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Considerations for the development of guidance on dose level selection for developmental and reproductive toxicity studies

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ABSTRACT

In 2022, the European Chemicals Agency issued advice on the selection of high dose levels for developmental and reproductive toxicity (DART) studies indicating that the highest dose tested should aim to induce clear evidence of reproductive toxicity without excessive toxicity and severe suffering in parental animals. In addition, a recent publication advocated that a 10% decrease in body weight gain should be replaced with a 10% decrease in bodyweight as a criterion for dose adequacy. Experts from the European Centre for Ecotoxicology and Toxicology of Chemicals evaluated these recent developments and their potential impact on study outcomes and interpretation and identified that the advice was not aligned with OECD test guidelines or with humane endpoints guidance. Furthermore, data analysis from DART studies indicated that a 10% decrease in maternal body weight during gestation equates to a 25% decrease in body weight gain, which differs from the consensus of experts at a 2010 ILSI/HESI workshop. Dose selection should be based on a biological approach that considers a range of other factors. Excessive dose levels that cause frank toxicity and overwhelm homeostasis should be avoided as they can give rise to effects that are not relevant to human health assessments.

1. Introduction and background

In order to best protect human health, toxicity studies need to provide information on the relevant hazards associated with a material and need to identify a point of departure from normality which can be used as a basis for a risk assessment. A critical factor in achieving these goals is the specification of the study and in particular the selection of dose levels. Advice and guidance on dose level selection is provided in Organization for Economic Cooperation and Development (OECD) test guidelines (TGs) and allied OECD guidance documents (GDs) but can be unclear particularly around selection of the highest dose tested.

To make dose level selection even more challenging there are very different ways in which information from toxicity studies is used in risk management. For risk assessment, the critical data from repeat dose toxicities studies is the no observed adverse effect level (NOAEL). This is compared to estimated or predicted human exposure to provide a risk assessment, to subsequently allow a risk management judgment to be made on human safety for that chemical application or exposure. When data are used for hazard-based classification the focus is purely on the effect (hazard), independent of toxicological potency and relevance to human exposure. This means that hazard-based classification may be considered of more limited value in protecting human health as there is little consideration of the degree of hazard (potency) or the relevance of the dose levels used in toxicity studies to human exposures.

The concepts developed in a technical report by the European Centre

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Abbreviations

ALAT	Alanine Amino Transferase
ASAT	Aspartate Amino Transferase
AST	accessory sex tissue
ATP	adenosine triphosphate
BUN	Blood Urinary Nitrogen
BWt	body weight
DART	developmental and reproductive toxicity
EPA	Environmental Protection Agency
EOGRTS	extended one generation reproductive toxicity study
GD	guidance document
gd	gestation day
ILSI	International Life Sciences Institute
IPCS	International Programme on Chemical Safety
IUGR	intrauterine growth restriction
HESI	Health and Environmental Sciences Institute
LOAEL	lowest observed adverse effect level
MTD	maximum tolerated dose
NOAEL	no observed adverse effect level
OECD	Organization for Economic Cooperation and Development
SD	Sprague Dawley
TG	test guideline
TK	toxicokinetic
VO	vaginal opening

for Ecotoxicology and Toxicology of Chemicals (ECETOC Technical Report 138; ECETOC, 2021) and accompanying publication (Sewell et al., 2022), recommend pragmatic and scientifically based approaches to dose level selection taking into account regulatory requirements, animal welfare and state of the art scientific approaches. In contrast to the principles proposed by Sewell et al. other approaches take a more theoretical and mathematical path and focus more on the need to increase the top dose levels tested to identify all potential hazards (van Berlo et al., 2022). These differing approaches primarily apply to repeat dose systemic toxicity studies, including carcinogenicity and reproductive toxicity studies. Recently, concerns have been expressed that insufficient dosing in assessments of reproductive toxicity may provide inadequate data for classification and labelling purposes (Hellsten et al., 2023). The overriding concern being that by missing elements of hazard it may not be possible to fulfil the precautionary protection goal served by classification and labelling. This is reflected in recent advice on dose level selection for developmental and reproductive toxicity (DART) studies from the European Chemicals Agency (ECHA 2022; Hellsten et al., 2023) which take a more conservative approach suggesting that the highest dose tested should aim to cause a greater degree of toxicity and clear evidence of effects on reproduction. Here the advice states that study designs should ensure the data generated are adequate for hazard identification and risk assessment with use of *'appropriately high dose levels'*, stating that in OECD TGs 414, 421/422 and 443 whilst the highest dose level should avoid death or severe suffering the aim is to observe toxicity, specifying that 'some' developmental or systemic toxicity is needed to provide clear evidence of adverse effects on reproduction.

Additionally, the recent advice issued by ECHA, and others supporting the use of such high dose levels (Heringa et al., 2020; Woutersen et al., 2020), demonstrate clear views on the use of other scientifically based aids to dose level selection and reinforce the need to observe toxicity. With ECHA stating that whilst all existing information should be considered *'setting the dose level by toxicokinetic considerations only is not allowed under REACH because dose-level selection should be based on toxicity to ensure that the data generated are adequate for hazard identification'*.

Although mindful of animal welfare ECHA proposes levels of toxicity that may be considered globally as too high, stating that the top-dose selection should aim to induce reproductive toxicity without excessive other toxicity or severe suffering that would compromise the interpretation of co-occurring reproductive effects. Examples of severe suffering given include prostration, severe lack of appetite and excessive mortality (though it is clear to the authors of this paper that excessive mortality is more than severe suffering and that mortality exceeds the maximum tolerated dose (MTD)). However, not all chemicals are reproductive toxicants, and in the absence of reproductive toxicity or other generalised toxicity, it is suggested in the ECHA advice that testing go up to the limit dose.

In view of these divergent approaches to dose level selection, the purpose of this paper is to further develop the ECETOC recommendations to cover DART studies. Due to the complexity of reproductive and developmental processes, these studies are uniquely vulnerable to high dose level-induced toxicity and the generation of findings not relevant to human exposures. DART studies are designed to detect and characterise hazard at all stages of the reproductive cycle from spermatogenesis, through mating, gestation, and post-natal development including mating to produce successive generations. The unique vulnerability of DART studies mentioned above arises largely from the consequences of maternal toxicity on normal *in utero* development. Excessive maternal toxicity can directly lead to significant adverse effects on *in utero* development and on subsequent post-natal development and function. Therefore, the study types that are within the scope of this paper are those that include dosing during gestation. The study that covers the whole of the reproductive cycle is described in OECD TG 416, the Two Generation Reproduction Toxicity Study. All other study types cover one or more phases of the reproductive cycle (OECD TG 414 Prenatal Developmental Toxicity Study; OECD TG 421 Reproductive/Developmental Toxicity Screening Test; OECD 422 Combined Repeat Dose Toxicity Study with the Reproduction/Developmental Screening Test; OECD TG 443 Extended One Generation Reproductive Toxicity Study (EOGRTS)).

