Workshop on Triggering and Waiving Criteria for the Extended One-Generation Reproduction Toxicity Study

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Triggering and Waiving Criteria for the Extended One-Generation Reproduction Toxicity Study

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1. EXECUTIVE SUMMARY

Under the new chemicals legislation in Europe, REACH (EU, 2006), the two-generation reproductive toxicity study, OECD TG 416, may be required for substances produced or imported at 100 tonnes per annum or more. At 1000 tonnes per annum, this study becomes a default requirement. The one-generation study design, OECD TG 415, is not a standard information requirement under REACH. A proposal has been developed for an extended one-generation study design—to be included in a tiered testing approach—that is intended to address the limitations of the standard design, while significantly reducing the numbers of animals required (Cooper *et al*, 2006).

The workshop was convened by ECETOC jointly with ECVAM to discuss triggers and waivers within a modular approach to the proposed extended one-generation reproduction toxicity study (ECETOC, 2008). Invited experts were from academia, regulatory authorities, contract research organisations, and industry.

The outcome will be provided to the OECD expert group on the 'extended one-generation reproductive toxicity guideline' for its consideration.

The results of the workshop indicate that on several issues a consensus exists and a common understanding for guidance can be given. These points include:

Study Design

1. If the second generation is an optional module, then the decision to conduct a second breeding should be made on the strength of the findings. If unequivocally positive or negative findings were obtained, no further work would be required and the substance can be classified accordingly.
2. The prenatal developmental toxicity (PDT) and developmental neurotoxicity (DNT) modules are based on methods included in guideline studies and, thus, the methods included in the modules are sufficiently validated.
3. A four weeks' pre-dosing period was considered to be adequate, since this study design is standard for pharmaceuticals (ICH, 2005) and because histopathology and testis weight (measured in the F_1 generation following > 70 days of exposure) are amongst the most sensitive endpoints.
4. If an F_1 mating is performed, it should start when the animals are at least 90 days of age.
5. A PDT module should not be a mandatory requirement of the OECD guideline. However, it may be included in the study design as long as it does not compromise the overall study objectives. In practice, it is likely that this study will be conducted standalone.

6. It may be premature to include a developmental immunotoxicity (DIT) module in the study design as there is no technical or validated guidance issued for this endpoint.

Triggers and Waivers

1. For practical reasons the number of triggers for the optional modules coming from within the study should be limited. Exceptions may consist of equivocal breeding results triggering a second generation and functional observation battery (FOB) / motor activity (MA) results triggering morphological and pathological evaluations in the optional DNT module. But a second generation could only be an optional module if questions over the additional information gained from the second breeding can be satisfactorily resolved.
2. There are no useful internal triggers for a DNT module. Consequently, the decision to include a DNT module needs to be made during the development of the guideline or during the study design phase. There was no consensus as to whether a DNT module should be a default requirement but, if it were to be, then it should be limited to FOB and MA.

Two reviews covering approximately 500 two-generation studies indicate that the second breeding in more than 95% of cases did not add significant new information over and above that found in the first generation (Makris, 2004; Janer *et al*, 2007). The question as to whether the two-generation study can be replaced by an extended one-generation study was heavily debated in all breakout groups. Two out of the three groups could not reach an agreement on this question. The third group concluded that there was enough information to support the use of the extended one-generation design as a replacement of the two-generation study. However, it was felt that a more thorough review of this information is needed. Several of the participants requested that the exceptions raised by Makris (2004) and Janer *et al* (2007) be evaluated carefully to more closely examine the endpoints concerned and the chemical classes of these compounds, and to evaluate what the overall outcome of an extended one-generation study design would have been for these compounds. A proposal was made for ECETOC to help facilitate a review of these data and to collect more data, e.g. through a survey of member companies and contract research organisations.

2. INTRODUCTION

Under the new EU Chemicals Regulation (REACH; EU, 2006), developmental and reproductive toxicity testing will be a key component for the risk assessment of chemicals; it is resource-intensive in terms of animal numbers, cost and time. However, there are currently no methods in sight that totally replace this kind of safety testing in animals, short- to mid-term. Consequently, new testing strategies leading to a reduction and/or refinement of animal use immediately should be applied as broadly as possible.

In 2006, the ILSI Health and Environmental Sciences Institute published a strategy for agricultural chemical safety assessment (ACSA) that included an adapted design for reproductive and developmental toxicity evaluation (Cooper *et al*, 2006.). The extended one-generation reproductive toxicity study design is currently under consideration as an alternative to the current one- and two-generation tests, with potentially widespread applicability and impact on chemical safety assessment across many industry sectors.

It is important to bear in mind that the ACSA testing proposal was designed in the context of an intelligent testing strategy for the evaluation of agrochemicals, aimed at addressing the inefficient development of data much of which is not used in the final risk assessment (Carmichael *et al*, 2006). It is a scientifically robust approach to the reduction and refinement of the package of studies required for agrochemical registration. However, it was not designed as a standalone study to replace existing protocols for the evaluation of reproductive toxicity, and adaptation of the ACSA extended one-generation study design for other uses should take into account the endpoints required for sector-specific risk assessments.

In an ECVAM workshop held in September 2006 and an EPAA (European Partnership for Alternative Approaches to Animal Testing) workshop held in November 2006, which was attended by more than thirty experts from industry and the regulatory community, it was discussed in detail and agreed that the extended one-generation study as developed by the ACSA project could, in principle, be applicable to safety testing under REACH. However, it was also agreed that the complex ACSA protocol should be modified in order to meet the current requirements for industrial chemical safety testing. This will deliver animal welfare benefits with regard to both refinement and a reduction in the number of animals used (more than 40% compared to the two-generation study).

