NATUR VÅRDS VERKET

Reproductive behaviour and environmental pollutants

Proceedings from a symposium in Stockholm, September 15-16, 2005

Editors Ulf Magnusson and Björn Brunström

REPORT 5561 • MAY 2006

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September 15-16, 2005

SWEDISH ENVIRONMENTAL PROTECTION AGENCY

Organized by ***ReproSafe***, a research programme
supported by the Swedish Environmental Protection Agency

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Internet: www.naturvardsverket.se

ISBN: 91-620-5561-5
ISSN 0282-7298

Layout: IdéoLuck AB (#60433)
Cover: Mating lions in Masai Mara, Kenya;, Photo copyright U.M.
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Foreword

For wild animals an appropriate sexual behaviour is necessary for the reproductive success. However, when discussing reproductive toxicology of environmental pollutants on animals we mainly consider the negative effects on the reproductive organs and gametes. Even so, it is becoming apparent that in many cases the reproductive behaviour is the most sensitive aspect of the reproductive process. One may therefore question whether we are focusing, scientifically as well as polcy-wise, on the right issues in reproductive toxicology.

During this symposium the reproductive behaviour in various classes of animals and how it is physiologically developed and regulated was described. Then examples were presented from the field and laboratory on how chemicals affect the reproductive behaviour. Finally, a very skilled panel discussed how effects on reproductive behaviour are, or should be, handled from a regulatory, environmental monitoring and policy-making perspective.

We in the ReproSafe programme are very pleased that leading scientists in the field as well as key persons in the policy-making process accepted to make presentations at this symposium. We also conclude that reproductive behaviour is a relevant end point that seems to be very sensitive for some chemicals and that it seems possible to include it in our test regimes.

Uppsala, April 2006

Ulf Magnusson and Björn Brunström,
editors and organizers

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Puberty and the Maturation of Male Reproductive Behavior: Recasting a Behavioral Potential

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Steroid hormones exert a profound influence on the development, structure and function of the nervous system. For example, steroid hormones influence such factors as neuronal survival, neurogenesis, synaptogenesis, receptor expression and neuronal excitability. During perinatal development, steroids act on the central nervous system to organize neural circuits, which remain relatively quiescent until the hormonal stimulation received in adulthood acts on these pathways to activate the appropriate adult physiology and behavior. However, the perinatal stage of maturation is not the only period of development when hormones can organize and influence the development of neural pathways and the behaviors that they will ultimately mediate. Recent research shows that puberty serves as another critical period of neural maturation when the pubertal rise in steroid hormones can further affect the development of adult behaviors, including reproductive behaviors.

In males, the pubertal rise in testosterone secretion precedes the increase in mating behavior, but elevated testosterone levels do not fully explain the expression of reproductive behavior in adulthood. For example, when exposed to doses of testosterone, or its androgenic (e.g. dihydrotestosterone) or estrogenic (e.g., estradiol) metabolites, that normally activate mating behavior in adults, prepubertal males engage in little or no sexual behavior. Thus, juveniles are behaviorally unresponsive to both the androgenic and estrogenic actions of testosterone prior to pubertal development. Steroid hormones affect behavior through specific intracellular receptors (e.g., androgen and estrogen receptors) in various limbic areas (e.g., hypothalamus and amygdala) that form the neural substrates mediating male sexual behavior. It is possible, therefore, that the absence of a behavioral response to steroids prior to puberty may be due to a paucity of these receptors. However, steroid-treated prepubertal males have similar numbers of androgen and estrogen receptors compared to adults in the neural circuit that controls mating behavior. Furthermore, additional studies have demonstrated these receptors are functional prior to puberty, indicating that the lack of mating behavior exhibited by steroid-treated prepubertal males must be mediated by processes other than the availability of steroids, the absence of their receptors, or the functionality of these receptors. Thus, pubertal development must alter the way in which the male responds to steroidal stimulation by reshaping the neural circuit that mediates mating behavior. Experiments are currently being conducted to elucidate the neural substrates that mediate these shifts in behavioral responsiveness during puberty, with preliminary data indicating morphological and phenotypical changes in hypothalamic and amygdalar brain regions.

Mating behavior can be activated in males castrated before puberty and treated with testosterone in adulthood, but the quantity and quality of the behaviors displayed are greatly compromised. For instance, severe impairments in mounting, intromissions, and ejaculatory behavior are observed in males castrated prior to puberty and treated with testosterone in adulthood when compared to males castrated after puberty for a similar length of time and treated with testosterone. Interestingly,

neither lengthier treatments with testosterone nor sexual experience reverse the impairments of mating behavior shown by animals castrated prior to puberty. Hence, the exposure to gonadal hormones during puberty leads to the further masculinization of the behavioral response to steroids in adulthood. Together, it appears that puberty is not merely a time when increasing levels of gonadal steroids activate neural circuits organized during perinatal development, but also a time of further shaping of the nervous system which allows for the appropriate behaviors to emerge in adulthood.

Environmental pollutants and sexually dimorphic behaviour in farm animals

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In a series of experiments designed to determine the effects of exposure to environmental levels of multiple EDCs, we studied the behaviour of adolescent sheep, born to ewes that had grazed pastures treated with sewage sludge, during pregnancy. The offspring had also grazed these pastures themselves until the time of testing. Animals were tested in a variety of situations to address various responses such as exploration of an unfamiliar environment (one year), and the activity (physical and vocal) during restraint in a weigh crate (two years). This allowed us to investigate two aspects of animal personality that have been shown to be sexually dimorphic, namely curiosity and emotional reactivity.

