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Vitenskapskomiteen for mattrygghet
Norwegian Scientific Committee for Food Safety

Opinion of the Scientific Panel on Food Additives, Flavourings, Processing Aids, Materials in Contact with Food and Cosmetics of the Norwegian Scientific Committee for Food Safety

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Assessment of four studies on developmental neurotoxicity of bisphenol A

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SUMMARY

The Norwegian Scientific Committee for Food Safety (Vitenskapskomiteen for mattrygghet, VKM) has on the request from the Norwegian Food Safety Authority (Mattilsynet) assessed four studies on developmental neurotoxicity following low dose exposure to bisphenol A (BPA) (Adriani *et al.*, 2003; Carr *et al.*, 2003; Negishi *et al.*, 2004; Ryan and Vandenbergh, 2006). The background for the request is uncertainties related to developmental neurotoxcity of BPA raised by the Nordic environmental agencies in Norway, Sweden and Denmark. VKM was asked to consider if these studies provide sufficient evidence to set a lower no observed adverse effect level (NOAEL) in the hazard characterisation of BPA. Further, a Norwegian exposure scenario based on available exposure data should be performed. The task has been assessed by the Scientific Panel on Food Additives, Flavourings, Processing Aids, Materials in Contact with Food and Cosmetics (Panel 4).

Bisphenol A (CAS number 80-05-7) is primarily used as a monomer in the production of polycarbonate, which is used to make food containers, such as beverage bottles, baby bottles, tableware and storage containers. It is also used as a precursor of certain epoxy resins used for protective coatings for food and beverage cans. BPA is permitted for use in food contact materials in the European Union (EU) with a specific migration limit (SML) of 0.6 mg/kg food. The migration limit in the EU regulations has yet to be modified according to an opinion from the European Food Safety Authority (EFSA) from 2006 where a new established tolerable daily intake (TDI) of 0.05 mg BPA/kg body weight (bw) was derived from a NOAEL of 5 mg/kg bw/day.

A European Union Risk Assessment Report (RAR) of BPA produced in accordance with Council Regulation (EEC) 793/93 has recently been updated (April 2008) reviewing a previously requested 2-generation study in mice (Tyl *et al*., 2007) and new data on human exposure and effects of BPA. A NOAEL of 50 mg/kg bw/day was suggested in this report. The Nordic environmental agencies (Norway, Sweden and Denmark) have participated in the discussions on this updated EU RAR of BPA and they strongly disagreed that this NOAEL also covers developmental neurotoxicity. According to the Nordic environmental agencies, the four above mentioned studies indicate a possible risk for developmental neurotoxicity of BPA at very low exposure levels (0.1-0.25 mg/kg bw/day). The position of the Nordic environmental agencies has been included as a footnote in the revised EU RAR.

Recently, in April 2008, the U.S. National Toxicology Program (NTP), Health Canada and Environment Canada have published draft reports on effects of BPA, including developmental effects (neural and behavioural effects) and expressed *some* concern for neural and behavioural effects in fetuses, infants and children at current human exposures. The European Commission has therefore asked EFSA to further assess possible age dependent toxicokinetics of BPA in animals and humans and their implications for hazard and risk assessment of BPA taken into account the most recent information and data available.

The present opinion from VKM Panel 4 is based on an evaluation of the design, conduct (or accomplishment) and the results in the four above mentioned studies. The study design has been evaluated in light of international recommendations given in relevant guidelines dealing with developmental neurotoxicity testing in animals. The recent international developments on BPA in the U.S. and Canada are not addressed in this opinion.

The report by Tyl and co-workers was central in the EFSA opinion from 2006 and the updated EU RAR from 2008. The Tyl study is a GLP compliant 2-generation reproductive toxicity evaluation in mice performed according to a modified OECD 416 guideline. However, the study did not include functional tests for developmental neurotoxicity.

VKM has reviewed the four above mentioned studies on neurodevelopmental toxicity of BPA as requested by the Norwegian Food Safety Authority. Although the design and reporting of these studies suffer from major and serious shortcomings, the overall findings may raise some concern.

It is the opinion of the VKM Panel 4 that the four studies do not provide sufficient evidence for setting a robust lower NOAEL for BPA than the current EFSA NOAEL of 5 mg/kg bw/day. The Panel is aware that the EU Commission recently has requested EFSA to re-evaluate the information available on BPA.

In order to eliminate any uncertainty regarding potential developmental effects of BPA at low doses, it is recommended that a GLP compliant study is carried out according to OECD guideline 426. Such a study should utilize a broad concentration range from the very low doses up to those with known maternal effects.

A Norwegian exposure scenario based on available data on exposure to BPA from food and beverages and via the environment was performed. In general, exposure levels of BPA in Norway are low. The estimated exposure of infants and children is in the range of 3.5 – 13.2 µg/kg bw/day, whereas the estimated aggregated exposure of adults is 1.5 µg/kg bw/day. As a result of the current use of BPA in food contact materials and other consumer products, infants and children are exposed to higher levels of BPA per kg body weight than the rest of the population.

SAMMENDRAG

Vitenskapskomiteen for mattrygghet (VKM) har på oppdrag fra Mattilsynet vurdert fire studier der bisfenol A (BPA) er undersøkt med hensyn til nevrotoksisitet ved utvikling av nervesystemet etter lavdoseeksponering av forsøksdyr (Adriani *et al.*, 2003; Carr *et al.*, 2003; Negishi *et al.*, 2004 og Ryan & Vandenbergh, 2006). Bakgrunnen for oppdraget er at forurensningstilsynene i Norge, Sverige og Danmark i en oppdatert risikovurdering fra EU har pekt på usikkerheter rundt lavdoseeksponering for BPA og mulige nevrotoksiske effekter ved utvikling av nervesystemet. VKM ble bedt om å ta stilling til hvorvidt disse studiene gir tilstrekkelig bevis for å fastsette en lavere nulleffektsdose (NOAEL) i farekarakteriseringen av BPA. Videre ble VKM bedt om å gjennomføre et eksponeringsscenario for Norge basert på tilgjengelige eksponeringsdata. Vurderingen er gjort av Faggruppen for tilsetningsstoffer, aroma, matemballasje og kosmetikk (Faggruppe 4).

Bisfenol A (CAS nummer 80-05-7) blir primært brukt som en monomer i produksjonen av polykarbonat, som benyttes til å lage beholdere for mat og drikke, slik som drikkeflasker, tåteflasker, servise og oppbevaringsbokser. Stoffet blir også brukt som en forløper til visse epoksyresiner brukt i beskyttelseslag i mat- og drikkebeholdere. BPA er regulert i EU-regelverket for matkontaktmaterialer med en migrasjonsgrense (SML) på 0,6 mg/kg næringsmiddel. Denne migrasjonsgrensen har ennå til gode å bli endret i samsvar med en vurdering fra det Europeiske mattrygghetsorganet (EFSA) fra 2006, hvor det ble fastsatt en ny verdi for tolerabelt daglig inntak (TDI) på 0,05 mg BPA/kg kroppsvekt utledet fra en NOAEL på 5 mg/kg kroppsvekt/dag.

EU har nylig (april 2008) oppdatert sin risikovurdering (Risk Assessment Report (RAR)) av bisfenol A innenfor rammene av rådsforordning (EØF) nr. 793/93 om vurdering og kontroll av risikoer ved eksisterende stoffer. I den reviderte risikovurderingen har de nå vurdert en tidligere etterspurt 2-generasjonsstudie i mus (Tyl *et al*., 2007), samt nye data på human eksponering og effekter av BPA. En NOAEL-verdien på 50 mg/kg kroppsvekt/dag er foreslått i denne vurderingen. De skandinaviske forurensningstilsynene (Norge, Sverige og Danmark) har deltatt i diskusjonen av denne reviderte risikovurderingen (EU RAR) av BPA, og de er sterkt uenige i at denne NOAEL-verdien også dekker nevrotoksiske effekter ved utvikling av nervesystemet. Med referanse til de fire ovennevnte studiene hevder de skandinaviske forurensningstilsynene at det ikke kan utelukkes effekter på læring og hukommelse i avkom ved eksponering for svært lave doser av BPA (0,1-0,25 mg/kg kroppsvekt/dag). Synspunktet til de skandinaviske forurensningstilsynene har blitt inkludert som en fotnote i den reviderte risikovurderingen fra EU.

U.S. National Toxicology Program (NTP), Health Canada og Environment Canada har i april 2008 publisert nye risikovurderinger (foreløpig utkast) hvor utviklingstoksikologiske effekter (effekter på nervesystemet og atferd) av BPA er omtalt. I konklusjonen i disse risikovurderingene uttrykkes det *noe* bekymring for effekter på nervesystemet og atferd hos foster, spedbarn og barn ved den nåværende humaneksponeringen for BPA. EU-kommisjonen har derfor bedt EFSA om å vurdere om det kan være mulige aldersavhengige toksikokinetiske forskjeller i metabolismen av BPA hos dyr og mennesker, og eventuelt deres betydning for fare- og risikovurdering av BPA tatt i betraktning de nye opplysningene fra USA og Canada.

Denne uttalelsen fra VKMs Faggruppe 4 er basert på en vurdering av design, gjennomføring og resultater i de fire ovennevnte studiene. Studienes design har blitt vurdert i lys av

internasjonale anbefalinger (OECD) gitt i relevante retningslinjer for testing av utviklingstoksikologiske effekter på nervesystemet i forsøksdyr. Den seneste utviklingen som er belyst i de nye risikovurderingene av BPA fra USA og Canada har ikke blitt vurdert nærmere i denne uttalelsen fra VKM.

Rapporten fra Tyl og medarbeiderne var sentral både i EFSAs vurdering fra 2006 og i den oppdaterte risikovurderingen (RAR) fra EU. Studien fra Tyl er en to-generasjons-reproduksjonstoksisitetsvurdering i mus utført i henhold til god laboratoriepraksis (GLP) og i overensstemmelse med den modifiserte retningslinjen OECD 416. Studien inkluderer ikke funksjonelle tester for å undersøke eventuelle nevrotoksiske effekter ved utvikling av nervesystemet.

VKM har vurdert de fire ovennevnte studiene relatert til nevrotoksisitet ved utvikling av nervesystemet etter lavdoseeksponering for BPA på oppdrag fra Mattilsynet. Selv om det er vesentlige mangler og svakheter i disse studienes design og presentasjon av resultater, kan de samlede funnene medføre noe bekymring.

VKMs Faggruppe 4 mener likevel at de fire studiene ikke gir tilstrekkelig grunnlag til å fastsette en lavere NOAEL for BPA enn den nåværende NOAEL-verdien på 5 mg/kg kroppsvekt/dag fastsatt av EFSA i 2006. Faggruppen er klar over at EU-kommisjonen nylig har bedt EFSA om å revurdere den tilgjengelige informasjonen for BPA.

For å eliminere usikkerheten knyttet til mulige nevrotoksiske effekter ved utvikling av nervesystemet ved eksponering for lave doser av BPA, anbefales det å gjennomføre en GLP-studie i overensstemmelse med retningslinjen OECD 426. En slik studie bør gjennomføres i et bredt konsentrasjonsområde, fra veldig lave doser og opp til doser hvor det er observert klare effekter på mødrene.

I vurderingen er det også gjennomført et norsk eksponeringsscenario, basert på tilgjengelige eksponeringsdata for BPA fra mat og drikke, og fra miljøet. Eksponeringen for BPA i Norge er generelt lav. Estimert eksponering hos spedbarn og barn er i området 3,5 – 13,2 µg/kg kroppsvekt/dag, mens den samlede estimerte eksponeringen hos voksne er 1,5 µg/kg kroppsvekt/dag. Som et resultat av dagens bruk av BPA i matkontaktmaterialer og andre forbrukerprodukter, eksponeres spedbarn og barn for høyere nivåer av BPA per kg kroppsvekt enn resten av befolkningen.

CONTENTS

1 BACKGROUND

Bisphenol A (BPA) (CAS number 80-05-7, reference number 13480) is regulated in the national legislation for food contact materials (Forskrift om materialer og gjenstander i kontakt med næringsmidler, 1993-12-21 nr 1381) with a specific migration limit (SML) of 0.6 mg/kg food. The European Food Safety Authority (EFSA) has recently published an opinion related to bisphenol A (EFSA, 2006). The migration limit in the EU regulations on food contact materials has yet to be modified according to the EFSA opinion and the new established tolerable daily intake (TDI) of 0.05 mg BPA/kg body weight (bw).

An European Union Risk Assessment Report (RAR) of 4,4'-isopropylidenediphenol (Bisphenol A) produced in accordance with Council Regulation (EEC) 793/93 was published in 2003 (EU, 2003). This risk assessment report has now been updated with United Kingdom as Rapporteur, reviewing a previously requested 2-generation study (Tyl *et al*., 2007) and new data on human exposure and effects of BPA that have become available since the original risk assessment report was completed. The Norwegian Pollution Control Authority (Statens forurensningstilsyn, SFT) and their sister organisations in Sweden and Denmark have participated in the discussions and given their comments to the revision of this EU RAR of BPA (EU, 2008).

The Nordic environmental agencies (Denmark, Norway and Sweden) strongly disagreed that the suggested no observed adverse effect level (NOAEL) of 50 mg/kg bw/day also covers developmental neurotoxicity. It is referred to four studies (Adriani *et al.*, 2003; Carr *et al.*, 2003; Negishi *et al.*, 2004; Ryan and Vandenbergh, 2006), which according to the Nordic environmental agencies indicate a possible risk for developmental neurotoxicity of BPA at very low exposure levels (0.1-0.25 mg/kg bw/day). The majority of the European Member States, however, support the NOAEL of 50 mg/kg bw/day. Thus, the position of the Nordic environmental agencies will only be included as a footnote in the revised EU RAR.

It should be noted that the NOAEL of 50 mg/kg bw/day for reproductive and general toxicity (effect on body weight, liver and kidney) stated in the revised EU RAR is based on the same study (Tyl *et al.*, 2007) as EFSA has used in their latest opinion (EFSA, 2006). However, EFSA considered the increased incidence of centrilobular hepatocyte hypertrophy as the most critical endpoint and their new established TDI is therefore based on a NOAEL of 5 mg/kg bw/day.

The request from the Norwegian Food Safety Authority to VKM is categorised as an urgent matter due to the situation that the Norwegian Ministry of the Environment at the moment are considering a ban on BPA in consumer products and the fact that the Norwegian Pollution Control Authority has disagreed upon the NOAEL for developmental toxicity in the revised EU RAR.

1.1 Exposure from polycarbonate baby bottles and other food contact materials

The Norwegian Food Safety Authority has recently commissioned an investigation of different polycarbonate baby bottles sold on the Norwegian market where realistic conditions of use were simulated (Biedermann-Brem *et al.*, 2007). The possibility of increased migration

of BPA under extreme washing conditions was given a special focus in the investigation. No increased levels of BPA were found compared with standard test procedures of new products.

Recently, it has also been shown that exposure of polycarbonate drinking bottles to boiling water (100°C) increased the rate of BPA migration (Le *et al*., 2008).

It should also be noted that BPA has been found to migrate in low levels from other food contact materials of polycarbonate and from epoxy resins used to coat metal products such as food cans.

1.2 Exposure from consumer products

The Norwegian Pollution Control Authority has investigated the exposure from some consumer products, such as mittens, where relatively high levels of free BPA have been measured (Molab, 2006). The exposure data from mittens was submitted to the revised EU RAR by the Norwegian Pollution Control Authority, but was not included as the use of mittens was not considered to be representative across the Member States in the European Union.

1.3 Environmental exposure

Within EUs program for the evaluation and control of the risks of existing substances (Council Regulation (EEC) 793/93) both a local and a regional exposure through the environment are estimated. A local exposure is here defined as exposure to food, water and air from polluted areas where a company has discharged BPA.