The existing recommendations of the ECETOC report (ECETOC, 2021) and of Sewell et al. (2022) represent approaches to selecting dose levels that allow for accurate risk assessment but also enable hazard-based classification based on identification of relevant hazards and are consistent with current regulatory frameworks. They can be summarised as follows. As currently recommended in OECD test guidelines and guidance documents, wherever practically possible, an understanding of systemic exposure (parent and/or major metabolites) should be gained through the use of toxicokinetic (TK) approaches to guide dose level selection and study interpretation. In most cases systemic exposure (blood and tissue) will be linear with externally applied dose, which demonstrates that the potential resulting biological effects (including any toxicities observed) represent true responses to increasing systemic exposure. In a minority of cases a less than proportional increase in systemic exposure may be demonstrated and this knowledge is critical in guiding approaches to dose level selection where plateaus of exposure or other non-linear kinetics can be taken into account. Where there are no or little data to make a dose selection decision based on systemic exposure, or where systemic exposure has a linear relationship with the externally applied/targeted dose, then signs of toxicity remain the main source of knowledge for selecting appropriate dose levels. As mentioned above, OECD Test Guidelines and associated Guidance Documents are often unclear about the level of toxicity required at the highest dose tested and it is recognised that there can be differing interpretations of the guidance leading to differing approaches. Guidance Document 116 (OECD, 2012) on chronic and carcinogenicity

testing illustrates differing approaches with regards to acceptable levels of toxicity at the high dose, and the use of the MTD versus the minimally toxic dose. The guidance document acknowledges that ambiguities around MTD definition and interpretation can mean a completed carcinogenicity bioassay that may be acceptable to one organization but not to another. However, the guidance document does recognise that excessive toxicity at the top dose level may compromise the usefulness of the study and/or quality of data generated, as well as the fact that the MTD is often used to decide whether the top dose tested was adequate to give confidence in a negative result.

There is no scientific justification or value in selecting the high dose in any repeat dose studies with the aim of causing overt/significant systemic toxicity (i.e., pain, distress, suffering) or lethality. In this paper the approaches to dose level selection outlined by OECD and by ECETOC are extended and developed to fully take into account the unique vulnerability of DART studies.

2. The holistic/toxicological approach to dose level selection

As stated above, the purpose of toxicity studies is twofold: to identify the potential hazards posed by a particular agent, and to provide an estimate of the dose level that produces no observable adverse effects. As the goal is to protect the human population from any adverse effect, even one that could occur at low frequency, the dose levels in animal studies are exaggerated. From a mathematical perspective, a response seen in one or two of 25 rodent litters can be extrapolated to predict the dose level that would confer a 1 in 10 000 or 1 in 100 000 risk by drawing a straight line from the 4% or 8% response level (1 or 2/25) to the 1/100 000 response level. The purely mathematical approach to maximising the chance of detecting a hazard would be to exaggerate the top dose level of a study to the maximum extent possible, i.e., be limited only by mortality, because exaggerated dose should be linearly related to exaggerated response. However, this ignores the biology. We know that the changes in metabolism, pharmacokinetics and/or physiology that occur at excessive dosages are clearly non-linear in their relationship with dose, and often produce effects on development and reproduction that have little or no relevance to even slightly lower dosages that are minimally toxic, let alone to typical population exposure levels. The extrapolations from these excessive dose levels, while mathematically feasible, are in fact meaningless in predicting adverse effects at environmentally relevant exposure levels.

There are many mechanisms by which effects on maternal health and homeostasis have secondary effects on embryonic development that are unspecific and not primary/genuine developmental effects. These have been the subject of multiple reviews over the years (e.g., Daston, 1994; Carney, 1997; Daston et al., 2018, and others). One apparently common mechanism of maternally-mediated developmental toxicity is the induction in rodents of the zinc-binding protein metallothionein, causing a transitory but systemic zinc deficiency. The transitory zinc deficiency is developmentally adverse (Taubeneck et al., 1994; Duffy et al., 1997, and others). This induction is part of a generalised acute phase response that occurs in response to systemic infection or inflammation, and also to intoxication by many chemicals. Because it is a high-dose phenomenon, it has no relevance for hazard or risk characterisation, but is a side effect of excessive maternal toxicity.

There are also numerous examples of saturation of pathways of elimination (especially metabolism) that have also been recently reviewed (Sewell et al., 2022). Ethylene glycol is a well-studied example in pregnant animals. Developmental toxicity is attributable to the glycolic acid metabolite. Both the metabolism of ethylene glycol and of glycolic acid are saturable, which leads to a supralinear relationship between administered dosage and systemic concentration (Corley et al., 2005; Carney et al., 2011a). Because of this, dosages above the lowest observed adverse effect level (LOAEL), even though they would be considered not to produce excessive toxicity, are contraindicated because the results would be irrelevant to predict risk or hazard for any relevant human exposure scenario, including accidental ingestion.

These examples provide ample evidence that there are dose levels that are too excessive to provide valid information on the hazard or risk of a chemical at relevant exposures. This was understood very early on in the existence of formalised toxicity testing and led to the concept of maximally tolerated dose in chronic toxicity studies, or minimally toxic dose in reproductive and developmental toxicity studies, with heuristics about body weight and body weight gain being the most common limiters of dose. For example, the US Environmental Protection Agency (EPA) Guidelines for Developmental Toxicity Risk Assessment (US EPA, 1991), which first appeared in the 1980s, state that the minimally toxic level should cause marginal but significantly reduced (maternal) body weight or reduced weight gain. US EPA's guidelines for developmental neurotoxicity explicitly state that a 20% decrement in maternal weight gain over the period of gestation and lactation is excessive (US EPA, 1998). Palmer (1978) cites a WHO report from 1967 stating that ideally the highest dose level in a developmental toxicity study should cause minimal signs of maternal toxicity, "e.g., a slight retardation of maternal weight gain". As pharmacokinetic and metabolism data have become more available, these have been increasingly used to inform dose setting to avoid dosages above saturating levels of absorption, metabolism and excretion.

3. A brief review of the evolution of the guidance on top dose level selection for DART studies

To ensure adequate dosing of pregnant maternal animals, current test guidelines (e.g., OECD 414; OECD, 2018a, with similar wording in 2001 version) advocate the highest dose should induce some developmental and/or maternal toxicity but not death or severe suffering. Dose levels that induce maternal toxicity presumably were included to increase sensitivity of the test, assuming that effects seen at high doses (often with bolus administration) are relevant to lower dose levels. To meet high-dose requirements, dose selection strategies have remained largely unchanged for decades; however, these dosing requirements have made it difficult for both registrants and regulators to separate maternal and developmental toxicity as it is difficult to determine direct causal effects on development from secondary effects due to altered maternal health (Carney et al., 2011b). It has long been known that maternal toxicity leads to reduced litter size, total litter losses and increased incidence of foetal pathology findings. Among reduced maternal body weight gain, also epigenetic and protein alterations can be causative for malformations. For review, see Rogers et al. (2005) or Tyl and Marr (2012).

