Slikker Jr. et al., 2005, Festing and Altman, 2002).

In view of that, housing variables including diet, single vs. group housing, wire vs. plastic cages, and whether animals of the opposite sex were housed in the same room. Depending on the endpoints of the study, the type of housing condition must be taken into consideration since they could have great implications for the interpretation and validity of results from toxicological assays and the number of animals needed to detect significant effects of toxic compounds (Verwer et al., 2007). To ensure the scientific validity of experimental data, scientists must be aware of the complex nature of the environment in which their animals are maintained (Weed and Raber, 2005).

Diet was identified as an important factor to be examined, as illustrated by the potential effects of soy (high estrogen content) in diets or in food rewards/reinforcers/treats as confounders in behavioral/hormonal studies. Group housing of same sex subjects seems to be the preferred choice, as it was thought to be a more humane and natural environment (Slikker Jr. et al., 2005, Festing and Altman, 2002). Counterbalancing the order of testing among test groups is a critical control point to reduce the bias on results. The variability introduced by the experimenter may arise from two sources: the conduct of procedures involving the animal (e.g., injection, oral dosing and surgical intervention) and lack of precision with measurements (Slikker Jr. et al., 2005).

Thus, regarding the tests with animals it owes pay attention at a) the number of animals used b) the biological meaning of the observed alterations, c) doses used are generally established between those that do not cause effects and those that produce the studied toxic effect (dose-answer); carrying in consideration the exposure real probability in the environment, and d) animal age or developmental critical period that affects the experimental planning. Since several factors can affect the experimental result (Aldridge et al., 2003), it is important during the tests accomplishment to standardized the correct animal manipulation and a data control that allows the identification of the animal used.

Predicted effects following exposure to various mixtures of chemicals are initially evaluated for the non-interaction of toxicants. The dose levels combinations may be selected based on selected effect as reproductive toxicology studies previously conducted in laboratory with each pesticide separately (Presibella et al., 2005). It also may be selected based on the presence of pesticides in biological samples as urine (Perry et al., 2007), food, water and the environment. Beyond that, the estimated dose to be used during mixture pesticides tests may

be calculated based on mathematical models as those using DL_{50} and toxic-unit concept calculated by a dose-response curve for each chemical. The toxic-unit was then used to classify each mixture response as additive, greater than additive, or less than additive (Mahar and Watzin, 2005).

Other important aspect is to define comparability factors before initiate the evaluation, verifying if the profiles used in the study are the same. Thus, the results obtained with the same experimental delineation can be compared. If a scientist wishes to explore measurements or assessments of several types of behavior (or any other information), it is important to realize that the information types could be correlated (Howard, 2002). Sometimes responses to the same essential questions are sought in several independent experiments or trials from different investigators.

It is important that the observed effects occur due to exposure to the substance test and not mother toxicity. In mammals, the normal performance of the maternal functions for the nestling health is indispensable. The development of major regulatory systems underlying behavior and physiology in the neonatal rat is primarily determined by the mother, who serves as the primary source of nutrition, grooming and warmth required for immediate survival (Huot et al., 2004), thereby playing a crucial role in the development of the architecture of the brain after birth. The quality of mother–infant relationships and the juvenile's subsequent social milieu are considered to be crucially formative for adjustment and social competence in adulthood. In most mammals, the mother–infant relationship is terminated at weaning (Ferdman et al., 2007). It is also important to note that the neurobehavioral genetics and some almost undetectable environmental differences may have large behavioral consequences (Smith and Corrow, 2005).

In the same direction, given the close integration of HPA function and behavior and particularly behavioral responses to stress; prenatal glucocorticoid manipulation leads to modification of behavior, brain and organ morphology, as well as altered regulation of other endocrine systems. Exposure of the fetal brain to excess glucocorticoid can have life-long effects on neuroendocrine function and behavior (Owen et al., 2005). The development of the HPA-axis, limbic system, and the prefrontal cortex are likely to be affected by antenatal maternal stress and anxiety (van der Bergh et al., 2005).

The experimental delineation have to consider the impact of maternal toxicity on hazard identification and/or risk assessment in reproductive toxicology; since maternal toxicity, particularly with the use of a maximum tolerated dose, has an impact on the various studied endpoints (Ladics et al., 2005). The traditionally signals observed of maternal toxicity in experimental studies are decrease in the weight gain (the females weight generally is observed during the whole test period), gestational period, water and food consumption decrease, clinical signals and mortality. For that, there are some methods described to evaluate the maternal behavior (Champagne et al., 2003, Slamberová et al., 2005).

The influence of litter must be taken into account in the allocation of test animals as well as the statistical analyses in developmental neurotoxicity studies (Holson et al., 2007). In respect of the litter, it is consider as the experimental unit. It can be used in case of time or cost limitations, one animal for sex per litter randomly chosen (in the risk evaluation of a substance) or to consider the average response of two or three pups as the unit of the experimental answer (Buelke-Sam et al., 1985; Holson and Pearce, 1992; Haseman et al., 2001); due to the response variability among pups of the same litter (Ulbrich, 2001). Litter must remain a factor in analysis throughout the study, not just in young animals.

The method validation is designed to determine the operational characteristics of a test, that is, its reliability and relevance, in addition to its strengths and limitations. For a method validation, it is important to verify, using test subjects, that the results are reproducible across time. The performing laboratory should maintain a historical control database to track any changes in the data over time in the animals and/or in the equipment. The value of historical data depends on its quality and its reliability. Many factors (e.g., strain, origin, associated microflora, housing, husbandry, and methods of measurand each outcome) can influence individual results so that in nearly all studies, contemporary controls are almost essential, and historical data, particularly from another laboratory should be treated with considerable caution.

Positive control data are instrumental in evaluating laboratory proficiency in detecting chemically-induced changes in measured endpoints of developmental neurotoxicity testing that involve functional and neuropathological assessments in offspring during and following maternal exposure. Positive control data are valuable in a weight-of-evidence approach to help determine the biological significance of results and provide confidence in negative results from these studies. Comparison of historical controls levels and effects of positive control, both within and between laboratories over time, will assist in interpretation of results, and will serve to further increase the level of confidence in the proficiency of the testing laboratory (Krofton et al., 2007).