The Norwegian Pollution Control Authority has performed comprehensive analyses of BPA in the environment, and BPA levels in fish from lakes/rivers (Mjøsa, Vorma, Øyeren) and fjords (Drammensfjorden) have been measured (Fjeld *et al.*, 2004a; 2004b). The highest levels from these analyses is around 10 times higher than the estimated levels of BPA in fish used in the revised EU RAR for the regional exposure to humans through the environment. The data used are intended to give a conservative (high) estimate for the exposure. The Norwegian Pollution Control Authority is of the opinion that the levels measured in fish are representative for regional exposure through the environment in Norway, since the data are from relatively large water systems and no local emissions have been detected related to the analyses.

2 TERMS OF REFERENCE

The Norwegian Food Safety Authority (Mattilsynet) has requested the Scientific Panel on Food Additives, Flavourings, Processing Aids, Materials in Contact with Food and Cosmetics of VKM to:

- Assess the relevant studies on developmental neurotoxicity following low dose exposure to bisphenol A, which are debated in the scientific literature (Adriani *et al.*, 2003; Carr *et al.*, 2003; Negishi *et al.*, 2004; Ryan and Vandenbergh, 2006). Based on these studies, consider if it is necessary to set a lower NOAEL in the hazard characterisation, due to the uncertainties related to developmental neurotoxicity.

- Perform a Norwegian exposure scenario based on available exposure data. The Norwegian Food Safety Authority considers it relevant to include the exposure data proposed by the Norwegian Pollution Control Authority to the ongoing work with the revised EU Risk Assessment Report (RAR) of bisphenol A in the assessment.

The assessment should make the Norwegian Food Safety Authority able to establish a new risk-based migration limit for bisphenol A in the national legislation on food contact materials. The Norwegian Food Safety Authority will, until the opinion from VKM is finished, act in accordance with the EFSA opinion from 2006 (EFSA, 2006).

The Norwegian Food Safety Authority assumes that VKM will coordinate their work with the department of the Norwegian Institute of Public Health giving scientific advice to the Norwegian Pollution Control Authority on this matter, in such a way that the risk management in Norway could be as uniform as possible, independent of the source of exposure.

2.1 Recent international developments on bisphenol A

Recently, in a draft brief on bisphenol A dated 14 April 2008, the U.S. National Toxicology Program (NTP) has concluded that there is *some* concern for neural and behavioural effects in fetuses, infants, and children at current human exposures. The NTP also expressed *some* concern for bisphenol A exposure in these populations based on effects in the prostate gland, mammary gland, and an earlier onset of puberty in females (NTP, 2008).

The Government of Canada has recently announced that they are planning to take action on BPA according to their Chemicals Management Plan. Reports from Health Canada and Environment Canada have raised concerns over possible harmful effects on newborns and infants, and in particular, on the elimination of BPA from the bodies of newborns and infants (Environment Canada and Health Canada, 2008). A 60-day public comment period on whether to ban the import, sale and advertising of baby bottles which contain BPA in Canada began on 19 April 2008.

The European Commission has in a request from 16 May 2008 (corrigendum to an initial letter dated 30 April 2008) asked EFSA to further assess possible age dependent toxicokinetics of BPA in animals and humans and their implications for hazard and risk assessment of BPA taken into account the most recent information and data available in the reports from U.S. NTP, Health Canada and Environment Canada. EFSA expects to provide further advice on the issue of BPA by July 2008.

3 OPINION

The present opinion is based on a thorough evaluation of the design, conduction of and the results in the four studies mentioned in the terms of reference (Adriani *et al.*, 2003; Carr *et al.*, 2003; Negishi *et al.*, 2004; Ryan and Vandenbergh, 2006). The design of the studies has been evaluated in light of recommendations given in relevant guidelines dealing with developmental neurotoxicity testing in animals. A review of the relevant guidelines for assessment of developmental neurotoxicity is included as appendix I.

The most important period for development of the nervous system is during *in utero* and early life. During this period, the nervous system is particularly susceptible for injuries.

Developmental neurotoxicity refers to any adverse effects of perinatal exposure to a toxic substance on the normal development of structure and/or function of the nervous system. Behaviour can be used for testing the integrity of almost all parts of the nervous system and behavioural testing have become central in the identification of potential neurotoxicants. Regulatory agencies have during the later years included functional assessments as a means for screening potentially neurotoxic compounds (Hass, 2006). A developmental neurotoxicity study gives information about whether the neurotoxicity seen is a part of the chemical's toxicity profile but may not be sufficient to determine whether the neurotoxicity is due to a direct or indirect effect of the exposure.

Developmental neurotoxicity studies are usually conducted in rodents with administration of the test substance to the dams during gestation and lactation. The neurotoxicity evaluation of offspring is based on observations to detect gross neurological and behavioural abnormalities, including the assessment of physical development, behavioural ontogeny, motor activity, motor and sensory function, learning and memory; and the evaluation of brain weights and neuropathology during postnatal development and adulthood. It is considered of vital importance that behavioural tests are designed and conducted in agreement with recommended guidelines and good scientific standard and practice.

The recent international developments on bisphenol A described in section 2.1 are not addressed in this opinion from VKM Panel 4.

3.1 Assessment of developmental neurotoxicity studies

3.1.1 Brief summary of the studies

A short overview of the most essential constituents of the four studies in question is shown in table 1 (page 17).

Adriani et al. 2003

Mated Sprague-Dawley (SD) rats (n = 9) were exposed to BPA dissolved in arachis oil at a concentration of 0.04 mg/kg by micropipette from mating day until PND 25. Control females (n=9) received arachis oil without BPA. Offspring were thus exposed to BPA *in utero* and through dams' milk until weaning at postnatal day (PND) 25. One male and one female per group (n=9/sex/group) was tested for novelty preference (PND 30-45), impulsivity (PND > 70) and response to d-amphetamine (PND > 70). Results were analysed by 3-4 ways ANOVA (analysis of variance between groups) without any adjustments for repeated measures (repeating data from same animals).

The **Novelty preference test** (PND 35-42) was carried out in a plexiglas box (70x30x35cm) divided in two parts by a partition wall with a door in. The rats were habituated to one part of the box (the familiar side) for 3 days. On day 4 the door was opened and time spent in the novel part of the box and activity in the novel vs familiar part (measured as line crossings) during a 24-min session (three 8-min time intervals), was video recorded and analysed. For **activity**, 4-ways ANOVA (**time** x **sex** x **treatment** x **side)** showed no main effect of "sex", "treatment" or "side". For novelty, 3-ways ANOVA (**time** x **sex** x **treatment**) showed no main effect of "sex" nor "treatment". "Time" was the most determinant single factor for both

variables. For "**activity**" a statistically significant "sex x time"-interaction and for "**novelty**" a statistically significant "sex x treatment"-interaction, appeared. Based on these interactions**,** "sex" was analysed separately. BPA-exposed rats of both sexes showed less decreased **activity** with time compared to their respective controls, particularly during the last 8-min of the 24-min session. In this respect, BPA-exposed rats showed higher activity than controls - in absolute counts BPA-exposed rats did about 10 more line crossings than controls. BPA-exposed females spent less time exploring the **novel** part during the first and last 8-min of the 24-min session than control females (approximately 1.5 vs 3 min and 3 vs 5 min, respectively) which was interpreted as BPA-exposed females showed tendency to avoid novelty, whereas BPA-exposed males did not.

For the **Impulsivity test** (PND >70), computer-controlled operant chambers were used. A chamber was provided with a house light, two nose-poking holes, a feeder device, a tray light and a tray where feed was delivered. The rats were feed-deprived prior to the 30-min daily sessions. Testing consisted of a training phase (1 week) and a testing phase (1 week). During the *training week*, rats were trained to achieve a feed reward in response to nose-poking. Nose-poking resulted in 1 or 5 pellets depending on nose-poking in the hole designated "immediate and small" (IAS) or "large and delayed" (LAD). The house light was lit (1 sec) during feed delivery. After feed delivery the tray light was lit for 25 sec, and during this period additional nose-poking did not result in feed delivery, but was recorded as "inadequate responding". During the training week, all rats developed a preference for the LAD hole – for both adequate and inadequate responses. During the *testing week*, feed was still delivered in response to nose-poking, but increasing delays (0-10-20-40-60-80-100 sec) was introduced for the LAD hole. The delay was fixed for a daily session. During the "delay period" the house light was on and additional nose-poking did not result in feed delivery, but was recorded as "inadequate responding".

Variables were the percentage choice between the LAD- and IAS holes, and frequency of inadequate responding. Results were analysed by 4-ways ANOVA (**sex** x **treatment** x **hole** x **delay factor**). For **percentage choice between the LAD- and IAS holes**, a main effect of "treatment" showed that BPA-exposed rats chose the LAD hole in more cases than the controls, even during the testing week when delays were introduced for the LAD hole and all rats progressively shifted towards the IAS hole which delivered 1 pellet immediately in response to a nose-poke. Concerning **inadequate responding**, no main effect of treatment appeared, but main effect of "sex" and a "sex x treatment"-interaction was seen. When the delay was 1 min or longer, BPA-exposed *males* had marked preference for the LAD hole compared to control males, e.g. BPA-exposed males showed *reduced* impulsivity and managed to withhold responses and wait for a reward. Control males were more active than both control females and BPA-exposed males/females. There was no difference between groups of females.

One week after impulsivity test, 4 rats/sex and 5 rats/sex received saline (1 ml/kg bw) and amphetamine (1 mg/kg bw) in the control and BPA-group, respectively, 15 min before a 30-min session in an **open-field** apparatus (plexiglas box 70x30x35cm). The behaviour was video-recorded and analysed (crossing and rearing) by ANOVA (**drug** (saline/amphetamine) x **treatment** (BPA/control) x **sex** (female/male)).

For both "crossing" and "rearing", a main effect of "drug" appeared, which showed that amphetamine caused increased activity level independent of "treatment" and "sex". For "crossing", a main effect of "sex" and a "sex x treatment"-interaction additionally appeared

and sex was analysed separately. Amphetamine caused more increased crossing in control males (about 100%) than in BPA-exposed males (about 50%), but no difference in increase between females (both groups increased about 50%). For "rearing", a "sex x treatment"-interaction additionally appeared and sex was analysed separately. Amphetamine caused less increased rearing in BPA-exposed males (about 50%) than in controls (about 100%), but no difference in increase between females (both about 50%). In sum, BPA-exposed **male** rats responded less to amphetamine than control males, in particular concerning number of crossings.

The authors concluded that perinatally exposure to BPA caused increased neophobia in female rats and reduced impulsive behaviour in male rats. The latter is possible related to changes in brain monoaminergic functioning because amphetamine-induced increased activity was less in male rats exposed to BPA compared to controls.

Comments from VKM Panel 4:
No positive control, no dose-response to BPA, and no parameters on reproductive toxicity was included in the study design. Concerning the dosing of BPA, it is not known whether the concentration given is per kg oil or per kg body weight of rats. The authors state that the administered dose is "within the range of human exposure". Based on this, VKM will assume that the dose is given as mg/kg bw/day. No control of cyclicity in females was included in the study, and thus not adjusted for in the statistical analysis. It is known that motor activity varies with the cyclic period in females with a peak phase of activity that corresponds to the cornification phase of estrus. Statistics: Results were analysed by 3-4 ways ANOVA. A repeated measure design was presumably added to the ANOVA when repeated measures from the same rat were utilized.

For study details, see **Appendix II.**

Carr et al. 2003:
Effects of performance in the Morris Water Maze were investigated in rats dosed with bisphenol A (BPA) or 17-beta-estradiol (E_2) from delivery to postnatal day (PND) 14. Male and female Fischer 344 rat was used for breeding and fed a casein-free diet in order to avoid natural phytoestrogens. The day after delivery (PND 1) *pups within the same litter* were assigned to different treatments and daily administered by gavage, either safflower oil 0.5 ml per kg bw (control), 100 µg/kg bw bisphenol A (low BPA), 250 µg/kg bw bisphenol A (high BPA) or 72 µg/kg bw 17 beta-estradiol (E_2). Total number of replications of each treatment group for each sex was 10. This design implicate that all pups in each litter were indirectly exposed to **all** test compounds through urine and feces as well as through direct contact (greasy oil). One male and one female per group (n=10) was assigned for behavioural testing at PND 33-40. Results were analysed by mixed model ANOVA, which includes adjustments for repeated measures (repeating data from the same animals).

Testing on PND 33 for swimming ability and motivation in a straight swim channel (15x150cm with an escape ramp in one end) showed no difference between groups. In the **Morris water maze** (PND 34-40) which tests spatial learning and memory, normally male rats perform better than females. During acquisition, exposure to E_2 or BPA-low eliminated the normal gender differences. This was due to worsen *male* performance and not facilitated female performance and unlike what was expected. Exposure to BPA-high exaggerated the normal gender differences during acquisition, due to worsen *female* performance. Females

exposed to E_2 (and BPA) were expected to perform as well as males and in such a way eliminate the normal gender differences because exposure to androgen-derived estrogens during brain development is supposed to *masculine the female brain*. Also unlike what was expected, exposure to BPA-high worsens the retention of spatial information in particular in females (statistically significant) but also in males (not statistically significant).

Comments from VKM Panel 4:
Although positive control and two dose levels of BPA were included in the study design, the exposure regimen in which all dose groups were represented in each litter leave behind huge uncertainties about the results. Presumably only 10 litters were used totally. Test animals in different treatment groups were littermates. There was no verification of pup exposure, e.g. chemical analysis of blood or tissue residues included in this study. Thus, the cause of the behavioural differences which appeared is unclear. Less emphasis is therefore placed on this study.

For study details, see **Appendix III.**

Negishi et al. 2004
Effects of response to fear-provoking stimuli and trans-2-phenylcyclopropyl-amine hydrochloride (tcy) were investigated in male rats perinatally exposed to bisphenol A (BPA) or nonylphenol (NP). Mated Fischer 344/N rats were daily exposed to corn oil 2 ml per kg bw (control), 0.1 mg/kg bw nonylphenol (NP low) or 10 mg/kg bw nonylphenol (NP high), or 0.1 mg/kg bw bisphenol A (BPA) by gavage from gestational day (GD) 3 to postnatal day (PND) 20 (n = 10-11/group). Offspring were exposed *in utero* and through dams' milk until weaning at PND 21. In the preweaning period, body weight of offspring was recorded and the dams were controlled for toxic signs. At weaning on PND 21, dams and redundant pups were sacrificed and subjected to pathological examination. At the age of 2 to 6 months one male per group and litter (n = 7-10/group) were behaviourally tested in the following devices: Open field, Spontaneous motor activity, Passive avoidance test, Elevated plus maze, Active avoidance test, and Monoamine-disruption test. The results were analysed by ANOVA.

There were no adverse effects of treatment on the reproductive parameters. No significant effect of treatment appeared for locomotion or rearing (**Open field**) or the rhythm or total counts of activity or immobile time (**Spontaneous motor activity**). No fear-provoking reactions in the **passive avoidance** test or in the **elevated plus maze** were seen. In the **active avoidance** test a main effect of treatment showed that exposure to BPA and low dose of NP caused fewer avoidance responses compared to the controls. In the **monoamine-disruption** test, BPA males and NP low dose males also failed to show a significant increase in locomotion following tcy-injection compared to controls.

The authors conclude that perinatal exposure to BPA and NP cause adverse behavioural effect when the animals were forced to avoid fear-provoking stimuli. In other words, NP/BPA-exposure disrupted the reception of intolerable stress, possibly due to alterations in the monoaminergic system.