In untreated animals, differences between sexes were identified in the duration of exploratory behaviour and in the number of vocalisations while in the weigh crate (one year only). In both cases, exposure to EDCs and other pollutants in sludge affected the males, making their behaviour more similar to that of the females (de-masculinisation effect?). In the second year of testing the animals in the weigh crate, the likelihood to vocalise revealed no sex-differences in the untreated animals, but a treatment effect on both sexes, in the same direction.

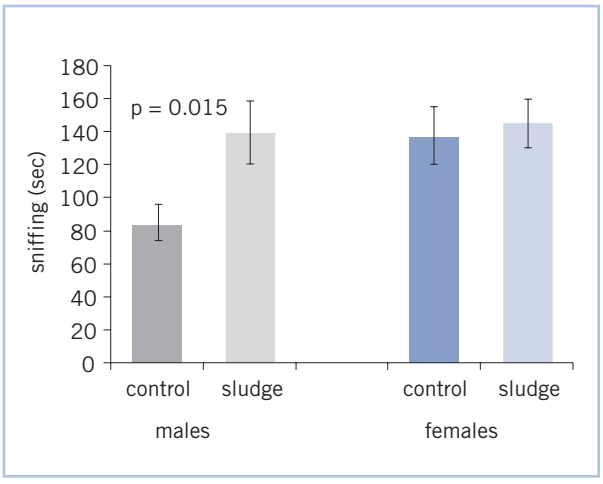

Figure 1: Exploratory behaviour during 5 minutes in an unfamiliar environment

The presence and absence of sex-differences does not only depend on the treatments imposed, but also on aspects of the test situation and on the previous experience of the animals, both affecting the mood of the animals when in the test. While behavioural tests can be a relatively quick and cheap way of studying the effects of treatments, they are not always very robust to small changes in the methods. This will be discussed using aggressiveness and tonic immobility in pigs as an example.

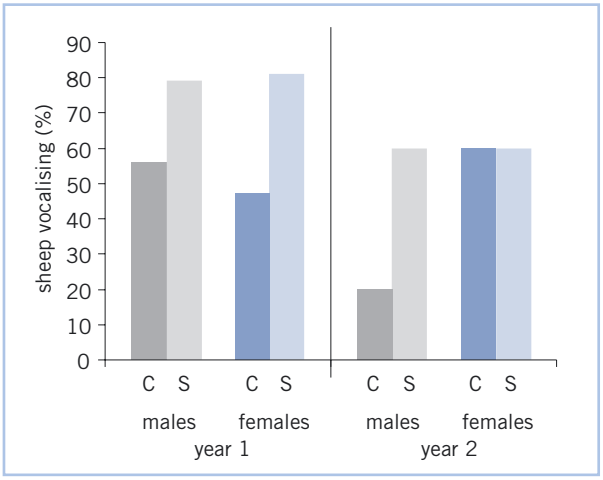

Figure 2: Vocalisations of sheep while restraint in a weigh crate; a different data set for each year

Environmental pollutants affect sex specific behaviour in mice

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The exposure to a large number of environmental pollutants classified as endocrine disrupters (EDs) raises a health concern to animals and humans. Since EDs mimic endogenous molecules, new experimental methodologies are needed to detect functional changes, especially in the case of neuroendocrinological damage. Because steroid hormones are a critical element of the process of sexual differentiation, exposure to EDs that mimic, antagonize or in other ways interfere with these hormonal signals at sensitive developmental stages in the life cycle is likely to impact subsequent reproductive, neuroendocrine and behavioral functions. Many laboratory studies on animal models, as some epidemiological data from human studies, now support the conclusion that levels of EDs exposure that have been dismissed as "background" and thus "safe" can have deleterious effects when low level exposure occur during fetal and/or neonatal development. Sexually dimorphic behaviors are particularly useful to study the effects of low concentrations of EDs like those found in the environment because they are highly sensitive to alterations of the endocrine milieu. Behavior is the endpoint of complex, integrated systems and therefore is a good biomarker of neuroendocrine and neurobiological alterations. In the conceptual frame of evolutionary theory,

sex-differences in behavior are thought to reflect adaptive differences of behavioral strategies in coping as resulting from sexual selection (Darwin 1871). Disruption of the normal processes of masculinization of males and feminization of females may undermine the survival and reproductive success (i.e., fitness) of exposed individuals.

I present here the recent work of our group in house mice showing subtle behavioral effects of the exposure to environmental-like, low doses of bisphenol A (BPA, an endocrine-like compound of large use in food industry) and of the pesticide methoxychlor (MXC) during critical developmental phases (prenatal, postnatal, and pregnancy). We examined the effects of developmental exposure to estrogenic EDs on the occurrence and pattern of a wide range of behaviours required for reproduction such as direct sexual behaviors, social behaviors (e.g. aggression, parental behavior), and non-social behaviors (e.g., learning, memory, exploration, emotionality) in adult life. Pregnant female mice were trained to spontaneously drink daily doses of corn oil with or without chemicals on gestation day 11 to postpartum day 8 (perinatal exposure). Part of the control and BPA-treated offspring were cross-fostered in order to discriminate prenatal (*via placenta*) and postnatal (*via lactation*) BPA exposure effects. The exposed offspring were examined at different ages in a series of behavioral tests. Furthermore, we examined the possible changes in the noradrenergic systems in the locus coeruleus and the preoptic area of the hypothalamus by in vitro receptor binding autoradiography of brain slices. A consistent effect of the maternal exposure to BPA, and partially to MXC, is that in different experimental settings, while a sex difference was observed in the control group, exposure to BPA decreased the sexual dimorphism of several behavioral responses as well as of the noradrenergic receptors density and/or affinity. The behavioural alterations observed in adult animals, males and females, that had been treated during the perinatal period suggest that estrogenic EDs acted on the early phases of behavioral differentiation. In particular, males and females showed differing sensitivities to estrogenic EDs, based on sex differences in the action of endogenous steroid hormones. We have found that BPA and MXC exposure mostly affected female mice on exploration, emotional and cognitive behaviors, and maternal behavior. These data confirm that exposure to weak environmental estrogens in the period of sexual differentiation can influence adult behavior.