To compare results obtained at different occasions and/or laboratories, methods of meta-analysis may be appropriate in some cases (Trksak et al., 2007). Formal methods of "meta-analysis" have been developed that attempt to combine the results of different experiments taking account of sample sizes and apparent quality of the data. Meta-analysis provides a tool to statistically aggregate data from existing experiments, so that the results can be summarized across a range of conditions and an increased pool of experimental data can be subjected to statistical analysis (Moher et al., 1999). However, when similar experiments are performed repeatedly in the same laboratory with the standardization of the same strain and sex and using a standard protocol that includes contemporary controls; there will often be scope for using historical data.

The sensibility of a test refers to the ability of this test in detect alterations in the answer that is being evaluated, preferably in doses below of that produces evident signals of toxicity by the agent in study. The behavioral biological responses can present great variability. In fact, some parameters present great variation among individuals.

Discussion of how to define a desirable/achievable level of variability in control responses in common behavioral studies proved to be too specific to address without having specified a set of tests or having provided data for evaluation (Slikker Jr. et al., 2005). Because of their size and complexity, traditional validation studies can consume valuable resources and use large numbers of research animals. This is particularly true when prenatal exposures to chemicals are used to validate multiple behavioral tests in the context of developmental neurotoxicity studies (Marable and Maurissen, 2004).

VARIABILITY OBTAINED IN THE EXPERIMENTAL DATA

In case of experimental data with high variability of response, the difference among treatments cannot be easily detected. In order to work with a considered high variability it should used an experimental delineation in which the variation between individuals is minimized in order to evidence the statistical meaning of the interest effects for the study (Haseman et al., 2001).

The approaches developed for chemical analyses in relation to uncertainty estimation and method validation are not directly applicable to biological essays. Therefore, if rigorous/statistically valid calculation of uncertainty is not possible, the uncertainty components are to be identified, and to be reasonably estimated.

Uncertainty in variables of an exposure model comes from many sources including instrument error and population variability; and can be grouped as systematic error (i.e., bias) and random error. The systematic error refers to the exactness that a determination is made. The exactness lack promotes the called bias or tendencies. It can cite as example problems with the calibration, equipment or reagents, etc. Then, the random error refers to the precision of a method, evaluated by the repeatable analysis performance of a sample. He provokes inconsistent changes in the system, causing dispersion around the respective average value. The examples are sampling mistake, pipetting, mistaken annotations, etc. However in neurobehavioral methods, it is difficult to establish a consistent uncertainty estimative due to the large variability of the population. It should be developed a control plan to assure that the variability keeps inside limits allowed for each methodology to establish the control of important experimental parameters.

The factors related to data variability were seen as the same variables that broadly influence behavioral testing in general, other than the obvious parameters of the test procedure (Slikker Jr. et al., 2005). A question was raised about defining a desirable/ achievable level of variability in control responses in behavioral studies.

A process can be considered reliable if the registered data is situated inside the control limits and the variability of the results is stable. In order to accomplish the conformity evaluation of the control data it should be know the analysis process and establish the criteria to verify whether the observed alterations are caused by inherent variations stable through time of the system. Checking on a determined day of test if all controls remain inside of a selected criterion of variability, the possibility of detecting anything other than the normal animal behavior variability would be negate.

The point is establish what are the principal confounds to be considered in behavioral studies and how are they to be dealt with and what are the principal variables and how they can be evaluated and controlled. Following, are presented some considerations about of the theme.

The standardized maintenance of the controls organism test and the experimental conditions aid the retrospective comparison of the method data through different occasions; facilitating the analysis of the observed response variability in the groups control accomplished at the same laboratory (Festing and Altman, 2002). Due to several possible interpretations, it is important to supply specific instructions for the controls analysis, explaining how to interpret the results and what to do with them. For that, it is necessary to define rules of data acceptance and rejection by the clear establishing of these limits. Thus, a

laboratory can build historical control graphs to measure and to analyze the variability in the procedures and methods used.

Biomarkers are used in numerous areas of research. The biomarkers use presupposes the determination of what the measurand is and your numeric referential for its comparison. The biomarkers owe preferentially reflect an increasing answer as the exposure dose increases, considering the frequency normal distribution (Gaussian) in a population, where the answer is small arriving to a maximum and declining again.

In general, the superior and inferior limits are established as 3 deviations-standard around of the average, with 99.73 % of chance of all measured are inside this band according to the normal distribution. Results with values outside the established limits will be considered as outside the standard after data careful evaluation.

However, in a complex biological system, sometimes it is not possible to obtain monotonic or biphasic curve in response to a toxicant. The absence of such a response may result from reactions of a complex biological system to a toxicant, nonmonotonic (biphasic) dose-effect relationships (Conolly and Lutz, 2004), since not all brain regions develop in the same time course. Indeed, there are gradients of maturation across modalities and hierarchical levels of processing within a given pathway and neuronal circuits are shaped by experience during critical periods of early postnatal life (Costa et al., 2004, Hensch 2004). The dose-response curves of different endpoints may be best described with nonlinear models or have different shapes or regions of activity (e.g. increasing and decreasing functions, different slopes or plateaus, etc.) (Coffey et al., 2007). Therefore, it is not always possible to express direct correlations between the observed effects and the exposure (Alonso et al., 2004) since multiple types of outcomes are sometimes measured on each animal in toxicology dose-response experiments.

Example of Quantifying a Neurobehavioral Test: Open-field

There are several parameters used to characterize motor performance. Of these, the open field seems to be able to provide a good measure of the approach response toward novelty (exploration) and describe influences of drug exposure (Prut and Belzung, 2003, Ehman and Moser, 2006), even if exploratory activity and emotional reactivity can interfere with one another (Lehmann et al., 2000, Zimmermann et al., 2001). A number of potential confounders could have produced or masked differences among the experimental groups. In addition, motor and/or sensory changes could either mask or exaggerate cognitive dysfunctions (Ehman and Moser, 2006).

One can observe that so many factors can affect the response in this test. For example, in a crowding cage that is considered to be a model of social stress, it appears that the effect of stocking density differs depending on the developmental stage of the animal: Juvenile rats increased anxiety following limiting space, whereas adult rats increased activity following increase in social tension (Arakawa, 2005). Also, litter is a significant source of variation in results obtained from all behavioral measures either on physical endpoints or during the pre-weaning period by potential maternal behavior components (Buelke-Sam et al., 1985; Holson and Pearce, 1992).

As to the face and construct validity of the open-field model, one may propose that it is fulfilled into the criteria of predictive, face and construct validity to be a relevant model of human behavior. In fact, face validity implies that the anxiety response (the phenomenological aspect) observed in the animal is identical to the one observed in humans. In the open field, the observed behavior is avoidance of threatening places, which can also be observed in humans. In rodents, forced confrontation with novelty is stressful. Stress induces anxiety-like behaviors, as it does in humans. So, the model may also fit construct validity (similar etiology) and may be a rodent model of normal anxiety (Prut and Belzung, 2003).