Comments from VKM Panel 4:
The study design did not include a positive control or dose-response of BPA, but some parameters on reproductive toxicity. Test animals were males only, which excludes evaluation of possible sex differences in response to BPA or NP exposures.

The animals went through a set of different tests and bad experience in one test may influence on the performance in the following ones. Results were analysed by ANOVA and for the active avoidance test adjustment for repeated measures was included. There is however concern about the lack of information about how the data were recorded (e.g. manually, blinded to the tester) in the elevated plus maze and the passive and active avoidance tests.

Developmental exposure to BPA did not influence on the animals' level of activity or on the tolerability for anxiety in general, but in situations with extreme stress the tolerability seems to be raised. The interpretation that this may be related to alterations in the monoaminergic system is questioned because alterations in locomotion that is evident only after pharmacological manipulations must be interpreted with caution.

For study details, see **Appendix IV.**

Ryan and Vandenbergh, 2006

Effects on anxiety and spatial memory were investigated in ovariectomised female mice perinatally exposed to bisphenol A (BPA) or ethinyl estradiol (EE). Mated C57/B1-6 mice were daily exposed to tocopherol-stripped corn oil 40 µl per dose (control), 2 µg/kg bw BPA, 200 µg/kg bw BPA, or 5 µg/kg bw ethinyl estradiol by gavage from gestational day (GD) 3 until weaning on postnatal day (PND) 21. Offspring was exposed to BPA *in utero* and through dams' milk until weaning on PND 21. At weaning, litter size, anogenital distance and pup weight were measured. Twenty-one females were assigned for puberty onset by daily checking for vaginal opening and subsequently cornified cells in vaginal smear (indicative of cycling). One week after weaning one female offspring per litter (n=14-16) were surgically ovariectomised and assigned for behavioural testing of **anxiety** (Elevated-plus maze and the light/dark preference chamber) and **spatial memory** (Radial-arm maze and Barnes maze) which started on PND 42. Results were analysed by ANOVA and a repeated measure design was added in the tests for spatial memory.

There were no effect of exposure found on the anogenital distance, litter size or body weight measured at weaning. Exposure to EE and highest dose BPA significantly accelerated onset of puberty in female mice as measured as day of first cornified smear.

In the **elevated-plus maze** the EE-exposed mice spent less time (15 sec) in open arms than controls (55 sec). No effect of BPA-exposure appeared although the mice in the highest dose BPA group spent marginally less time (30 sec vs 55 sec) in open arms ($p=0.06$). In the **light/dark preference chamber**, EE- and highest dose BPA mice spent significantly less time (75 sec and 120 sec, respectively) in the light part compared to controls (240 sec). No differences in number of transitions between light and dark parts or latency to first enter dark part of the chamber appeared. In the **radial-arm maze**, EE-exposed mice performed significantly less errors than controls in the last 5 out of 10 trials. The BPA-exposed animals differed from the controls in single trials. EE-exposed mice did fewer errors than controls in the **Barns maze**, but no significant effect of BPA-treatment was seen.

The authors concluded that perinatally exposure to 5 µg/kg bw ethinyl estradiol or 200 µg/kg bw BPA accelerated onset of puberty in female offspring mice. Furthermore, that ethinyl estradiol was found to masculinise behaviour in ovariectomised mice in all behavioural assays

used and that BPA increased anxious behaviour in a dose-dependent fashion, but had no effect on short-time spatial memory.

Comments from VKM Panel 4:
A positive control, two dose levels of BPA and some parameters on reproductive toxicity were included in the study design. However, the reproductive parameters were assessed at weaning and not at delivery, which is an incomplete assessment. The test animals were ovariectomised females only which excludes evaluation of possible sex differences in response to BPA or EE exposures. Additionally, even if the use of ovariectomised mice removes the potential confounding factors of cyclicity on behaviour, it also eliminates the evaluation of possible hormonal interactions of the test substance that may influence on behaviour.

With regard to puberty onset, the number of females per group was limited, 5-4-5-7 for the control, low dose BPA, high dose BPA and the EE groups, respectively, and it is not known whether the animals in each group represent different litters. The result is thus questioned.

Effects interpreted as anxiety-related behaviour was only shown in one (light/dark) of two tasks and only in mice exposed to the highest BPA dose. There is concern about the lack of information about how the data were recorded (e.g. manually or automatically) in all the behavioural tests.

For study details**, see Appendix V.**

Table 1. Short overview of essential constituents of the studies

Parameters	**Adriani *et al.*, 2003**	**Carr *et al.*, 2003**	**Negishi *et al.*, 2004**	**Ryan and Vandenbergh, 2006**
Species/strain/route	Rat/SD/oral by micropipette	Rat/Fisher/oral by gavage	Rat/Fisher/oral by gavage	Mice/C57/BL-6/oral by gavage
Dose groups	1) Control/arachis oil 2) BPA in arachis oil at a concentration of 0.04 mg/kg[1]	1) Control /safflower oil 0.5ml/kg bw 2) 17 beta-estradiol E2 3) BPA 0.10 mg/kg/day 4) BPA 0.25 mg/kg/day	1) Control /corn oil 2ml/kg bw 2) BPA 0.10 mg/kg/day 3) NP 0.10 mg/kg/day 4) NP 10 mg/kg/day	1) Control/corn oil 0.4 µg/dose 2) BPA 0.002 mg/kg/day 3) BPA 0.20 mg/kg/day 4) EE 5 µg/kg/day
Dosing period	GD 0 - PND 25 (mating to weaning)	PND 1 to PND 14 (directly to pups)	GD 3 to PND 20	GD 3 to PND 21
Number (N) dams	9 /group	Not given – presumably 10 totally	10-11/group	Probably 14-16/group
Offspring exposed	*In utero* and through lactation	Directly	*In utero* and through lactation	*In utero* and through lactation
Offspring tested	1 male and 1 female per litter Totally: 9/sex /group	All exposure groups within the same litter. Totally: 10/sex/group	1 male per litter Totally: 7-10 males/group	1 ovariectomizied female per litter Totally: 14-16 females/group
Reproductive and developmental parameters	Not performed	Not performed	Body weight of dams and pups Pathological exams of dams and pups	At weaning: anogenital distance, body weight of pups, litter size. Assessment of puberty
Activity	Not performed	Not performed	- Open field behaviour (PNW 8) - Spontaneous motor activity (PNW 12)	Not performed
Anxiety-related behaviour	Novelty preference test (PND 35-45)	Not performed	Elevated plus-maze test (PNW 14)	Elevated plus-maze (PND 42) Light/dark preference chamber
Learning and memory	Impulsive behaviour (PND > 70) : - Schedule-controlled test (nose poking holes, increased delay in food delivery)	Spatial memory: - Swim channel test (on PND 33) - Morris water Maze test (on PND 34)	Learning and memory: - Passive avoidance test (PNW 13) - Active avoidance test (PNW 15)	Short-time spatial memory: - Radial-arm maze - Barnes maze
Pharmacological challenge	Open field response to amphetamine challenge (PND> 70)	Not performed	Open field response to tcy-challenge (Monamine disruption test)	Not performed

BPA – bisphenol A, NP – nonylphenol, EE – ethinyl estradiol, tcy - trans-2-phenylcyclopropyl-amine hydrochloride, GD – gestational day, PND – postnatal day, PNW – postnatal week

[1] It is not known whether the concentration given is per kg oil or per kg body weight of rats. The authors state the administrated dose is "within the range of human exposure". Based on this, VKM will assume that the dose is given as mg/kg bw/day.

3.1.2 Comments to studies design and results from VKM Panel 4

All four studies discussed have been published in international peer reviewed journals. However, none of the studies were performed according to Good Laboratory Practice (GLP). The main emphasis in the present opinion has been placed on the studies by Adriani *et al.*, 2003; Negishi *et al.*, 2004, Ryan and Vandenberg, 2006. Less emphasis has been placed on the study by Carr *et al*. 2003, which will be commented on in the end of this section.

The studies by Adriani *et al.*, 2003 and Negishi *et al.*, 2004 were performed with rats, while the study by Ryan and Vandenberg, 2006 used mice as test specie. In all studies, the main endpoint for BPA induced developmental neurotoxicity was behavioural alterations in offspring and the litter was used as the statistical unit. The number of litters per treatment group in the various studies varied from 9 to 16. According to OECD 426 – developmental neurotoxicity, testing for motor activity and associative learning and memory, should be conducted in 20 offspring/sex (1/sex/litter) and 10 offspring/sex (1/sex/litter), respectively for each treatment group.

In all three studies, dams were directly exposed to BPA from mating until weaning of the offspring by gavage. The maternal BPA exposure level was in the range 0.002 – 0.20 mg/kg bw/day. Dose-response of BPA (two dose levels) and a positive control (ethinyl estradiol) was included in the study of Ryan and Vandenberg, but not in the other two. Preferentially, in order to achieve dose-response at least three dose levels and a concurrent control should be used.

Some parameters on reproductive toxicity were included in two of the studies. Negishi *et al.*, 2004 recorded body weight of offspring and controlled dams for toxic signs during the lactation period. Additionally, necropsy of dams and redundant pups were performed at weaning. No effects of BPA exposure were found. Ryan and Vandenberg, 2006 measured litter size, anogenital distance and pup weight at weaning in addition to onset of puberty in female mice only. They reported "accelerated puberty" in female mice developmentally exposed to the highest dose of BPA. The "accelerated puberty" is questioned because there are uncertainties whether the animals in each group represented different litters and the number per group was small (4-7 mice). The study of Adriani *et al.*, 2003 included no such parameters. Preferentially, litter size and anogenital distance is to be measured at birth and at weaning and the calculated viability of pups should be reported in addition to body weight during the pre weaning period.

In all three studies, the offspring were indirectly exposed to BPA *in utero* and through dams' milk. Both sexes were behaviourally tested in the study of Adriani *et al.*, whereas Negishi *et al.*, used male rats only and Ryan and Vandenberg ovariectomised female mice only, as test animals. Preferentially, both sexes should be tested in parallell.

The three studies utilized tests classified in the same categories; anxiety-related behaviour and learning and memory (see table 1). Negishi *et al.,* 2004 additionally ran two tests for motor activity.

The tests utilized differed across studies except for one (Elevated plus-maze) in two studies (Negishi *et al.*, 2004 and Ryan and Vandenberg, 2006). Because no references were given for the test conduction it remains unknown whether the Elevated plus-maze was conducted

equally in these two studies. The behavioural tests were mainly conducted according to acceptable methods in all studies.

Testing of the animals started at different ages in the different studies: at postnatal days 35, 42 and 56 for the studies of Adriani *et al.*, 2003, Negishi *et al.*, 2004 and Ryan and Vandenbergh, 2006, respectively. The elevated plus-maze, which was used for anxiety-related behaviour in two of the studies, was conducted in male rats at the age of 98 days (PNW 14) (Negishi *et al.*, 2004) whereas it was conducted in ovariectomized female mice at the age of 42 days (Ryan and Vandenberg, 2006). Comparison of behavioural results across species and ages must be done with caution. According to OECD 426 – developmental neurotoxicity, testing for motor activity and associative learning and memory should be conducted both in adolescent (PND 23-27) and in young adults (PND 60 and older) and preferentially not in the same animals in order to avoid confounding effects of age and prior training.

Information about how the behavioural data were recorded (e.g. manually, blinded to the tester) are insufficient in all studies; either for some or for all of the tests used. In all studies, results were mainly analysed by ANOVA and a repeated measure design was mainly included when repeated measures for the same animal were utilized.

Anxiety-related behaviour was shown in female animals developmentally exposed to PBA in the studies that included females (Adriani *et al.*, 2004, Ryan and Vandenberg, 2006). Female rats, but not males, developmentally exposed to BPA showed some neo-phobia (spend less time than control female in the novel part of a novelty preference test), however, both male and female rats showed some increased novelty-induced stress (increased activity in the novel part) (Adriani *et al.*, 2004). The novelty preference test started when the animals were 35-45 days of age which also is the period for puberty onset. No control of cyclicity in females was included in the study, and thus not adjusted for in the statistical analysis. It is known that motor activity varies with the cyclic period in females. Ryan and Vandenberg showed that ovariectomized female mice developmentally exposed to the highest BPA dose showed anxiety-related behaviour in a light/dark preference chamber (spend less time in light place compared to controls). The use of ovariectomized mice removes the potential confounding factors of cyclicity. No anxiety-related effects of BPA were shown in the elevated plus-maze for male rats (Negishi *et al.*, 2004), or ovariectomized mice (Ryan and Vandenberg, 2006).

Concerning the **learning and memory** tests, adverse effects of developmental exposure to BPA in male animals appeared in the studies that included males (Adriani *et al.*, 2003, Negishi *et al.*, 2004). In the study of Adriani *et al.*, 2003, BPA exposed males showed decreased impulsiveness in a schedule-controlled nose-poking test, but BPA exposure had almost no implications on female behaviour. In the study of Negishi *et al.*, 2004, which only included males, no effect of BPA exposure in male rats appeared in the passive avoidance test but effects were seen in the active avoidance test. Apparently the BPA-exposed male rats failed when they were forced to avoid fear-provoking stimuli; they acquired avoidance response to an electrical input more slowly than control males but did develop adequately escape performance. In the study of Ryan and Vandenberg, 2006, which only included females, no effects on spatial memory in ovariectomized mice developmentally exposed to BPA were found.

No effects on **motor activity** appeared in 56-85 days old male rats developmentally exposed to BPA (Negishi *et al.*, 2004).

In order to detect the neurological cause behind the behavioural alterations found, two of the studies (Adriani *et al*., 2003, Negishi *et al.*, 2004) utilized chemical challenge and motor activity was measured previous to and after the chemical challenge. Adriani *et al*., 2003 showed that male rats, but not females, developmentally exposed to BPA were less sensitive for amphetamine-induced hyperactivity than control. Negishi *et al.*, 2004 showed tendency to increased activity in BPA-exposed male rat following injection of trans-2-phenylcyclopropyl-amine hydrochloride, which may indicate alterations in the monoaminergic system. One has to be aware that behavioural alterations which are evident only after pharmacological manipulations must be interpreted with caution.

Based on the reported results, the studies indicate that maternal exposure to 0.04 mg/kg bw or 0.2 mg/kg bw BPA during gestation and lactation may cause anxiety-related behaviour in female offspring but not in males (Adriani *et al.*, 2003 and Ryan and Vandenbergh, 2006). Correspondingly, depending on the test procedure maternal exposure to 0.04 mg/kg bw or 0.1 mg/kg bw BPA during gestation and lactation may cause behavioural alterations consistent with decreased impulsiveness/facilitated learning (schedule-controlled test) or learning deficits (active avoidance test) in male offspring (Adriani *et al.*, 2003 and Negishi *et al.*, 2004), but no spatial memory deficits were reported in females (Ryan and Vandenbergh, 2006). However, comparison of behavioural results across species, ages and methods must be done with caution.

Less emphasis is placed on the study by Carr *et al.*, 2003 due to several shortcomings in the experimental design and reporting. Presumably only 10 litters were used totally. The test animals in different treatment groups were littermates. There was no verification of pup exposure, e.g. chemical analysis of blood or tissue residues included in this study. Thus, the cause of the behavioural differences which appeared is unclear.

The four studies are reviewed in more detail in **Appendices II-V.**

3.2 Exposure assessment

Bisphenol A is primarily used as a monomer in the production of polycarbonate (PC) and as a precursor of certain epoxy resins used for coatings. PC is widely used in products such as tableware, food and drink packaging including infant bottles. Epoxy resins are used to coat metal products such as food cans, beverage containers, lids for glass jars and bottles as well as water supply pipes. With respect to children, mouthing of items with residual levels of BPA, such as mittens, may further contribute to oral exposure. Other potential sources for oral exposure are intake via drinking water as well as from regional and/or local contamination of the environment.