Effects of estrogens on the sexual differentiation and activation of male sexual behavior in birds

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Estrogens have widespread effects on brain and behavior and affect a variety of functions including namely reproduction, memory, nociception, neuronal plasticity and excitability. Effects of estrogens related to reproduction (in particular male sexual behavior control) are however best characterized. In birds, effects of estrogens have been extensively investigated in a number of species including the

Japanese quail and several songbirds. The actions of estrogens on male sexual behavior can be divided in three main classes based on the underlying mechanisms and time-course of appearance. A: Long-term effects. During ontogeny, exposure of male embryos or neonates to small doses of estrogens causes a permanent demasculinization of male sexual behavior that will last for the entire life of the subjects. Males treated with estrogens early in life will be unable to display male-typical copulatory behavior in adulthood even after treatment with exogenous testosterone. Females are spontaneously demasculinized by the endogenous estrogens secreted by their ovary. The brain correlates of this demasculinization are poorly understood but a few candidate brain features have been identified in quail (e.g., neuronal size in the lateral preoptic medial nucleus) that correlate with this behavioral demasculinization. Interesting, the sexual differentiation of the song control system in songbirds does not seem to depend on the same endocrine mechanisms and may be, at least in part, under direct genetic control. B: Short-term effects. In adult birds, male sexual behavior is activated by the action in the brain of testosterone secreted by the testes. In the brain, testosterone is metabolized into estrogens by the enzyme aromatase and this enzymatic conversion is critical for the production of behavioral effects. Pharmacological inhibition of brain aromatase activity or blockade of estrogen receptors by anti-estrogens markedly decrease or completely block the effects of testosterone on most aspects of male sexual behavior. These estrogen-mediated behavioral effects of testosterone result largely from an activation of estrogen receptors resulting in the transcription of a variety of genes involved namely in neurotransmission (e.g., vasotocin). Brain aromatase transcription is also finely regulated by androgens (mostly in mammals) and estrogens (mostly in birds) and quantification of the aromatase mRNA or protein provides in birds a good measure of estrogen exposure. Well-defined neuroanatomical and neurochemical changes that appear and vanish within a few hours or days are thus associated with the activation of male sexual behavior by estrogens in birds. C: Ultra-short term effects. Recent work in quail has demonstrated that brain aromatase activity can additionally be modulated within minutes by calcium-dependent phosphorylation processes. Changes in intracellular calcium concentration such as those associated with neurotransmission are thus likely to affect rapidly brain aromatase activity and consequently brain estrogen concentration. These changes in estrogen bioavailability affect with relatively short latencies (15-30 min) the expression of male sexual behavior by mechanisms that presumably do no involve changes in transcriptional activity and could probably be detected only by electrophysiological techniques or through the analysis of changes in intracellular second messenger systems. Effects of estrogens on brain and behavior thus provide multiple endpoints that could be used to assess exposure of animal subjects to estrogenic compounds present in the environment

The Japanese Quail Model: Neuroendocrine and Behavioral Responses to Environmental Contaminants

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The Japanese quail has become an avian model for evaluating adverse effects of endocrine disrupting chemicals (EDCs). The male quail is exquisitely sensitive to the effects of exogenous estradiol, particularly during embryonic development. Moreover, the neural systems that modulate behavior are well characterized, including maturation and age-related changes in th endocrine and behavioral components of reproduction. The focus of our studies has been to address the following questions: 1) When is exposure most detrimental and what is the potential range of adverse effects, including those that are distinct from the reproductive circuit, 2) What type of EDC has the most potential for disrupting neuroendocrine and behavioral responses, and 3) What mechanisms are involved in these effects when considering various classes of EDCs?

Neuroendocrine and behavioral variables were evaluated in Japanese quail following embryonic administration of estrogen- or androgen-active EDCs. Selected doses were administered at embryonic day 4 or 11 by egg injection, either into the yolk or air cell, depending on the experiment. Estrogen-active EDCs included estradiol (E; positive control in a one and a two generation experimental paradigm) and methoxychlor (MXC). These experiments showed that estradiol demasculinized male sexual behavior, altered hypothalamic neurotransmitters, and impaired reproductive function. Exposure over two generation paradigms suggested transgenerational effects. Dietary MXC, even at very low environmentally relevant levels also disrupted neuroendocrine and behavioral systems.