A major modification of the ACSA test protocol for use under REACH could be to design reliable triggering and/or waiving criteria for the components of the protocol as modules. An ECETOC task force has developed criteria (ECETOC, 2008) and a subsequent workshop, jointly organised by ECETOC and ECVAM, was held to discuss the triggering and waiving criteria and agree on their relevance and on possible validation needs. The workshop was an active event

with invited participants representing industry, academia, and the regulatory community (programme and list of participants are given in Appendices 1 and 2, respectively).

Structure of the workshop

After initial presentations to give the background to the ACSA project, and present the findings of the ECETOC task force, the workshop participants were divided into three groups for breakout sessions. The three groups were given the same questions to address (below), with a plenary session after the breakout sessions to identify areas of agreement and disagreement.

The following questions were to be addressed during the breakout sessions:

To address validation criteria:

1. Is sufficient information available to rely on an extended one-generation study for the evaluation of reprotoxicity?
2. Are there any endpoints identified as triggers that are less sensitive than the modules that they are expected to trigger?
3. Can endpoints that are proposed as triggers, be rated with respect to their specificity (low, medium, high) in order to avoid unnecessary confirmatory testing?
4. Can specific guidance be given with respect to the mechanistic relevance of triggers (e.g. *in vitro* endpoints)?
5. What validation is required for the modules themselves?

To address study conduct:

6. Should the final guideline be a core design with all modules included as a default, some of which may be waived, or one with a reduced default design and in which additional modules can be triggered? What are the implications for endpoints as waivers and triggers in these cases?
7. If post-weaning exposure interferes with the conduct or interpretation of a module (e.g. potential for pre-implantation loss in a PDT module, or difficult interpretation of DNT findings):
    a. Is it acceptable to cease exposure at weaning (and recommence maternal exposure at GD6 in the case of a PDT module)? If not, would a standalone study be required?
    b. What information requirements would have to be satisfied to support the decision to cease exposure at weaning? Should this be the default approach?

8. If a PDT module is added to the study design, should this be conducted in a set of the F_1 generation selected at weaning, or by further mating of the P generation?

To address risk assessment and data poor substances:

9. Reduced data generation for risk assessment:
   a. Is it acceptable to waive a triggered module in favour of additional uncertainty factors being added in the derivation of a DNEL?
   b. What guidance should be given for the application of such uncertainty factors?
10. In the absence of data, are the modules automatically triggered (depending on the information requirements within the appropriate regulatory framework)?

Question added by the workshop participants:

11. Should the design of the core reproductive guideline be changed and, if so, how?

3. BREAKOUT SESSIONS

3.1 Breakout Group I

Chair: Jochen Buschmann
Rapporteur: Matt Dent
Sandra Coecke
Gunilla Ericsson
Alexius Freyberger
Betty Hakkert
Christa Hennes
Ulrich Hübel
Miriam Jacobs
Hennicke Kamp
Lo Meisters
Ulrike Reuter
Roland Solecki

To address validation criteria:

Q1: Is sufficient information available to rely on an extended one-generation study for the evaluation of reproductive toxicity?

The majority of time in the breakout group was spent discussing this question, reflecting the fact that participants had strong opinions on this topic.

At least two participants in this breakout group had concerns about what was termed the 'deliberate abandonment' of the two-generation study, since this is the only test that investigates the effects of a xenobiotic from pre-conception to reproductive competence. Another participant pointed out that although the textbook view of reproductive toxicology is that a two-generation study is necessary for the evaluation of reproductive toxicity, we need to evaluate the historical data to see if this is true in practice. Several analyses have been conducted on available data showing that the second generation does not appear to add significant value to the risk assessment of the chemicals studied. Some discussion focussed on the chemical domain of the databases used as well as the bias introduced when only data from successfully marketed chemicals are evaluated. It was therefore recommended that this evaluation be extended to new as well as existing chemicals. One idea involved asking companies how often reproductive effects that are only manifest at the second breeding or present at a lower dose than the first generation resulted in a chemical not being further developed for marketing. This could help judge the significance of the bias that results from using data from currently-marketed chemicals. It was also mentioned

that it is inadequate to simply compare the results of the second breeding with that of the first, since all available data (including repeat-dose toxicity and in vitro data) should be taken into account for a fully integrated approach.

During the discussions the group was reminded that the application of the extended one-generation study to safety assessment does not mean that the second generation is not needed. It means that in some cases a robust safety assessment can be made without the second generation, and the task ahead is therefore to define where it can and cannot be used. This point was made in various ways during the discussions:

- How many negative results are needed before the appropriate level of confidence is reached that a chemical is not a reproductive toxin/toxicant?
- For a given chemical which study design is needed to give enough evidence that a compound is not toxic to reproduction?

The group agreed that the burden of proof is on showing that for a given chemical the F_1 generation mating phase is unnecessary.

The overall conclusion of the group was that there is some evidence that, in practice, a properly designed extended one-generation study may be adequate for safety assessment in some cases. But there is currently not enough information to be certain that it is appropriate for general use.

Q2: Are there endpoints identified as triggers that are less sensitive than the modules that they are expected to trigger?

The group agreed that, for practical reasons, triggers for the optional modules should not come from within the study.

Second generation module:
Many thought that since not enough information is currently available to rely on first generation mating for a complete assessment of reproductive effects, this module should be standard and only waived if there is a good justification. The group discussed that, scientifically, the decision to perform a second mating could be a risk-based judgement, and not based on hazard. For example, if human exposure to a chemical is well below the DNEL for that chemical and no reproductive effects are seen after the first mating, is continuation of the study with a second breeding scientifically justified? Many felt that this is an acceptable principle that is already captured under REACH and is not a question specific to reproductive toxicology. The principal of performing an extended one-generation study and only mating the F_1 generation if equivocal results are seen in the parental generation was discussed. This was termed the 'grey zone', since

most participants felt comfortable relying on an extended one-generation study for hazard identification and risk assessment if either clearly positive or clearly negative results are obtained. A minority view was that if equivocal results were seen in the parental generation it would be better to repeat the study than to investigate further by pairing the F_1 generation because the results of this pairing could give rise to more uncertainty.

