OECD/OCDE 421
Adopted:
29 July 2016

OECD GUIDELINE FOR TESTING OF CHEMICALS

Reproduction/Developmental Toxicity Screening Test

INTRODUCTION

1. OECD Guidelines for the Testing of Chemicals are periodically reviewed in the light of scientific progress. The original screening Test Guideline 421 was adopted in 1995, based on a protocol for a "Preliminary Reproduction Toxicity Screening Test" discussed in two expert meetings, in London in 1990 (1) and in Tokyo in 1992 (2).

2. This TG has been updated with endocrine disruptor relevant endpoints, as a follow up to the high-priority activity initiated at OECD in 1998 to revise existing Test Guidelines and to develop new Test Guidelines for the screening and testing of potential endocrine disruptors (3). TG 407 (Repeated Dose 28-Day Oral Toxicity Study in Rodents) for example, was enhanced in 2008 by parameters suitable to detect endocrine activity of test chemicals. The objective in updating TG 421 was to include some endocrine disruptor relevant endpoints in screening TGs where the exposure periods cover some of the sensitive periods during development (pre- or early postnatal periods).

3. The selected additional endocrine disrupter relevant endpoints, also part of TG 443 (Extended One Generation Reproductive Toxicity Study), were included in TG 421 based on a feasibility study addressing scientific and technical questions related to their inclusion, as well as possible adaptations of the test design needed for their inclusion (4).

4. This Guideline is designed to generate limited information concerning the effects of a test chemical on male and female reproductive performance such as gonadal function, mating behaviour, conception, development of the conceptus and parturition. It is not an alternative to, nor does it replace the existing Test Guidelines 414, 415, 416 or 443.

INITIAL CONSIDERATIONS

5. This Screening Test Guideline can be used to provide initial information on possible effects on reproduction and/or development, either at an early stage of assessing the toxicological properties of chemicals, or on chemicals of concern. It can also be used as part of a set of initial screening tests for existing chemicals for which little or no toxicological information is available, as a dose range finding study for more extensive reproduction/developmental studies, or when otherwise considered relevant. In conducting the study, the guiding principles and considerations outlined in the OECD Guidance Document n° 19 on the

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recognition, assessment, and use of clinical signs as humane endpoints for experimental animals used in safety evaluations (5) should be followed.

6. This test does not provide complete information on all aspects of reproduction and development. In particular, it offers only limited means of detecting post-natal manifestations of pre-natal exposure, or effects that may be induced during post-natal exposure. Due (amongst other reasons) to the relatively small numbers of animals in the dose groups, the selectivity of the end points, and the short duration of the study, this method will not provide evidence for definite claims of no effects. Moreover, in the absence of data from other reproduction/developmental toxicity tests, positive results are useful for initial hazard assessment and contribute to decisions with respect to the necessity and timing of additional testing.

7. The results obtained by the endocrine related parameters should be seen in the context of the "OECD Conceptual Framework for Testing and Assessment of Endocrine Disrupting Chemicals" (6). In this Conceptual Framework, the enhanced TG 421 is contained in level 4 as an *in vivo* assay providing data on adverse effects on endocrine relevant endpoints. An endocrine signal might not however be considered sufficient evidence on its own that the test chemical is an endocrine disruptor.

8. This Guideline assumes oral administration of the test chemical. Modifications may be required if other routes of exposure are used.

9. Before use of the Test Guideline on a mixture for generating data for an intended regulatory purpose, it should be considered whether, and if so why, it may provide adequate results for that purpose. Such considerations are not needed, when there is a regulatory requirement for testing of the mixture.

10. Definitions used are given in Annex 1.

PRINCIPLE OF THE TEST

11. The test chemical is administered in graduated doses to several groups of males and females. Males should be dosed for a minimum of four weeks and up to and including the day before scheduled kill (this includes a minimum of two weeks prior to mating, during the mating period and, approximately, two weeks post-mating). In view of the limited pre-mating dosing period in males, fertility may not be a particular sensitive indicator of testicular toxicity. Therefore, a detailed histological examination of the testes is essential. The combination of a pre-mating dosing period of two weeks and subsequent mating/fertility observations with an overall dosing period of at least four weeks, followed by detailed histopathology of the male gonads, is considered sufficient to enable detection of the majority of effects on male fertility and spermatogenesis.