Androgen-active EDCs included the antiandrogens vinclozolin and DDE (ethylene, 1,1-dichloro-2,2-bis(p-chlorophenyl) and an androgenic compound, trenbolone acetate. Androgenic active EDCs impaired male sexual behavior and impacted neuroendocrine systems. The impact on the vasotocin (VT) system was clear with decreased immunoreactive VT in the DDE exposed males. An addition behavioral assessment including a modified open field runway test in which 1 or 2 weeks old chicks were separated from their conspecifics and monitored as they rejoined the other chicks. This behavioral test revealed subtle effects of EDCs; chicks exposed to high doses of trenbolone appeared unable to vocalize. Interestingly, these chicks exhibited the stereotypic behavior characteristic of separation calling, without any sound and coincidently, their conspecifics also did not vocalize. Therefore, these data showed that both estrogenic and androgen active EDCs affect neural and behavioral responses in the quail model. Further, androgen- and estrogen-active compounds were both capable of disrupting endocrine and behavioral components of reproduction. In summary, embryonic EDC exposure in the precocial Japanese quail interferes with steroid dependent mechanisms involved in the sexual differentiation of neural systems that direct reproduction in the Japanese quail.

Supported by EPA #R826134010 (Star Grant), NSF #9817024, EPA R-2877801(MAO).

The neuroendocrinology of fish sexual behaviour: mechanisms and implications for endocrine disruption studies

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Teleost fish are the most diverse vertebrate taxa with over 24,000 living species. Teleosts exhibit the widest range of modes of reproduction among vertebrates. The diversity in reproductive patterns includes gonochoristic species, male-to-female sex changing species, female-to-male sex changing species, serial (i.e. male-to-female-to-male) sex-changing species, simultaneous hermaphrodites, and asexual reproduction in some parthenogenic species. The fertilization mode also varies among teleosts: while most species are external fertilizers, live-bearers also occur in phylogenetic independent lines. Mating systems can vary from monogamous, to polygamous to promiscuous species. Also the patterns of parental care are the most diverse among vertebrates, with most species showing no care, to species with bi-parental, paternal or maternal care. The high variability in reproductive modes is also present within species, ranging from inter-population differences in mating systems, to the occurrence of alternative sexual phenotypes within the same sex. This wide variation in their modes of reproduction makes teleosts a group of election for the study of the proximate causes of reproductive behaviour in vertebrates. However, it also raises problems when one needs to extrapolate results across different taxonomic units (e.g. fish families/ vertebrate classes). The basic neuroendocrine mechanisms underlying teleost reproduction will be described and briefly compared to those of other vertebrates. The role of sex steroids and forebrain neuropeptides on the differentiation of reproductive phenotypes and on the expression of sexual behaviour will then be addressed. Finally, a brief review of studies on endocrine disruptors that have used fish reproductive behaviour as an indicator will be presented.

From this brief review I should stress the following points: (1) due to the great diversity among fish and to between species variations in reproductive physiology and how it responds to environmental stressors, it is advisable to use more than one species as a bioindicator; (2) most EDS studies have concentrated on few freshwater species (mainly poecilids); there is a need for the development of studies in costal and estuarine species, which are potential areas of contamination by EDS. A potential model for the use of toadfish vocalizations as a tool for the evaluation of contamination of such areas will be presented; (3) Most studies have concentrated on male sexual behaviour, since females usually do not exhibit conspicuous behavioural patterns. It will also be proposed to direct future studies to species with sex-role reversal, in which females are the active courting sex (e.g. peacock blenny). These new approaches may open new avenues of research for a more efficient use of fish behaviour as an indicator of endocrine pollution.

Measuring complex behaviour patterns in fish – effects of endocrine disruptors on the guppy reproductive behaviour

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Animal behaviour is potentially a unique biomarker of chemical stress. On the one hand, chemical-induced changes of behaviour are intrinsically linked to neural, hormonal and metabolic processes within the animal. On the other hand, the behaviour of an animal is involved in such vital life processes as feeding, predator avoidance, reproduction and migration, all events of great importance to the individual's health and population maintenance. Accordingly, behaviour can be considered as a functional interface between the individual and the population.

A major obstacle in behavioural research is in obtaining unbiased measurements and presenting them in numerical terms, independent of human interpretation and observational endurance. Simple locomotory behaviour can be rather easily quantified with existing computerized vision systems, whereas it is much more difficult to obtain unbiased measurements of complex behaviour patterns. We have developed a computerized vision system (DISPLAY), which identifies and quantifies complex behaviour patterns and interactions in fish, with the initial aim of quantifying the reproductive behaviour of the guppy (*Poecilia reticulata*). The system will be demonstrated by example at the meeting. In short, the fish scenery is viewed from above by a progressive scan camera which is connected to a framegrabber. At a frame-rate of 12 s^{-1}, the fish silhouettes are identified in each 8-bit image and converted to a 1-bit image, which is stored in a frame file. This significant data reduction allows prolonged recordings to be stored on disk. Subsequently, the position and orientation of each fish relative to the other(s), the distance between them and their body curvature are determined in each frame. Further, frame-to-frame comparisons enable calculation of speed and direction of fish movements. With these measurements available, composite behaviour patterns can now be broken down into its constituting elements which can be entered graphically by the user as distinct *classifiers*. Basically, a given behaviour pattern is recognized and recorded when all classifiers are fulfilled simultaneously. The number and duration of the given behaviour during the recording period are finally written to file for subsequent statistical analysis.

The system has been used for studying the effects of endocrine disruptors on the reproductive behaviour of the male guppy. Both estrogenic and antiandrogenic substances significantly suppress the number and duration of *sigmoid displays*, the characteristic courtship behaviour of male guppies. Some of these results will be presented at the Stockholm meeting.