In view of the complexity and inter-dependencies of the experimental model illustrated above, there have been a number of initiatives to develop scientifically robust approaches to dose level selection in DART studies. In 2009, the International Life Sciences Institute (ILSI) Health and Environmental Sciences Institute (HESI) held a workshop on 'Developmental Toxicology – New Directions' with a goal to improve relevance and predictivity of animal studies (Brannen et al., 2011). Workshop discussions included the proposal to use kinetic data in dose selection to avoid nonlinear kinetics that can occur at high (irrelevant) maternally toxic doses, with the majority of attendees concluding that a more rational upper limit should be adopted. A further ILSI/HESI workshop in 2010 sought consensus on the impact of maternal toxicity on developmental toxicity study designs (Beyer et al., 2011). While the goal of harmonisation was not realised, there was some consensus on maximum maternal toxicity with respect to altered maternal body weight gains during gestation (see Beyer et al., 2011). For general toxicity studies, 5–10% decrease in body weight gain was considered as possibly adverse by some participants, whereas for developmental and reproductive toxicity studies there was no consensus, although a 20% decrease in maternal body weight gain was deemed too much. Based on these discussions, a decrease in body weight gain during the treatment period in the dose-range finding study of 10–15% should be justified as a

suitable high dose in reproductive toxicity studies. Beyer et al. (2011) reported another consensus opinion among workshop attendees that the occurrence of maternal mortality indicated that the MTD was exceeded.

In 2012, committee members reviewing pesticide registrations for the Italian Ministry of Public Health (Giavini and Menegola, 2012) proposed that maximum dose levels for environmental chemicals should not produce maternal toxicity in developmental toxicity studies. They argued that this approach to dose setting would improve interpretation of developmental toxicity findings while avoiding inconclusive results, chemical misclassifications, use of massive dose levels, and generation of erroneous results due to saturation of kinetics or other non-linear relationships. In 2018, Scialli et al. (2018) advocated for an evolution to hypothesis-driven developmental toxicity testing with dose setting based on internal dose and mode-of-action/critical windows information, arguing that considerably more information is available to develop intelligent study designs rather than using a standardised protocol with excessive dose levels. Despite these appeals for more rationale dose selection, some European regulators, for example ECHA, advocate that the selection of the top dose should aim to achieve the highest possible dose level in the parental generation without severe suffering or death. Adding that if the concept of avoiding death was also applied to the filial generation, death of the developing organism cannot be investigated in developmental toxicity studies (Hellsten et al., 2023). Hellsten et al. (2023) also noted that if exposure covers developmental and mature life stages (e.g., EOGRTS) then effects seen in the filial generation adults are considered developmental toxicity; however, in interpreting these findings, it is important to consider dose level (e.g., during growth phases, offspring consume more diet and may have greater exposure if dosed by the dietary route), duration (i.e., filial generation typically is dosed for a longer period than parental generation), and effect (e.g., target organ toxicity that has been reported in adult animals previously with a similar dosing scenario) when interpreting whether effects indicate developmental toxicity.

The suggested criteria for top dose selection also raise concern with regard to changing the definition of acceptable levels of animal suffering. Guiding principles in the OECD Humane Endpoints Guidance Document (Guidance Document 19; OECD, 2000) states *'Studies must be designed to minimise any pain, distress or suffering experienced by the animals, consistent with the scientific objective of the study';* as described in this document, the scientific objectives of DART studies cannot be achieved with excessive dose levels. In contrast, the recent dose selection guidance from ECHA states that the top dose should not induce "severe suffering". This opens the question as to what constitutes animal "suffering" versus "severe suffering". The "degree of suffering" is likely to be subjective between laboratories, registrants, regulations, and regions. Examples of severe suffering in the ECHA advice on top dose selection include excessive mortality, indicated as >10% mortality, which is not in line with OECD TGs 414, 443 and 421/422 and Humane Endpoints Guidance Document where it is indicated that death should be avoided (Table 1). The ECHA recommendation to now avoid only "severe suffering" (as opposed to suffering) could be considered counter to the 3R s (i.e., refinement to minimise suffering; https://nc3rs.org.uk/who-we-are/3rs), which are generally supported by regulatory agencies globally. It could also lead to challenges in appropriate consideration of societal pressures to lessen animal suffering. Lastly, such significant levels of toxicity are identified as confounding data in many OECD test guidelines and guidances. It may be more appropriate that mortality (rather than 'excessive' mortality) be considered as more than severe suffering, and this could be extended to animals that are moribund and/or display signs signalling euthanasia, as these could be considered as equivalent to the death of an animal, and therefore also in excess of severe suffering.

4. Concerns and deficiencies regarding the approach proposed by van Berlo et al

In the paper by van Berlo et al. (2022), the authors reviewed how a MTD criterion for 90-day studies (i.e., a 10% decrease in body weight) had been modified for use in other toxicity study types based on an initial adaptation (i.e. a 10% decrease in body weight *gain*) for carcinogenicity studies. Specifically, the authors state that a '*10% decrease in body weight gain criterion also ended up in other test guidelines and guidances for toxicity endpoints other than carcinogenicity, so outside the context it was intended for*'. This statement espouses the view that a 10% decrease in body weight gain is not sufficient for MTD for other test guidelines or guidances but without considering other study-related parameters that can impact MTD selection criteria. This approach has attracted comment and has raised concerns from other stakeholders (Arts et al., 2023).

First, when defining MTD, the physiological status of the animals should be considered. As noted above, DART studies that include gestational and lactational phases warrant greater consideration when selecting MTD criteria because the reproductive outcome and health of the offspring are closely associated with maternal wellbeing before, during and after gestation. Van Berlo et al. (2022) referenced the OECD TG 426 Developmental Neurotoxicity study as having an MTD based on a 10% reduction in body weight gain but did not appreciate these studies having a vulnerable gestational and lactational phase.

Gestational Body Weight/Gains: During gestation, a 10% decrease in body weight gain in pregnant animals is a more suitable MTD criterion than a 10% or greater decrease in body weight as proposed by van Berlo et al. (2022). In a representative dataset shown in Fig. 1A and B, a 10% change in body weight in maternal animals during gestation (e.g., gestation day (gd) 6–21 as in the OECD 414 study) would be equivalent to a 24% decrease in body weight gain, a decrement that exceeds the consensus recommendations of DART experts (Beyer et al., 2011). Thus, a 10% change in body weight in pregnant animals in the absence of significant *in utero* foetal loss is an indicator of excessive toxicity and exceedance of the MTD. Statistical differences in net body weight gain (terminal maternal body weight minus gravid uterine weight) also can indicate maternal toxicity in rats as this is the weight of the dam without contribution by the conceptuses (although some maternal organ weights also increase in size during pregnancy; Tyl and Marr, 2012).

During the last trimester, maternal body weight gain is primarily driven by increases in foetal body weights (Fig. 2). Thus, with oral gavage studies, there is some concern that maternal animals are receiving higher doses of test compound based on body weight during the last third of pregnancy. If the maternal liver cannot compensate for the increased dose, there may be greater toxicity to both the dams and foetuses during the last third of gestation. Thus, there may be more profound foetal body weight reductions at term and/or increased foetal death due to continued direct or indirect toxic insult or stress. Furthermore, maternal toxicity occurring during this foetal growth stage often is associated with developmental effects such as decreased foetal body weights and/or developmental delays (e.g., delayed ossification) (Carney et al., 2011b). Frequently, the relationship between developmental outcomes and maternal toxicity are difficult to assess, particularly given background incidences of variations and malformations and restrictions on the use of historical control data.

Animals that lose weight late in gestation during the period of greatest foetal growth tend to have smaller foetuses and this has also been shown experimentally by Garofano et al. (1998). Maternal food restriction (50%) from day 15 of pregnancy resulted in intrauterine growth retardation in the offspring.