12. Females should be dosed throughout the study. This includes two weeks prior to mating (with the objective of covering at least two complete oestrous cycles), the variable time to conception, the duration of pregnancy and at least thirteen days after delivery, up to and including the day before scheduled kill.

13. Duration of study, following acclimatisation and pre-dosing oestrous cycle evaluation, is dependent on the female performance and is approximately 63 days, [at least 14 days premating, (up to) 14 days mating, 22 days gestation, 13 days lactation].

14. During the period of administration, the animals are observed closely each day for signs of toxicity. Animals which die or are killed during the test period are necropsied and, at the conclusion of the test, surviving animals are killed and necropsied.

DESCRIPTION OF THE METHOD

Selection of animal species

15. This Test Guideline is designed for use with the rat. If the parameters specified within this TG 421 are investigated in another rodent species a detailed justification should be given. In the international validation program for the detection of endocrine disrupters on TG 407, the rat was the only species used. Strains with low fecundity or well-known high incidence of developmental defects should not be used. Healthy virgin animals, not subjected to previous experimental procedures, should be used. The test animals should be characterised as to species, strain, sex, weight and age. At the commencement of the study the weight variation of animals used should be minimal and not exceed 20% of the mean weight of each sex. Where the study is conducted as a preliminary study to a long-term or a full-generation study, it is preferable that animals from the same strain and source are used in both studies.

Housing and feeding

16. All procedures should conform to local standards of laboratory animal care. The temperature in the experimental animal room should be 22 °C (± 3°). Although the relative humidity should be at least 30% and preferably not exceed 70% other than during room cleaning, the aim should be 50-60%. Lighting should be artificial, the photoperiod being 12 hours light, 12 hours dark. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water. The choice of diet may be influenced by the need to ensure a suitable admixture of a test chemical when administered by this method.

17. Animals should be group housed in small groups of the same sex; animals may be housed individually if scientifically justified. For group caging, no more than five animals should be housed per cage. Mating procedures should be carried out in cages suitable for the purpose. Pregnant females should be caged individually and provided with nesting materials. Lactating females will be caged individually with their offspring.

18. The feed should be regularly analysed for contaminants. A sample of the diet should be retained until finalisation of the report.

Preparation of the animals

19. Healthy young adult animals are randomly assigned to the control and treatment groups. Cages should be arranged in such a way that possible effects due to cage placement are minimized. The animals are uniquely identified and kept in their cages for at least five days prior to the start of the study to allow for acclimatisation to the laboratory conditions.

Preparation of doses

20. It is recommended that the test chemical be administered orally unless other routes of administration are considered more appropriate. When the oral route is selected, the test chemical is usually

administered by gavage; however, alternatively, test chemicals may be administered via the diet or drinking water.

21. Where necessary, the test chemical is dissolved or suspended in a suitable vehicle. It is recommended that, wherever possible, the use of an aqueous solution/suspension be considered first, followed by consideration of a solution/emulsion in oil (e.g. corn oil) and then by possible solution in other vehicles. For vehicles other than water the toxic characteristics of the vehicle should be known. The stability and homogeneity of the test chemical in the vehicle should be determined.

PROCEDURE

Number and sex of animals

22. It is recommended that each group be started with at least 10 males and 12-13 females. Females will be evaluated pre-exposure for oestrous cyclicity and animals that fail to exhibit typical 4-5 day cycles will not be included in the study; therefore, extra females are recommended in order to yield 10 females per group. Except in the case of marked toxic effects, it is expected that this will provide at least 8 pregnant females per group which normally is the minimum acceptable number of pregnant females per group. The objective is to produce enough pregnancies and offspring to assure a meaningful evaluation of the potential of the test chemical to affect fertility, pregnancy, maternal and suckling behaviour, and growth and development of the F_1 offspring from conception to day 13 post-partum.