Environmental pollutants and sexual dimorphic behaviour in children

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Polychlorinated biphenyls (PCBs) and dioxins are known as neurotoxic compounds that may modulate sex steroid hormones. Steroid hormones play a mediating role in brain development and may influence behaviors that show sex differences, such as childhood play behavior. In our study, we evaluated the effects of perinatal exposure to environmental levels of PCBs and dioxins on childhood play behavior and whether the effects showed sex differences.

As part of the follow-up to the Dutch PCB/dioxin study at school age we used the Pre-School Activity Inventory (PSAI) to assess play behavior in the Rotterdam cohort (n=207). The PSAI assesses masculine or feminine play behavior scored on three subscales: Masculine, Feminine, and Composite. Prenatal exposure to PCBs was defined as the sum of PCB 118, 138, 153, 180 in maternal and cord plasma, and breast milk. For breast milk we measured additional PCBs as well as 17 dioxins.

Respondents returned 160 questionnaires (age 7.5 years (+ 0.4)). Effects of prenatal exposure to PCBs, measured in maternal and cord plasma, on scores on the masculine and composite scales were different for boys and girls. In boys, higher prenatal PCBs levels were related with less masculinized play, whereas in girls higher PCB levels were associated with more masculinized play. Higher prenatal dioxin levels were associated with more feminized play in boys as well as girls, assessed by the Feminine scale.

We conclude that the results of this exploratory study indicate that prenatal steroid hormone imbalances caused by prenatal exposure to environmental levels of PCBs, dioxins and other related organochlorine compounds might influence later play behavior in children at school age.

Reference:

Hestien J.I. Vreugdenhil, Froukje M. E. Slijper, Paul G.H. Mulder, Nynke Weisglas-Kuperus, Effects of perinatal exposure to PCB's and dioxins on play behavior in Dutch children at school age, Environmental Health Perspectives 2002;110 (10): A593-8

Investigations of reproductive behaviour in current reproductive toxicity test guidelines

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Reproductive behaviour comprises maternal behaviour that facilitates suckling by young animals and mating behaviour in males and females. These types of behaviour are under neuroendokrine control and sex differences generally result from perinatal action of sex hormones in the developing nervous system.

The regulatory test methods for reproductive toxicity testing within the OECD Test Guideline Program are the prenatal developmental toxicity study (TG 414), the one-generation study (TG 415), the two-generation study (TG 416), the reproduction/developmental toxicity screening tests (TG 421 and 422) and the developmental neurotoxicity study (proposed TG 426). In the prenatal developmental toxicity study, mated female animals are used and the pregnant animals are sacrificed on the day prior to the expected birth. Therefore, assessment of mating and maternal behaviour is not possible in this study design. The developmental neurotoxicity study also starts with mated female animals and consequently mating behaviour cannot be studied.

The generation studies and the screening studies include the periods of mating and maternal care for the offspring. The two-generation study is unique as it is the only study where mating and maternal behaviour of animals exposed during development can be studied. In the section on initial considerations, it is stated that these guidelines is designed to provide information concerning effects on reproductive performance, including mating behaviour. The method sections, however, mentions only that behavioural abnormalities or changes have to be recorded throughout the test period. This means that in practise, maternal and mating behaviour is not studied as such using these guidelines. In some cases, effects indicating affected reproductive behaviour may be reported, but the effects most probably have to be rather marked to be observed, e.g. severe impairment of maternal behaviour leading to pup mortality or markedly increased time to mating.

Methods for studying maternal and mating behaviour have been used to show effects of various chemicals and appear relatively similar among studies. Assessment of maternal behaviour normally comprises observations of nest building, nursing time and posture, and maternal licking and grooming of the pups, as well as the pup retrieval test. Recordings of these endpoints could be included in e.g. the two-generation study without any changes in the general design of the study. Effects on maternal behaviour as such are relevant for risk assessment of chemicals and may also contribute to relevant interpretation of effects observed on pups, e.g. help elucidating whether increased postnatal mortality of pups are due to developmental toxicity effects or may be attributed to impaired maternal care. Assessment of mating behaviour in males conventionally uses ovariectomized females rendered sexually receptive by injection of estradiol. Inclusion of this method in e.g. the two-generation study may be rather labour-intensive and also requires additional operated females, as other females have to be mated to produce the next generation. In an optimised approach proposed by Chahoud et al (1998), the sexual cycles of female rats are determined, and only those in oestrus are

selected and mated. This method could, after some further development in relation to female mating, be included in the two-generation study.

In conclusion, the current OECD Test Guidelines for reproductive toxicity mentions observations of reproductive behaviour without specific guidance on methods. Some marked effects may be observed, but a sensitive assessment would require inclusion of specific methods. Such methods are available in the literature, can be included and it is recommended to consider this in relation to future updating of the OECD one- and two-generation study.

Endocrine disruption & reproductive effects assessment in aquatic life – progress of the OECD Validation Management Group

Thomas H. Hutchinson (AstraZeneca and VMG Co-Chair), Gerald T. Ankley (USEPA and VMG Co-Chair) & Anne Gourmelon (OECD Secretariat, rue André Pascal, Paris)