DNT module:
The trigger for DNT testing is usually neurotoxic effects in adults, under the assumption that offspring may be more sensitive to the neurotoxic effect. One participant noted that DNT studies have not yet contributed to regulatory risk assessments or classification and labelling and questioned the value of this study or the way they are currently performed.

PND module:
If a littering study is needed for a chemical, it is likely that a PND study would already be available or would need to be performed. In the context of a littering study this would not be a module triggered by effects. The potential of waiving a PND study/module was discussed if no adverse effects were seen in an extended one-generation study and a rabbit PND study. There was confusion regarding how this would be viewed within REACH.

DIT module:
Due to the uncertainties that surround these assessments it was considered unnecessary to discuss DIT at this time.

Q3: Can endpoints that are proposed as triggers, be rated with respect to their specificity (low, medium, high) in order to avoid unnecessary confirmatory testing?

Q4: Can specific guidance be given with respect to the mechanistic relevance of triggers (e.g. in vitro *endpoints)?*

These questions were dealt with together during a brief discussion at the end of the breakout session.

The principle that triggers should come from the available database rather than from within the study was reiterated during the discussion. The group also agreed that it is difficult to formulate specific guidance to rate triggers since each chemical should be dealt with on a case-by-case basis. One example of avoiding confirmatory testing could be accepting a classification and/or additional uncertainty factor on the basis of a trigger such as neurotoxicity in adult animals rather than performing a DNT study or module. Proving that a classification or additional uncertainty factor is not necessary could involve providing mechanistic data to prove that the effect is either

not relevant to human use of a chemical or that developing organisms would not be more sensitive, but no specific examples were given during the session.

Q5: What validation is required for the modules themselves?

The PND study is already considered a definitive test and the DNT study is already in regular use and is requested by regulators. Thus, no validation is needed for these studies/modules. The group agreed that it is too early to consider validating a DIT module since there is no agreed format for this, but it is likely that this is one module where some experimental validation will be needed.

To address study conduct:

Q6: Should the final guideline be a core design with all modules included as a default, some of which may be waived, or one with a reduced default design and in which additional modules can be triggered? What are the implications for endpoints as waivers and triggers in these cases?

The group agreed that it would be difficult and unadvisable to create a study design that relies on its own triggers. This was in part due to the logistical problems associated with major last-minute changes to complex study designs.

Second generation module:
Due to the answers given to Question 1, the overall recommendation of the group was that the study should include a second generation which could be waived if the first generation was clearly positive. Some participants also felt that the second generation could also be waived if the first generation was clearly negative.

In the event that the extended one-generation study is validated and accepted for use under REACH, then the discussion turned to the possibility of using this design at 100 tonnes per annum should a reproductive study be required. Some participants indicated that this could be a useful role for the extended one-generation study. The idea that there could be specific roles for this study design came across strongly. For example, the application of this study design could be very different for new chemicals than for existing chemicals with a history of (presumed) safe use. Therefore, the modules that are included as default for any given chemical will be different and the applicability of the chosen study design should be considered and justified.

DIT module:
This group unanimously agreed that this should not be a default module since there are no accepted procedures to assess developmental immunotoxicity and no standard regulatory requirement to assess this endpoint.

PND module:
Although there are pros and cons for including this module in the extended one-generation study design (as described in the ECETOC report), the group felt that, on balance, it was better to use a tiered testing strategy that involved performing this shorter study before (and separate to) the littering study.

DNT module:
The view of some participants was that this module, or at least FOB and MA evaluations, should be included as a default for chemicals that are not neurotoxic to adults. Others stated that this would lead to unnecessary testing, and since this approach is not required by REACH it should not be followed.

The overall consensus reached was that for practical reasons, this module should only be included if triggered by the available data. However, it was suggested that a screen for neurotoxicity could be included within the extended one-generation study design to determine whether a separate DNT study is needed. Such a screen might include functional observations, locomotor activity, brain weights and standard (non-perfused) brain histology.

Q7: If post-weaning exposure interferes with the conduct or interpretation of a module is it acceptable to cease exposure at weaning and recommence maternal exposure at a later stage?

There was unanimous agreement that in principle the answer to this question is yes. However, the group recognised that most of the time a PDT study would already be available, and advised against including an extensive DNT module within the extended one-generation study design unless it was triggered.

Q8: If a PDT module is added to the study design should this be conducted in a set of the F_1 generation at weaning or by further mating of the P generation?

There was unanimous agreement that if a PDT module is included this should be derived from the F_1 generation to avoid complicating data interpretation for parental animals.

To address risk assessment and data poor substances:

Q9: Reduced data for risk assessment: Is it acceptable to waive a triggered module in favour of additional uncertainty factors being added in the derivation of a DNEL?

It was strongly felt that if data are needed to complete the safety assessment they should be generated. If it is decided that a littering study is required (due to high tonnage or alerts from other data sources), it would presumably be difficult to argue that certain data should not be generated. However, this opinion should be viewed in the context of the exposure-based waiving that is possible under REACH and the ability to waive testing that is not scientifically justified.

The only exception to this, considered scientifically justifiable by the group, was in the context of a chemical that is a known neurotoxin in adults. In this case, an additional uncertainty factor could be included to waive a DNT study or module. The reason for this is that if a chemical is neurotoxic in adults then neurotoxic effects would be expected in offspring, but they should be assumed to occur at lower doses in offspring unless proven otherwise. However, the consequences that waiving data generation may have on classification and labelling should also be considered. If a chemical is neurotoxic in adults, the burden of proof is on the registrant to prove the chemical is not a developmental neurotoxin, otherwise classification and labelling for developmental toxicity would need to be accepted.

Q10: In the absence of data are the modules automatically triggered (depending on the information requirements within the appropriate regulatory framework)?