Dosage

23. Generally, at least three test groups and a control group should be used. Dose levels may be based on information from acute toxicity tests or on results from repeated dose studies. Except for treatment with the test chemical, animals in the control group should be handled in an identical manner to the test group subjects. If a vehicle is used in administering the test chemical, the control group should receive the vehicle in the highest volume used.

24. Dose levels should be selected taking into account any existing toxicity and (toxico-) kinetic data available. It should also be taken into account that there may be differences in sensitivity between pregnant and non-pregnant animals. The highest dose level should be chosen with the aim of inducing toxic effects but not death or severe suffering. Thereafter, a descending sequence of dose levels should be selected with a view to demonstrating any dosage related response and no-observed-adverse effects (NOAEL) at the lowest dose level. Two to four fold intervals are frequently optimal for setting the descending dose levels and addition of a fourth test group is often preferable to using very large intervals (e.g. more than a factor of 10) between dosages.

25. In the presence of observed general toxicity (e.g. reduced body weight, liver , heart, lung or kidney effects, etc.) or other changes that may not be toxic responses (e.g. reduced food intake, liver enlargement), observed effects on endocrine sensitive endpoints should be interpreted with caution.

Limit test

26. If an oral study at one dose level of at least 1000 mg/kg body weight/day or, for dietary or drinking water administration, an equivalent percentage in the diet, or drinking water using the procedures described for this study, produces no observable toxic effects and if toxicity would not be expected based upon data from structurally related substances, then a full study using several dose levels may not be considered necessary. The limit test applies except when human exposure indicates the need for a higher oral dose level to be used. For other types of administration, such as inhalation or dermal application, the physical chemical properties of the test substances often may dictate the maximum attainable concentration.

Administration of doses

27. The animals are dosed with the test chemical daily for 7 days a week. When the test chemical is administered by gavage, this should be done in a single dose to the animals using a stomach tube or a suitable intubation cannula. The maximum volume of liquid that can be administered at one time depends on the size of the test animal. The volume should not exceed 1 ml/100 g body weight, except in the case of aqueous

solutions where 2 ml/100 g body weight may be used. Except for irritating or corrosive test chemicals which will normally reveal exacerbated effects with higher concentrations, variability in test volume should be minimized by adjusting the concentration to ensure a constant volume at all dose levels.

28. For test chemical administered via the diet or drinking water, it is important to ensure that the quantities of the test chemical involved do not interfere with normal nutrition or water balance. When the test chemical is administered in the diet either a constant dietary concentration (ppm) or a constant dose level in terms of the animals' body weight may be used; the alternative used should be specified. For a test chemical administered by gavage, the dose should be given at similar times each day, and adjusted at least weekly to maintain a constant dose level in terms of animal body weight.

Experimental schedule

29. Dosing of both sexes should begin at least 2 weeks prior to mating, after they have been acclimatised for at least five days and females have been screened for normal oestrous cycles (in a 2 weeks pre-treatment period). The study should be scheduled in such a way that oestrous cycle evaluation begins soon after the animals have attained full sexual maturity. This may vary slightly for different strains of rats in different laboratories, e.g. Sprague Dawley rats 10 weeks of age, Wistar rats about 12 weeks of age. Dams with offspring should be killed on day 13 post-partum, or shortly thereafter. The day of birth (viz. when parturition is complete) is defined as day 0 post-partum. Females showing no-evidence of copulation are killed 24-26 days after the last day of the mating period. Dosing is continued in both sexes during the mating period. Males should further be dosed after the mating period at least until the minimum total dosing period of 28 days has been completed. They are then killed, or, alternatively, are retained and continued to be dosed for the possible conduction of a second mating if considered appropriate.

30. Daily dosing of the parental females should continue throughout pregnancy and at least up to, and including, day 13 post-partum or the day before sacrifice. For studies where the test chemical is administered by inhalation or by the dermal route, dosing should be continued at least up to, and including, day 19 of gestation, and dosing should be re-initiated as soon as possible and not later than PND 4.

31. A diagram of the experimental schedule is given in Annex 2.

Mating procedure

32. Normally, 1:1 (one male to one female) matings should be used in this study. Exceptions can arise in the case of occasional deaths of males. The female should be placed with the same male until evidence of copulation is observed or two weeks have elapsed. Each morning the females should be examined for the presence of sperm or a vaginal plug. Day 0 of pregnancy is defined as the day on which mating evidence is confirmed (a vaginal plug or sperm is found). In case pairing is unsuccessful, re-mating of females with proven males of the same group could be considered.