Van Berlo et al. (2022) requested to remove the 10% decrease in body weight gain as an MTD criterion for top dose selection in test guidelines and guidances for toxicity endpoints other than carcinogenicity. However, it is clear that for MTD criteria, "one size doesn't fit all" and the proposal to incorporate ≥10% change in body weight into all test guidelines cannot be supported for DART studies.

Table 1
Selected text from recent ECHA advice, OECD Test Guidelines (TGs), and OECD Guidance Documents (GDs) on Top Dose Selection, Use of TK and Data Interpretation.

Considerations for top dose selection	ECHA recent top dose selection advice 2022	OECD Guidance Documents 19 (Humane endpoints), 43 (Reproductive toxicity testing) and 150 (Evaluating chemicals for endocrine disruption)	OECD TG 414 developmental toxicity study	OECD TG 443 EOGRTS and GD 151 on EOGRTS	OECD TG 421/422 Repro screening studies
Top dose guidance	For the highest dose level, it should be demonstrated that **the aim is that it is the highest possible dose level without severe suffering or death**, or the limit dose concept shall be used. … the top-dose selection should demonstrate an aim to induce clear evidence of reproductive toxicity without excessive other toxicity and severe suffering in parental animals (e.g. prostration, severe inappetence (lack of appetite), **excessive mortality as signs of severe suffering)** that would compromise the interpretation of co-occurring reproductive effects. **Excessive mortality is defined as, "More than 10% mortality** (Section 3.7.2.4.4 of Annex I to the CLP Regulation)."	GD 43 on Repro Tox: Ideally, unless limited by the physico-chemical nature or biological effects of the test substance, **the highest dose level should induce toxicity** but **not mortality in the parental animals**. Studies intended to assess prenatal hazard are generally designed to include at least one dose group that elicits some degree of maternal toxicity. GD 19 on Humane Endpoints: A humane endpoint can be defined as the earliest indicator in an animal experiment of severe pain, severe distress, **suffering**, or impending death **(note severe suffering is not indicated)**. GD 19 on Humane Endpoints: **Studies must be designed to minimise any pain, distress or suffering experienced by the animals, consistent with the scientific objective of the study** GD 19 on Humane Endpoints: Annex 3 - Clinical signs indicating the **need for closer observation or humane killing** (This annex includes many clinical signs such as dehydration, difficulty breathing, jaundice, altered motor activity, abnormal vocalization, abnormal posture, decreased grooming, abortion, agalactia, etc.) … Body weight loss or emaciation: Particularly **when bodyweight has decreased by more than 20% compared with control animals**, or bodyweight has decreased by more than 25% over a period of 7 days or more.	the highest dose should be chosen with **the aim to induce some developmental and/or maternal toxicity** (clinical signs or a decrease in body weight) but **not death** or severe suffering. For other types of administration, such as inhalation or dermal application, the physical chemical properties of the test chemical often may indicate the maximum attainable level of exposure (for example, dermal application should not cause severe localised toxicity)	OECD TG 443: If dose levels are based on toxicity, the highest dose should be chosen with **the aim to induce some systemic toxicity,** but **not death** or severe suffering of the animals.	The highest dose level should be chosen with **the aim of inducing toxic effects** but **not death** or severe suffering.
Notes on interpretation	**The focus of the OECD TG 443 study in the REACH annexes is on sexual function and fertility**, which should be prioritised in the study design of the OECD TG 443 study. Regarding the highest dose level, **it is important to ensure that sufficient severity of toxicity in both female and male animals is achieved to ensure that potential effects on sexual function and fertility in either gender is not overlooked**. As the study should be designed to ensure adequate assessment of the effects on sexual function and fertility, the **dose levels should not be reduced**	GD 43 on DART studies: In some cases, however, the presence of maternal toxicity may impact the interpretation of study data. For example, **when maternal toxicity is so severe (e.g., mortality) that foetal well-being is compromised, information on developmental effects may be difficult to interpret**. GD 43 on DART studies: Both absolute and relative weights of the male reproductive organs should be considered as a decrease in absolute weight may occur and may not necessarily be related to a reduction in body weight gain. However, **care should be taken in**		GD 151 on EOGRTS: The **EOGRTS is designed to assess fertility and to evaluate the pre- and postnatal effects of chemicals on development.** Based on weight of evidence and/or specific regulatory authority's requirements, **evidence of systemic toxicity or reproductive toxicity may be required at the highest dose level** in order to ensure that the test system is optimised to be able to investigate any reproductive toxic property of a substance measured in the test system. As noted in paragraph 19, **toxicokinetics may also be considered in dose selection** … It is	OECD TG 422: Generally, it is assumed that there are differences in sensitivity between pregnant and non-pregnant animals. Consequently, it may be more complicated to determine dose levels in this combined test that are adequate to evaluate both general systemic toxicity and specific reproduction/ developmental toxicity, rather than when the individual tests are conducted separately. Moreover, **interpretation of the test results with respect to general systemic toxicity may be more difficult than when conducting a separate repeated-dose study.** OECD TGs 421/422**: In the presence**

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Table 1 (*continued*)

Considerations for top dose selection	ECHA recent top dose selection advice 2022	OECD Guidance Documents 19 (Humane endpoints), 43 (Reproductive toxicity testing) and 150 (Evaluating chemicals for endocrine disruption)	OECD TG 414 developmental toxicity study	OECD TG 443 EOGRTS and GD 151 on EOGRTS	OECD TG 421/422 Repro screening studies
	to get enough offspring for the assessment of developmental toxicity.	**interpreting data where a substantial bodyweight effect is evident.** GD 150 on Endocrine Disruptors: It is also important to know whether an in vivo endocrine disruption test has been performed at doses or concentrations which would not be expected to cause **systemic toxicity that could mask endocrine effects, or which could cause misleading endocrine changes secondary to general or specific (non-endocrine) organ toxicities.** GD 150 on Endocrine Disruptors: **The top dose or concentration should be sufficiently high to give clear systemic (i.e. non-endocrine specific) toxicity** in order to ensure that a wide range of exposures (high to low) is tested. However, **endocrine effects observed solely in the presence of clear systemic toxicity should be interpreted with caution and may be disregarded when sufficiently justified to be caused by secondary effects which are unlikely to be due to endocrine activity.** GD 150 on Endocrine Disruptors: Endpoints for hormonal-mediated activity and endpoints potentially sensitive to, but not diagnostic of, hormonal-mediated activity listed in Table B1 **can be affected by a variety of non-endocrine factors, such as marked systemic toxicity, handling stress or infections**.		recognised that **some dose levels of the test substance may affect fertility**, such that an insufficient number of pups may be produced for assessment of the F1 generation. **In situations where fertility is affected**, the lower dose levels should therefore be carefully selected to ensure the objectives of the study can be met. GD 151 on EOGRTS: Where there is clear toxicity to the offspring, first signs of which appear during the lactation phase, it may be related to the transfer of the test substance to the offspring via the milk … However, **reduced offspring growth, relative to controls, may also be a consequence of reduced milk production or quality or other maternal toxicity, and therefore such results must be interpreted with caution.** GD 151 on EOGRTS: Organ weights are key endpoints in TG 443 but **reductions in body weight may confound the interpretation of organ weight changes**. GD 151 on EOGRTS: Both VO and first oestrus can be influenced by body weight and condition (potential consequences of toxicity to the dam during gestation and/or lactation). **Mothers exposed to high doses of a test material often have offspring with lower body weights. This will affect offspring maturation and hence the pubertal process.** GD 151 on EOGRTS: **Interpretation of TG 443 DNT test results should take into account available information on** mechanisms of action, **toxicokinetics, maternal toxicity and potential indirect effects on offspring,** as well as any available data on neurotoxic effects of the specific test chemical. GD 151 on EOGRTS: The interpretation of the results of the EOGRTS in the context of other studies on the substance should be considered in a wider weight of evidence evaluation … Some advice on interpretation of the EOGRTS and stress the importance of considering all	**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.**