With the exception of the second breeding, modules should not automatically be triggered. To include all modules as a default could result in the generation of data that are not needed for regulatory or safety purposes. Conversely, if data are needed for specific regulatory purposes, fewer animals could be used by including certain modules within this study rather than by performing separate tests. This could be an automatic trigger, but only if the objectives of the study are not compromised by including the extra modules. This type of approach would not be covered in a test guideline but in the context of the overall testing strategy for each individual chemical.

Q11: Should the design of the core reproductive guideline be changed and, if so, how?

The group agreed this was an important question that warranted further debate, but the breakout session ended before it could be discussed.

3.2 Breakout Group II

Chair: Aldert Piersma
Rapporteur: Beate Holzum
Karin Aschberger
Peter Boogaard
Audrey Bottomley
Bengt Danielsson
Nathalie Delrue
Ulla Hass
Sara Lloyd
Nigel Moore
Hiroshi Ono
Patricia Pazos
Troy Seidle
Ben van Ravenzwaay

General remark:

Opinions in the group differed whether the modules should be included as defaults or whether the basic study design should be a limited one with the modules triggered. Mainly for DNT, a minority of participants asked for a standard inclusion of this module. As a possible compromise, a standard limited functional testing (FOB, MA) in the progeny was discussed.

To address validation criteria:

Q1: Is sufficient information available to rely on an extended one-generation study for the evaluation of toxicity to reproduction?

Published reviews of 176 studies (Janer *et al*, 2007) and over 350 unpublished data submissions to EPA (Makris, 2004) are available to allow an evaluation to be made of the information gained by a second generation. They indicate that in less than 5% of studies sensitivity was increased by a second generation, in all cases without new qualitative results. Another study cited to the group indicated that in three out of 22 two-generation studies conducted in one laboratory, the second generation was more sensitive than the first, but that the second mating would have been triggered by assessments made earlier in the study (Myers *et al*, 2008).

There was some concern expressed that reliance on published data or data submitted to agencies as part of a registration may result in bias as only negative or minimal findings of low concern

would be reported. To some extent, this is negated by a longitudinal evaluation of 22 studies selected without bias from one laboratory, in which adverse treatment-related effects on reproductive performance were not observed in 18 of 22 cases (Myers *et al*, 2008). Nevertheless, the group considered that it may increase confidence to evaluate unpublished data as some participants thought that there is additional uncertainty because some classes of chemicals may have been underrepresented in the published reviews. The question arose as to which grade of uncertainty would be acceptable in order to rely on an extended one-generation study only.

The conclusion of the group was that there was enough information to support the use of an extended one-generation study design. However, some participants wished to review the published data more thoroughly, particularly with reference to the few exceptions (for example, those cases where the F_2 generation is more sensitive than the F_1 generation).

Q2: Are there any endpoints identified as triggers that are less sensitive than the modules that they are expected to trigger?

Some participants expressed concern that the nervous and the immune systems may be more sensitive in the developing organism than in adults, and that triggers from adult animals may sometimes be less sensitive than findings in the progeny. However, this was not a consensus view.

A HESI survey of 113 studies that included a behavioural assessment of F_1 generation animals showed that in 15% of cases the DNT data along with other data defined the NOEL, and in 2.6% of cases it solely defined the NOEL (Middaugh *et al*, 2003). However, as the review predominantly comprised pharmaceutical compounds the incidence for industrial chemicals alone is unknown.

There was disagreement whether some DNT screening (FOB, MA) should generally be included in the study or whether DNT testing should only be performed in case of triggers. The majority of the group felt that more data are needed to finally answer this question.

Q3: Can endpoints that are proposed as triggers be rated with respect to their specificity (low, medium, high) in order to avoid unnecessary confirmatory testing?

The following points were made during the discussion.

Mode of action and *chemical structure* may act as triggers, not as waivers. There are not enough data available to answer the question for these two parameters.

Toxicity in adults is an example where rating is possible. Convulsion would be a trigger of high specificity while ataxia is considered of low specificity.

Effects on the thyroid are indicative of developmental neurotoxicity and, thus, of high specificity. However, thyroid effects may result in classification in the absence of a specific DNT.

In vitro tests may add to *in vivo* data and could contribute to triggering decisions as part of a weight of evidence approach. However, there are insufficient data available to rate triggers based on *in vitro* testing.

The group found it difficult to give more examples of triggers, and was unable to identify or agree on criteria for waiving a module (e.g. DNT). However, there was agreement that triggers should always be considered in a weight of evidence approach.

Q4: Can specific guidance be given with respect to the mechanistic relevance of triggers (e.g. in vitro endpoints)?

Currently there is not enough information available to use mechanistic relevance for triggering or waiving.

Regarding *in vitro* tests for each class of compounds (i.e. for each mechanism) a proper *in vitro* model (like micromass for retinoids) would be necessary. Further, *in vitro* data would generally be superseded by *in vivo* data.

Q5: What validation is required for the modules themselves?

For most of the modules there are sufficient data available to adequately design the modules.

Prenatal developmental toxicity is already validated as there are a lot of studies with a broad historical data base.

DNT can be considered as validated as a sufficient data base is available with the US EPA, and there is an OECD guideline.

Regarding *DIT,* some members felt that it is premature to be discussing endpoints for which validated international guidelines and regulatory data requirements do not currently exist, while others expressed a desire to have a guidance paper dealing with suitable methods to be able to decide on the need for validation.

To address study conduct:

Q6: Should the final guideline be a core design with all modules included as a default, some of which may be waived, or one with a reduced default design and in which additional modules can be triggered? What are the implications for endpoints as waivers and triggers in these cases?

Reproductive toxicity:
The reproductive module should be included as default.

DNT:
Regarding DNT, three options were discussed. One was that it may be useful to include a limited screen (FOB, MA) as default. The other options were that the whole module would be default or triggered. The question was raised as to which triggers would determine the latter option.

DIT:
Regarding DIT, some members felt strongly that it is premature to include DIT even as a triggered module, while others felt differently. Some members felt that they lacked sufficient knowledge on this subject to express a definitive opinion.