According to the terms of reference, the Norwegian Food Safety Authority has asked VKM to include relevant exposure data proposed by the Norwegian Pollution Control Authority to the work with the revised EU RAR. An overview of this data is shown in Table 2.

Table 2. Daily intake of Bisphenol A from different sources (estimated by the Norwegian Pollution Control Authority (SFT) on the basis of EU RAR 2003)

Source of exposure*	**Daily intake of BPA (mg/kg bw/day)**
Exposure via food and beverages	
Canned food and beverage (infant 6 – 12 months) (EU RAR)	0.0043
Canned food and beverage (young child 1.5 – 4.5 years) (EU RAR)	0.009
Polycarbonate tableware and food storage containers (young child 1.5 – 4.5 years) (EU RAR)	0.0009
Canned food and beverages including wine, and polycarbonate tableware and food storage containers (adults) (EU RAR)	0.0015 (0.00125 + 0.00025)
Exposure via the environment	
Local BPA exposure (EU RAR)	0.007
Regional exposure (EU RAR)	0.0000093
Regional exposure (Mjøsa, Drammensfjorden etc.) (SFT)	0.000029
Mittens (SFT)	0.0033

*Data are based on the following concentrations of BPA in food or beverages:
Canned food and beverages (infant 6-12 months) – 100 µg/kg
Canned food and beverages (child 1.5-4 years) – 50 µg/kg
Canned beverages (adult) – 10 µg/l (including wine)
Canned food (adult) – 50 µg/kg
Polycarbonate tableware and food storage containers – 5 µg/kg foodstuff

3.2.1 Estimates of daily intake of BPA from polycarbonate and epoxy-resin food contact applications

Consumer exposure to BPA may occur via the oral or dermal route. In this opinion, only oral exposure is considered. The highest potential for human oral exposure is through migration of BPA from products directly in contact with food and beverages.

Table 3. Potential daily intake of BPA from food contact materials and mittens (µg/kg bw/day) (based on EFSA 2006, EU RAR 2003/2008, SFT)

	BPA concentration in food/mittens (µg/kg) or liquid (µg/l)	Dietary exposure to BPA (µg/kg bw/day)				
		0-3 months infant	4-6 months infant	6-12 months infant	Child 1.5 – 4 years	Adult
Migration of BPA from PC bottles to liquid (EFSA, 2006)	50/10[a]	8.7/1.7[b]	5.9/1.2[c]	5.9/1.2[c]		
Migration of BPA from PC bottles to liquid (EU RAR, 2003)	50	8[d]	7[e]	7[e]		
Migration from epoxy-resin cans to powdered formula (EFSA/EU RAR)	100	2.3[b, f]/2.1[f]	2.3[b,f]/1.9[f]	1.6[c, f]/-		
Migration from epoxy-resin cans to commercial food and beverages (EFSA/EU RAR)	100/50/10[g]			5.2[c]/4.3[h]	4.2[i]/9[i]	1.2/1.2
Migration from PC tableware and storage containers (EFSA/EU RAR)	5			0.3/0.3	0.9[i]/0.9[i]	0.25/0.25
Mittens (SFT)	98.2				3.3[j]	

[a] EFSA: a conservative scenario based on the highest concentration observed in realistic conditions of use vs. a more typical migration concentration
[b] EFSA: based on an average body weight of a 3 months infant of 6.1 kg and consumption of 174 ml/kg bw/day of infant formula reconstituted from 23 g/kg of powder
[c] EFSA: based on an average body weight of a 6 months infant of 7.8 kg, consumption of 118 ml/kg bw/day of infant formula reconstituted from 16 g/kg of powder and 52 g/kg bw/day of canned food and beverages
[d] EU RAR: based on an average body weight of 4.5 kg and consumption of 0.7 l/day (1-2 months)
[e] EU RAR: based on an average body weight of 7 kg and consumption of 0.98 l/day (4-6 months)
[f] EFSA: data based on samples from a non-EU market; EU RAR: not used in EU risk characterisation since EU infant formulae are not packed in food cans
[g] EFSA: 100 µg/kg food or beverage for infants, 50 µg/kg for food and 10 µg/l beverages for adults and children. EU RAR: 100 µg/kg food or beverage for infants, 50 µg/kg food or beverage for children and 50 µg/kg for food and 10 µg/l beverages for adults
[h] EU RAR: based on body weight 8.7 kg and intake of 0.375 kg canned food and beverages
[i] EFSA/EU RAR: based on an average body weight of 11 kg and consumption of 2 kg food and beverages (1/3 food and 2/3 beverages)
[j] SFT: based on a body weight of 15 kg and 50 g of mittens ingested over a period of 100 days/year

Potential exposure of formula-fed infants (0 - 6 months)

Dietary exposure of infants 0-6 months depends on their consumption pattern. This includes situations where babies are exclusively breastfed to situations where babies are exclusively fed infant formulae. Dietary sources would be BPA migrating from PC bottles into liquid (50/10 µg/l, Table 3) and BPA migrating from cans with epoxy-phenolic coating into infant formula powder (100 µg/kg).

Based on the migration values of 50 µg/l BPA from PC bottles and 100 µg/kg BPA from cans with epoxy-phenolic coating used by EFSA (Table 3), a conservative potential dietary exposure for a 3 month formula-fed infant would be 11 µg/kg bw/day (8.7 + 2.3 µg/kg bw/day). Based on the more typical migration of 10 µg/l BPA from PC bottles which also is suggested by EFSA, a potential dietary exposure for a 3 month formula-fed infant would be 4 µg/kg bw/day (1.7 + 2.3 µg/kg bw/day). The corresponding dietary exposures for a 6 month infant would be 8.2 µg/kg bw/day (5.9 + 2.3 µg/kg bw/day) and 3.5 µg/kg bw/day (1.2 + 2.3 µg/kg bw/day), respectively.

The potential dietary exposure based on estimation from EU RAR (Table 3) results in a total intake of 8 µg/kg bw/day for a 2-4 months infant and 7 µg/kg bw/day for a 4-6 months infant.

The overall potential dietary exposure of formula-fed infants aged 0-3 and 4-6 months would be in the range of ***4 - 11*** *and* ***3.5 - 8.2 µg/kg bw/day****, respectively.*

Potential exposure of formula-fed infants (6 - 12 months)

Dietary sources of exposure to BPA for infants in the age group 6 - 12 months would be through intake of commercial baby food and drinks as well as from PC tableware and storage containers and from PC bottles. Based on migration values of BPA used by EFSA (table 3), intake of canned food and beverages would lead to an exposure of 5.2 µg/kg bw/day. Additional exposure would be 0.3 µg/kg bw/day from PC tableware and storage containers, 5.9 µg/kg bw/day from PC bottles and 1.6 mg/kg bw/day for use of canned powdered formula, resulting in a total conservative exposure of 13.0 µg/kg bw/day (5.2 + 0.3 + 5.9 + 1.6 µg/kg bw/day). If the more typical migration value of 10 µg/l from PC bottles to liquid is used, a total exposure of 8.3 µg/kg bw/day (5.2 + 0.3 + 1.2 + 1.6 µg/kg bw/day) is obtained. Based on values used by EU RAR, estimated exposure would be 4.3 µg/kg bw/day from canned food and beverages (food intake = 0.375 kg/day, bw = 8.7 kg), 0.3 µg/kg bw/day from PC tableware and storage containers and 7.0 µg/kg bw/day from PC bottles resulting in a total exposure of 11.6 µg/kg bw/day (4.3 + 0.3 + 7.0 µg/kg bw/day).

The overall potential dietary exposure of formula-fed infants aged 6 – 12 months would be in the range of ***8.3 - 13.0 µg/kg bw/day.***

Potential dietary exposure of young children (1.5 - 4.5 years)

Estimations made by both EFSA and EU RAR are based on a total daily intake of 2 kg of commercial foods (one third solid and two-thirds beverages) and the assumption that all foods and beverages comes from sources that lead to BPA exposure (Table 3). This results in an overestimation of the actual intake through dietary sources, but the degree of overestimation

is unknown. An additional source for exposure of young children would be through mouthing of mittens used outdoors during winter season.

Daily exposure of BPA from mittens estimated by the Norwegian Pollution Control Authority is based on a value of 98.2 µg residual BPA/g mittens and the assumption that the complete amount of residual BPA from 50 g mittens is ingested over a period of 100 days per year. This estimation represents a worst case scenario and is based on analysis of residual ("freely available") BPA in selected products presented in the report "Miljøgifter i utvalgte produkter" (Molab AS, 2006). In short, test material from three different types of mittens was extracted in dichloromethane for 48 hours and treated with ultrasound three times during the extracting period before BPA was analysed by GC/MS. Levels of residual BPA in the three types of mittens tested were 3.9, 23.1 and 98.2 mg/kg test material.

Based on migration values used by EFSA (50 µg/kg for food and 10 µg/l for beverages), the estimated exposure from canned food and beverages and the use of PC tableware and storage containers would be 5.1 µg/kg bw/day (4.2 + 0.9 µg/kg bw/day). Estimated exposure based on migration values used by EU RAR (50 µg/kg for food and 50 µg/l for beverages) would be 9.9 µg/kg bw/day (9.0 + 0.9 µg/kg bw/day). Migration from mittens would result in an additional exposure of 3.3 µg/kg bw/day.

*The overall potential dietary exposure of young children (1.5 - 4.5 years) would be in the range of **8.4 - 13.2 µg/kgbw/day** when migration from mittens is included.*

Potential dietary exposure of adults

Estimations made by both EFSA and EU RAR are based on a total daily intake of 1 kg canned food, 2 litre canned beverages (including wine) and the use of PC tableware and storage containers. Based on migration values by EFSA and EU RAR (50 µg/kg for food and 10 µg/l for beverages) (Table 3), this would result in a potential exposure of 1.2 µg/kg bw/day (0.83 + 0.33 µg/kg bw/day). Migration from PC tableware and storage containers would add a potential exposure of 0.25 µg/kg bw/day.

*The overall potential dietary exposure of an adult would be **1.45 µg/kg bw/day.***

The overall potential dietary (consumer) exposure for different age groups is summarised in Table 4.

Table 4. Summary of the overall potential dietary (consumer) exposure to BPA

Sources	Estimated dietary exposure to BPA (µg/kg bw/day)				
	0-3 months infant	4-6 months infant	6-12 months infant	Child 1.5 – 4 years	Adult
Food and beverages	4.0 – 11.0	3.5 – 8.2	8.3 – 13.0	5.1 – 9.9	1.45
Food, beverages and mittens				8.4 – 13.2	

3.2.2 Migration of bisphenol A from polycarbonate bottles – recent studies

Numerous studies have been reported on migration of bisphenol A from polycarbonate bottles into water or food stimulant. Some studies were designed to estimate migration from simulated real use conditions, others intended to estimate maximum migration of BPA from PC under extreme conditions or migration from long-lasting use. Data from these studies have been summarised and are published in various opinions/risk assessments (EFSA, 2006; EU RAR 2003; EU RAR 2008; NTP 2007; Environment Canada and Health Canada, 2008). A conservative concentration of 50 μg/l BPA migrating from PC bottles to liquid has been used to estimate daily intake of BPA in infants in the assessments made by EFSA and EU RAR.

Three recent studies investigated migration of BPA from PC bottles under conditions representative of actual use (Ehlert *et al.*, 2008, Le *et al.*, 2008; Maragou *et al.*, 2007). In the study by Le *et al.*, migration of BPA from new and used PC bottles filled with water and left for up to 7 days at room temperature was measured. The mean concentration of BPA in water after 24 hours was 0.24 μg/l. Mean concentrations across all sampling days ranged from 0.08 to 1.33 μg/l for both new and used PC bottles. The rates of BPA migration at room temperature were in the range of 0.20 ng/h to 0.79 ng/h. When bottles were filled with boiling water (100°C) and left for 24 hours at room temperature, the rate of migration increased by 15- to 55-fold and the final concentrations of BPA in water ranged from 1.92 μg/l up to 7.67 μg/l (Le *et al.*, 2008).

In the study by Ehlert *et al.*, residual content of BPA was measured in 18 different brands of polycarbonate baby bottles and migration of BPA was determined by placing the bottles filled with water in a microwave oven and heating to 100 °C. Average migration of BPA into water after three microwave cycles ranged from < 0 – 0.7 μg/l, and there was no difference in the amount of BPA after the three consecutive heating cycles. Furthermore, there was no correlation between the amount of residual BPA in the bottles and migration of BPA.

In the study by Maragou *et al.*, migration from new PC bottles under a variety of conditions was assessed. No detectable migration of BPA was reported when bottles were cleaned, rinsed and sterilised prior to being filled with water and incubated at 70°C for 2 hours (detection limit 2.4 ppb). When bottles, after repeated cycles of cleaning and sterilisation, were filled with boiling water and left at room temperature for 45 minutes, the average BPA concentration was 10 ppb (ranged from the detection limit 2.4 ppb up to 14.3 ppb). A fourth recent study investigated the migration of BPA from PC bottles under extreme washing conditions (Biedermann-Brem *et al.*, 2007). They concluded that after alkali washing solutions at concentrations typical for dishwashers, the amount of BPA released and transferred into the beverage was unlikely to exceed 10 μg/l.

The estimated contribution to daily intake of BPA per kg body weight for infants based on migration values from these studies are shown in Table 5. All concentrations were found to be below the conservative estimations used by EFSA and EU RAR.

Table 5. Contribution from PC bottles to potential daily intake of BPA for infants (μg/kg bw/day)

Infant age group (months)	Migration of BPA from PC bottles into water at room temperature (0.24 μg/l)[a]	Migration of BPA from PC bottles into water during microwave heating (0.7 μg/l)[b]	Migration of BPA from PC bottles into water when filled with boiling water (10 μg/l)[c]	Migration of BPA from PC bottles into liquid (EFSA/EU RAR) (50μg/l)[d]
1-2[e]	0.04	0.11	1.6	7.8
4-6[f]	0.03	0.10	1.4	7.0
6-12[g]	0.02	0.07	1.0	5.2

[a] Migration value from Le *et al.*, 2008
[b] Migration value from Ehlert *et al.*, 2008
[c] Migration value from Maragou *et al.*, 2007
[d] Migration value from EFSA 2006, EU RAR 2003/2008
[e] Based on an average body weight of 4.5 kg and consumption of 0.7 l/day (1-2 months) (EU RAR)
[f] Based on an average body weight of 7 kg and consumption of 0.98 l/day (4-6 months) (EU RAR)
[g] Based on a body weight of 8.7 kg (EU RAR) and an assumed volume drink taken in from PVC bottles of 0.9 l/day (additional to the 0.375 kg canned food and beverages) (6 - 12 months)

3.2.3 Exposure via the environment

The main route of environmental exposure to BPA is the oral route. Local exposure estimated by EU RAR (2008) varies from 0.0058 μg/kg bw/day to 41 μg/kg bw/day. The highest level is found in the vicinity of plants producing BPA. From regional sources, the average total daily exposure to BPA via the environment is estimated to be 0.0093 μg/kg bw/day. Regional exposure includes intake from drinking water, fish, crops, meat, milk and air. Regarding regional sources in Norway, consumption of fish from certain lakes and fjords would result in an increased exposure compared to the average regional exposure estimated by EU RAR (2008). Based on concentration of BPA registered in fish from lake Mjøsa, Drammensfjorden etc. (0.014 mg/kg), and by using standard defaults for indirect exposure of humans applied by EU, the Norwegian Pollution Control Authority (SFT) has estimated a total regional exposure of 0.029 μg/kg bw/day (Fjeld *et al.*, 2004a; 2004b; EC TGD, Part 1). Based on Norwegian data on fish consumption, the total regional exposure would be 0.019 μg/kg bw/day (VKM, 2007) (Table 6).