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Table 1 (*continued*)

Considerations for top dose selection	ECHA recent top dose selection advice 2022	OECD Guidance Documents 19 (Humane endpoints), 43 (Reproductive toxicity testing) and 150 (Evaluating chemicals for endocrine disruption)	OECD TG 414 developmental toxicity study	OECD TG 443 EOGRTS and GD 151 on EOGRTS	OECD TG 421/422 Repro screening studies
				available data, including physico-chemical data, TK data, … The IPCS mode of action framework	
Toxicokinetics	**Setting the dose level by toxicokinetic considerations only is not allowed under REACH** because dose-level selection should be based on toxicity to ensure that the data generated are adequate for hazard identification. For substances under REACH, the available toxicokinetic data is typically insufficient to conclude on toxic dose levels and, therefore, guide on dose-level selection. However, toxicokinetic information may provide reasons to adjust, for example, the dosing route and regime.	GD 43 on DART studies: **An understanding of the pharmacokinetic** and pharmacodynamic profile of a test substance in the developing system and of the complexities of direct and indirect developmental exposures during pregnancy, lactation, and to neonates by various routes of exposure **is critical to study design, dose selection, and the interpretation and extrapolation of reproductive toxicity data.**	**Dose levels should be selected taking into account any existing toxicity data as well as additional information on metabolism and toxicokinetics of the test chemical or related materials.** This information will also assist in demonstrating the adequacy of the dosing regimen	OECD TG 443: **When selecting appropriate dose levels, the investigator should consider all available information, including** the dosing information from previous studies, **TK data from pregnant or non-pregnant animals,** the extent of lactational transfer, and estimates of human exposure. **If TK data are available which indicate dose-dependent saturation of TK processes, care should be taken to avoid high dose levels which clearly exhibit saturation, provided of course, that human exposures are expected to be well below the point of saturation.** In such cases, the highest dose level should be at, or just slightly above the inflection point for transition to nonlinear TK behaviour. **Interpretation of the results of the study should take into account all available information on the substance, including** physico-chemical, **TK and toxicodynamic properties**, available relevant information on structural analogues, and results of previously-conducted toxicity studies with the test chemical (e.g. acute toxicity, toxicity after repeated application, mechanistic studies and studies assessing if there are substantial qualitative and quantitative species differences in vivo/*in vitro* metabolic properties). (see also paragraph 21) GD 151 on EOGRTS: **Knowledge of the absorption, distribution, metabolism and excretion characteristics of a substance in the test species may help dose selection.** For example, absorption of a substance may be saturated at a certain dose level. If toxicokinetic data are available beforehand, then the highest dose level could be set with the intention of avoiding saturation of toxicokinetic processes, as any higher dose level may not ENV/JM/MONO (2013)10 15	**Dose levels should be selected taking into account any existing toxicity and (toxico-) kinetic data available.**

(*continued on next page*)

Table 1 (*continued*)

Considerations for top dose selection	ECHA recent top dose selection advice 2022	OECD Guidance Documents 19 (Humane endpoints), 43 (Reproductive toxicity testing) and 150 (Evaluating chemicals for endocrine disruption)	OECD TG 414 developmental toxicity study	OECD TG 443 EOGRTS and GD 151 on EOGRTS	OECD TG 421/422 Repro screening studies
				increase systemic exposure unless other factors, such as loss of the integrity of intestinal lining or microbial activity in intestine contribute in case of oral absorption. **Saturation of TK processes may be included in the rationale for dose setting** (see paragraph 27) provided that human exposures are expected to be well below the point of saturation. (Creton et al., 2012; Saghir et al., 2012; McCoy et al., 2012; McFadden et al., 2012) Please refer to GD 151 for cited references	
Reference	ECHA (2022)	OECD (2000) OECD (2008) OECD (2018b)	OECD (2018a)	OECD (2018c) OECD (2013)	OECD (2016a) OECD (2016b)

Fig. 1A. Representative OECD 414 dataset showing mean gestational body weight (±SD) in control CD®(Sprague Dawley; SD) dams with a dotted line depicting a 10% decrease in gestational body weight over the course of gestation after initiation of dosing on gd 6. This magnitude of body weight decrease translates to maternal body weights that are 39 g lower than control dams on gd 21. The control data used for this graph were taken from a guideline-compliant study in a company database and showed intermediate increases in body weight gain during gestation (i.e., studies reporting the largest and smallest gestational body weight gains were not used).

Fig. 1B. Representative OECD 414 dataset showing mean gestational body weight gains in control CD®(SD) dams (blue line; same dataset as Fig. 1A) with dotted lines depicting a hypothetical 10% (green) and 20% (orange) decrease in gestational body weight gain over the course of gestation after initiation of dosing on gd 6. The red dotted line depicts gestational body weight gain that corresponds with a 10% decrease in gestational body weight (see Fig. 1A), which translates to a 24% decrease in gestational body weight gain over gestation (gd 0–21). This magnitude of decrease in gestational body weight gain exceeds DART expert guidance for maternal toxicity per Beyer et al. (2011).

Lactational Body Weight/Gains: Maternal animals generally lose body weight at some intervals during lactation and these intervals may not be entirely consistent across animals. Thus, comparisons of maternal body weight/gains during lactation can be complex and should incorporate other indicators of toxicity to aid in data interpretation.

In summary, a biological approach, using available data that takes into account the complexity of a multicompartment model (maternal-placental-foetal) leads to different conclusions compared to the mathematical/theoretical approach of van Berlo et al. (2022). Thus, a maternal body weight gain decrease of 10%–15% during gestation is considered as an appropriate parameter for selection of a suitable high dose level in reproductive/developmental toxicity studies. A 20% deficit in maternal body weight gain during gestation is considered too high as

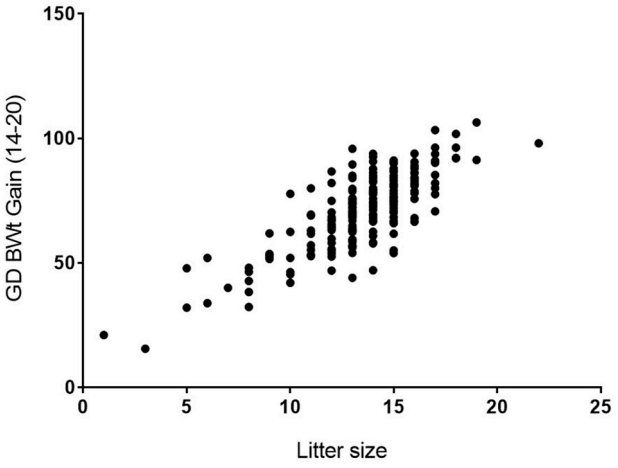

Fig. 2. OECD 421/422 data on litter size vs. maternal bodyweight gains during the last trimester (gd 14–20) in the rat, showing the positive association between litter size and maternal body weight gain during this period. Data points represent individual maternal bodyweight gains and corresponding litter sizes. R2 = 0.632; p < 0.0001 by linear regression (n = 214 pairs). BWt = Body weight.

agreed by many experts representing academia, government and industry as a 'tripartite consensus' (Beyer et al., 2011). Higher dose levels would only add noise and make a proper evaluation of the real results more difficult.