The open-field test is also interesting because locomotor's activity can serve as a tool to evaluate the effects of stressors that alter the functioning of the hypothalamic-pituitary-gonadal (HPG) axis (McCormick et al., 2005), that is one of the targets of chemical endocrine disruptors. In addition, the medial hypothalamus could play an important role in the modulation of defense responses measured by rat exploratory behavior in open-field and elevated plus-maze tests (Jardim and Guimarães, 2004).

The results of the open-field arena locomotion test can reveal changes related to fungicide fenarimol exposure during the perinatal and lactation periods, with a consequent decrease in locomotion, mainly during lactation (Castro et al., 2007). Fenarimol acts as a genotoxin, and possesses estrogenic properties and acts both as an estrogen agonist and as an androgen antagonist (Castro et al., 2005; 2007). These findings could indicate long-term neurotoxicity (Viberg et al., 2004), although they do not shed light on the question of whether the lower number of units recorded is related to a motor effect or to other factors such as alterations to the physiological systems that control anxiety, exploratory activity and emotional reactivity (Zimmermann et al., 2001).

However, some important analytical key steps need to be controlled in order to avoid many sources of error and bias. It is important to control full range of laboratory quality control measures, to limit variability and demonstrate that methods are in control. Keeping these points in view, it is important to determine the normal fluctuation of a tested behavior, as the general activity in an open-field arena. The normal fluctuation can be considered as the variability in behavior due to animal biology and not due to some non-random cause as a stressful situation or environment. So, this fluctuation can be established from the laboratory historical data in each behavioral test.

To determine the normal fluctuation of a control group in open-field behavior, one can use the coefficient of variation (CV). However, the CV is good only for comparing variability of samples, independently of the time of the observations. As we are studying effects of pesticides during several days, although the CV detects changes in the average, it cannot show the real modifications; for example, a mean equal to 100 and standard deviation (SD) 10 give a CV of 10%. If the mean changes to 1000, and the SD to 100, both mean and variability have changed but the CV remains the same.

For this reason, we propose a new approach for interpreting the results of behavioral experiment, as provided by the present example of open-field data. We know that animal behavioral variability depends on several causes, and we need to simplify the problem. So, after the exposure to the same test condition, if the behavior of a group of rats is different from another group of rats not exposed to chemicals or other physical and environmental parameters in the same experimental conditions, we need to investigate if there is an assignable cause or it is only a natural variation. The aim is to propose a limit for this decision.

In this experiment, rearing, locomotion and immobilized time frequencies were observed during the gestational period in two phases: Phases 1, rats exposed during days 1 to 6 of pregnancy, and Phase 2 exposed during days 6 to 15 of pregnancy.

After understanding the experiment, we begin search a statistical model for modeling the data, and the first one was to observe the individual behavior of the rats. For example, the rearing frequency, data of 44 rats whose mothers were manipulated during the gestational period are showed in the Figure 1, where D21, D30, D60 and D90 are the days of observation.

However, how we deal with rearing frequency that surpasses other observations for the same group? For this reason, as we have studied, initially, the parameter rearing, observing the rats' behavior, and constructing the following behavior models:

(a) individual by using a control-chart for individual measurements,
(b) litter average, using a control-chart for grouped data, and
(c) litter averages, using an analysis of variance (ANOVA).

We have studied individual behaviors for days 21, 30, 60 and 90, Phases 1 and 2, and Phase 1 for the litter averages and ANOVA, as following described.

a. Individual as an Experimental Unit

For individual observations, the sample size is 1, and for measuring the parameter we have used the moving range of two successive observations.

The moving range is defined as

$$MR_i = |x_i - x_{i-1}|$$

which is the absolute value of the first difference of the data (the difference between two consecutive data points).

The control-chart lines for individual measurements are calculated using the following expressions:

$$UCL = \bar{x} + 3\frac{\overline{MR}}{1.128}$$

$$Center\ \text{Line} = \bar{x}$$

$$LCL = \bar{x} - 3\frac{\overline{MR}}{1.128}$$

UCL stands for upper control limit, LCL for lower control limit, $\bar{x}$ is the average of the rearing frequency measurements of all rats, and $\overline{MR}$ is the average of all moving ranges of two rats observed in a random manner.

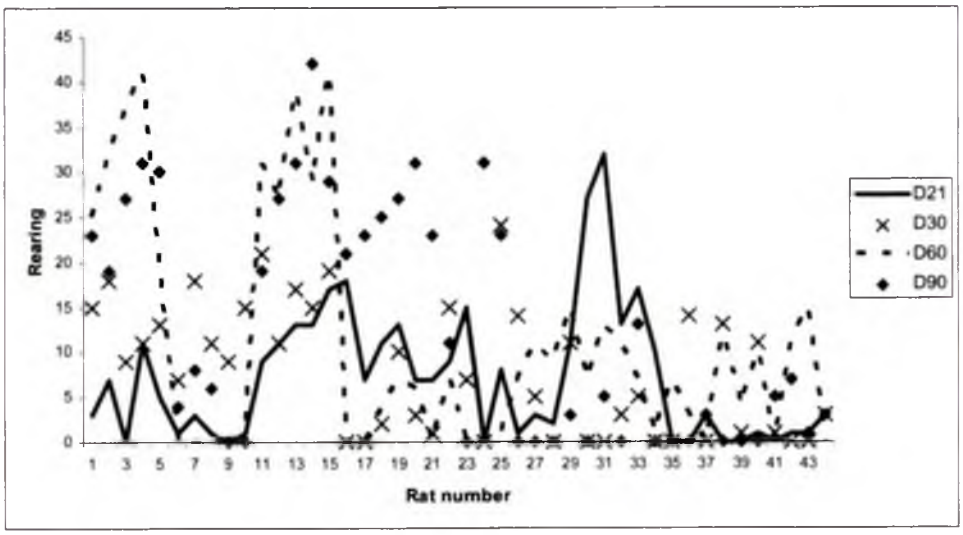

Figure 1. Rearing of rats in an open-field arena at different postnatal days (individual observations).

Using the data for rearing, D21, Phases 1 and 2, we have: $\bar{x}$ =7.16 and $\overline{MR}$ = 4.65. Then,

UCL = 16.3211, rounded to 17.
Center Line = 5.505495
LCL = 0 (if the calculation is negative, LCL is set to zero)

Figure 2 illustrates this control-chart for individual measurement for rearing, Day 21, Phases 1, exposed during days 1 to 6 of pregnancy, and Phase 2 exposed during days 6 to 15 of pregnancy, whose data are on the Annex.