Second generation:
Capacity for the second generation needs generally to be reserved in the laboratory at the start of the study. Due to the high need of capacity for a second generation, it may otherwise not be possible for logistical reasons to include a second generation.

The use of a second (F_1) generation is to evaluate equivocal findings (e.g. fertility) seen in the first (P) generation. For practical/scientific reasons it may be more suitable to use F_1 animals than mating the P animals for a second time. An exception, where a second generation may not be useful in any case, is perinatal mortality. Susceptible F_1 animals may have died as pups and are thus not available to create the second generation. Therefore, perinatal mortality may not be seen in the second generation.

In any case, specific study data are necessary to decide on the need of a second generation. It would be advisable to have real life examples to decide whether a second generation is necessary.

Prenatal developmental toxicity:
Arguments for inclusion of the PDT module into the extended one-generation study are a reduction in the number of animals needed for a separate PDT study and an increase in the amount of information that can be gained from these animals during the course of the study.

Arguments against inclusion of a PDT module include the standard practice to conduct a PDT study in rats before embarking on a reproductive toxicity study, and logistical reasons (the setting

of the high dose will be limited to cover the needs of the other modules; about 100 females are needed; a staggered start has to be performed to be able to handle the caesarean sections). It is also not considered in the Cooper publication (Cooper *et al*, 2006).

Thus, the conclusion of the group was that developmental toxicity should generally be tested in a separate study. However, in specific cases it may be sensible to include it in the extended one-generation study.

The question arose if negative findings in a rabbit developmental toxicity study together with negative findings in an extended one-generation study could waive the rat developmental toxicity study. However, loss of information has to be considered because malformations in the generation study may be hidden by perinatal mortality with subsequent cannibalism. In addition, as stated above, the rat PDT study might have already been performed before the start of the extended one-generation study.

To cover the needs of all modules included in an extended one-generation study, careful dose spacing is important.

Q7: If post-weaning exposure interferes with the conduct or interpretation of a module (e.g. potential for pre-implantation loss in a PDT module, or difficult interpretation of DNT findings), a. Is it acceptable to cease exposure at weaning (and recommence maternal exposure at GD6 in the case of a PDT module)? If not, would a standalone study be required?

For the DNT module, the distinction between developmental effects and direct (acute) effects is not possible when treatment of F_1 animals is continued beyond weaning. In addition, in case of continued treatment the study design would be different from the separate guideline DNT study. The group discussed the two following two options.

One option would be to perform the complete DNT module only if triggered. However, the breakout group did not define triggers beyond those proposed in the ECETOC Document 45 (ECETOC, 2008). The other option would be to include a limited DNT module (MA, FOB only, as in the OECD 407 study) as default in F_1 animals. In this case, the dams will have been treated during lactation (without continued treatment of F_1 animals). This would mean a different design for pesticides (no study or a triggered full DNT study) than for chemicals (MA, FOB). However, it would allow a comparison between findings in directly dosed animals based on data from the OECD 407 study, and in animals indirectly exposed during development.

Questions 7b to 10 were not addressed by the breakout group due to time constraints.

3.3 Breakout Group III

Chair: Peter Ridgway
Rapporteur: Steffen Schneider
Pauline Bingham
Susanne Bremer
Neil Carmichael
Gerard Cooke
Paul Foster
Wassila Gaoua-Chapelle
Antonio Lacerda de Queiroz
Walter Lichtensteiger
Richard Vogel
Hans-Werner Vohr
Christopher Willoughby
Marc Willuhn

To address validation criteria and general study design:

Q1: Is sufficient information available to rely on an extended one-generation study for the evaluation of reprotoxicity?

Q11: Should the design of the core reproductive guideline be changed and, if so, how?

These two questions were discussed together. The breakout group had a lively debate on whether or not the F_1-extended one-generation study is an adequate replacement of the two-generation study according to OECD TG 416, and about the duration of pre-mating treatment in particular of male animals.

No consensus was reached about these issues.

Adequate replacement of OECD TG 416:
In terms of an adequate replacement of OECD TG 416, one argument brought forward by those opposing this suggestion was that in the life stages concept (Cooper *et al*, 2006) the F_1-extended one-generation study is part of a more comprehensive data package. This is not the case for industrial chemicals; for the chemicals it would be a standalone study. Thus, the position of the participants in favour of this suggestion was that this complex study is never carried out standalone; the amount of available data depends on REACH requirements. For compounds at a tonnage level of > 100 t/a data will likely be available from previous sub-chronic and prenatal developmental studies. With regard to the value of the second generation, reference was made to

the Janer study, which comprises a detailed analysis of retrospective two-generation data (Janer *et al*, 2007). However, some objected to the uncritical use of this analysis because of its potential or perceived bias towards reporting of effects of low concern. Further to this, there was an extensive discussion about the practicability of internal triggers for a second generation coming from the first breeding in the F_1-extended one-generation study.

Duration of pre-mating treatment:
The second major issue in the debate was the duration of pre-mating treatment of four weeks in the F_1-extended one-generation study versus ten weeks in the two-generation study according to OECD TG 416. Ten weeks' duration of pre-mating treatment is supported by the argument that, if one wants to use fertility as an internal trigger for a second generation, such treatment duration would be needed to cover the entire spermatogenic cycle. Four weeks' duration of pre-mating treatment in the P generation is supported by the fact that comprehensive histopathological data of the sexual organs will be obtained at the end of the F_1 breeding, which is the most sensitive endpoint in terms of male fertility. This approach is also backed up by the experience with pharmaceuticals, where the shortened pre-mating treatment proved its value since the adoption of the ICH guideline in 1993 (ICH, 2005). There was agreement that 70 days of breeding for the F_1 generation, as proposed by Cooper *et al* (2006) were not long enough in biological and practical terms; this should be extended to 90 days.