Table 6. Concentration of exposure to humans via the environment estimated by EU RAR (2008), SFT and VKM

Source of exposure	**Daily intake of BPA (μg/kg bw/day)**
Local BPA exposure (EU RAR, 2008)	41
Regional exposure (EU RAR, 2008)	0.0093
Regional exposure (Mjøsa, Drammensfjorden etc.) (SFT)[1]	0.029
Regional exposure (Mjøsa, Drammensfjorden etc.)[2]	0.019

[1] Based on intake of 115 g fish/day, body weight 70 kg (TGD on Risk Assessment, EUR 20418EN/1)
[2] Based on intake of 65 g fish/day, body weight 70 kg (Fish and seafood consumption in Norway, VKM, 2006)

3.2.4 Aggregated exposure

For adults in certain areas of Norway, a potential aggregated exposure would be from dietary intake of BPA (Table 3) and an additional increased environmental exposure due to consumption of fish from contaminated water sources (Table 6). An estimated aggregated intake would be **1.5 μg/kg bw/day of BPA** (1.45 + 0.029 (0.019) μg/kg bw/day). No local exposure is included. For young children (1.5 - 4.5 years), an additional regional exposure from eating contaminated fish would be of minor significance compared to the estimated potential exposure from dietary sources and mittens (Table 3).

4 CONCLUSION

The report by Tyl and co-workers was central in the EFSA opinion (EFSA, 2006) and the updated EU RAR (EU, 2008). The Tyl study is a GLP compliant 2-generation reproductive toxicity evaluation in mice performed according to a modified OECD 416 guideline (Tyl *et al.*, 2007). However, the study did not include functional tests for developmental neurotoxicity.

VKM Panel 4 noted that, although not addressed in this opinion, U.S. National Toxicology Program (NTP) and Canadian authorities recently published draft reports on effects of BPA, including developmental effects (neural and behavioural effects) and expressed *some* concern for neural and behavioural effects in fetuses, infants and children at current human exposures.

VKM has reviewed the four studies on neurodevelopmental toxicity of BPA (Adriani *et al.*, 2003; Carr *et al.*, 2003; Negishi *et al.*, 2004; Ryan and Vandenbergh, 2006) as requested by the Norwegian Food Safety Authority. Although the design and reporting of these studies suffer from major and serious shortcomings, the overall findings may raise some concern.

It is the opinion of the VKM Panel 4 that the four studies mentioned above do not provide sufficient evidence for setting a robust lower NOAEL for BPA than the current EFSA NOAEL of 5 mg/kg bw/day. The Panel is aware that the EU Commission recently has requested EFSA to re-evaluate the information available on BPA.

In order to eliminate any uncertainty regarding potential developmental effects of BPA at low doses, it is recommended that a GLP compliant study is carried out according to OECD guideline 426. Such a study should utilize a broad concentration range from the very low doses up to those with known maternal effects.

A Norwegian exposure scenario based on available data on exposure to BPA from food and beverages and via the environment was performed. In general, exposure levels of BPA in Norway are low. The estimated exposure of infants and children is in the range of 3.5 – 13.2 µg/kg bw/day, whereas the estimated aggregated exposure of adults is 1.5 µg/kg bw/day. As a result of the current use of BPA in food contact materials and other consumer products, infants and children are exposed to higher levels of BPA per kg body weight than the rest of the population.

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6 APPENDICES

6.1 Appendix I - Guidelines for reproduction toxicity, including developmental toxicity

Three guidelines which include parameters for observing possible developmental neurotoxicity in animals have been selected and are briefly summarised:

1. OECD guideline 416. Two-generation reproduction toxicity study (Adopted January 2001)
2. OECD guideline 426. Developmental neurotoxicity study (Adopted October 2007)
3. ICH Topic S5 (R2) Detection of toxicity to reproduction for medicinal products & toxicity to male fertility (Adopted March 1994)

Table 7 gives a brief comparison of the three relevant guideline-studies with focus on parameters relevant for observing developmental neurotoxicity.

6.1.1 OECD guideline 416: Two-generation reproduction toxicity study

The aim of this two-generation reproduction test is to provide general information concerning effects of a test substance on the integrity and performance of the male and female reproductive systems, including gonadal function, the oestrus cycle, mating behaviour, conception, gestation, parturition, lactation and weaning, and the growth and development of the offspring.

The study design includes three generations (P, F1 and F2) and both sexes of the parental (P) and first offspring (F1) generations are dosed. In the assessment, the F1 generation is particularly important as this has been exposed to the test article, first *in utero* and through milk from their dams. From weaning, the test substance is administered directly to the F1 generation during growth and development to adultness and mating, and further to the F1 females through gestation to weaning of the next generation (F2). The guideline recommends assessment of developmental neurotoxicity by functional observations (reflex ontogeny, sensory function, motor activity) before and/or after weaning of the F1 offspring if such investigations not are included in separate studies.

6.1.2 OECD guideline 426: Developmental neurotoxicity study

This guideline, which recently has come into force, is specifically aimed at assessment of the potential of chemicals to exert developmental neurotoxicity. A study designed according to this guideline will provide data including dose-response characterisation on the potential functional and morphological effects on the developing nervous system of the offspring that may arise from exposure *in utero* and during early life. Dosing to the parental generation female rats is from gestational day (GD) 6 to weaning of the offspring at postnatal day (PND) 21. The offspring are selected and kept for various evaluations up to young adultness (PND 60-70).

Neuropathology and various test categories for neurological function and performance *in vivo* are defined, and within each category specific tests are proposed (Table 7). The tests described in OECD guideline 426 are designed specifically for assessing developmental

neurotoxicity, but it is also proposed that the neurotoxicity tests can be conducted as a combination with the study described in OECD guideline 416.

6.1.3 ICH Topic S5 (R2): Detection of toxicity to reproduction for medicinal products and toxicity to male fertility

This guideline includes all studies recommended to be performed in order to evaluate possible toxicity to reproduction of medicinal products. One of the studies described in this guideline is a study to evaluate effects on pre- and postnatal toxicity. The design of this study is quite similar to that of OECD guideline 426 with regard to animal numbers, groups and dosing period, but the offspring is kept undosed until adultness to assess reproductive performance and the F1 females are kept until parturition of offspring. This study includes assessment of developmental neurotoxicity by carrying out specific tests on sensory function and behaviour on the F1 offspring (Table 7).

It is concluded, that out of the three summarised guidelines, OECD 426 is specifically dedicated to assess developmental neurotoxicity and is best suited for such investigations.

Table 7. Overview of the three relevant guidelines for assessment of effects on developmental neurotoxicity

Guideline	**OECD 416 – Two-generation reproduction toxicity study**	**OECD 426 – Development Neurotoxicity Study**	**ICH Topic S 5 (R2) Detection of Toxicity to reproduction for Medicinal Products and Toxicity to Male Fertility**
Design and group size	Control and ≥ 3 test groups ≥ 20 animals/sex/group P and F1 generations dosed	Control and ≥ 3 test groups ≥ 20 animals/group Only P generation ♀ dosed	Control and ≥ 3 test groups 16- 20 animals/group Only P generation ♀ dosed
Preferred species	Rat	Rat	Rat
Dosing period	P ♂: 70 days before mating P ♀: several cycles before mating and until weaning of offspring F1 ♂: Weaning to mating F1 ♀: Weaning to weaning of offspring (F2)	Dosing of P ♀ only from GD6 to PND21 (weaning) Offspring selected for testing are kept until young adult (PND 60-70)	Dosing of P ♀ only from GD6 (implantation) to PND21 (weaning) After weaning, offspring selected for mating to assess reproductive competence
Offspring (F1) evaluated on	• Clinical observations, morbidity, mortality • Food/water consumption • Body weight/growth • Oestrus cycle/sperm parameters • Number of pups, sex, live/stillbirths, gross anomalities • Age of vaginal opening/perputial separation and for F2 anogenital distance • Necropsy w/organ weights • Histopathology	• Clinical observations, morbidity, mortality • Body weight/growth • Number of pups, sex, live/stillbirths, gross anomalities • Anogenital distance and age of vaginal opening/perputial separation	• Clinical observations, morbidity, mortality • Food/water consumption • Body weight/growth • Number of pups, sex, live/stillbirths, gross anomalities • Age of vaginal opening/perputial separation • Necropsy w/organ weights • Histopathology • Physical development
Specific assessment on developmental neurotoxicity	• Functional observations (recommended but optional) o motor activity, sensory function, reflex ontogeny	• Brain weight and neuropathology (macro/microscopic) • Behavioural ontogeny eg.: o righting reflex, negative geotaxis • Motor activity (automated recording) • Motor and sensory function eg.: o extensor thrust, righting reflex, auditory startle habituation, evoked potentials • Learning and memory eg.: o passive avoidance, delayed matching to position, olfactory conditioning, Morris water maze, Biel or Cincinnati maze, radial arm maze, T-maze, retention of schedule controlled behaviour	• Sensory functions and reflexes eg.: o Surface righting o Auditory startle o Air righting o Response to light • Behaviour o Motor activity o Learning and memory

P: parental generation, F1: offspring of the parental generation, F2: offspring of the F1 generation, GD: gestation day, PND: postnatal day

6.2 Appendix II (Adriani *et al.*, 2003)

Altered Profiles of Novelty Seeking, Impulsive behavior and response to D-amphetamine in rats perinatally exposed to bisphenol A

SUMMARY OF STUDY DESIGN:

Animals:

- Mated Sprague-Dawley (SD) rats (observation of vaginal plug=[1]GD 1)
- Treatment group and control (n=9 per group)

Dose:

- bisphenol A (BPA) dissolved in arachis oil at a concentration of 0.04 mg/kg ("within the range of human exposure")
- arachis oil

Exposure period:

- Dams were exposed from mating day until [2]PND 25 by micropipette
- Pups were exposed *in utero* and through dam's milk

Delivery:

- No information

Lactation/suckling period:

- No information

Weaning:

- PND 25

Pathological examination:

- No information

Test animals:

- Indirect exposure
- One male/female per litter (n=9/group)
- Housed in groups of 3 (same sex)

Test period:

- Starts 3 weeks after weaning and when adult (>70 days old)

Test procedures:

- Test 1: Novelty preference test (PND 30-45)
- Test 2: Impulsivity test (operant test procedure) (PND > 70)
- Test 3: Open field with amphetamine (PND > 70)

[1] GD – gestational day

[2] PND – postnatal day

Statistics:

- General design of the ANOVA was: two **sex** (male vs female) x **treatment** (control vs BPA) x "subject"
- In addition for Test 1: x **side** (familiar vs novel) and x **time** factors
- In addition for Test 2: x **delay factor** (0-100 sec)
- In addition for Test 3: x **drug factor** (saline vs amphetamine)
- Multiple comparisons within significant interactions: Tukey HSD test

COMMENTS FROM VKM PANEL 4:

Exposure data is given imprecisely: Bisphenol A is administrated to rats in a concentration of 0.04 mg/kg, but it is not clear whether this is per kilo oil or per kilo rats' (body weight). The volume of oil administered is not given; but it was "depending on body weight".

No information of endpoints concerning toxicity (e.g. body weight) is given and information about reproductive parameters in general (length of pregnancy, litter size, litter sex-ratio, culling, body weight increase in pups and dams, etc.) are very scarce.

The statistics: It is not stated whether repeated measure design was added to the ANOVA when repeated measures from the same rat was utilized, but presumably it is included. 3/4-ways ANOVAs may be informative but may also be difficult to interpret.

Short information about the behavioural tests used:

Novelty preference test (PND 35-42)

Plexiglas box (70x30x35cm) divided in two parts by door. The two parts had wide-mesh and narrow-mesh floor, respectively.

Familiarization phase (day 1-3): each animal 20 min in one part (named "the familiar compartment")

Novelty preference test (day 4): 5 min in familiar part, 24 min in the whole box (door open)

Behavior video-recorded and analysed: time spent in each part, number of times the rat crossed (with both forepaws) the lines of the three floor-sections (on the video screen the floors were subdivided into three sections).

Impulsivity test (PND >70)

Operant chamber: two nose poking holes, a feeder device, tray where feed was delivered and tray light, house light. Schedule-controlled behavior is measured.

Rats were food deprived prior to testing (80% of normal diet)

30-min sessions

<u>Training phase (1 week):</u>

- Nose-poking in one of the two holes caused delivery of immediate and small pellet (IAS, one pellet) or large and delayed pellet (LAD, five pellets)
- House light was lit (1 sec) during feed delivery
- After feed delivery, tray light was on for 25 sec. In this period nose-poking did not result in feed delivery, but was recorded
- Any nose-poking resulted in feed delivery except when house light was on

Testing phase (1 week):

- Delayed delivery of feed reinforce in response to nose-poking in the LAD hole (few, large pellets)
- During the delay, the house light was on and additional nose-poking did not result in feed delivery, but was recorded ("inadequate responding")
- The delay for the LAD hole was fixed during a daily session but gradually increased (0-10-20-40-60-80-100 sec) over days/sessions
- Dependent variable:
  - percentage choice between LAD- and IAS holes
  - frequency of inadequate nose-poking

COMMENTS FROM VKM PANEL 4:
Regarding the impulsivity test: For the" training phase", there are not given any endpoint/cut off value to ensure that all animals had learned the procedure equally, e.g. a certain number of feed pellets delivered and consumed. After a "training phase", all animals should be on the same level in order to avoid the introduction of systematic errors in the testing phase. The percent choice for the LAD and IAS hole is given under "Results". According to [3]Fig. 2, when delay is 0 it seems that both groups had established a preference for the large hole (about 70% nose-poking). Even the percent nose-poking for the LAD- and IAS holes, respectively, is equal between groups, the absolute number of nose-pokings and of reinforcers (pellets) obtained should have been given as it influences on the learning (positive feedback).

Open field with amphetamine (1 week after impulsivity test)
Plexiglas box (70x30x35cm)
Floor subdivided in three sections (lines on the video screen)
One 30-min session
Video-recorded behavior
15 min prior to testing, d-amphetamine or saline was injected subcutaneously to 5 and 4 rats, respectively, per group.
d-amphetamine: 1 mg/kg dissolved in saline and injected subcutaneously (1 ml/kg bw)
Saline: 1 ml/kg bw
Variables: latency, frequency and duration of rearing (rat in vertical position), grooming (mouth or paw on body), and crossing the floor sections (both forepaws cross lines placed on video screen). Behavior was video recorded and analysed in a treatment-blinded manner by means of computer (software "The observer").

Results:

Novelty preference test

Activity (e.g. number of times the rat crossed (with both forepaws) lines on video screen which illustrated three sections of the floor)
4-ways ANOVA (**time** x **sex** x **treatment** x **side)**:

[3] With regard to figures, it is referred to figures in the original paper

Main effect of time, e.g. time had a significant influence on the activity, which decreased in all groups with advancing time.
Sex x time: e.g. sex had different activity depending on time.
Sex was therefore analysed separately by 3-ways ANOVA (**time** x **treatment** x **side)**:

Males: "time by side"-interaction, "treatment x time x side"-interaction (e.g. the two groups "activity" varied with "session time" and "side of the chamber".)
The authors interpret this as when males were in the novel compartment, *males in general* were more active early in session than later (e.g. "time", Fig. 1c). Initially (the first 0-8 min) there was no group difference, and both groups showed *decreased* activity throughout the 24-min session. BPA kept higher activity than controls during the second interval (9-16 min) and the last interval (17-24 min) of the session. However, only activity during the last 8 min (difference in absolute count about is 10) yields statistically significance.
In the familiar compartment, males of both groups showed almost no activity (data not shown).