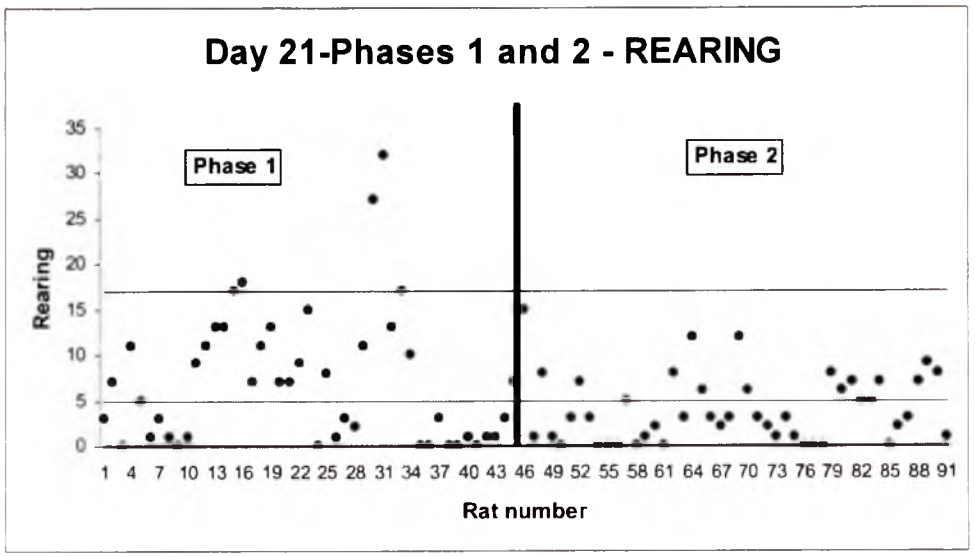

Figure 2. Control-chart for individual measurement of rearing frequency for Day 21. Phases 1 and 2.

It's reasonable to consider that the limits of decision are 0 and 17, that is, we accept a maximum of 17 and a minimum of 0 for a normal behavior. Using these limits, the rats with a non-normal behavior are three, quantifying 3.3% (3/91 x 100%0) of all rats. If we define that a maximum of 5% of the rats' out-of-control is acceptable, then we do not need to redo the experiment.

b. Grouped Data by Litter as an Experimental Unit

Control limits for grouped data is similar as we get samples of size n, when use n=4 in this example.

The control-chart lines are calculated using the following expressions:

$$\text{UCL} = \bar{\bar{x}} + 3\frac{\bar{s}}{\sqrt{n}}$$

$$\textit{Center}\ \text{Line} = \bar{\bar{x}}$$

$$\text{LCL} = \bar{\bar{x}} - 3\frac{\bar{s}}{\sqrt{n}}$$

UCL stands for upper control limit, LCL for lower control limit, $\bar{\bar{x}}$ is the average of all litter averages, and $\bar{s}$ is mean of all standard deviations of the rearing measurements of all rats observed in a random manner.

Using the data for rearing, D21, Phases 1 and 2, for litter whose mothers were manipulated during the gestational period, we have: $\bar{\bar{x}}$ = 7.30 and $\bar{s}$ =.3,65. Then,

$$\text{UCL} = 7.30 + 3\frac{3.65}{\sqrt{4}} = 7.30 + 5.48 = 12.78, \text{ rounded to } 13.$$

Center Line = 5.505495

$$\text{LCL} = 7.30 - 3\frac{3.65}{\sqrt{4}} = 7.30 - 5.48 = 1.82, \text{ rounded to } 1.$$

Figure 3 illustrates this control-chart for 4 pups from the same litter, Day 21.

It's reasonable to consider that the limits of decision are 1 and 13, that is, we accept a maximum of 13 and a minimum of 1 for an average normal behavior. Using these limits, and considering a sample of nine litters (litter 8 was excluded, as it had only 3 observations), there is only one without a normal behavior. So, the percentage of litters with a non-uniform behavior is 11% (1/9 x 100%) of all of them.

If we define that a maximum of 5% for litters' out-of-control is acceptable, then we need to redo the experiment.

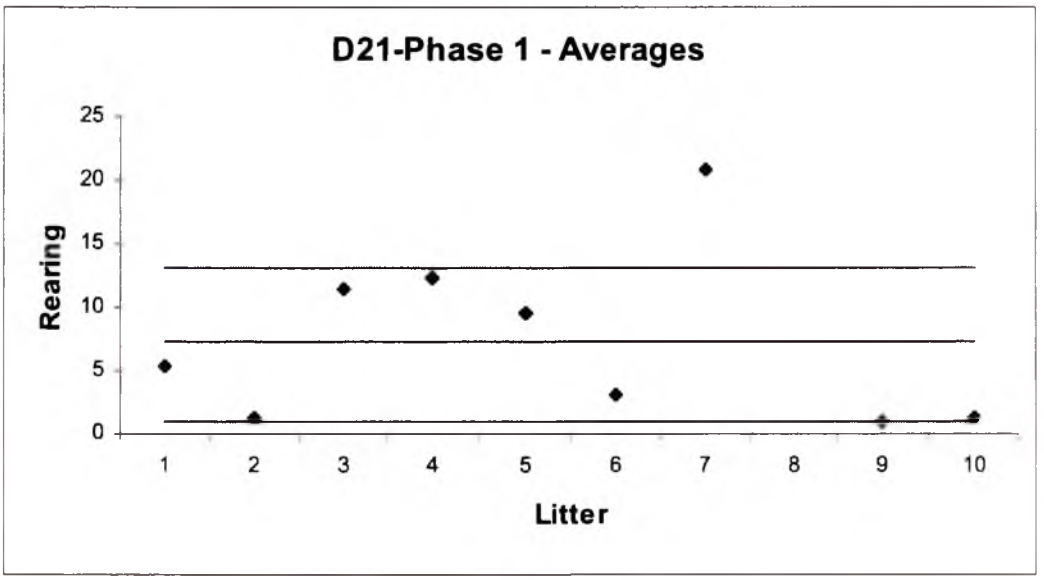

Figure 3. Control-chart for litter averages rearing frequency for D21.

c. Analysis of Variance (ANOVA) for the Litter Averages

The results for this line of thought is on Table 1.