It should be noted that a *proposal for an alternative study design* was made. In this design, pregnant dams exposed from GD6 onwards are placed into the study, their offspring are carried beyond sexual maturity into adulthood, then paired, and the resulting pregnant F_1 females are examined mid pregnancy. This design would substantially contribute to solve the issues raised in the preceding debate.

Q2: Are there any endpoints identified as triggers that are less sensitive than the modules that they are expected to trigger?

Q3: Can endpoints that are proposed as triggers be rated with respect to their specificity (low, medium, high) in order to avoid unnecessary confirmatory testing?

Second generation:
There was consensus that equivocal findings in the outcome of the P breeding should be defined, i.e. in terms of whether they are treatment related or in terms of severity. This means that if there is clear evidence of an effect one would classify, and if there was clearly no effect one would not classify. If there is ambiguity, a second breeding would follow to confirm equivocal findings.

DNT module:
In the life stages concept as proposed by Cooper *et al* (2006) the DNT module was included to fulfil a requirement for pesticide testing. In contrast, DNT is currently not required for chemicals. For the > 100 t/a level supporting information from other studies should be available. There was consensus that the absence of information from other studies is not a waiver for the module. From the list of triggers mentioned in the ECETOC Document 45 (2008), the focus should be on the following:

- Changes in motor function (e.g. disturbances of gait, abnormal posture, muscle tone, or stereotypic movements);
- effects on level of arousal (e.g. hyperactivity, lethargy);
- effects of automatic functions (e.g. salivation, lacrimation, urination, defecation);
- emotional effects (e.g. stereotypy, aggression, biting, licking, self-mutilation);
- effects on thyrotropin and thyroxine;
- effects on thyroid weight, supported by hispathological findings;
- hispatological findings in the thyroid.

Data on them would most likely be available. Following this, an extensive discussion came up about the practicability of combined testing, i.e. in terms of dose setting, time constraints and resources. In this context, the question was raised whether the continuous treatment beyond the sensitive phase for neurodevelopment would interfere with the assessment of developmental neurotoxicity at the end of F_1 breeding. There was consensus that for animals earmarked for DNT, dosing could be stopped at PND 21.

PDT module:
The value of this module was generally questioned, because it was assumed that no one will carry out an F_1-extended one-generation study without having information from embryo-foetal development. Another issue raised in the debate was whether lower dose levels and exposure via feed are acceptable to assess prenatal developmental toxicity. There was no consensus, because sometimes peaks and sometimes 'area under the curve' determine the effects caused by test compounds. There was consensus that the module should be seen in the context of an integrated testing strategy.

DIT module:
The consensus was that there is no requirement for chemicals to have this module, and that there are neither guideline nor consensus about the needs to adequately cover this endpoint. Changes in immunoglobulines, as proposed by the ECETOC Document 45 (2008), are a poor trigger because this is an insensitive trigger. Only in combination with histopathology findings and phenotyping of immunocompetent cells it may give adequate information to induce further

immunotoxicity testing. The question was raised, which studies should show these triggers because immunotoxicity is not addressed in standard regulatory studies.

Q4: Can specific guidance be given with respect to the mechanistic relevance of triggers (e.g. in vitro *endpoints)?*

The group did not feel sufficiently knowledgeable to answer this question.

Q5: What validation is required for the modules themselves?

It was reported, that a number of feasibility studies for the F_1-extended one-generation protocol as proposed by Cooper *et al* (2006) are currently underway. They are, however, not meant to be validation studies. They rather address the feasibility of this complex protocol under standard laboratory conditions. There was consensus, that if feasibility of the entire F_1-extended one-generation study works, the modules do not require an additional separate validation.

To address study conduct:

Q6: Should the final guideline be a core design with all modules included as a default, some of which may be waived, or one with a reduced default design and in which additional modules can be triggered? What are the implications for endpoints as waivers and triggers in these cases?

There was fundamental disagreement in the group about how to approach the F_1-extended one-generation study for the testing of chemicals in the frame of REACH legislation. The majority of the breakout group was in favour of running the study with a reduced default design and trigger the modules; the others were in favour of a core design with all modules as default and of waiving modules. Some participants were of the opinion that streamlined versions or core elements of the modules should be default investigations.

During the debate, a number of individual questions were raised and thoughts shared. These are listed below:

- Triggers within a study are difficult to handle, in terms of resources and logistics.
- If the study is originally planned as a two-generation study with the option to waive the second generation, the investigator would need histopathology data of F_1 offspring to make this decision. This is impractical.
- For practical reasons, one should not decide halfway through a study to add or waive a module; one would have to know beforehand if it is triggered or waived. Thus, it is

recommended to run a study with a reduced core design and trigger the modules based on existing data.

- Companies would accept to repeat a study on equivocal findings, rather than having a maximum approach all the time.
- Some DIT elements should be a default requirement in the core study, like a differentiation of lymphocyte sub-populations using FACS (fluorescence-activated cell sorting) analysis, in 5-weeks old F_1 animals, as a potential trigger for further DIT testing. There is no other study type to investigate potential effects on the developing immune system; all other testing is done in young adult animals.

Q7: If post-weaning exposure interferes with the conduct or interpretation of a module (e.g. potential for pre-implantation loss in a PDT module, or difficult interpretation of DNT findings),
a. Is it acceptable to cease exposure at weaning (and recommence maternal exposure at GD6 in the case of a PDT module)? If not, would a standalone study be required?
b. What information requirements would have to be satisfied to support the decision to cease exposure at weaning? Should this be the default approach?

There was consensus that, as for a DNT protocol, it should be possible to cease exposure at weaning, but it is not recommended.

Q8: If a PDT module is added to the study design, should this be conducted in a set of the F_1 generation selected at weaning, or by further mating of the P generation?

There was agreement to add this module to the F_1 generation.

To address risk assessment and data poor substances:

Q9: Reduced data generation for risk assessment:
a. Is it acceptable to waive a triggered module in favour of additional uncertainty factors being added in the derivation of a DNEL?
b. What guidance should be given for the application of such uncertainty factors?