Females: "side x treatment"-interaction: e.g. the females in general were most active in the novel compartment compared to the familiar one and most active during the first two 8-min intervals. BPA-exposed showed statistically significantly *higher* activity than controls (and males) during the second (9-16 min) and last (17-24 min) interval of the session (difference in absolute count is about 10). Initially (0-8 min) there was no group difference (Fig. 1d).
In the familiar compartment, no group difference appeared.
Sum: BPA-exposed rats of both sexes showed higher activity in the last 8-min of a 24-min session than their respective control sex.

COMMENTS FROM VKM PANEL 4:
Neither main effect of "treatment" nor main effect of "sex "appeared in the overall analysis, which imply that neither of these factors had any significant implication on the behavior observed. "Time" was actually the most determinant single factor on activity, which also influenced on sex: "sex x time" -interaction. Sex was therefore analysed separately. For none of the sexes no main effect of the main variables (**time, treatment, side)** appeared, but significant interactions. For males, "treatment" appeared in a three-ways interaction and influenced on rats "activity" together with "time" and "side". For female, "treatment" influenced together with "side".

BPA-exposed males/females showed less decreased activity *with time* compared to their respective controls. This may indicate that BPA-exposed rats need longer time than controls to be familiar with a new environment (e.g. changed habituation profile), **or** that they were more curious **or** more active in general than controls. The absolute difference in line-crossing during each of the two last 8 min time-interval (no difference during the first 8 min) is about ten (10). The author denotes this finding as "novelty-induced hyperactivity", but it is questioned if this activity level can be characterized as "hyper".

"Activity" varies with the cyclic period in females. No control of cyclicity was included in the study and thus not adjusted for in the statistical analysis.

Novelty preference: (% time spent in "novel part" during 8-min interval of the 24 min session)
3-ways ANOVA (**time** x **sex** x **treatment):** Main effect of "time" and for the "sex x treatment"-interaction
"Time" had a significant influence on the preference for novelty, which increased with increasing time.
Sex x treatment: e.g. sex reacted different on novelty depending on treatment.
Sex was therefore analysed separately by 2-ways ANOVA (**time** x **treatment**):
Females: main effect of "treatment"; then multiple comparisons showed that BPA-exposed spend less time in the first (approx. 1.5 vs 3 min) and last 8 min (approx. 3 vs 5 min) of the 24 min session than controls.
Males: No difference between BPA-exposed and control males in time spent in novel compartment
Sum: BPA-exposed females showed tendency to avoid novelty, whereas BPA-exposed males did not.

COMMENTS FROM VKM PANEL 4:
No main effects of "treatment" or "sex" appeared in the overall analysis, but of "time". "Time" was actually the most determinant single factor. Sex was analysed separately based on the interaction "sex x treatment", and for females "multiple comparisons" was applied. BPA-exposed females showed a different novelty-seeking pattern across time compared to controls which is interpreted as a ***tendency*** to avoid novelty, whereas BPA-exposed males did not.

Impulsivity test
Choice between reinforcers: ("large and delayed" (LAD) or "immediate and small" (IAS))
Rats of both groups and sexes established a preference for the large reinforcers during the training period (LAD hole) based on % choices (Fig. 2). With increasing delays before delivery of the reinforces in response to nose-poking in the LAD hole, rats started to nose-poke for the small and immediate ones (IAS hole).
No sex differences were observed.
Main effect of "treatment": BPA-exposed rats (both sexes) had marked preference for the LAD hole during the whole test, e.g. reduced impulsivity.

COMMENTS FROM VKM PANEL 4:
During the training phase, all nose-poking except when house- and tray light was on resulted in a reward. Thus, this is a schedule that promotes activity. If the rats had or developed unequal activity during the training phase they also achieved different learning curves.

As a measure of the feed-back on the nose-poking behavior, the number of reinforcers (feed pellet) achieved should have been included and mentioned in the text, both for the testing phase and for the training phase. Additionally, no information about how much the rats eat in cage after testing is given, nor is the rats' body weight gains. The possibility that BPA-exposed rats were less hungry than the controls and therefore managed to wait for several pellets (LAD hole) instead of taking one (IAS hole), cannot be excluded.

According to Fig. 2 (both groups, both sexes), when the delay was at its maximum of 100 sec: BPA-rats and controls chose to nose-poke the LAD hole in 40% and 35% of all nose-poking, respectively. The reminding per cent nose-poking was presumably in the IAS hole.

Inadequate responding: (nose-poking in either hole during the delays – obtained no reinforcers)
With increasing duration of the delay, the nose-poking in the LAD hole *decreased* whereas that in the IAS hole increased (e.g. inability to inhibit an inadequate response - *measure of impulsivity*)
Main effect of "delay": e.g. –"delay" was a main factor that influenced on inadequate responding
"delay x hole"-interaction: e.g. - the inadequate responding in the LAD hole was reduced with increasing delays whereas that in the IAS hole was increasing
Main effect of "sex": e.g. – "sex" influenced on inadequate responding and "sex" was then analysed separately.
"sex x treatment"-interaction: e.g. - dependent on treatment, sex behaved differently

Males: main effect of "group", "delay x treatment"- interaction, "delay x hole x treatment"-interaction

As duration of delay increased, the inadequate nose-poking behavior increased in both groups. However, when delays were 1-min or longer (rats had to wait for reward), BPA-exposed rats did significantly *less* nose-poking in the hole for small and immediate reinforcers (IAS hole) when the house light was on (Fig. 3 a) than control males. This was interpreted as BPA-males showed *less impulsive behavior* than control males. Control males were more active than control females/BPA-exposed males/females.

Females: no main effects or interactions

COMMENTS FROM VKM PANEL 4:
BPA-exposed males did clearly less inadequate nose-poking in the IAS hole with increasing delays, but how was the absolute number of inadequate nose-poking in the LAD hole? (Fig. 2 shows only the per cent choices of the LAD hole). The variable "inadequate responding" was defined as nose-poking without reward in either holes – and in order for the reader to get a more complete picture concerning the rats behavior, the absolute number of poking into the LAD hole, as well as number of reinforces achieved, should have been presented. The "number of reinforces" is essential to ensure that all groups have had equal learning. Differences in this parameter indicate that different groups have had different feed-back on their behavior which will influence on the interpretation of the results.

Concerning the absolute number of BPA-exposed males nose-poking in the IAS hole when the delay was 1 min and more, it seems to level that of females, e.g. BPA-males showed a female-like nose-poking frequency (Fig. 3).

This reduced nose-poking may as well be interpreted as BPA-exposure makes the male rats smarter; they have learned that nose-poking when house light is on is not reinforced and manage to withhold inadequate responding – providing that there was no difference in inadequate nose-pokings in the LAD hole.

Open field with amphetamine
Crossing (no of crossing of the three subdivided floor-sections; horizontal movements)
3-ways ANOVA: drug (saline vs amphetamine) x **treatment** (BPA vs control) x **sex**
Main effect of "drug", e.g. amphetamine caused more "crossing" than saline in all groups
Main effect of "sex", e.g. males and females showed different activity level
"Sex x treatment"-interaction, e.g. sex showed different activity dependent on treatment

Sex analysed separately:
Males: main effect of "drug", main effect of "treatment", "drug x treatment"- interaction
Amphetamine caused increased line crossing in both groups of males, but more in control males (about 100%) than in BPA-exposed males (about 50%) (Fig. 4c).
Females: main effect of "drug", e.g. females in both groups showed increased activity in response to amphetamine (about 50%) compared to saline (Fig. 4d)

Rearing (frequency and duration when body is in vertical position)
3-ways ANOVA: drug (saline vs amphetamine) x **treatment** (BPA/control) x **sex**
Main effect of "drug", e.g. amphetamine caused increased "rearing" in all groups compared to saline
"Sex x treatment"-interaction, e.g. sex showed different "rearing" in the control- and BPA-groups
Sex analysed separately:
Males: main effect of "drug", main effect of "treatment": e.g. amphetamine caused increased rearing in all males, but the increase was less marked in BPA-exposed males (about 50%) than in controls (about 100%) (Fig. 4a)
Females: main effect of drug e.g. females in both groups showed increased activity (about 50%) (Fig. 4b)

COMMENTS FROM VKM PANEL 4:
The number of animals per drug (saline and amphetamine) is small: 4 rats/sex group and 5 rats/sex group received saline and amphetamine, respectively. The results of an ANOVA based on such a small number are questioned.

Concerning "rearing", there was no main effect of "sex" or "treatment" in the overall analysis but a significant "sex x treatment"-interaction.

In sum, BPA-exposed **male** rats responded differently on amphetamine than control males, in particular concerning number of crossings in an open field device. BPA-exposed **males** showed less increased crossings than control males.

Results of the variable "grooming" listed along with "crossing" and "rearing" under "Material and Methods" is not shown or mentioned in the text.

One has to be aware that behavioural alterations which are evident only after pharmacological manipulations must be interpreted with caution.

Conclusion:

The authors concluded:
1. Developmental exposure to BPA caused increased novelty-induced stress/reduced habituation to novelty during adolescence. BPA-exposed females showed neophobia (spend less time in novel than familiar department)
2. Developmental exposure to BPA caused decreased impulsivity (more preference for the LAD hole during testing) in adult rats. BPA-males exhibited a female-like frequency of nose poking when the delay before the next reinforce (feed) was 1 min or longer

3. Male rats developmentally exposed to BPA were less sensitive for amphetamine-induced hyperactivity than control. Female BPA-rats, on the other hand, showed similar response to amphetamine as control female.
4. The effects seen were sex dependent

Both novelty-induced stress and decreased impulsivity may be seen as indexes of a reduced reactivity or readiness to react to environmental changes.

COMMENTS FROM VKM PANEL 4:

1. Concerning "activity" in the Novelty preference test, BPA-exposed rats of both sexes showed *higher* activity than their respective control sex during the last 16 min of a 24 min interval. However, the absolute difference in activity between BPA-rats and controls is low (about 10 crossings) per 8-min interval and the biological implications are questioned. For none of the sexes no main effect of the main variables (**time, side** or **treatment**) appeared, but significant interactions. For males, "treatment" appeared in a three-ways interaction and influenced on the rats activity along with "time" and "side". For females, "treatment" influenced together with "side". It might be a bit too strong to characterize this finding as "hyperactivity/novelty-induced stress/reduced habituation".

   Concerning "novelty" in the Novelty preference test, "time" was actually the most determinant single factor. BPA-exposed females showed a different novelty-seeking pattern across time compared to controls which is interpreted as a tendency to avoid novelty in the first and last 8 min parts of a 24-min session, whereas BPA-exposed males did not.

   In sum, female BPA-exposed rats spend somewhat less time than control female in the novel part, but are more active when they do. Male BPA-exposed rats spend as much time as control males in the novel part, but are more active during the last 8 min spend. The author has interpreted this as "increased novelty-induced stress". "Activity" varies with the cyclic period in females. No control of cyclicity was included in the study and thus not adjusted for in the statistical analysis.

2. BPA-exposed males showed marked preference for the LAD hole compared to controls, which may be interpreted as *reduced* impulsivity **or** facilitated learning (learned that when light is lit, no reward is given in response to nose-poking). During the training phase, all nose-poking except when house- and tray light was on resulted in a reward. Thus, this is a schedule that promotes activity. The author mentions that a baseline is established during the training phase. As a measure of the feed-back on nose-poking behavior, the number of reinforcers (feed pellet) achieved should have been included and mentioned in the text. It is crucial for the interpretation of the results that all rats experienced equal learning during the training phase. Additionally, no information about how much the rats eat in cage after testing is given, nor is the body weight gain. The possibility that BPA-exposed males were less hungry than the controls and therefore managed to wait for several pellets (LAD hole) instead of taking one (IAS hole), cannot be excluded.

   Additionally, absolute number of inadequate nose-poking (during the delay, when nose-poking was without any consequences) is only given for the IAS hole. In order to get a more complete picture of the rats' behavior, number of nose-poking for the LAD hole

during the delay should have been given as well. The result of the nose-poking schedule is statistically significant for the BPA-exposed males, but it is asked for more results in order to get a complete picture of the behavior.

3. In control rats, amphetamine injection caused significantly increased rearing and crossing in an open field device. In particular concerning number of "crossing", BPA-exposed male rats showed less response to amphetamine than control male. No difference appeared in female rats following amphetamine injections. However, the number of animals per drug (saline and amphetamine) is small: 4 rats/sex and 5 rats/sex received saline and amphetamine in the control and BPA-group, respectively. The results of an ANOVA based on such a small number are questioned. One has to be aware that behavioural alterations which are evident only after pharmacological manipulations must be interpreted with caution.

4. In conclusion, in the schedule-controlled behavioral test and the amphetamine test, BPA-exposed males responded differently from male control. BPA-exposure had almost no implications on female behavior.

   No positive control or dose-response to BPA was included in the study design.

6.3 Appendix III (Carr *et al.*, 2003)

Effects of Neonatal Rat Bisphenol A Exposure on Performance in the Morris Water Maze

Summary of study design:

Animals:

- For breeding: Adult male and female Fischer 344 rat
- Casein free diet in order to avoid natural phytoestrogens
- After parturition: pups *within the same litter* were assigned to *different treatment* groups. There was a member of each treatment group for each sex per litter. Litters were not standardized. Total number of replications of each treatment group for each sex was 10.

Dose:

- Safflower oil (control), 0.5 ml per kg body weight
- 72 µg/kg body weight 17beta-estradiol (E_2)
- 100 µg/kg body weight bisphenol A (low BPA)
- 250 µg/kg body weight bisphenol A (high BPA)

Exposure period:

- Postnatal day (PND) 1 to 14 (day of delivery assigned PND 0)
- Orally by gavage to pups

Delivery:

- No information

Lactation/suckling period:

- Pup body weight was recorded in relation to dosing

Weaning:

- PND 22

Pathological examination:

- No information

Test animals:

- One male/female pup per group, n=10 (exposure groups *within same* litter)

Test period:

- PND 33 - 40

Test procedures:

- Straight swim channel
- Morris water maze

Statistics:

- SAS package using ANOVA mixed model

- ANOVA general linear model used prior to analysis: data were subjected to sphericity test for compound symmetry. For nonspherical data, the Greenhouse-Geisser adjusted F ratios were used
- Probe trial data were analysed using ANOVA general linear model

COMMENTS FROM VKM PANEL 4:

Number of mated females is not given, neither number of dams that delivered.
Number of litters per group is not given, Under Material and methods it says that "pups within the same litter were assigned to different treatment groups" and that "the total number of replications of each treatment group for each sex was 10". Presumably only 10 litters were used. Different exposure groups **within** the same litter. Even though casein free diet was used in order to eliminate natural phytoestrogens, all pups in each litter were exposed to **all** test compounds through urine and feces as well as through direct contact (greasy oil). Additionally, the dams would be exposed through cleaning the pups and elimination through milk cannot be excluded. Thus, there is no control of the test animal's exposure in this study and its use in toxicological evaluation of BPA is questioned. It is in particular not possible to rely on data from the control group.

Dosing to the pups starts on PND 1 which is one day after delivery. It is well known that disturbances during/soon after delivery stress the dam and may e.g. elicit cannibalism. In reproductive studies, handling of pups usually starts on PND 3.

The design does not include essential reproductive parameters which are indicative of the environmental and social factors during development that may influence on the later adult behavior.

Statistics: A mixed ANOVA model adjusts for repeating data from the same rat (includes a repeated measure design).