Litter size

33. On day 4 after birth, the size of each litter may be adjusted by eliminating extra pups by random selection to yield, as nearly as possible, four or five pups per sex per litter depending on the normal litter size in the strain of rats used. Blood samples should be collected from two of the surplus pups, pooled, and used for determination of serum T4 levels. Selective elimination of pups, e.g. based upon body weight, or anogenital distance (AGD) is not appropriate. Whenever the number of male or female pups prevents having four or five of each sex per litter, partial adjustment (for example, six males and four females) is acceptable. No pups will be eliminated when litter size will drop below the culling target (8 or 10

pups/litter). If there is only one pup available above the culling target, only one pup will be eliminated and used for blood collection for possible serum T4 assessments.

34. If litter size is not adjusted, two pups per litter are sacrificed on day 4 after birth and blood samples are taken for measurement of serum thyroid hormone concentrations. If possible the two pups per litter should be female pups to reserve male pups for nipple retention evaluations except in the event that removing these pups leaves no remaining females for assessment at termination. No pups will be eliminated when litter size will drop below 8 or 10 pups/litter (depending on the normal litter size in the strain of rats used). If there is only one pup available above the normal litter size, only one pup will be eliminated and used for blood collection for possible serum T4 assessments.

In life observations

Clinical observations

35. Throughout the test period, general clinical observations should be made at least once a day, and more frequently when signs of toxicity are observed. They should be made preferably at the same time(s) each day, considering the peak period of anticipated effects after dosing. Pertinent behavioural changes, signs of difficult or prolonged parturition and all signs of toxicity, including mortality, should be recorded. These records should include time of onset, degree and duration of toxicity signs.

Body weight and food/water consumption

36. Males and females should be weighed on the first day of dosing, at least weekly thereafter, and at termination. During pregnancy, females should be weighed on days 0, 7, 14 and 20 and within 24 hours of parturition (day 0 or 1 post-partum) and at least day 4 and 13 post-partum. These observations should be reported individually for each adult animal.

37. During pre-mating, pregnancy and lactation, food consumption should be measured at least weekly. The measurement of food consumption during mating is optional. Water consumption during these periods should also be measured when the test chemical is administered via drinking water.

Oestrous cycles

38. Oestrous cycles should be monitored before treatment starts to select for the study females with regular cyclicity (see paragraph 22). Vaginal smears should also be monitored daily from the beginning of the treatment period until evidence of mating. If there is concern about acute stress effects that could alter oestrous cycles with the initiation of dosing, laboratories can expose test animals for 2 weeks, then collect vaginal smears daily to monitor oestrous cycle for a minimum of two weeks during the pre-mating period with continued monitoring into the mating period until there is evidence of mating. When obtaining vaginal/cervical cells, care should be taken to avoid disturbance of mucosa, which could induce pseudopregnancy (7) (8).

Offspring parameters

39. The duration of gestation should be recorded and is calculated from day 0 of pregnancy. Each litter should be examined as soon as possible after delivery to establish the number and sex of pups, stillbirths, live births, runts (pups that are significantly smaller than corresponding control pups) and the presence of gross abnormalities.

40. Live pups should be counted and sexed and litters weighed within 24 hours of parturition (day 0 or 1 post-partum) and at least on day 4 and 13 post-partum. In addition to the observations described in paragraph 35, any abnormal behaviour of the offspring should be recorded.

41. The AGD of each pup should be measured on the same postnatal day between PND 0 through PND 4. Pup body weight should be collected on the day the AGD is measured and the AGD should be normalized to a measure of pup size, preferably the cube root of body weight (9). The number of nipples/areolae in male pups should be counted on PND 12 or 13 as recommended in OECD GD 151 (10).