This approach was discussed as clearly being not scientific, but only pragmatic. The question was raised how much non-testing is penalised. The consensus opinion was that this should be a case by case decision. Generating data should generally be the preferred option, and science should be the basis for decisions rather than pragmatism.

Q10: In the absence of data, are the modules automatically triggered (depending on the information requirements within the appropriate regulatory framework)?

There was consensus that a complex and expensive study would only be initiated based on some available data. Particularly, if information on triggers for the DNT module as listed above (under Q3) is lacking, this module should be carried out.

4. PLENARY SESSION

The plenary session focussed on the major points of agreement and disagreement from the breakout sessions, with the aim at obtaining a majority consensus. Six questions were discussed.

1. Should the study design (guideline) be an extended one-generation study with the possibility of triggering extra modules, or should it be a two-generation study with all these modules built in and which will be waived on the basis of existing data?

In essence, the question relates to whether the modules should be included by default and how, or if, their inclusion or exclusion may be determined by the analysis of internal or external triggers. There was a difference of opinion as to whether the DNT module should be mandatory or not, and, if not mandatory, whether it should be included by default or through triggers. It was agreed that in any event the DNT module should include the functional observation battery and motor activity assessment, while morphometry and pathological assessment could be a further triggered requirement. In contrast, the PDT module was seen to be an optional component of the study design—with prenatal toxicity more likely to be evaluated in a standalone study—and it was considered premature to recommend inclusion of a DIT module.

With regard to the second generation module, there was some debate as to whether the second breeding could be abandoned. Two reviews of data for many chemicals have indicated that in most cases the second breeding—to produce the F_2 generation—does not add significant new information over and above that found for a combination of other studies (Makris, 2004; Janer *et al*, 2007). However, it was questioned as to what would have been lost in the small minority of cases where the F_2 generation did provide additional information. Would the additional information be sufficient to alter the classification (e.g. qualitative findings not seen in the F_1 generation) or risk assessment (e.g. lower NOAEL or greater margin of safety)? In those cases where the F_2 generation added quantitative or qualitative data to the hazard or risk assessment, would a second breeding have been triggered in an extended one-generation study to identify these effects? Further evaluation of these few exceptional cases was called for, including closer examination of the endpoints concerned, chemical class of test substance, and overall available data for each substance, as well as a prediction of the outcome had an extended one-generation study been conducted instead of the two-generation study.

Another concern was raised over the potential for bias in the data sets used for the evaluations (Makris, 2004; Janer *et al*, 2007)—e.g. studies on agricultural chemicals that were not sent for registration, or otherwise unpublished and unavailable studies—as it was felt that data submitted to review either by regulatory agencies or peer-reviewed publications may not represent the 'worst case'. In reality, it is rare that a dossier on an agricultural chemical, which progresses to a

two-generation reproduction study in its development phase, is not submitted for registration. Furthermore, a longitudinal survey of 22 two-generation reproductive toxicity studies, selected without bias, conducted over a ten-year period in one laboratory demonstrated that in more than 80% of cases there were no adverse effects of treatment upon reproductive performance (Myers *et al*, 2008). However, a proposal was made for ECETOC to gather more data—e.g. through a survey of member companies and contract research organisations—and co-ordinate a data evaluation exercise, perhaps in co-operation with RIVM and the US EPA.

2. Is the proposed pre-dosing period of four weeks (Cooper et al*, 2006) adequate, or is a longer pre-mating dosing period required in the context of an extended one-generation study?*

The pharmaceutical industry has for many years used a four week pre-mating exposure period (ICH, 2005), and this exposure duration is considered adequate for the evaluation of effects upon male fertility (Takayama *et al*, 1995; Ulbrich and Palmer, 1995). There was consensus among the participants that, since histopathology and weight of the testis were among the most sensitive endpoints and could be the basis of classification in the absence of other findings, four weeks of exposure prior to mating would be sufficient. One participant expressed the opinion that ten weeks of exposure would increase the confidence in a negative outcome to the study, especially in the event that a second breeding was not undertaken.

Concern was expressed that the considerations of pre-mating exposure with respect to detecting effects on male fertility did not explicitly take into account effects on female fertility, although the duration of the oestrus cycle in rats is typically about five days. The impact of exposure duration on the detection of effects on female fertility requires further evaluation to ensure that changes to the pre-mating exposure period do not impair detection. Any deficiencies in this regard would be common to the guidelines for pharmaceutical evaluation (ICH, 2005), which may offer some guidance or background information.

3. Is it useful to include a PDT module in the extended one-generation study, or will this always be conducted as a standalone study?

Some participants felt that the conduct of a PDT study might jeopardise the conduct of the overall study, but there was agreement that such a module could be a useful approach to reducing the overall use of animals in the evaluation of toxicity to reproduction should it be feasible, on a case-by-case basis. In practice, it was considered more likely that such a study would be conducted as a standalone study within a tiered testing strategy, but incorporation as a module may be appropriate in a testing framework if tonnage requirements dictated the need for both a PDT and a generation study. Consensus was reached that the possibility for such a module

should be raised in the opening discussions of the guideline, and its inclusion could be suggested in the event that it would not compromise the study as a whole, but that it should not be a mandatory module.

4. Should the decision on triggering or waiving the DNT module be entirely based on external, i.e. pre-existing, triggers?

The consensus opinion was that there was no need for internal triggers for a DNT module, as they would occur too late in the conduct of a study to include the DNT evaluations as a module. Furthermore, resource planning considerations mandate that provision must be made for DNT evaluations sufficiently in advance to ensure availability of general and specialised resources. Therefore any decision to include a DNT module—in the event that it was agreed to be optional or triggered—should be made before the study is started.

There was no consensus as to whether a DNT module should be included as a default in the core study design. However, agreement was reached that a DNT module should focus on a functional observation battery and assessment of motor activity, which would allow comparison of findings in juveniles with data from adults in 28- or 90-day repeated dose studies (OECD 1995, 1998). Morphometry and pathological examinations should be triggered from treatment-related findings in the in-life observational tests.