As part of the Endocrine Disrupter Testing & Assessment (EDTA) activity, the OECD has established a Validation Management Group for mammalian test guidelines ('VMG-mamm') and a similar group for ecotoxicology test guidelines ('VMG-eco'). Today a major part of the VMG-eco work is addressing aquatic species (amphibians, fish and invertebrates). Based on recommendations from OECD technical expert meetings held since 1998, the VMG-eco activity includes the validation of a frog metamorphosis assay (see Opitz et al., 2005, Environ Toxicol Chem 24:653-64) and a 21d fish endocrine screening assay in freshwater fish species (eg Panter et al., 2004, Aquat Toxicol 70: 11-21). Recently an international ring-test has been conducted to assess the performance (reliability and reproducibility) of this screening assay using a range of positive and negative control chemicals. An update will also be given on other candidate OECD test guidelines, including a short-term fish reproduction test (Ankley et al., 2001, Environ Toxicol Chem 20: 1276–1290) and a 60d fish sexual development test (eg Örn et al., 2003. Aquat Toxicol 65: 397-411). Finally, an update will given on the protocol optimization (pre-validation) work on lifecycle tests with copepod crustaceans (eg Breitholtz and B.-E. Bengtsson, 2001, Mar Poll Bull 42: 879-886). The technical and logistical challenges involved in validating such assays will be summarized, especially comparing the reproducibility of biomarkers and apical endpoints for developmental and reproductive toxicity.

Round table discussion

Participants:
Ulf Magnusson (moderator)
Ingvar Andersson, European Environment Agency
Ulla Hass, Danish Institute for Food and Veterinary Research
Tom Hutchinson, AstraZeneca
Monique Matrrec-Fairley, CEFIC
Mary Anne Ottinger, University of Maryland
Peter Pärt, DG Joint Research Centre, European Commission

Introduction of the members of the panel that were not speakers at the conference

Three of the members of the panel, *Monique Matrrec-Fairley*, *Ingvar Andersson* and *Peter Pärt*, were not among the speakers and therefore introduced themselves before the discussion.

Monique Matrrec-Fairley is from CEFIC, an organization representing the chemical industry in Europe. She is in charge of a research programme called the Long Range Research Initiative.

Ingvar Andersson is at the European Environment Agency. He is working on environment and health issues and is together with Peter Pärt writing a report on environment and health.

Peter Pärt is at the Joint Research Centre of the European Commission in Ispra, Italy. He is an advisor in the Institute for Environment and Sustainability and is co-ordinating a programme on environment and health within the JRC.

Is reproductive behaviour an end point that is generally of high sensitivity?

The moderator *Ulf Magnusson* asked the scientists in the panel if it is a general feature that reproductive behaviour is a sensitive end point.

Mary Anne Ottinger answered that concerning birds, researchers have been pushing for behavioural tests over and over again. When *MAO* and her colleagues began working in this area about ten years ago, there were virtually no behavioural test paradigms that were even being considered, especially under the old EPA toxicology test guidelines. *MAOs* group has been involved in a whole series of behavioural studies and now behaviour is beginning to emerge as a potential regulatory test. She pointed out that the criteria for behavioural tests are going to be difficult because in order to put tests into regulatory application, they have to be very repeatable.

According to *MAO*, two types of tests that might lend themselves to regulatory application are the reproductive behaviour test, which is well worked out in many laboratories, and potentially some sort of a motor open field test. She emphasized that quite a lot more work is still needed to make these tests very "test guideline-friendly" so that it would be easy to carry out the tests in a consistent manner between testing labs according to quality assurance levels. Her opinion was that these tests would be very sensitive, and for younger animals they would give information about survival characteristics. Reproductive behaviour is very "test-friendly" and it would also give some indication of the reproductive capability in

the wild. A range of chemicals probably impact both reproductive behaviour and motor activity according to data from Dr Ottinger's lab.

Ulf Magnusson then asked those working with mammals if behaviour in general is more sensitive than other end points?

Ulla Hass replied that the question is a very difficult one to answer. For some chemicals, like those that are neurotoxicants or endocrine disrupters, she expected that behaviour would be a very sensitive end point because such compounds may influence the sexual differentiation of the brain. The question is how the effects can be assessed. For these kinds of chemicals, she considered changes in behaviour or brain structure to be the most sensitive effects to examine. When it comes to reproductive behaviour, *UH* emphasized that more studies have to be done and more data is needed to show whether reproductive behaviour is more sensitive than for example motor activity.

Ulla Hass also explained that behavioural end points actually are included in the guidelines for developmental neurotoxicity. There is a sexual dimorphism in motor activity where females are showing higher activity than males and *UH* expected effects on motor activity to be sensitive when studying endocrine disrupters. It may even be as sensitive as mating behaviour and nursing behaviour. *UH* concluded that at present she doesn't know whether looking at reproductive behaviour would be more sensitive than looking at activity.

Tom Hutchinson pointed out that it depends very much on the group of chemicals looked at. As an example he mentioned that several publications suggested that methoxychlor is a model environmental oestrogen. However, to induce a vitellogenic response in rainbow trout, *TH* and co-workers had to expose the fish to concentrations of methoxychlor that were almost lethal because of the neurotoxic response. The primary mode of action of methoxychlor is as a neurotoxicant and that's why it is used as an insecticide. For compounds like insecticides, *TH* felt that behavioural end points should be considered more.

A second point by *TH* was that there is not a body of data that has been collected using the same exposure route and the same chemical so that an apples to apples comparison can be made. He suggested that if you, e.g., do waterborne exposure on fish, including some of the species that have been used for other end points, using some of the OECD reference chemicals, then you actually could make the comparisons. But according to *TH* there is presently not data available to do these comparisons.

How does your organization respond to signals that reproductive behaviour may be a sensitive end point?

Ulf Magnusson asked *Ingvar Andersson* how his organization will respond to signals from scientists at a meeting like this that reproductive behaviour is a very sensitive end point.