Short information about the behavioural test used:

Straight swim channel (15x150cm with an escape ramp in one end) **(PND 33)**

- Test of swimming ability and motivation
- Rat placed in one end with head facing the wall, thus the rat has to turn and swim to the other end in order to reach the escape platform.
- 4 consecutive times on **one** day (1-min intervals)
- Latency (sec) recorded (from one end to the other)

Morris water maze (PND 34-40)

- Test of spatial learning and memory
- Black circular tank (D=183 cm) visually divided in four quadrants with four starting points. An escape platform 1 cm below the water level was situated in one of the quadrants. Visual cues placed on walls in the room. Video camera mounted above the maze.
  - **Acquisition phase** (PND 34-37)
    - Four 60-sec trials per day for 4 days
    - 10 sec rest between the 4 daily trials
    - Different starting point in each trial (randomly chosen)

- Reaching the platform was rewarded by 10-sec rest
- If not finding the platform within 60-sec trial, rat was placed on platform for 10 sec
- Latency (sec) to find platform was recorded manually using a stopwatch

o **Probe trial** (PND 40)

- Performed 72 h after the acquisition phase
- Purpose: evaluation of memory of platform location
- One 60-sec trail
- Latency (sec) to find platform was recorded manually using a stopwatch

Results:

Body weight during the dosing period:

- No main effect of treatment (data no shown)

Straight swim channel

- No main effect of treatment/gender or interactions

Acquisition data

- Main effect of gender and statistically significant interaction "gender x treatment". Further analysis was therefore performed for each sex separately
- Females: no main effect of treatment ([4]Fig. 1). BPA-high *tended* to use longer time to find the platform
- Males: males exposed to E_2 tended to use longer time to locate the platform than controls but the difference was only statistical significance on day 3 (Fig. 1)
- Normal gender-dependent pattern (Fig. 2) changed by exposure:
  - Control: male perform better than female (males > females)
  - E_2 eliminates the gender difference (males ≈ females)
  - BPA-low nearly eliminates the gender difference (males ≥ females)
  - BPA-high exaggerates the gender difference (males >> females)

Probe data

High BPA-females spent significantly *less* time than controls in the escape quadrant. High BPA-males tended to spend less time in the escape quadrant (not statistically significant).

Conclusion:

The authors conclude:
Acquisition of maze performance was significantly better in control males than in control females. Postnatal exposure to E_2 or BPA did not negatively affect **acquisition** of the Morris water maze, but

- Exposure to E_2 or BPA-low eliminated the normal gender-differences during acquisition due to worsen male performance and not facilitated female performance. Exposure to BPA-high did not disrupt the normal gender differences during acquisition, but appeared to result in some negative effects on female performance. Exposure to BPA-high worsened the retention of spatial information in rats (longer time to locate the platform: statistically significant in females, but also decreased in males).

[4] With regard to figures, it is referred to the original paper's figures

The authors point out that gender differences in the acquisition of Morris water maze previously have been reported but so has lack of gender differences. The gender difference found in the present study was mainly due to E_2-males (and BPA-low male) using longer time to locate the platform than control males. This is unlike what expected, because exposure to androgen-derived estrogens during brain development is supposed to *masculine the female brain*. Thus, females exposed to E_2 (and BPA) were expected to perform as well as males in this task and in such a way eliminate the normal gender differences. The author refers to other studies and suggests that female performance in the Morris water maze may not be strongly influenced by developmental exposure to E_2.

COMMENTS FROM VKM PANEL 4:
Less emphasis is placed on this study due to several shortcomings in the experimental design and reporting. Presumably only 10 litters were used totally. The test animals in different treatment groups were littermates. There was no verification of pup exposure, e.g. chemical analysis of blood or tissue residues included in this study. Thus, the cause of the behavioural differences which appeared is unclear.

6.4 Appendix IV (Negishi *et al.*, 2004)

Behavioral Alteration in Response to Fear-Provoking Stimuli and Tranylcypromine Induced by Perinatal Exposure to Bisphenol A and Nonylphenol in Male Rats

Summary of study design:

Animals:

- Adult male and female Fischer 344/N rat
- Sperm in vaginal smear (examined daily) was assigned [5]GD 0
- Mated dams were randomly assigned to treatment groups, n= 10-11 per group

Dose:

- Corn oil (control), 2 ml per kg body weight per day
- 0.1 mg/kg body weight nonylphenol (NP low)
- 10 mg/kg body weight nonylphenol (NP high)
- 0.1 mg/kg body weight bisphenol A (BPA)

Exposure period:

- GD 3 to [6]PND 20 (day of delivery assigned PND 0) by gavage
- Daily recording of maternal body weight

Delivery:

- Pups counted, weighed
- Litters were culled to 6 (equal sex ratio as far as possible)

Lactation/suckling period:

- Dams controlled for clinical signs of toxicity
- Pups body weight recorded on PND 3-7-14-21 (and at 8 and 13 weeks of age)

Weaning:

- PND 21
- Male pups housed in group according to treatment (n=7-8 per cage)

Pathological examination:

- At weaning: organ weight (liver, kidney, spleen, thymus)
  - Dams
  - Some of the males not used for behavioral test
- At 8 week of age:
  - The rest of the males not used for behavioral test

Test animals:

- One male offspring per group and litter (n=7-10/group)

[5] GD - gestational day
[6] PND - postnatal day

Test period:

- Postnatal week (PNW) 8-24 (e.g. 2 to 6 months old rats)

Test procedures:

- Open field
- Spontaneous motor activity
- Passive avoidance test
- Elevated plus maze
- Active avoidance test
- Monoamine-disruption test

Statistics:

- StatView, Version 5.0
- Body weight: ANOVA with "treatment" as between-subject factor and "day" as repeated measure factor
- No of pups and organ weight: one-way ANOVA
- Behavioral test measures (except avoidance test): one-way ANOVA
- Active avoidance test concerning percentage correct avoidance: repeated measures of ANOVA over days (sessions)
- Passive avoidance test concerning latency, data were logarithmically transformed
- Post hoc analysis: Fischer's protected least-significant difference test for comparisons between groups

COMMENTS FROM VKM PANEL 4:
Number of mated females is not given, neither number of dams that delivers.

Housing males in groups may influence on the arrangement of range between males.

Pups were examined and litters were culled at the day of delivery. This is usually done at PND 2-3 in order to leave the dam and her litter in peace the first days.

The statistics: control for repeated measures (repeating data from same animals) for the Active avoidance test is included.

Short information about the behavioral test used:

Open field (PNW 8)

- Male housed individually 24 h before test
- Performed during the dark phase (21:30-23:00)
- Rectangular field (56 x 39 cm) above which a video camera was mounted
- 5-min session
- Behavior automatically recorded and analysed by computer-assisted system
- Variables: locomotion, rearing, "other behaviour"

Spontaneous motor activity (PNW 12)

- Male housed individually in the test cage 24 h before test

- Sensor monitor mounted above test cage which used body heat as an indirect measure of activity
- 12-h register period during dark phase
- All counts were automatically totaled and recorded in 2-min intervals
- An 2-min interval with no signal was defined "immobile time" (count = 0)

Passive avoidance test (PNW 13)
- Male housed individually during test
- Light and dark compartment
- Session one:
  - Animal placed in the light part, when entering the dark part an electric foot shock was given
  - Latency period before entering the dark part recorded
- Session two (24 h later): retention trial where no shock in dark part occurred
  - Animals placed in the light part
  - Test terminated when animal entered dark part or after 20 min
  - Recorded
    - Latency period before entering the dark part
    - Frequency and percentage of poking into the dark part until complete entrance

Elevated plus maze (PNW 14)
- Two open and two closed arms (same type of arm opposite each other) and an central square platform
- Rat placed in the central square facing an open arm
- 5-min session
- Recorded: Frequency of entries into open/closed arms recorded (arm entering=moving the head into an open arm)

Active avoidance test (PNW 15)
- Male housed individually during test
- Two way shuttle box: two compartments separated by wall with hole in
- Rats had a 5-min habituation to the box before every trial
- Acquisition test
  - 5-sec buzzer tone and light (conditioned stimuli, CS) was followed by a 5-sec electrical foot shock (unconditioned stimuli, UCS) in one part of the box, the rat could avoid the shock by moving to the other part
  - 25 daily trials in four consecutive sessions/days (totally 100 trials)
- Extinction test (on the day after the fourth session)
  - The same as described above but without UCS (totally 25 trials)
- Each trial separated by variable intertrial intervals (10-90 sec; total 1,250 sec/session)
- Variables:
  - correct avoidance responses (rat moves to "safe" compartment within the 5-sec CS) recorded in each block of 25 daily trials (both the acquisition *and* the extinction phase)

- percent failure to avoid the 5-sec stimulus (shock) within its 5 sec duration (in the four acquisition phase)
- latency periods associated with both CS and UCS (in the four acquisition sessions)

Monoamine-disruption test (PNW 22-24)

- Single intraperitoneal injection of saline challenge one day prior to the tcy[7]-challenge
- Single intraperitoneal injection of 5 mg/kg bw tcy
- Rats were subjected to 4 min test in the open-field apparatus 5.5 h after both injections

COMMENTS FROM VKM PANEL 4:

No details on how the variables were recorded in the passive and active avoidance tests or the elevated plus maze are given (e.g. manually, blinded to the tester).

The "Passive avoidance test" is a simple method for assessing learning and memory, but substantial intersubject variability makes the use of large groups necessary. According to the information given under "Results", only 8 males per group were tested.

In the "Active avoidance test" the animals can avoid the shock by making a specific response (e.g. the term "active" avoidance) which in this case was to move to the "safe" compartment within 5 sec. This was presumably a two-ways conditioned avoidance response; no fixed "safe" compartment but the animal had to return to the compartment where it was just shocked when the signal occurred. It is not given details whether the same apparatus as in the passive test was used. According to the information given under "Results", 9-10 males per group were tested, which is more than that for the passive test. This implies that some of the males in each group had not attended the passive test and were novel to the association "shock and escape".

Results:

Maternal toxicity and reproductive results

- No statistical significant findings on maternal bw, litter size, organ weight (**data not shown**)

Development of male offspring

- No statistical significant findings on body weight gain or organ weight

Open-field test (PNW 8)

- No effects of treatment on locomotion or rearing (**data not shown**)

Spontaneous motor activity (PNW 12)

- No main effect of treatment on the rhythm or total counts of activity, or immobile time (**data not shown**)

Passive avoidance test (PNW 13, n = 8 per group)

[7] trans-2-phenylcyclopropyl-amine hydrochloride

- No main effect of treatment. In session 1, all groups readily entered the dark part. In the retention session 2 (no shock) all groups showed hesitation to enter the dark part ([8]Fig. 1).

Elevated plus maze (PNW 14)
- No main effect of treatment

Active avoidance test (PNW 15, n = 9-10 per group)
Acquisition phase (4 sessions):
Repeated measure one-way ANOVA showed main effects of "treatment" and "sessions", and the interaction "treatment x session". One-way ANOVA and post hoc multiple comparisons for *each session* showed:
- BPA showed significantly less per cent "correct avoidance responses" than controls in session 1 to 3 ([8]Fig. 2A, about 20% less), and NP-low showed significantly less per cent "correct avoidance responses" than controls in session 1 ([8]Fig. 2B, below 10% less)
- BPA-exposed had significantly higher per cent of failure of avoidance during a shock presentation of 5-sec duration than controls (about 2.5% vs 0.2%, Fig. 2C). NP-low dose showed a similar tendency (about 0.4% vs 0.2%)
- No effects of the latency periods associated with CS or UCS (Fig. 2E, D)

Extinction phase (1 session):
- BPA- and NP-exposed tended to have less "correct avoidance responses" (not statistically significant) e.g. didn't moves to "safe" compartment within 5 sec.

COMMENTS FROM VKM PANEL 4:
Concerning the Passive avoidance test, the number of animals per group may be too small for group differences to appear. Under "Discussion" the author also points out "the large individual differences in the experimental conditions used".

During the acquisition phase in the Active avoidance test, BPA-males responded less to light and buzzer-tone which warned about the coming electrical shock, than controls. BPA-males also more often than controls failed to escape from the ongoing 5-sec shock. However, it is questioned whether a difference of about 2.3 % in failure of avoidance across 100 trials is biologically significant, although statistically significant.

A usual interpretation of this test is that failure to acquire avoidance while adequately developing escape performance indicates learning deficit. In the acquisition phase, the BPA-males showed a slower learning curve during the first three out of four sessions compared to controls. However, during the extinction phase all rats showed escape performance.

Monoamine-disruption test (PNW 22-24, n=7-9 per group)
Confirmation of effect: 5 mg/kg tcy caused increased motor activity 5.5 hr after injection in extra control males (n = 3-4/group) which confirmed the schedule for the disruption test (Fig. 3A).
Results for open field for BPA-and NP-exposed rats were injected with saline and tcy, respectively:

[8] With regard to figures, it is referred to figures in the original paper

- Locomotion:
  - Tcy/Saline injections:
    - NP-high dose responded equally to controls to both types of injections; tcy caused a significant increase in locomotion compared to the saline-injection (Fig. 3B)
    - BPA-males and NP-low dose failed to show a significant increase in locomotion following tcy-injection compared to the saline-injection.
- Rearing
  - Tcy injection:
    - All groups showed significant decreased rearing following tcy-injection
  - Saline injection:
    - NP-low dose showed increased rearing compared to controls
    - BPA showed *tendency* to increased rearing compared to controls

COMMENTS FROM VKM PANEL 4:
Concerning locomotion: The per cent increase in locomotion was about equal in all groups (20-25%) following tcy-injection. But, BPA-males and NP-low responded **more** to the saline injection (18-20%) than controls (not known if statistically significant), thus the differences in increase between the saline- and tcy-challenges were less than for the controls. Different response to saline-injection between groups may indicate high individual variation in sensitivity to the injection in itself.

Toxicant-related alterations in locomotion that are evident only after pharmacological manipulations must be interpreted with caution. Altered drug responsiveness may reflect compromised neural function; however, it is also possible that drug distribution and/or metabolism are substantially different from control and toxicant-exposed groups as a consequence of some non-neuronal influence of the compound studied. Additionally, it is imperative to take into account the behavioral baseline before concluding that a treatment has altered sensitivity to a pharmacological probe. When a baseline measure is elevated, an apparent decreased response to motor stimulant might actually reflect a system that has reach its maximum output capacity (max. locomotion 25%). That is, behavioral as well as pharmacological factors can contribute to changes in drug sensitivity.

Conclusion:

The authors conclude:
Perinatal exposure to BPA and NP exposure

- cause adverse behavioral effect when the animals were forced to avoid fear-provoking stimuli. In other words, NP/BPA-exposure disrupted the reception of intolerable stress, possibly due to alterations in the monoaminergic system.

COMMENTS FROM VKM PANEL 4:
There are some concerns about how the data for the different variables are recorded in the passive and active avoidance test as well as in the elevated plus maze (e.g. manually?, blinded to the tester?).

In the active avoidance test, BPA-males responded less to the light and buzzer-tone which warned about a coming electrical shock, than controls. BPA-males also more often than controls failed to escape from the ongoing 5-sec shock. However, it is questioned whether a difference of about 2.3 % in failure of avoidance across 100 trials is biologically significant, although statistically significant.

BPA-males and NP-low dose failed to show a significant increase in locomotion following tcy-injection compared to the saline-injection. However, BPA-males and NP-low responded **more** to the saline injection (18-20%) than controls (not known if statistically significant), thus the differences in increase between the saline- and tcy-challenges were less than for the controls. Different response to saline-injection between groups may indicate high individual variation in sensitivity to the injection in itself. The different response in behavior to saline is not discussed by the authors. The authors' conclusion that alterations in the monoaminergic system may lay behind the behavioral alteration seen may be a bit far reached. Toxicant-related alterations in locomotion that are evident only after pharmacological manipulations must be interpreted with caution.