Clinical biochemistry

42. Blood samples from a defined site are taken based on the following schedule:
- from at least two pups per litter on day 4 after birth, if the number of pups allows (see paragraphs 33-34)
- from all dams and at least two pups per litter at termination on day 13, and
- from all adult males, at termination,

All blood samples are stored under appropriate conditions. Blood samples from the day 13 pups and the adult males are assessed for serum levels for thyroid hormones (T4). Further assessment of T4 in blood samples from the dams and day 4 pups is done if relevant. As an option other hormones may be measured if relevant. Pup blood can be pooled by litter for thyroid hormone analyses. Thyroid hormones (T4 and TSH) should preferably be measured as 'total'.

43. The following factors may influence the variability and the absolute concentrations of the hormone determinations:
- time of sacrifice because of diurnal variation of hormone concentrations
- method of sacrifice to avoid undue stress to the animals that may affect hormone concentrations
- test kits for hormone determinations that may differ by their standard curves.

44. Plasma samples specifically intended for hormone determination should be obtained at a comparable time of the day. The numerical values obtained when analysing hormone concentrations differ with various commercial assay kits.

Pathology

Gross necropsy

45. At the time of sacrifice or death during the study, the adult animals should be examined macroscopically for any abnormalities or pathological changes. Special attention should be paid to the organs of the reproductive system. The number of implantation sites should be recorded. Vaginal smears should be examined in the morning on the day of necropsy to determine the stage of the oestrous cycle and allow correlation with histopathology of ovaries.

46. The testes and epididymides as well as prostate and seminal vesicles with coagulating glands as a whole, of all male adult animals should be trimmed of any adherent tissue, as appropriate, and their wet weight taken as soon as possible after dissection to avoid drying. In addition, optional organ weights could include levator ani plus bulbocavernosus muscle complex, Cowper's glands and glans penis in males and paired ovaries (wet weight) and uterus (including cervix) in females; if included, these weights should be collected as soon as possible after dissection.

47. Dead pups and pups killed at day 13 post-partum, or shortly thereafter, should, at least, be carefully examined externally for gross abnormalities. Particular attention should be paid to the external reproductive genitals which should be examined for signs of altered development. At day 13 the thyroid from 1 male and 1 female pup per litter should be preserved.

48. The ovaries, testes, accessory sex organs (uterus and cervix, epididymides, prostate, seminal vesicles plus coagulating glands), thyroid and all organs showing macroscopic lesions of all adult animals should be preserved. Formalin fixation is not recommended for routine examination of testes and epididymides. An acceptable method is the use of Bouin's fixative or modified Davidsons for these tissues (11). The tunica albuginea may be gently and shallowly punctured at the both poles of the organ with a needle to permit rapid penetration of the fixative.

Histopathology

49. Detailed histological examination should be performed on the ovaries, testes and epididymides (with special emphasis on stages of spermatogenesis and histopathology of interstitial testicular cell structure) of the animals of the highest dose group and the control group. The other preserved organs including thyroid from pups and adult animals may be examined when necessary. The thyroid weight could be determined after fixation. Trimming should also be done very carefully and only after fixation to avoid tissue damage. Tissue damage could compromise histopathology analysis. Examinations should be extended to the animals of other dosage groups when changes are seen in the highest dose group. The Guidance on histopathology (11) details extra information on dissection, fixation, sectioning and histopathology of endocrine tissues.

DATA AND REPORTING

Data

50. Individual animal data should be provided. Additionally, all data should be summarised in tabular form, showing for each test group the number of animals at the start of the test, the number of animals found dead during the test or killed for humane reasons, the time of any death or humane kill, the number of fertile animals, the number of pregnant females, the number of animals showing signs of toxicity, a description of the signs of toxicity observed, including time of onset, duration, and severity of any toxic effects, the types of histopathological changes, and all relevant litter data. A tabular summary report format that has proven to be very useful for the evaluation of reproductive/developmental effect is given in Annex 3.

51. Due to the limited dimensions of the study, statistical analyses in the form of tests for "significance" are of limited value for many endpoints, especially reproductive endpoints. If statistical analyses are used then the method chosen should be appropriate for the distribution of the variable examined, and be selected prior to the start of the study. Statistical analysis of AGD and nipple retention should be based on individual pup data, taking litter effects into account. Where appropriate, the litter is the unit of analysis. Statistical analysis of pup body weight should be based on individual pup data, taking litter size into account. Because of the small group size, the use of historic control data (e.g. for litter size), where available, may also be useful as an aid to the interpretation of the study.