Ingvar Andersson pointed out that the European Environment Agency (EEA) is a minor player in this field compared with for instance the European Chemicals Bureau. He then explained that EEA can, for example, use their state of the environment report where there are assessments made on environment and health. They have also highlighted the importance of observations in wildlife as an early warning for reproductive effects. A long list of different wildlife species, called sentinels or early warning species, has been put together by EEA. In their

report, EEA can also highlight any effects on wildlife populations as well as presenting human bio-monitoring studies and time series for levels of contaminants in humans and the environment.

IA also mentioned that EEA discusses research projects that they think should be initiated and bring these discussions to the research management organisation within the EU. Furthermore, workshops, conferences and seminars are arranged. He stressed that EEA is not a risk assessment body but rather a body between research and policy.

Ulf Magnusson then asked *Monique Matrrec-Fairley* about the response of her organization to outputs from meetings like this one and how CEFIC handles new information about the effects of chemicals.

Monique Matrrec-Fairley said that the industry at the moment has to respond quite quickly because of the EU legislation and REACH. She emphasized that there are 30,000 chemicals to be tested that we know hardly anything about and we need to understand how risk assessments can be done in the best way. She also pointed out that the industry is funding research and is working closely with the OECD. Funding is given to make sure that tests developed within OECD are robust and properly validated. Moreover, meetings and workshops are held to respond to the regulatory process and to make sure that the chemicals are as safe as possible for the environment and for health.

Ulf Magnusson asked *Peter Pärt* how a change is achieved at the regulatory and political level.

Peter Pärt answered that the regulators, particularly the regulatory risk assessors that do human risk assessment, are very conservative. *PP* has been involved through the Commission in developing something called integrated risk assessment together with the US EPA and WHO IPCS (International Programme on Chemical Safety). In 2002 they produced a framework for integrated risk assessment. The information both from the ecological side and the human health side for a certain compound is merged and then a risk assessment is made based on all the information available. This idea particularly met a lot of resistance from the regulatory risk assessors that do human risk assessments. *PP* considered ecotoxicological risk assessors to be more open-minded in this context and that they appreciate the idea of using human health data to make ecotoxicological risk assessments. The human risk assessors often say that they have their models and they know how to use them and they don't understand why the situation should be complicated. It was indicated by *PP* that the area still is quite controversial.

Can more "intelligent" testing strategies be used?

Ulf Magnusson pointed out that increased testing and animal welfare are big issues within REACH and no one likes unnecessary testing on animals instead of using existing data. He then asked the panel whether existing data can be used in a more "intelligent" way.

Peter Pärt answered that the concept "intelligent testing strategies" is discussed within the EU. This strategy means that theoretical models are used to make predictions and consequently experimental animals are not used. Information from a quite simple system is used and it is assumed that the same class of chemicals will respond in the same way in another test system. *PP* meant that this strategy of course is very tempting to use because it simplifies things. It is also related to that we already have good fundamental information from animal experiments

and in vitro systems that can be used. *PP* stressed that lack of new information is a problem if this strategy is to be used for all new chemicals. Therefore, tests on animals or in other systems have to be carried out to provide data for the theoretical models.

Mary Anne Ottinger agreed and referred to that the expert group on avian tests within OECD had to deal with that two-generation tests require large investments and take time. She suggested an approach to cut down on animal numbers by designing a test paradigm that would focus in on responsive end points, i.e. to identify measures, possibly behaviour, that are more sensitive and more reliable. She referred to a document prepared by the expert group which contains a table in which they identify classes of chemicals and then determine which kind of test would, for example, be responsive to thyroid effects or sensitive to oestrogenic activity. She explained that the idea would be not to use all tests for every chemical but to have some sort of a subset of tests or end points measured and to cut way down on animal numbers.

Ulla Hass considered it to be a very good idea to use some kinds of biomarkers instead of incredibly long studies but she stressed that it requires regulatory acceptance. These strategies have to lead to regulation of the chemicals because otherwise the long studies still have to be done. *UH* also commented on the use of ecotoxicological data for human toxicology and she asked how, for instance, a change in the reproductive behaviour of a bird can be extrapolated to humans. The question is whether the answer should be given by the "rat people" or the "bird people". She also pointed out that one of the reasons that bird data is not used for human risk assessment is because there are differences between birds and mammals. In mammals there is a placenta, and a dose is given to a pregnant animal. In birds, the chemical can be put into the egg so the problem is how to translate the exposure of the egg to an exposure during pregnancy in a mammal. When it comes to fish, *UH* said that she certainly would like risk assessment to be based on vitellogenin induction and use that for protecting humans against oestrogens. She then pointed out the problem with calculating from what is put in the water to the doses in rats and how the lack of placenta in fish should be dealt with. Her conclusion was that it is not only conservatism that is the reason for the lack of integration between the two areas.

Monique Matrrec-Fairley commented that integrated, or intelligent, testing really is needed but that tests to replace animal testing are not yet validated. The industry find the total replacement of animal testing as a conflict of interest, because on the one hand you have to make sure that all children are very safe and on the other hand use fewer animals. She pointed out that it is not easy for the industry at the moment to determine the best risk assessment, so an intelligent or integrated approach has to be used.

The view of *Tom Hutchinson* was that the biomarkers are the linkage tools for efficient use of animals. You link mechanisms to get savings of animals, of test compound, and of money, and that is something that the pharmaceutical industry is presently doing. As an example, he mentioned that zebra fish are used to screen for drugs instead of rats or dogs.