5. Wider perturbations of homeostasis; further endpoints useful for determining a suitable high dose level in DART studies

Aside from changes in body weight gain, there are numerous other parameters that should be considered when selecting the high-dose level for DART studies. Embryo-foetal development is contingent upon a healthy internal environment in the maternal animal. Thus, it is important to consider potential maternal target organ toxicity and other mechanisms of maternal toxicity or stress that can influence gestational or lactational outcomes in the offspring. The following sections provides some examples of changes other than bodyweight that need to be taken into account in dose level selection.

5.1. Maternal clinical signs of toxicity

Aside from effects on maternal body weight/gain, clinical signs during gestation or lactation (e.g., increased or decreased activity, altered maternal caregiving/nursing behaviour, incoordination, altered respiration) may indicate maternal toxicity and that an MTD has been met or exceeded. Excessive dose levels may result in significant toxicity and extend to marked effects on animal health (e.g., tremors/convulsions, lateral recumbency).

5.2. Effects on food consumption/nutritional intake

Food consumption is a marker of homeostasis and decreased food consumption during any study phase may indicate systemic toxicity (Stump et al., 2012). Feed restriction studies have shown that effects on body weight can alter reproductive performance in adult animals. With up to 17 weeks of feed restriction, Chapin et al. (1993) reported a decrease in absolute accessory sex tissue (AST) weights and percent motile sperm in adult male Sprague-Dawley rats weighing 12% less than controls. Effects on AST weights, spermatogenesis (degeneration of pachytene spermatocytes) and decreased plasma testosterone were reported in a feed restriction study in younger male rats (e.g., feed restriction starting at 6 weeks of age) (Rehm et al., 2008). In female rats, a 25% decrease in feed consumption during a two-week premating period led to a 16% decrease in body weights, prolonged dioestrus and reduced fertility associated with decreased corpora lutea (Terry et al., 2005). Decreased food consumption during gestation has been shown to affect foetal growth, alter weanling organ weights and delay development (Carney et al., 2004). Maternal feed restriction to 20, 15, 10 and 7.5 g diet/day from gd 6–17 resulted in decreases in foetal body weights by 5%, 7%, 10% and 24% despite reinstating *ad libitum* access to feed during gd 17–21 when the greatest acceleration in foetal growth occurs (Fleeman et al., 2005). At the highest level of feed restriction, foetal skeletal variations were increased. Taken together, these studies clearly show that decreased feed consumption can affect reproductive and foetal parameters in otherwise healthy animals; however, these reports likely underestimate the effects that would be seen when decreased food consumption and body weights are due to toxicity. Notably, decreased food consumption can be a sign of the systemic toxicity but is seldom seen in isolation.

Nutrition in pregnant and lactating animals is critical to healthy offspring as deficiencies in key nutrients can alter maternal physiology and affect development. Compounds inducing tissue damage and cytokine release can lead to increased maternal metallothionein synthesis in the liver (Coyle et al., 2009). Metallothionein leads to sequestering of Zn ions in the liver with consequently decreased maternal Zn blood concentrations and decreased placental transfer of Zn ions to the embryo (Daston, 1994.). This ultimately induces abnormal embryo-foetal development. Chemicals acting in this way are for example alcohol, valproic acid and 2-ethylhexanoic acid. Supplementation of Zn in the diet of the dam ameliorates embryotoxicity.

Reproductive outcomes in rabbits, a second species used in developmental toxicity assessments, also are sensitive to decreases in feed intake. A feed restriction study during organogenesis (gd 7–19) in pregnant rabbits (150 g feed/day in controls vs. 110, 75, 55, 35 and 15 g feed/day in restricted groups) resulted in significant decreases in foetal body weight at ≤ 75 g feed/day despite only a 2% decrease in maternal body weight on gd 20 and control levels of diet from gd 20–29 (C-sections on gd 29) (Cappon et al., 2005). Decreased ossification was seen in foetuses at these same levels of feed restriction. Pregnant rabbits are especially sensitive to gastrointestinal disturbances and may suffer enteropathy or pregnancy toxemia, a nutritional disorder that occurs secondary to insufficient food intake and metabolic effects (Patton et al., 2008). Thus, decrements in food intake may indicate maternal toxicity prior to significant body weight changes. Interestingly, maternal rabbits often have negative net bodyweight gain, making this endpoint less useful to indicate maternal toxicity in this species. Moxon et al. (2023) reviewed the challenges of interpreting rabbit developmental toxicity studies and the difficulty distinguishing maternal toxicity from specific offspring effects given the rabbit's sensitivity to stress.

Maternal food consumption during lactation also warrants careful examination in reproductive toxicity studies (e.g., OECD TGs 421/422, 426, 416, 443), wherein rats deliver offspring and dosing continues during lactation. Sustained reductions in food and/or water consumption during lactation may indicate that an MTD has been achieved or exceeded. The lactation phase is accompanied by large increases in maternal feed consumption in rats (e.g., 2–3 times increase relative to non-pregnant adult females; Saghir et al., 2013). In rodents, maternal nutritional intake during lactation must be adequate to support the large metabolic demands of milk synthesis to maintain litters and support pup growth rates.

Thus, reduced food intake can affect reproductive parameters in adult male and female rats. Furthermore, decreased maternal food intake during gestation, regardless of the cause, will result in maternal undernutrition which is known to decrease foetal growth and may compromise milk production during lactation. Thus, it is incumbent on registrants, regulators and study personnel to exercise good judgment in dose selection so as not to limit the amounts of nutrients available to the mother during these demanding life stages. Most laboratories have

criteria defining when altered food consumption affects animal welfare in non-pregnant adult animals; however, these limits are more difficult to define in reproductive studies where duration and timing (e.g., embryonic vs. foetal growth period in late gestation; first vs. second week of lactation) can significantly impact the effects of feed consumption deficits.

5.3. Maternal toxicants affecting clinical chemistry parameters

Diflunisal, an anti-inflammatory and analgesic drug, caused defects in the axial skeleton in rabbits. Clark et al. (1984) could demonstrate that the mechanism of this effect was anaemia and a depletion of adenosine triphosphate (ATP) in erythrocytes. Administration of diflunisal before implantation (gd 5) caused a long-lasting anaemia until Day 15. The drug was eliminated from the blood at Day 9, the timepoint where axial defects are induced in this species and where the peak of haemoglobinuria occurred. This proved that the defects were caused by maternal toxicity and not a direct action of the test compound to the embryo. Thus, when haematology and clinical chemistry parameters are examined in maternal toxicity dose range finding studies, anaemia in the range of 10–15% should be judged as dose limiting.

The loop diuretic indacrinone induced wavy ribs, and defects at scapula and humerus in rat foetuses. These effects at scapula and humerus were no longer observed after supplementation with potassium, and the incidence of wavy ribs were reduced. This demonstrated that maternal hypokalaemia was the underlying cause of teratogenicity (Robertson et al., 1981).