If the null hypothesis is rejected, significance level of 5%, the decision rule is the following: we do not to redo the experiment.

As we see in this example, the null hypothesis that all the litter averages are equal is rejected. For this reason, at least one of the averages is different from the others, and we need to redo the experiment.

A summary for the rearing frequency data, whose mothers were manipulated during the gestational period on D21, is the following:

Individual behavior, Phases 1 and 2	do not repeat
Litter averages, Phase 1	repeat
ANOVA, Phase 1	repeat

However, for not losing all the data, we have applied the Tukey's test to identify which litter has an average different from the others. For this example, the results were: L1 different from L6, L7, L8, L9, and L10; L2 different from L7, L8, L9, and L10; L3 different from L8, L9, and L10, and L4 not different from L5 to L10. For this reason, we can make a partition in two groups: (L1, L2, L3) and (L4, L5, L6, L7, L8, L9 and L10). For this reason, the candidates for a new experiment are L1, L2 and L3, because their group is smaller than the other one.

Table 1. ANOVA for rearing, Day 21, Phases 1 and 2

Source	SS	df	MS	F	P-value	Critical F
Between groups	1725,119697	9	191,6799663	9,833202518	3,18795E-07	2,169562317
Within groups	662,7666667	34	19,49313725			
Total	2387,886364	43				

Based on these findings from the data of the samples for rearing, we can write the following methodology with an overall decision rule for the acceptable behavior presented by the rats and, in consequence, if we redo or not an experiment:

Step 1. Observe the rats regarding the characteristics being studied, and register the results of them.

Step 2. For each characteristic, calculate the control limits for the individual observations and litter average, according the following expressions:

individual rats

$$UCL = \bar{x} + 3\frac{\overline{MR}}{1.128}$$

$$Center\ \text{Line} = \bar{x}$$

$$LCL = \bar{x} - 3\frac{\overline{MR}}{1.128}$$

litter

$$\text{UCL} = \bar{\bar{x}} + 3\frac{\bar{s}}{\sqrt{n}}$$

$$Center\ \text{Line} = \bar{\bar{x}}$$

$$\text{LCL} = \bar{\bar{x}} - 3\frac{\bar{s}}{\sqrt{n}}$$

Step 3. Verify how many rats for individual control or by litter are out of the limits, and calculate the respective percentage.

Step 4. If the percentage is greater than 5 (five) % in both situations, the experiment must be performed again.

Step 5. If the percentage is less than 5 (five) % in one or both situations, perform an analysis of variance (ANOVA) regarding the litter averages.

Step 6. If the ANOVA null hypothesis is rejected, perform the Tukey's test, and identify the different sets of behavior. Redo all the experiment for the smaller set.

Step 7. Continue the study in such a way that the assignable cause for the difference in the litters can be found

This chapter emphasizes that it is necessary to have a clear decision rule, based on the data, because statistics is a real experimental science. For this reason, if the model diverges from the reality, the model is wrong, not the nature. It can be concluded that on neurobehavioral tests, the diminution of the variability in the animal performance response to a stimulus is a big challenge to the scientists. In a near future, criteria related to the accepted behavioral needs improve when more data are available. The approach to be used to estimate the behavior change obtained during mixture pesticides tests and its potential applicability in additive and synergistic responses needs careful evaluation.

PERSPECTIVES OF NEUROBEHAVIORAL PERINATAL STUDIES

It can be conclude that the utilization of an experimental protocol containing indices related to reproduction and animal development can identify initial damages due to the environmental pollutants exposure. The protection of possible damages due to pesticides at the development of the young organisms, like the newborn and children; requires an integrated strategy able to monitor the standard use of these products and the integration in the potential effects evaluation improvement.

The near future requires the ability to see how individual compounds affect signaling networks and how mixtures of compounds affect a common physiological endpoint by either similar or diverse modes of action in the body (Andersen and Dennison, 2004). In this sense, the study of subtle effects through new batteries of experimental tests promoting biomarkers more specific should be a complement of the traditional evaluation approach for pesticides in young animals. Sharing this information is important for the improvement of the perinatal evaluation, in order to the implementation of a regulatory approach that minimizes the possible risks to what the young organisms.

In order to reduce the uncertainty of the estimates regarding the exposure to these products in order to protect adequately the children, it can be suggest to address these aspects in various areas of research as: a) kinetic differences of an agrochemical in young and adult in order to subsidize information regarding the exposure of these products in children; b) appropriated investigation to sustain the safety use of the compounds regarding the habits of the infantile population, for example, the diet (Pennycook et al., 2004); c) improve the pre and postnatal developmental parameters evaluation; d) promote more specific biomarkers study; and e) promote advances in ecogenetics through the interaction gene-environment-health study (Hubal et al., 2000).

ANNEX:
DATA FOR REARING, DAY 21, PHASE 1 AND 2

Control Group			Rat	D21	D30	D60	D90
Phase	Dose	Litter					
1	1	1	1	3	15	25	23
		1	2	7	18	32	19
		1	3	0	9	37	27
		1	4	11	11	41	31
		1	5	5	13	19	30
		2	1	1	7	0	4
		2	2	3	18	0	8
		2	3	1	11	0	6
		2	4	0	9	0	0
		2	5	1	15	0	0
		3	1	9	21	31	19
		3	2	11	11	27	27
		3	3	13	17	39	31

		3	4	13	15	29	42
		3	5	17	19	41	29
		4	1	18	0	0	21
		4	2	7	0	0	23
		4	3	11	2	4	25
		4	4	13	10	7	27
		4	5	no data	no data	no data	no data
		5	1	7	3	6	31
		5	2	7	1	0	23
		5	3	9	15	7	11
		5	4	15	7	0	0
		5	5	no data	no data	no data	no data
		6	1	0	0	0	31
		6	2	8	24	1	23
		6	3	1	14	7	0
		6	4	3	5	11	0
		6	5	2	0	9	0
		7	1	11	11	15	3
		7	2	27	0	7	0
		7	3	32	0	13	5
		7	4	13	3	11	0
		7	5	17	5	7	13
		8	1	10	0	1	0
		8	2	0	0	7	0
		8	3	0	14	3	0
		8	4	no data	no data	no data	no data
		8	5	no data	no data	no data	no data
		9	1	3	0	0	3
		9	2	0	13	12	0
		9	3	0	1	4	0
		9	4	1	11	10	0
		9	5	no data	no data	no data	no data
		10	1	0	1	0	5
		10	2	1	0	11	7
		10	3	1	0	15	1
		10	4	3	3	0	3
		10	5	no data	no data	no data	no data
2	1	1	1	7	12	27	5
		1	2	15	8	13	15
		1	3	1	9	19	27
		1	4	8	3	32	21
		1	5	no data	no data	no data	no data
		2	1	1	3	15	0
		2	2	0	2	18	0
		2	3	3	2	9	0
		2	4	7	9	32	0