Evaluation of results

52. The findings of this toxicity study should be evaluated in terms of the observed effects, necropsy and microscopic findings. The evaluation will include the relationship between the dose of the test chemical and the presence or absence, incidence and severity of abnormalities, including gross lesions, identified target

organs, infertility, clinical abnormalities, affected reproductive and litter performance, body weight changes, effects on mortality and any other toxic effects.

53. Because of the short period of treatment of the male, the histopathology of the testes and epididymides should be considered along with the fertility data, when assessing male reproductive effects. The use of historical control data on reproduction/development (e.g., for litter size, AGD, nipple retention, serum T4 levels), where available, may also be useful as an aid to the interpretation of the study.

54. For quality control it is proposed that historical control data are collected and that for numerical data coefficients of variation are calculated, especially for the parameters linked with endocrine disrupter detection. These data can be used for comparison purposes when actual studies are evaluated.

Test report

55. The test report should include the following information:

Test chemical:

- source, lot number, limit date for use, if available
- stability of the test chemical, if known.

Mono-constituent substance:

- physical appearance, water solubility, and additional relevant physicochemical properties;

- chemical identification, such as IUPAC or CAS name, CAS number, SMILES or InChI code, structural formula, purity, chemical identity of impurities as appropriate and practically feasible, etc.

Multi-constituent substance, UVBCs and mixtures:

- characterised as far as possible by chemical identity (see above), quantitative occurrence and relevant physicochemical properties of the constituents.

Vehicle (if appropriate):

- justification for choice of vehicle if other than water.

Test animals:

- species/strain used;
- number, age and sex of animals;
- source, housing conditions, diet, etc.;
- individual weights of animals at the start of the test.
- justification for species if not rat

Test conditions:

- rationale for dose level selection;
- details of test chemical formulation/diet preparation, achieved concentrations, stability and homogeneity of the preparation;
- details of the administration of the test chemical;

- conversion from diet/drinking water test chemical concentration (ppm) to the actual dose (mg/kg body weight/day), if applicable;
- details of food and water quality;
- detailed description of the randomisation procedure to select pups for culling, if culled.

Results:

- body weight/body weight changes;
- food consumption, and water consumption if available;
- toxic response data by sex and dose, including fertility, gestation, and any other signs of toxicity;
- gestation length;
- toxic or other effects on reproduction, offspring, post-natal growth, etc.;
- nature, severity and duration of clinical observations (whether reversible or not);
- number of adult females with normal or abnormal oestrous cycle and cycle duration;
- number of live births and post-implantation loss;
- pup body weight data
- AGD of all pups (and body weight on day of AGD measurement)
- nipple retention in male pups,
- thyroid hormone levels, day 13 pups and adult males (and dams and day 4 pups if measured)
- number pups with grossly visible abnormalities, gross evaluation of external genitalia, number of runts;
- time of death during the study or whether animals survived to termination;
- number of implantations, litter size and litter weights at the time of recording;
- body weight at sacrifice and organ weight data for the parental animals;
- necropsy findings;
- detailed description of histopathological findings;
- absorption data (if available);
- statistical treatment of results, where appropriate.

Discussion of results.

Conclusions.

Interpretation of results

56. The study will provide evaluations of reproduction/developmental toxicity associated with administration of repeated doses (see paragraphs 5 and 6). It could provide an indication of the need to conduct further investigations and provides guidance in the design of subsequent studies. OECD Guidance Document 43 should be consulted for aid in the interpretation of reproduction and developmental results (12). OECD Guidance Document 106 on Histologic Evaluation of Endocrine and Reproductive Tests in Rodents (11) provides information on the preparation and evaluation of (endocrine) organs and vaginal smears that may be helpful for this TG.

LITERATURE

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11) OECD (2009). Guidance Document for Histologic Evaluation of Endocrine and Reproductive Tests in Rodents. Environment, Health and Safety Publications, Series on Testing and Assessment (No.106), Organisation for Economic Cooperation and Development, Paris.