5.4. Evaluation of metabolome data

Metabolomic patterns were evaluated in 44 studies using plasma of pregnant rats at gestation day 20 (Keller et al., 2019). Metabolomic data were compared to the routinely assessed parameters of body weight and food consumption. Metabolome-derived No Observed Effect Levels were below the classic maternal NOAELs. These data suggest that using the classic maternal parameters may be too crude. Physiological imbalance may occur at lower dose levels, and maternal toxicity may be overlooked.

A compilation of the same endpoints for maternal toxicity studies, OECD TG 414, OECD TG 421 and OECD TG 422 studies (total of 127 studies) revealed that 31 compounds had significant changes in the metabolome below the NOAEL. Additionally, 37 compounds showed non-significant changes below the NOAEL (BASF, internal data). Thus, metabolomic data may provide information on mode-of-action when effects are observed or indicate that physiological changes have occurred even if effects are not grossly apparent.

Metabolomics data clearly shows perturbations to a range of measures in addition to the endpoints traditionally assessed in DART studies. As scientific understanding continues to develop in this area these data may provide additional information to guide dose level section.

5.5. Maternal circulatory changes and cardiovascular active compounds

It has been known for some time that interruption of the oxygen supply to the embryo is teratogenic. Clamping of the uterine vessels in rats on gd 14 caused foetal deaths, limb anomalies and cleft palate in rats (Leist and Grauwiler, 1974). Later it was shown that also cardiovascular active drugs caused hypoxia to the pregnant animal due to their pharmacological mechanism. Vasoconstricting agents caused malformations in rats due to hypoxia, particularly in digits when preceded by haemorrhage (Webster and Abela, 2007). Digital defects in rat (Yoshida et al., 1988) and rabbit foetuses (Danielsson et al., 1989, 1990) were also observed after treatment of the mothers with vasodilating calcium antagonists. A probable underlying contributor is an alteration of the disposition of blood from central to peripheral compartments. The anti-depressant Phenytoin caused decreased heart rate and teratogenic effects in A/J mice, but not in C57Bl/6 J mice, a strain resistant to heart rate effects (Watkinson and Millicovsky, 1983); maternally mediated embryotoxicity was proposed as the mode of action.

5.6. Maternal histopathology observations

Pathological signs of irritation/corrosion at the dosing site (skin, gastro-intestinal or respiratory tract) may indicate that an MTD has been reached or exceeded; suffering or distress to the animal must be avoided. In case of histopathological examinations, changes indicating impairment of liver function (necrosis, elevated Alanine Amino Transferase (ALAT), Aspartate Amino Transferase (ASAT)) or kidney function (necrosis, degeneration/regeneration, increased Blood Urinary Nitrogen (BUN), increased creatinine) can be considered as dose-limiting (ECETOC Technical Report 138; ECETOC, 2021).

5.7. Role of maternal stress

Environmental factors, such as exposure of pregnant rats to noise, has been shown to result in lower litter size and increased incidence of malformations in rats (Geber, 1966). Embryonic deaths, but no malformations were observed in mice after noise exposure (Kimmel et al., 1976). Treatment of pregnant mice with diazepam and phenytoin led to increased blood levels of corticosterone (Barlow et al., 1980; Hansen et al., 1988). In the case of diazepam the lowest dose causing increased cortisone levels in the dams was also the lowest dose level causing cleft palate in the foetuses.

Burgueño et al. (2020) conducted 14 separate meta-analyses of the role of maternal stress and administration of corticosteroids during pregnancy on foetal parameters in rodent studies. Both maternal stress and administration of corticosteroids were associated with low birth weights. Offspring body weights remained lower in later life, indicating no rapid postnatal recovery.

Usually range-finding studies for developmental toxicity studies use relatively crude parameters (body weight, body weight gain, food consumption, and in some rare cases also clinical chemistry and haematological data). As shown above, there are many other factors that impact implantation and embryofoetal growth. This demonstrates how sensitive the pregnancy and lactation phases are, and strengthens the argument not to go too high with respect to the top dose level. Of course, it is not known if, for example, metabolome changes are adverse or not, but doses that markedly perturb maternal physiology should be avoided. It should also be considered that effects on body weight and food consumption can affect the amount of nutrients and micronutrients available to the dam and the foetuses, which are especially important during gestation and lactation.

6. Animal studies in the context of the protection of human health

Reproductive toxicity studies are designed to apply to laboratory animals, mainly rats. However, the purpose of testing is to predict the outcome of such studies for *humans*. Whilst animal studies are used as surrogates for humans, it is important to acknowledge that studies in animals have their own limitations and deficiencies and may not always replicate the situation in humans. It may be informative to consider how a physician would respond to the situation where a pregnant woman would have a 10% lower body weight at the end of gestation compared to a normal gestation. Let us assume that to have such a situation food consumption would need to be 25% lower than under normal circumstances. Most likely the physician would conclude that this could potentially have several consequences for the unborn child. It's important to note that individual circumstances can vary, and the effects on the unborn child may depend on other factors such as the overall health of the mother and the specific cause of the reduced body weight. However, here are some general possible consequences.

- Restricted foetal growth: Maternal undernutrition or inadequate weight gain during pregnancy can lead to restricted foetal growth. Insufficient maternal nutrition may limit the availability of essential nutrients required for the development of the foetus, potentially resulting in low birth weight or intrauterine growth restriction (IUGR).
- Impaired foetal development: A reduced body weight in the mother may indicate inadequate nutrient intake during pregnancy. This can result in deficiencies of vital nutrients such as protein, iron, folate, calcium, and others, which are crucial for the development of the foetus. Nutrient deficiencies may lead to impaired organ development, increased risk of birth defects, and long-term health issues for the child.
- Increased risk of preterm birth: Inadequate maternal weight gain or low body weight can increase the risk of preterm birth.
- Compromised immune system: Poor maternal nutrition can impact the development of the foetal immune system. The child may have a weaker immune response, making them more susceptible to infections and diseases early in life.
- Cognitive and neurological effects: Proper nutrition during pregnancy is vital for the development of the foetal brain and nervous system. Inadequate weight gain or nutritional deficiencies may increase the risk of cognitive and neurological impairments in the child, potentially affecting their learning abilities and overall development.
- Long-term health implications: The consequences of reduced body weight during pregnancy may extend into the child's later life. Studies have suggested that poor maternal nutrition during gestation can increase the risk of chronic conditions, such as cardiovascular diseases, obesity, and type 2 diabetes, in adulthood.

Pregnancy in itself has a measurable effect on homoeostasis. For example, the effects of pregnancy on the internal, naturally occurring metabolites (<1.5 Da) in rats, using a blood-based metabolomics approach, were very pronounced. In addition, simple overnight fasting also had a significant effect on internal metabolite levels. What is important in the context of reduced body weight and pregnancy is that there also was an interaction between fasting and pregnancy mediated metabolome changes (Ramirez-Hincapie et al., 2021). This indicates that in developmental toxicity studies in which there is a pronounced reduction (>10%) in body weight, it is likely that this condition by itself will have an adverse effect. This may not be immediately evident but can for instance reduce homeostasis and defence mechanisms. So, with increasing maternal toxicity, the discriminative power of such studies to detect selective developmental toxicity is diminished.