Table. Continued

Control Group			Rat	D21	D30	D60	D90
Phase	Dose	Litter					
		2	5	no data	no data	no data	no data
		3	1	3	0	13	0
		3	2	0	0	7	0
		3	3	0	0	8	0
		3	4	0	0	9	0
		3	5	5	0	1	0
		4	1	0	1	3	1
		4	2	1	3	5	0
		4	3	2	7	9	0
		4	4	0	9	1	4
		4	5	8	1	0	4
		5	1	3	5	1	11
		5	2	12	9	3	1
		5	3	6	3	1	7
		5	4	3	8	5	1
		5	5	2	0	0	0
		6	1	3	5	31	18
		6	2	12	17	23	29
		6	3	6	0	0	15
		6	4	3	11	11	21
		6	5	2	10	9	17
		7	1	1	1	0	20
		7	2	3	3	8	11
		7	3	1	5	0	13
		7	4	0	4	0	9
		7	5	0	0	0	23
		8	1	0	11	27	13
		8	2	8	19	33	15
		8	3	6	7	0	21
		8	4	7	19	9	17
		8	5	no data	no data	no data	no data
		9	1	5	13	11	17
		9	2	5	17	10	1
		9	3	7	9	3	15
		9	4	0	8	1	19
		9	5	2	6	5	7
		10	1	3	0	0	11
		10	2	7	4	0	13
		10	3	9	0	0	17
		10	4	8	0	0	9
		10	5	1	4	1	3

REFERENCES

Aldridge, J., Gibbons, J., Flaherty, M., Kreider, M., Romano, J., Levin, E. (2003) Heterogeneity of Toxicant Response, Sources of Human Variability. *Toxicological Sciences, 76*, 3–20.

Allio, L., Ballantine, B., Meads, R. (2006) Enhancing the role of science in the decision-making of the European Union. *Regulatory Toxicology and Pharmacology, 44*, 4–13.

Alonso, A. & Geys, H., Molenberghs, G., Kenward, M., Vangeneugden, T. (2004) Validation on surrogate markers in multiple randomized clinical trials with repeated measurements: canonical correlation approach. *Biometrics, 60*, 845-853.

Andersen, M. & Dennison, J. (2004) Mechanistic approaches for mixture risk assessments—present capabilities with simple mixtures and future directions. *Environmental Toxicology and Pharmacology, 16*, 1–11.

Arakawa, H. (2005) Age dependent effects of space limitation and social tension on open-field behavior in male rats. *Physiology & Behavior, 84*, 429–436.

Araújo, A, Lima, J, Moreira, J, Jacob, S, Soares, M, Monteiro, M, Amaral, A, Kubota, A, Meyer, A, Cosenza, A, Neves, C, Markowitz, S. (2007) Exposição múltipla a agrotóxicos e efeitos à saúde: estudo transversal em amostra de 102 trabalhadores rurais, Nova Friburgo, RJ. *Ciência & Saúde Coletiva, 12(1)*, 115-130.

Birkhøj, M., Nellemann, C., Jarfelt, K., Jacobsen, H., Andersen, H., Dalgaard, M., Vinggaard, A. (2004) The combined antiandrogenic effects of five commonly used pesticides. *Toxicology and Applied Pharmacology, 201*, 10– 20.

Buelke-Sam, J., Kimmel, C., Adams, J., Nelson, C., Vorhees, C., Wright, D., ST Omer, V., Korol, B., Butcher, R., Geyer, M., Holson, J., Kutscher, C., Wayner, M. (1985) Collaborative behavioral teratology study. *Neurobehavioral Toxicology and Teratology, 7*, 691-624,.

Calamandrei G, Maranghi F, Venerosi A, Alleva E, Mantovani E. (2006) Efficient testing strategies for evaluation of xenobiotics with neuroendocrine activity. *Reproductive Toxicology, 22*, 164–174.

Castro, V.; Chiorato, S.; Pinto, N. (2000) Relevance of developmental testing of exposure to methamidophos during gestation to its toxicology evaluation. *Toxicology Letters, 118(1-2)*, 93 – 102.

Castro, V.; Mello, M.A.; Diniz, C.; Morita, L.; Zucchi, T.; Poli, P. (2007) Neurodevelopmental effects of perinatal fenarimol exposure on rats. *Reproductive Toxicology, 23*, 98-105.

Castro, V.; Mello, M.A.; Poli, P.; Zucchi, T. (2005) Prenatal and perinatal fenarimol-induced genotoxicity in leukocytes of in vivo treated rats. *Mutation Research, 583(1)*, 95-104.

Castro, V.; Silveira, M.; Perez, M. (1999) Application of clinical indicators of exposition in the evaluation of family agriculture health: the Sumaré case – Brazil. *The International Journal of Sustainable Development and World Ecology, 6 (3)*, 172 – 184.

Champagne, F., Francis, D., Mar, A., Meaney, M. (2003) Variations in maternal care in the rat as a mediating influence for the effects of environment on development. *Physiology and Behavior, 79*, 359-371.

Clewell H. Gentry P, Covington T, Sarangapani R, Teeguarden J. (2004) Evaluation of the potential impact of age- and gender-specific pharmacokinetic differences on tissue dosimetry. *Toxicology Science, 79*, 381-393.

Coffey, T., Gennings, C., Moser, V. (2007) The simultaneous analysis of discrete and continuous outcomes in a dose-response study: using desirability functions. *Regulatory Toxicology and Pharmacology, In Press, Corrected Proof, Available online 12 January 2007.*

Cohen Hubal EA, Sheldon LS, Burke JM, McCurdy TR, Berry MR, Rigas ML, et al. (2000) Children's exposure assessment: a review of factors influencing children's exposure, and the data available to characterize and assess that exposure. *Environmental Health Perspectives, 108*, 475–86.

Conolly, R. & Lutz, W. (2004) Nonmonotonic dose-response relationships, mechanistic basis, kinetic modeling, and implications for risk assessment. *Toxicological Sciences,* 77, 151-157.

Cory-Slechta, D. (2005) Studying Toxicants as Single Chemicals: Does this Strategy Adequately Identify Neurotoxic Risk? *NeuroToxicology, 26*, 491–510.