12) OECD (2008). Guidance Document on Mammalian Reproductive Toxicity Testing and Assessment. Environment, Health and Safety Publications, Series on Testing and Assessment (No. 43), Organisation for Economic Cooperation and Development, Paris.

ANNEX 1

DEFINITIONS (see also (6) OECD GD 150)

Androgenicity is the capability of a chemical to act like a natural androgenic hormone (e.g. testosterone) in a mammalian organism.

Antiandrogenicity is the capability of a chemical to suppress the action of a natural androgenic hormone (e.g. testosterone) in a mammalian organism.

Antioestrogenicity is the capability of a chemical to suppress the action of a natural oestrogenic hormone (e.g. oestradiol 17ß) in a mammalian organism.

Antithyroid activity is the capability of a chemical to suppress the action of a natural thyroid hormone (e.g. T_3) in a mammalian organism.

Developmental toxicity: the manifestation of reproductive toxicity, representing pre-, peri- post-natal, structural, or functional disorders in the progeny.

Dosage is a general term comprising of dose, its frequency and the duration of dosing.

Dose is the amount of test chemical administered. The dose is expressed as weight of test chemical per unit body weight of test animal per day (e.g. mg/kg body weight/day), or as a constant dietary concentration.

Evident toxicity is a general term describing clear signs of toxicity following administration of test chemical. These should be sufficient for hazard assessment and should be such that an increase in the dose administered can be expected to result in the development of severe toxic signs and probable mortality.

Impairment of fertility represents disorders of male or female reproductive functions or capacity.

Maternal toxicity: adverse effects on gravid females, occurring either specifically (direct effect) or not specifically (indirect effect).

NOAEL is the abbreviation for no-observed-adverse effect level. This is the highest dose level where no adverse treatment-related findings are observed due to treatment.

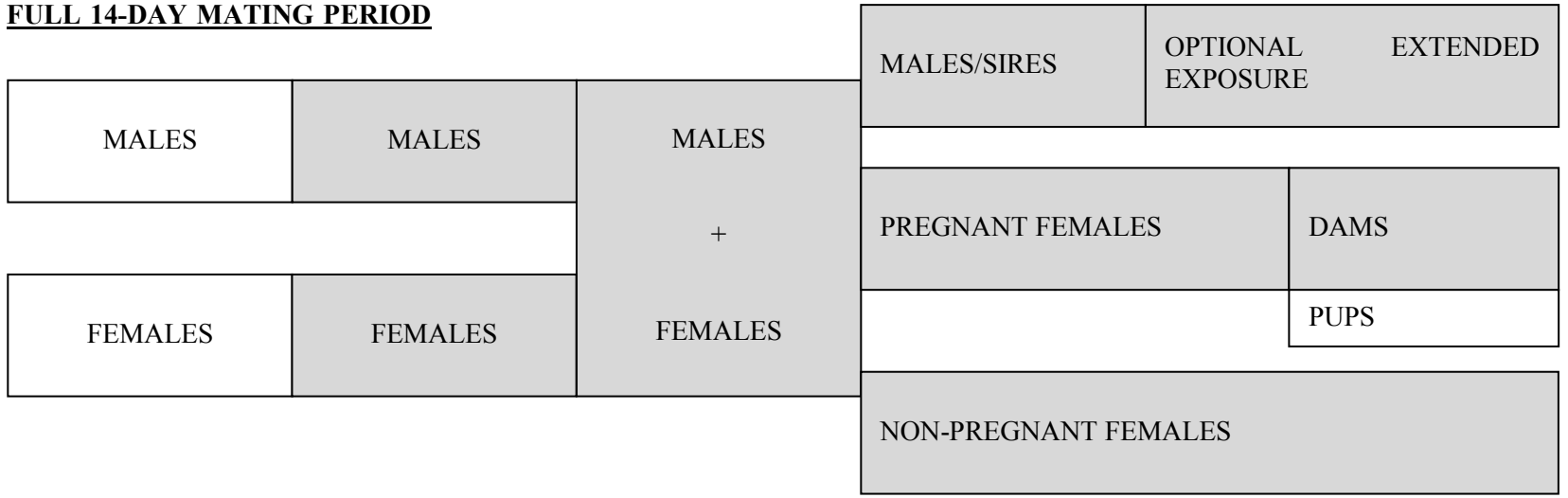
Oestrogenicity is the capability of a chemical to act like a natural oestrogenic hormone (e.g. oestradiol 17ß) in a mammalian organism.