Lastly, as mentioned by Scialli et al. (2018), more information is available for dose setting than in the past, including *in silico*/read across data, *in vitro* data, toxicokinetic modelling, that can be used to inform dose selection in both in vivo and *in vitro* studies. As our technology and scientific knowledge develop, new approach methodologies may be able to supplement or eventually replace approaches in animals, though *in vitro* to *in vivo* extrapolation and appropriate dose selection will likely remain challenging. In the meantime, test guidelines that include additional study endpoints can be used to better characterise toxicity in the offspring and in some study types, the parental animals. Better use of these additional information sources will likely mean that excessively toxic high dose levels are not needed to detect adverse outcomes.

7. Conclusions and recommendations

Overall, the existing general concepts, recommendations and approaches to dose level selection elaborated in ECETOC Technical Report 138 (2021) and in Sewell et al., (2022), are equally applicable to DART studies, and represent approaches to selecting dose levels that allow for accurate risk assessment and enable hazard-based classification based on identification of relevant hazards.

- OECD test guidelines currently recommend the use of toxicokinetic to avoid dosing above the non-linear range. However, there is no internationally agreed guidance on how to use toxicokinetic data in setting the top dose. Where there are no or little data to make a dose selection decision based on systemic exposure, or where systemic exposure has a linear relationship with the externally applied/targeted dose, then signs of toxicity remain the main source of knowledge for selecting appropriate dose levels.
- Changes in metabolism, pharmacokinetics and/or physiology that occur at excessive dosages may be non-linear in their dose-relationship, and often produce effects on development and reproduction that have little or no relevance to even slightly lower dosages that may be considered minimally toxic. The extrapolations made from excessive dose levels are meaningless in predicting adverse effects at environmentally relevant exposure levels. The complexity and inter-dependencies of DART experimental models have led to the development of scientifically robust approaches to dose level selection in DART studies (Brannen et al., 2011; Beyer et al., 2011), including consensus limits on reductions in body weight gain, with >20% considered excessive and 10–15% reduction adequate for the high dose level. Thus, the proposal to remove the MTD criterion of a 10% decrease in body weight *gain* from test guidelines for DART studies is not supported.
- Mathematically/theoretically focussed approaches (as opposed to holistic approaches) developed with the intention of maximising the chance of detecting a hazard by exaggerating the top dose level of a study to the greatest extent possible are not recommended and should be avoided as such approaches fail to appreciate the integrated biology of the test organism.

The reason for conducting DART studies is to determine the potential for a chemical to produce reproductive toxicity, and if so, to provide a starting point for human risk assessment. In order to fulfill that purpose, dose levels should be selected such that every dose group produces interpretable data, thereby maximising the utility of the study for both hazard and risk evaluation. While the argument has been made that exaggerating the dose levels in these studies to the maximum extent possible increases the chances of detecting a hazard, we have demonstrated here that effects observed at these high dose levels can be produced by secondary mechanisms that are not relevant for prediction of real-world hazard or risk. Conducting studies at dose levels that cause frank toxicity and overwhelm homeostasis leads to misclassification of chemicals that are not reproductive hazards. Although some might view a high rate of false positives as precautionary, in reality it is the opposite because chemicals falsely identified as reproductive toxicants become a high priority for replacement. As a consequence of this, chemicals that are well-studied and pose low risk are deselected in favour of newer chemicals that have data gaps.

Animal toxicity studies have limitations, but increasing the dose levels in these studies does not address those limitations. In fact, increasing dose levels to excessively toxic levels may further increase the limitations and applicability of the studies to the human health hazard and risk assessment. While there are no alternative methods available at this time to replace the animal tests for DART, there are many tools available that can provide additional information that address some of the limitations. These include pharmacokinetic information, which is routinely used in the pharmaceutical industry for dose setting, as well as *in vitro* and *in silico* tools that shed light on mode of action, and the appropriateness of a given animal model for predicting human toxicity (Scialli et al., 2018, others). We believe that it would be more reasonable to explore these approaches to supplement the animal models rather than to advocate for increasing dose levels to the point where the results are not interpretable.

In summary, a biological approach to dose level selection using all available and relevant data that takes into account the complexity of a multicompartment model (maternal-placental-foetal) leads to a holistic

consideration of dose. Embryo-foetal development is dependent on a healthy internal environment in the maternal animal. A wide range of additional factors such as maternal target organ toxicity, the effects of nutritional status and the effects of maternal stress have all been shown to have a detrimental effect on normal development.

As our knowledge grows in this area, we are becoming even more aware of the multiple subtle changes in maternal homeostasis (that can be described through the use of metabolomics), caused by xenobiotics at even low doses (well below the MTD), all with the potential to adversely affect development. These advances in understanding should be taken into account wherever possible in dose level selection. As our scientific understanding advances, there is more information available for dose setting than in the past, including *in silico*/read across information, *in vitro* data, toxicokinetic modelling. Furthermore, test guidelines include additional study endpoints to better characterise toxicity in the offspring and in some study types, the parental animals. These information sources mean that excessively toxic high dose levels are simply not needed to detect adverse outcomes.

Finally, we must consider the impact of the way in which studies have to take into account animal welfare. Examples of severe suffering in the ECHA advice on top dose selection include excessive mortality, indicated as >10% mortality, which is not in line with OECD TGs 414, 443 and 421/422 and Humane Endpoints Guidance Document where it is indicated that death should be avoided. The ECHA recommendation to avoid only "severe suffering" could be considered counter to the 3R s (in this case a refinement to minimise suffering) which are generally supported by regulatory agencies globally. It could also lead to challenges in appropriate consideration of societal pressures to lessen animal suffering. Moreover, it is a key responsibility of investigators using animal models to ensure that the outputs of a test are interpretable and able to provide data that contribute to the overall goal of protecting human health. The use of excessive dose levels is incompatible with this protection goal and will lead to the generation of data that are difficult to interpret and the reliability and relevance of which are doubtful and misleading. In addition, this may well lead to further unnecessary studies on animals to clarify that findings produced at excessive dose levels are of no relevance to humans.

Any guidance on dose level selection should take into consideration the themes developed in this paper around the need to consider wider changes in homeostasis, and not be limited to an overly simplistic reliance on body weight reductions alone.

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CRediT authorship contribution statement

R.W. Lewis: Writing – review & editing, Writing – original draft, Conceptualization. **A.K. Andrus:** Writing – review & editing, Writing – original draft, Conceptualization. **J. Arroyo:** Writing – review & editing, Writing – original draft. **S. Brescia:** Writing – review & editing, Writing – original draft, Conceptualization. **P.A. Botham:** Writing – review & editing, Writing – original draft, Conceptualization. **M. Corvaro:** Writing – review & editing, Writing – original draft, Conceptualization. **G.P. Daston:** Writing – review & editing, Writing – original draft, Conceptualization. **T. Hofmann:** Writing – review & editing, Writing – original draft, Conceptualization. **C. Rodriguez:** Writing – review & editing, Writing – original draft, Conceptualization. **F. Sewell:** Writing – review & editing, Writing – original draft, Conceptualization. **B. van Ravenzwaay:** Writing – review & editing, Writing – original draft, Conceptualization. **K. Wiench:** Writing – review & editing, Writing – original draft, Conceptualization. **S. Marty:** Writing – review & editing, Writing – original draft, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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