Costa, L., Aschner, M., Vitalone, A., Syversen, T., Soldin, O. (2004) Developmental neuropathology of environmental agents. *Annual Review of Pharmacology and Toxicology, 44*, 87-110.

Crofton K.M., Makris S.L.,. Sette W.F, Mendez E., Raffaele K.C. (2004) A qualitative retrospective analysis of positive control data in developmental neurotoxicity studies. *Neurotoxicology and Teratology, 26*, 345–352.

Crofton, K.M., Foss, J.A., Hass, U., Jensen, K., Levin, E.D., Parker, S.P. (2007) Undertaking positive control studies as part of developmental neurotoxicity testing. *Neurotoxicology and Teratology, doi: 10.1016/j.ntt.2007.06.002*

Dam K, Seidler F, Slotkin T. (2000) Chlorpyrifos exposure during a critical neonatal period elicits gender-selective deficits in the development of coordination skills and locomotor activity, *Developmental Brain Research, 121*, 179-187.

Das, R. (2002) Role of ancillary variables in the design, analysis, and interpretation of animal experiments. *ILAR Journal, 43 (4)*, 214-222.

Dinham, B. & Sapna Malik, L. (2003) Pesticides and Human Rights. *International Journal of Occupational and Environmental Health, 9*, 40–52.

Donnelly, K., Lingenfelter, R., Cizmas, L., Falahatpisheh M.H., Qian, Y, Tang, Y., Garcia, S., Ramos, K., Tiffany-Castiglioni, E., Mumtaz, M. (2004) Toxicity assessment of complex mixtures remains a goal. Environmental *Toxicology and Pharmacology, 18*, 135–141.

Ehman K.D. & Moser V.C. (2006) Evaluation of cognitive function in weanling rats: A review of methods suitable for chemical screening. *Neurotoxicology and Teratology, 28*, 144 – 161.

Festing, M. & Altman, D. (2002) Guidelines for the design and statistical analysis of experiments using laboratory animals. *ILAR Journal, 43 (4)*, 244-258.

Ferdman, N., Murmu, R.P., Bock J., Braun, K., Leshem, M. (2007) Weaning age, social isolation, and gender, interact to determine adult explorative and social behavior, and dendritic and spine morphology in prefrontal cortex of rats. *Behavioural Brain Research, 180*, 174–182.

Groten, J., Heijne, W., Stierum, R., Freidig, A., Feron, V. (2004) Toxicology of chemical mixtures: a challenging quest along empirical sciences. *Environmental Toxicology and Pharmacology, 18*, 185–192.

Haseman, J., Bailer, A., Kodell, R., Morris, R., Portier, K. (2001) Statistical issues in the analysis of low-dose endocrine disruptor data. *Toxicological Sciences, 61*, 201-210.

Hass U. (2006) The need for developmental neurotoxicity studies in risk assessment for developmental toxicity. *Reproductive Toxicology. 22*, 148–156.

Hensch, T. (2004) Critical period regulation. *Annual Review Neuroscience*, 27, 549–79.

Holson, R. & Pearce, B. (1992) Principles and pitfalls in the analysis of prenatal treatment effects in multiparous species. *Neurotoxicology and Teratology, 14*, 221-228.

Holson, R., Freshwater, L., Maurissen, J., Moser, V., Phang, W. (2007) Statistical issues and techniques appropriate for developmental neurotoxicity testing. *Neurotoxicology and Teratology, doi: 10.1016/j.ntt.2007.06.001*

Howard B. R. (2002) Control of Variability. *ILAR Journal, 43(4)*, 194-201.

Hubal, E., Sheldon, L., Burke, J., Mc Curdy, T., Berry, M., Rigas, M., Zartarian, V., Freeman, N. (2000) Children's Exposure Assessment, A Review of Factors Influencing Children's Exposure, and the Data Available to Characterize and Assess That Exposure. *Environ Health Perspectives, 108*, 475-486.

Huot R, Gonzalez M, Ladd C, Thrivikraman K, Plotsky P. (2004) Foster litters prevent hypothalamic-pituitaryadrenal axis sensitization mediated by neonatal maternal separation. *Psychoneuroendocrinology, 29*, 279–289.

Jacobsen, H., Østergaard G., Lam H.R., Poulsen M.E, Frandsen H., Ladefoged O., Meyer O. (2004) Repeated dose 28-day oral toxicity study in Wistar rats with a mixture of five pesticides often found as residues in food: alphacypermethrin, bromopropylate, carbendazim, chlorpyrifos and mancozeb. *Food and Chemical Toxicology, 42*, 1269–1277.

Jadhav, S, Sarkar, S, Kataria, M, Tripathi, H. (2007) Subchronic exposure to a mixture of groundwater-contaminating metals through drinking water induces oxidative stress in male rats. *Environmental Toxicology and Pharmacology, 23*, 205–211.

Jardim M. & Guimarães F. (2004) Role of glutamate ionotropic receptors in the dorsomedial hypothalamic nucleus on anxiety and locomotor behavior. *Pharmacolology,Biochemistry and Behaviour; 79(3),* 541-546.

Ladics, G., Chapin, R., Hastings, K., Holsapple, M., Makris, S., Sheets, L., Woolhiser, M., Burns-Naas, L. (2005) Developmental Toxicology Evaluations—Issues with Including Neurotoxicology and Immunotoxicology Assessments in Reproductive Toxicology Studies. *Toxicological Sciences, 88(1),* 24–29.

Laessig, S., Auger, A., McCarthy, M., Silbergeld, E. (2007) Effects of prenatal chlordecone on sexually differentiated behavior in adult rats. *Neurotoxicology and Teratology, 29*, 255–263.

Lehmann J, Stohr T, Feldon J. (2000) Long-term effects of prenatal stress experiences and postnatal maternal separation on emotionality and attentional processes. *Behaviour Brain Research, 107*,133 - 144.

Lengyel, Z., Szabó, A., Papp, A., Nagymajtényi, L. (2007) Functional neurotoxic effects in rats acutely exposed to insecticide combinations. *Pesticide Biochemistry and Physiology, 87*, 94–102

Marable, B.R. & Maurissen, J.P.J (2004) Validation of an auditory startle response system using chemicals or parametric modulation as positive controls. *Neurotoxicology and Teratology, 26*, 231–237

Mahar, A.M. & Watzin, M.C. (2005) Effects of metal and organophosphate mixtures on Ceriodaphnia dubia survival and reproduction. *Environmental Toxicology and Chemistry, 24(7)*, 1579-1586.