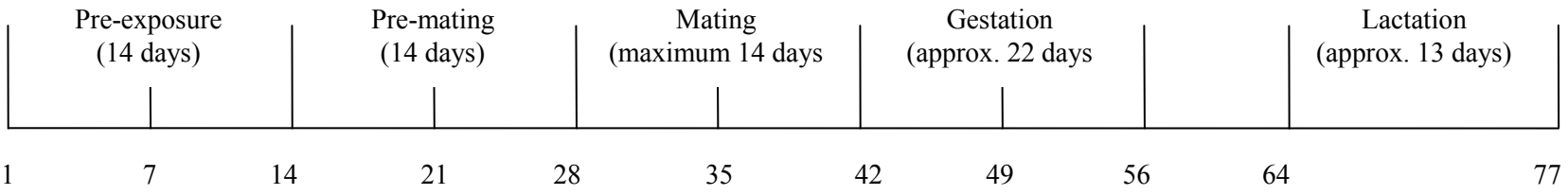
Reproduction toxicity represents harmful effects on the progeny and/or an impairment of male and female reproductive functions or capacity.

Thyroid activity is the capability of a chemical to act like a natural thyroid hormone (e.g. T_3) in a mammalian organism.

Validation is a scientific process designed to characterise the operational requirements and limitations of a test method and to demonstrate its reliability and relevance for a particular purpose.

ANNEX 2 DIAGRAM OF THE EXPERIMENTAL SCHEDULE INDICATING THE MAXIMUM STUDY DURATION, BASED ON A FULL 14-DAY MATING PERIOD





Start of the study

Pre-exposure evaluation of oestrous cyclicity, followed by daily monitoring of vaginal smears from the beginning of treatment until evidence of mating

Dosing

Without dosing

Necropsy males/sires (after a dosing period of at least 4 weeks)

Parturition (PND 0) to PND 4: AGD in all pups (PND 0-PND 4; same day)
Termination of 2 pups per litter for T4 (PND 4)

Day 13 post-partum
Necropsy females and pups
Nipple retention in male pups
Necropsy males/sires (optional)

OBSERVATIONS	VALUES				
Dosage (units).....	**0 (control)**	**. . .**	**. . .**	**. . .**	**. . .**
Pairs started (N)					
Oestrus cycle (at least mean length and frequency of irregular cycles)					
Females showing evidence of copulation (N)					
Females achieving pregnancy (N)					
Conceiving days 1 - 5 (N)					
Conceiving days 6 - . . .[1] (N)					
Pregnancy ≤ 21 days (N)					
Pregnancy = 22 days (N)					
Pregnancy ≥ 23 days (N)					
Dams with live young born (N)					
Dams with live young at day 4 pp (N)					
Implants/ dam (mean)					
Live pups/ dam at birth (mean)					
Live pups/ dam at day 4 (mean)					
Sex ratio (m/ f) at birth (mean)					
Sex ratio (m/ f) at day 4 (mean)					
Litter weight at birth (mean)					
Litter weight at day 4 (mean)					
Pup weight at birth (mean)					
Pup weight at the time of AGD measurement (mean males, mean females)					
Pup AGD on the same postnatal day, birth – day 4 (mean males, mean females, note PND)					
Pup weight at day 4 (mean)					
Male pup nipple retention at day 13 (mean)					
Pup weight at day 13 (mean)					
ABNORMAL PUPS					
Dams with 0					
Dams with 1					
Dams with ≥ 2					
LOSS OF OFFSPRING					
Pre-natal/ post-implantations (implantations minus live births)					
Females with 0					
Females with 1					
Females with 2					
Females with ≥ 3					
Post-natal (live births minus alive at post-natal day 13)					
Females with 0					
Females with 1					
Females with 2					
Females with ≥ 3					

[1] last day of the mating period