The animals were run in a set of different test. No anxiety were seen in the passive avoidance test or in the elevated plus maze. Under "Discussion" the authors point out that it is possible that an experience in an earlier test may influence on the results of subsequent tests but this was similar for all groups.

Additionally, the possibility that physical differences may influence on the reactions to electrical shock are discussed, e. g. that non-neurological effects may underline the observed changes in this behavior.

In sum, male rats exposed to BPA *in utero* and during the suckling period responded less to the light and buzzer-tone which warned about a coming electrical shock in an active avoidance test than controls.

6.5 Appendix V (Ryan and Vandenbergh, 2006)

Developmental exposure to environmental estrogens alters anxiety and spatial memory in female mice

Summary of study design:

Animals:

- Mated C57/B1-6 mice (observation of vaginal plug = [9]GD 1)

Dose:

- 2 µg/kg/day, 200 µg/kg/day bisphenol A (BPA)
- 5 µg/kg/day ethinyl estradiol (EE)

Exposure period:

- Dams were randomly assigned to treatment groups on GD 3 and exposed daily from GD 3 until [10]PND 21 by gavage
- Pups were exposed *in utero* and through dams' milk until weaning

Delivery:

- No information

Lactation/suckling period:

- No information

Weaning:

- On PND 21
- Litter size, anogenital distance and weight of each pup were measured
- 21 non-ovariectomized females checked for puberty by daily checking for vaginal opening and subsequently checking vaginal smear for cornified cells (indicative of cycling)

Pathological examination:

- No information

Test animals:

- One female offspring/litter
- Surgically overiectomized 1 week after weaning. 2 weeks recovery period thereafter

Test period:

- Starts 3 weeks after weaning, eg. PND 42

Test procedures:

- Elevated-plus maze
- Light/dark preference chamber
- Radial-arm maze

[9] GD - gestational day

[10] PND – postnatal day

- Barnes maze

Statistics:

- PC SAS
- ANOVAs used to determine main treatment effect
- Two-tailed Student's t-test were used to determine specific group effects
- Repeated measure design was added to ANOVA analysis in tests for spatial memory. Additionally, the Barnes maze data were pooled by day and used as an alternative method for determining group effects over time

COMMENTS FROM VKM PANEL 4:

Dose-response and a positive control are included in the design.

Numbers of mated females or dams that deliver are not given. Neither is number of dams nor litters per treatment group.

Litter size is given under "Results" and only at weaning and not at delivery. Thus, loss of pups from delivery until weaning is not given and subsequently there are no measures of maternal stress, maternal/pup toxicity or nursing behavior.

Pup weight is only given at weaning and not during the preweaning period. Thus, information about possible toxic effects present is missing. It is not possible to follow the individual test animal's weight curves. Body weight is a rough, but important measure of normal development or possible toxic influence as well as an indication of maternal nursing behavior. The latter has not been considered. Anogenital distance (AGD) was only evaluated at weaning (usually also performed soon after delivery).

Puberty was only recorded with regards to females. Information about onset of male puberty is lacking as well as the possible sex difference. Additionally, there are no information given about which litters the chosen 21 females for puberty evaluation came from or which criterions that are used to pick just these animals. However, the method for detecting onset of cycling is approved.

Behavioral tests included ovariectomized females. The possible influence of BPA or EE on male behavior is missing, as well as the possible sex difference. It is possible that the use of ovariectomized females provide an adequate method to detect behavior disturbances caused by low dose toxic exposure.

Concerning the statistics, it is stated that repeated measure design was included for the Barnes and radial-arm mazes data because repeated measures on each animal were utilized.

Short information about the behavioral tests used:

Two sexually dimorphic, non-reproductive behaviors: anxiety and short term spatial memory

A) Anxiety-related behavior
   - Elevated-plus maze (4-armed; two open and two closed, e.g. tunneled, arms)
   - Light/dark preference chamber

B) Short-time spatial memory

- Radial-arm maze
- Barnes maze

A) Anxiety-related behavior:

Both assays provide both aversive and comfortable compartments." Anxiety" is quantified by measuring the amount of time an animal spends in the respective apartments.

Testing were performed in the beginning of the dark cycle when the mice activity is high

- Elevated-plus maze (4-armed):
  - One session of 15 min was performed.
  - Time spent in the centre, in the open or closed arms were recorded
- Light/dark preference chamber:
  - Each session lasted 15 min
  - The mouse was initially placed in the light department and the latency to enter the dark box was recorded.

COMMENTS FROM VKM PANEL 4 TO THE ANXIETY-RELATED BEHAVIOR TESTS

The elevated-plus maze is used for evaluation of general anxiety.

There is neither no information given on how "time" was measured in the two assays (e.g. manually?) nor whether the testing was blinded to the tester.

14 mice/group were tested in both tests.

B) Short time spatial memory

- Radial-arm maze: consist of a center with doors to each of the eight arms. A reward is placed in the end of each arm. Doors are closed when the mouse is in an arm, but opens when reward is collected. One session allowed visits to all the eight arms. Failure or error is recorded if mouse visits previous arm; then it got no reward but is trapped for 30s ("punished").
  - Animals were on a 23-h food deprivation schedule
  - Body weights were recorded
  - Two sessions per day for five consecutive days were run (e.g. totally 10 sessions)
  - Memory error (mouse entered a previously explored arm), total numbers of correct visits, and number of correct visits before an error, were recorded

- Barnes maze: The task consist of an illuminated round wooden platform covered with wet polyethylene. Along the border of the platform were 20 holes and beneath one only an escape box was placed. One daily test consisted of 10 trials. Between each trial the platform was rotated but the location of the escape box was constant. Thus the mouse had to use spatial landmarks (and *not olfactory ones*) in order to locate the box. Next day the location of the box was changed in order to measure short-time memory only.
  - Animals tested 10 daily trials for five consecutive days (50 trials).
  - Number of holes investigated, refusals to enter the box, and refusals to stay in the box, were recorded

COMMENTS FROM VKM PANEL 4 TO THE SPATIAL MEMORY TESTS

There is neither no information given on how the different variables were recorded (e.g. manually?) nor whether the testing was blinded to the tester.

16 mice/group were tested in both tests.

Results:

Anogenital distance (AGD), body weight and litter size

No effect of exposure

COMMENTS FROM VKM PANEL 4:

These parameters were only measured at weaning and not in the pre weaning period. According to OECD guideline 416, AGD is to be measured soon after delivery.

The number of litters in each treatment group is not given in [11]Table 1.

The litter size at delivery compared to the litter size at weaning is indicative of the environmental influence the test animals have been under before testing.

The body weight gain from delivery to weaning is essential in assessing the normal development.

Puberty:

Exposure to EE and BPA-200 caused significantly accelerated onset of puberty in female mice as measured as day of first cornified smear.

COMMENTS FROM VKM PANEL 4:

Under Material and methods it says that 21 individuals were checked daily for vaginal opening. The number of females per group is limited, 5-4-5-7 for the control, BPA-2, BPA-200 and the EE groups, respectively. There are no information on whether the females per group come from different litters or which criterions that are used to pick just these animals. In developmental studies, the litter is the statistically unit. Thus, mean day of puberty onset per litter and treatment group should have been used.

Presumably it is corrected for the day of delivery, but information is scarce.

ANXIETY:

Elevated plus maze:

The EE-exposed mice spent less time (15 sec) in open arms than controls (55 sec). No effect for BPA-exposed although BPA-200 spent marginally less time (30 sec) in open arms ($p=0.06$)

[11] With regard to tables and figures, it is referred to the original paper

Light/dark preference:
EE- and BPA 200-exposed mice spent less time (75 sec and 120 sec, respectively) in light place compared to controls (240 sec). No differences in number of transitions between light/dark parts or latency to first enter dark part of the chamber.

COMMENTS FROM VKM PANEL 4:
Ovariectomized female mice exposed to BPA-200 during development showed anxiety-related behavior in one (light/dark) of the two tests used. However, there is concern about the lack of information about how the data is recorded. The author comments on that the same animals were used in both tests, potentially altering the effects in the second assay which was the light/dark preference chamber. A small follow-up study with different mice showed the same tendency, but the data is not shown.

SHORT TIME SPATIAL MEMORY

Radial-arm maze (8 arms):

- Data analysed by trial (2 sessions/day x 5 days = 10 trials):
  - Main effect of treatment on "mean no of errors" (entering a previously explored arm): EE-exposed mice committed less error than controls in the last 5 trials (Fig. 4). In some of the trials (4, 7, 9, 10), EE-exposure had an effect on the latency until first error (e.g. had more correct arm entering before first error occurred) (Fig. 5).
  - Rate of improvement *within each* group (defined as comparing the "total no of error on each trial" between the 10 trials when the number of error committed in trial-1 was baseline) (Fig. 4):
    There was main effect of trial on total no of error in the EE-mice, BPA-200 and BPA-2 mice, but not control. Compared to baseline, the EE-mice, BPA-200 and BPA-2 mice performed less error from trial-3, -2 and -8, respectively.
    Main effect of trial on the first error: As the trials progressed it did take longer time before the EE-mice (trial 3 onwards) and BPA-200 mice (trial 8 onwards) performed an error (e.g. chose the wrong arm) (Fig. 5).

COMMENTS FROM VKM PANEL 4:
The variable/parameter "Latency before the first error was made" is not described in Material and Methods, but it is probably a deviation of the variable "number of correct visits before error occurred".

Even though "total no of errors" may mirror "total no of correct choices", this is not explained in the text. The results of "total no of correct visits" are not shown.

As far as rate of improvement within each group is concerned, there was no main effect of trial for the controls. This may be interpreted as the controls didn't improve and may question this maze as relevant for evaluation of short term memory. It may also prove that the individual variance in behavior is huge and that the number of animals used is too small to achieve a reliable result. BPA-200 mice committed less error from day 2, but not onwards, as on day 4 more errors were committed. The absolute counts is however small, and even though statistical significant differences is shown, the biological significance is questioned. Furthermore, the total number of errors initially committed is about 3.5 for all groups (Fig. 4), which decrease across the 10 trials to about 2.5 for all groups except the EE-exposed mice which commit about one error in the two last trials. It really may be questions whether the statistical significant results achieved has any biological significance.

Concerning Fig. 5, the only group that shows what may be interpreted as a "learning-curve" is the EE-exposed. The other groups enter averagely 4 new arms in all trials before they returned to a previously visited arm. The EE-exposed mice enter steadily more new arms in each of the 10 trials. In the last trial, these animals enter nearly 7 of 8 possible arms before returning to a previously visited one. The BPA-200-exposed mice show some improvement (about 5 subsequent correct arms visits) in trial-8 and -9, but are back to basic (around 4 subsequent correct arms visits) in the last session. This may very well be randomly just as in trial-4 where the BPA-200-exposed mice commit error early (arm 3.5). As stated for "no of error", the absolute count of correct arm visits is however small, and even though statistical significant differences is shown the biological significance is questioned. The BPA-200 exposed showed more variation across the 10 trials than controls (Fig. 5), but such variation is not unusual in behavioral tests. However, the authors put considerable weight on the BPA-200 exposed performances in trial-8 and -9 and conclude it proves facilitated spatial memory. This conclusion is as argued above based on weak evidence and is not agreed upon.

In sum, EE-exposed performed significantly less error than controls in the last 5 trials (Fig. 4) which may be interpreted as a "learning-curve". The BPA-exposed animals did differ from the controls in single trials, which is no expression of a significant biological effect. This task reveals no altered spatial memory in BPA-exposed animals.

Barnes maze:

- Data analysed by all 50 trials:
  - Main effect of treatment on "no of errors":
    EE-exposed mice performed significantly less error than controls in nine separate trials. No effects of BPA-exposure.
- Data analysed by day (the 10 daily trials were pooled) (Fig. 6):
  - Main effect of treatment on "no of errors":

EE-exposed mice performed significantly less error than controls on each of the 5 days. BPA-exposed mice performed less error (12) than controls (15) on **one** day; day 1.

- Data analysed by day (the 10 daily trials were pooled) (Fig. 6):
  - Improvement over time within each group (defined as comparing the "no of errors per day" between days when number of error performed on day 1 was baseline):

    All groups performed better day by day (Fig. 6). From day 2 on: EE-exposed and controls. From day 3 on: BPA-exposed mice.
  - No main effect of escape hole location on the number of errors made.

COMMENTS FROM VKM PANEL 4:
The variable/parameter "no of errors" is not described in Material and Methods or defined under "Results", but is presumably the same as "number of holes investigated" before finding the escape box.

The description on how ANOVA analysis was set up is spare (2-, 3- or 4-ways ANOVA?). The rationale behind the sentence (p 90) "There was no main effect of escape hole location on the number of errors made" is difficult to follow. As the variable "escape hole location" is not explained, how is the analyse set up?

Results of some of the variables described under M&M are not given: refusals to enter the box, refusals to stay in the box.

When data were analysed by day, the 10 daily trials were pooled. BPA-200 exposed mice performed less error (12) than controls (15) on **one** day; day 1. The biological significance of a mean difference of 3 is questioned. Further, the author states that using a repeated-measure design didn't change the results (p 90). However, data is not shown.
Improvement over time: Each group was compared with itself and all groups improved. EE-exposed and controls improved from day 2, which may be interpreted as no difference in learning ability between these two groups. BPA-exposed used longer time (1 day) before improvement than controls which may as well be interpreted as <u>less</u> learning ability in using spatial memory than controls.

Improvement over time: The controls improved from about 15 errors at day-1 to about 10 errors at day-5, the BPA-2 from about 13 to about 10, the BPA-200 from about 12 to 10, and the EE-exposed from about 11 to about 5.

In sum: EE-exposed mice perform less error than controls in the Barns maze, but no effect of BPA-treatment.

Conclusion:

The authors conclude:
1. BPA and EE accelerated puberty in female mice.
2. Developmental exposure to EE and BPA-200 caused altered adult behavior in two tasks consistent with increased level of anxiety. The results were similar in both assays used and were independent of activity level. BPA increased anxious behavior in a dose-dependent fashion.

3. Developmental exposure to EE caused masculinized spatial ability as well as anxiety-related behavior in adult female mice
4. Developmental exposure to BPA had no effect on spatial memory

COMMENTS FROM VKM PANEL 4:

1. Number per group is small (5-4-5-7 for the control, BPA-2, BPA-200 and the EE groups, respectively) and there are uncertainties whether the animals in each group represent different litters.
2. Concerning BPA-exposure, effects interpreted as anxiety-related behavior was only shown in one (light/dark) of two tasks and only in mice exposed to the highest BPA dose. However, there is concern about the lack of information about how the data is recorded (e.g. manually?). The statement "independent of activity level" is presumably based upon no effect of treatment neither on latency to first enter the dark half of the chamber nor on the number of transitions between the two sections. Both "latency" and "transitions" are probably used as indirect measures of activity.
3. The claim that EE cause masculinized behavior in adult female mice is unsubstantial as there were no male controls.
4. Agree

The conclusions drawn by the authors are questioned because of several weaknesses in the study. Information about central facts for a reproductive study is too spare and leaves the reader unable to control for herself. In particular, information of the number of exposed dams and litter per groups is crucial for developmental behaviour studies.

For the behavioural tests, no information on how the different variables were recorded (e.g. manually?) is given, or whether the testing was blinded to the tester. Even though some *statistical* significant differences are shown in the behavioral tasks used, the *biological* significance is questioned.

The results in this study are not concurrent with that found by others. The authors argue that other studies on e.g. anxiety following developmental exposure to BPA have been performed in intact females. Furthermore, that different results concerning developmental exposure to BPA on spatial ability are due to different tasks, e.g. the study of Carr et al.

The authors conclude that use of ovariectomized mice removes the potential confounding factors of cyclicity. However, whether this represents a valid model for risk assessment remains to be discussed.