5. What are the appropriate triggers for a second breeding (F_2 generation) module?

The reference to 'equivocal findings' (ECETOC, 2008) was considered to be insufficiently clear. A pragmatic approach was agreed, in which the decision to conduct a second breeding would be made on the strength of the findings in terms of decision making for classification. If unequivocally positive findings were made, then no further work would be required and the substance could be classified. In the case of unequivocally negative findings, no further work would be required and the substance would not be classified. If there were treatment-related effects that were insufficient for classification (e.g. small changes in isolated sperm parameters), then a second breeding would be warranted. (It should be noted that this question presumes that the second breeding can be an optional module.)

6. Is it premature to include a DIT module at the current time?

Some participants declared insufficient expertise in the area of developmental immunotoxicity to comment on the state of the science. However, it was noted that there is currently neither

published consensus nor validated and accepted guideline for the conduct of DIT studies, nor are there regulatory requirements for this endpoint at present. It was agreed that a guidance document for DIT—e.g. an OECD technical guidance document—would be a pre-requisite for defining a DIT module in the extended one-generation study.

It was suggested that assessment of splenic and/or blood cell phenotype by flow cytometry could be fairly easily added and could provide useful information. However, the overall consensus view was that it was indeed premature to include a DIT module at this time.

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ABBREVIATIONS

ACSA	Agricultural chemical safety assessment
DIT	Developmental immunotoxicity
DNEL	Derived no effect level
DNT	Developmental neurotoxicity
EC	European Commission
ECVAM	European Centre for the Validation of Alternative Methods
EEC	European Economic Community
EPAA	European Partnership for Alternative Approaches to Animal Testing
EU	European Union
F_1	First generation of offspring
F_2	Second generation of offspring
FACS	Fluorescence-activated cell sorting
FOB	Functional observation battery
GD	Gestation day
HESI	Health and Environmental Sciences Institute
ICH	International Conference on Harmonisation
ILSI	International Life Sciences Institute
MA	Motor activity
NOAEL	No observed adverse effect level
NOEL	No observed effect level
OECD	Organisation for Economic Co-operation and Development
P	Parental (generation)
PDT	Prenatal developmental toxicity
PND	Postnatal day
REACH	Registration, evaluation, authorisation and restriction of chemicals
RIVM	National Institute of Public Health and the Environment
US EPA	United States Environmental Protection Agency

APPENDIX 1: WORKSHOP PROGRAMME

Monday 14 April 2008

13.00-14.00	Registration and lunch	
14.00-14.10	**Welcome**	Christa Hennes, ECETOC Susanne Bremer, ECVAM
14.10-14.20	**Introduction to the overall context of the project**	Neil Carmichael, ECETOC
14.20-15.10	**Overview of the ECETOC TF findings and questions that emerged during the development of the criteria**	
	- On triggers and waivers	Nigel Moore, Dow
	- On validation needs	Thomas Hartung, ECVAM
15.10-15.30	**Introduction to the breakout group sessions**	Nigel Moore
15.15-15.45	Coffee break	
16:00-18:00	**Breakout group sessions (each group is asked to discuss all the questions)**	

Group I	**Chair:**	**Jochen Buschmann, Fraunhofer Institut**
	Rapporteur:	**Matt Dent, Unilever**
Group II	**Chair:**	**Aldert Piersma, RIVM**
	***Rapporteur*:**	**Beate Holzum, Bayer HealthCare**
Group III	**Chair:**	**Peter Ridgway, HSE**
	***Rapporteur*:**	**Steffen Schneider, BASF**

19.00-22.00	Dinner	

Tuesday 15 April 2008

09.00-11.00	**Continuation of breakout group sessions**	
11.00-11.30	Coffee break	
11.30-12.15	**Reports from the three breakout groups**	Chair: Nigel Moore
12.15-13.00	**Plenary discussion**	Moderator: Neil Carmichael
13.00-14.00	Lunch	
14.00-15.00	**Conclusions of the workshop**	Chairs: Thomas Hartung / Nigel Moore
	Close of workshop	

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and other members of the ECETOC task force on *Triggering and Waiving Criteria for the Extended One-Generation Reproduction Toxicity Study* (Document No. 45).

ECETOC WORKSHOP REPORTS

No.	Title
No. 1	Workshop on Availability, Interpretation and Use of Environmental Monitoring Data. 20-21 March 2003, Brussels
No. 2	Strategy Report on Challenges, Opportunities and Research Needs Arising from the Definition, Assessment and Management of Ecological Quality Status as Required by the EU Water Framework Directive Based on the Workshop EQS and WFD versus PNEC and REACh - Are They Doing the Job? 27-28 November 2003, Budapest
No. 3	Workshop on Use of Human Data in Risk Assessment. 23-24 February 2004, Cardiff
No. 4	Influence of Maternal Toxicity in Studies on Developmental Toxicity. 2 March 2004, Berlin
No. 5	Workshop on Alternative Testing Approaches in Environmental Risk Assessment. 7-9 July 2004, Crécy-la-Chapelle
No. 6	Workshop on Chemical Pollution, Respiratory Allergy and Asthma. 16-17 June 2005, Leuven
No. 7	Workshop on Testing Strategies to Establish the Safety of Nanomaterials. 7-8 November 2005, Barcelona
No. 8	Workshop on Societal Aspects of Nanotechnology, 9 November 2005, Barcelona
No. 9	Workshop on the Refinement of Mutagenicity / Genotoxicity Testing. 23-24 April 2007, Malta
No. 10	Workshop on Biodegradation and Persistence. 26-27 June 2007, Holmes Chapel
No. 11	Workshop on the Application of *'Omics* in Toxicology and Ecotoxicology: Case Studies and Risk Assessment, 6-7 December 2007, Malaga

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