McCarty, L.S. & Borgert, C.J. (2006) Review of the toxicity of chemical mixtures: Theory, policy, and regulatory practice. *Regulatory Toxicology and Pharmacology, 45*, 119–143.

McCormick C, Robarts D, Kopeikina K, Kelsey J. (2005) Long-lasting, sex- and age-specific effects of social stressors on corticosterone responses to restraint and on locomotor responses to psychostimulants in rats. *Hormones and Behaviour, 48(1)*, 64-74.

Moher, D., Cook, D.J., Eastwood, S., Olkin, I., Rennie, D., Stroup, D.F. (1999) Improving the quality of reports of meta-analyses of randomized controlled trials: the QUOROM statement. *Lancet, 354*, 1896–1900.

Moser, V., Shafer, T., Ward, T., Meacham, C., Harris, M., Chapin, R. (2001) Neurotoxicological outcomes of perinatal heptachlor exposure in the rat. *Toxicological Sciences, 60*, 315-326.

Mumtaz, M., De Rosa, C., Cibulas, W., Falk, H. (2004) Seeking solutions to chemical mixtures challenges in public health. *Environmental Toxicology and Pharmacology, 18*, 55–63.

Owen, D. Andrews, M.H. Matthews, S.G. (2005) Maternal adversity, glucocorticoids and programming of neuroendocrine function and behaviour. *Neuroscience and Biobehavioral Reviews*, 29, 209–226.

Pennycook, F., Diamand, E., Waterson, A., Howard, CV. (2004) Modeling the dietary pesticide exposures of young children. *International Journal of Occupational and Environmental Health, 10*, 304-309.

Perry, M., Venners, S., Barr, D., Xu, X. (2007) Environmental pyrethroid and organophosphorus insecticide exposures and sperm concentration. *Reproductive Toxicology, 23*, 113–118.

Presibella, K., Kita, D., Carneiro, C., Andrade, A., Dalsenter, P. (2005) Reproductive evaluation of two pesticides combined (deltamethrin and endosulfan) in female rats. *Reproductive Toxicology, 20*, 95–101.

Prut, L. & Belzung C. (2003) The open field as a paradigm to measure the effects of drugs on anxiety-like behaviors: a review. *European Journal of Pharmacology, 463*, 3– 33.

Ricceri, L., Venerosi A, Capone F, Cometa M, Lorenzini P, Fortuna S, Calamandrei G. (2006) Developmental neurotoxicity of organophosphorous pesticides: fetal and neonatal exposure to chlorpyrifos alters sex-specific behaviors at adulthood in mice. *Toxicological Sciences, 93(1)*,105–113.

Richardson, J. and Chambers, J. (2005) Effects of repeated oral postnatal exposure to chlorpyrifos on cholinergic neurochemistry in developing rats. *Toxicological Sciences, 84*, 352 - 359.

Robinson, P. & MacDonell, M. (2004) Priorities for mixtures health effects research. *Environmental Toxicology and Pharmacology, 18*, 201–213.

Rohlman, D., Arcury, T., Quandt, S., Lasarev, M., Rothlein, J., Travers, R., Tamulinas, A., Scherer, J., Early, J., Marín, A., Phillips, J., MCCauley, L. (2005) Neurobehavioral

Performance in Preschool Children from Agricultural and Non-Agricultural Communities in Oregon and North Carolina. *NeuroToxicology, 26*, 589–598.

Scheuplein, R., Charnley, G., Dourson, M. (2002) Differential sensitivity of children and adults to chemical toxicity i. biological basis. *Regulatory Toxicology and Pharmacology. 35*, 429–447.

Slamberová, R., Charousová, P., Pometlová, M. (2005) Maternal behavior is impaired by methamphetamine administered during pre-mating, gestation and lactation. *Reproductive Toxicology, 20*, 103–110.

Slikker Jr., W., Acuff, K., Boyes, W., Chelonis, J., Crofton, K., Dearlove, G., Li, A., Moser, V., Newland, C., Rossi, J., Schantz, S., Sette, W., Sheets, L., Stanton, M., Tyl, S., Sobotka, T. (2005) Behavioral test methods workshop. *Neurotoxicology and Teratology, 27*, 417 – 427.

Smith, A.L. & Corrow, D.J. (2005) Modifications to husbandry and housing conditions of laboratory rodents for improved well-being. *ILAR Journal, 46*, 140–147.

Trksak, G., Glatt, S., Mortazavi, F., Jackson, D. (2007). A meta-analysis of animal studies on disruption of spatial navigation by prenatal cocaine exposure. *Neurotoxicology and Teratology, doi: 10.1016/j.ntt.2007.06.003*

Ulbrich, B. (2001) Litter variability in behavioural testing, is testing of one animal per litter enough? *Frontiers in Fetal Health, 3*, 273-274.

Van den Bergha, B.R.H., Mulderb, E.J.H., Mennesa, M., Gloverd, V. (2005) Antenatal maternal anxiety and stress and the neurobehavioural development of the fetus and child: links and possible mechanisms. A review. *Neuroscience and Biobehavioral Reviews, 29*, 237–258.

Verwer, C.M.; van der Ven, L.T.; van den Bos, R.; Hendriksen, C.F. (2007) Effects of housing condition on experimental outcome in a reproduction toxicity study. *Regulatory Toxicology and Pharmacology, 48*, 184–193.

Viberg H, Fredriksson A, Eriksson P. (2004) Investigations of strain and/or gender differences in developmental neurotoxic effects of polybrominated diphenyl ethers in mice. *Toxicological Sciences, 812*, 344-353.

Vinggaard A, Jacobsen H, Metzdorff S, Andersen H, Nellemann C. (2005) Antiandrogenic effects in short-term in vivo studies of the fungicide fenarimol. *Toxicology, 207*, 21–34.

Waissmann, W. (2002) Health surveillance and endocrine disruptors. *Cadernos de Saúde Pública, 18 (2)*, 511-517.

Weed, J.L. & Raber, J.M. (2005) Balancing Animal Research with Animal Well-being: Establishment of Goals and Harmonization of Approaches. *ILAR Journal, 46*, 118-128.

Weiss, B., Amler, S., Amler, R. (2004) *Pesticides Pediatrics, 113 (4)*, 1030-1036.

Zimmermann A, Stauffacher M, Langhans W, Würbel H. (2001) Enrichment-dependent differences in novelty exploration in rats can be explained by habituation. *Behaviour Brain Res*earch,*121*,11-20.