Peter Pärt claimed that in the area of environment and health he did not agree with what Ulla Hass said because there are a number of examples where we have learned from environmental effects that the same thing can occur in humans. He pointed out that in many physiological respects there are not such big differences between humans and mammalian wildlife. As an example he mentioned that if

you can find a correlation between some contaminants and bone decalcification in moose then there is a strong indication to look for the same kind of effects also in humans. According to *PP* this example shows the basic idea of the integrated way of thinking. There are animals in the environment that are naturally exposed to levels that we may, in certain situations, also be exposed to.

Ulla Hass pointed out the difference between animals in an animal study, animals in the environment and human beings. In animal studies you can directly test a certain chemical and see its effects whereas animals in the environment are exposed to mixtures of chemicals.

One in the audience commented that you have to try a hypothesis in wild animals and then prove it in experimental animals. That is a way to solve the puzzle because you can never make the experiment with human beings.

Mary Anne Ottinger agreed with the idea of integrating across animal species because the fundamental biology is at some level very analogous across species and classes of animals. She also pointed out the importance of determining the ecotoxicological risk to protect populations. The compounds that constitute a problem should be identified as well as their mechanisms of action. In the next step some of these chemicals could perhaps be used safely at certain times of the year and perhaps not at other times. She argued that we then begin to get a wiser, more intelligent use of these compounds.

Another in the audience commented that regarding wildlife the important question is what we want to do, namely to protect wildlife. He claimed that in wild populations, particularly with behaviour, things that are critically important might not necessarily be sensitive. In wildlife populations are, for instance, prey avoidance and sexual selection critical for health and safety. In laboratory strains, the animals are not sensitive at all to those things because they have lost the ability for sexual selection or prey avoidance. He further exemplified with own experiments on the vitellogenin response in fish, which was very positive with short-term exposure, but with long-term exposure there was no response at all. In that case, he explained, you can screen for long-term exposure and say there is no impact from the chemical because there is no vitellogenin response. However, with long-term exposure those fish have absolutely no reproductive potential at all.

Ulla Hass said that she also has the feeling that the effects observed in fish or quail, or whatever species, are relevant for evaluating the risk for humans. She would feel rather safe using the studies showing an effect, but was worried about the negative ones and she asked which criteria that should be used for prioritizing further testing of chemicals. Her opinion was that we would still have to test in an animal model with a placenta because some kinds of developmental effects occur because of very small changes in the dam, like changes in thyroid hormones. Such changes have absolutely no consequences for the adult animal but they have consequences for the developing brain. She stressed that we have to realize that small effects in the pregnant female can have effects on the foetus.

Ulla Hass then turned to an issue where she wanted to be a little bit provocative. Her view was that we shall use all the data we have but she doesn't agree when people feel that the new test systems in REACH should not use more animals. Animal rights people are upset because it has been estimated that REACH testing will actually lead to the use of three million animals for testing the 30,000 chemicals that we know nothing about. She emphasized that we are approximately 420 million people in the EU and that means that for each of us, once in our lifetime, we would have to use less than one percent of a rat to test

all the 30,000 chemicals. *UH* also exemplified with the number of pregnancies within the EU, presently around 3 million, which would be better protected if the 30 000 chemicals were tested using 3 million rats.

Can behavioural end points be included in test guidelines?

Ulf Magnusson then asked those working with test guidelines whether it is more complicated to include behavioural endpoints because these end points are less reproducible and not so robust compared with other end points.

Tom Hutchinson commented that it is scientific logic that as any parameter becomes more sensitive it automatically becomes more variable. He considered the systems presently used to be insufficiently robust and that systems such as image analysis are not used widely enough.

Ulla Hass on the other hand referred to a workshop in the US where they looked at the coefficient of variation in behavioural end points compared to other end points in studies of developmental toxicity. The conclusion was that there is not a larger variation in behavioural test results compared with the other end points included.

Mary Anne Ottinger considered a behavioural assay that is chosen carefully to be very repeatable. She also exemplified with vasotocin whose expression in the avian brain is affected by many compounds. An effect on vasotocin is likely to mean a behavioural implication and vice versa. To become more efficient in developing a guideline that identifies the chemicals of interest she argued that a change in for instance behaviour or morphology should be linked to a mechanism.

Concluding remarks

Ulf Magnusson made the conclusion that reproductive behaviour is a relevant end point that seems to be very sensitive for some chemicals and that it seems possible to include it in our test regimes. He also appreciated the important, but difficult, discussion about the need to use experimental animals to create a safe world for ourselves and the environment. Lastly, he pointed out that this meeting has shown that people working in different toxicological disciplines, studying various animal classes, can learn a lot from each other. On behalf of the organizers he then thanked all the participants.

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Reproductive behaviour and environmental pollutants

Proceeding from a symposium in Stockholm, September 15-16, 2005

REPORT 5561

SWEDISH ENVIRONMENTAL PROTECTION AGENCY

ISBN 91-620-5561-5
ISSN 0282-7298

The ***ReproSafe*** (Reproduction and Chemical Safety) programme is a research programme supported by the Swedish Environmental Protection Agency (Naturvårdsverket) during a five-year period (2001-2006).

The programme addresses the issue of a growing scientific and public concern that chemicals in the environment may impair human and wildlife reproduction. The programme is built on a comparative approach aiming to increase the knowledge about mechanisms of action for chemically induced reproductive impairment and to develop new and sensitive methods for recording such impairment.
A postgraduate school on reproductive toxicology is attached to the programme and regularly seminars and stakeholder meetings are arranged.

More info at
http://www-cru.slu.se/ReproSafe.htm

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