LANXESS Energizing Chemistry

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October 13, 2023

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Comments on the CLH report 'Proposal for Harmonized Classification and Labelling based on Regulation (EC) No 1272/2008 (CLP Regulation), 3,5-dimethylpyrazole (EC number: 200-657-5, CAS number: 67-51-6).

Reproductive toxicity:

Following a review by an independent external developmental and reproductive toxicology (DART) expert, we dispute the proposed reproductive toxicant category 1B classification. It is our understanding that the independent DART studies support the existing reproductive toxicant category 2 classification and a detailed analysis is provided in Annex 1.

In summary based on the available data from 3 independent DART studies, performed under recent guideline standards and under GLP conditions, and the definitions provided in the EU regulation, no classification in category 1 is justified. 3,5-Dimethyl pyrazole (DMP) is not a known reproductive toxicant (Cat. 1A) nor are the prerequisites of a presumed human reproductive toxicant provided.

Classification as "Presumed human reproductive toxicant" (Cat. 1B) requires clear evidence of an adverse effect on sexual function and fertility or on offspring development in the absence of other toxic effects or proof that the observed other toxic effects did not lead to the effects seen in the offspring.

Adverse effects on sexual function and fertility were not seen. In the OECD 443 (which has the most complex design, the highest number of animals and parameters assessed of the 3 DART studies and therefore has the highest statistical power), no effects on mating, fertility, or fecundity were noted, as comparison for both generation mating phases. The different reproductive indices in no instance dropped below 90%, and, consequently, is within normal physiological ranges across the study groups and assured enough live pups for subsequent evaluations. No consistent dose-dependency was seen in comparison of F0 and F1 results and reproductive success in the F1 generation was comparable or higher than in the parental generation (fertility index, live birth index, pup viability). Please see analysis on pages 4 and 5 of Annex 1 for more details.

No test article-related changes in sperm count, sperm mobility, velocity, or morphology were noted following DMP administration for the males in the cohort 1A

(see analysis on page 6 of Annex 1). Oestrous cycles were unaffected by the test article at any dose level for F0 females and 1A and 1B cohort females (see analysis on page 6 of Annex 1). A slight but statistically significant increase in primordial follicles was observed in the high dose (HD) group of cohort 1A females and resulted in a statistically significant increase in total follicle numbers (see analysis on page 7 of Annex 1). However, as reproductive performance was unaffected in the F1 generation, the increased primordial follicle numbers in the HD group should not be misinterpreted as effect on follicle maturation but seen as an incidental finding.

Mean live litter size was not affected in the F1 generation, even when the calculated postimplantation loss was higher. Early postnatal mortality (PND 0-4) was higher in the high dose group, mainly triggered by a single litter and the cause of death in these pups could not be determined. A treatment-related effect cannot be excluded. Later postnatal mortality up to weaning (PND 5-21) was higher but attributed to a litter where the dam died on lactational day 5 and the entire litter of 14 remaining pups had to be sacrificed as they were too young for independent survival. Details on early life mortality and pup development to weaning are discussed further on pages 7 to 9 of Annex 1. In summary, mortality after the initial direct *post partum* period (the so-called viability index) was not increased by treatment of DMP, neither by maternal transfer nor by direct dosing of the pups starting at PND 14.

Based on natural variation in the control groups, the variation in terms of age and body weight at sexual maturation in DMP treated male and female pups was comparable (see pages 9 to 11 of Annex 1). The HD female with the longest period of sexual maturation developed a regular oestrus cycle, mated successfully at the first day of cohabitation, became pregnant and had an all-live litter without any postimplantation loss. Although the litter was small the pups were healthy and upon necropsy, the reproductive organs were unremarkable. The oldest HD male pup at achieving preputial separation also mated successfully at the first cohabitation day and sired a healthy litter of 13 pups. No post-implantation loss was observed. Although the male had a high food consumption, upon necropsy there were no anomalies in the reproductive organs. Therefore, even with delayed sexual maturation, reproductive capacity was not affected and the delay should be considered as incidental findings. This is discussed on page 9 and 10 of Annex 1.

No effect on acoustic startle response, motor activity, or grip strength was noted in cohorts 2A+B, and no overt differences were noted following functional observational batteries in cohort 2A animals around weaning or at around 10 weeks of age. No DMP related changes in brain measurements, macroscopic or neuropathological findings were noted or effects on brain weight, brain measurements, macroscopic or microscopic changes were recorded. Microscopic findings were generally infrequent, of a minor nature, and consistent with the usual pattern of findings in rats of this strain and age.

No DMP related differences in lymphocyte populations were evident within the spleen and no spleen weight changes were observed. Additional haematology assessments in F0 parental animals and the additional F1 cohorts did not display changes in white blood cell count that could suggest detrimental effects on immune function after DMP treatment. More analysis on developmental immunotoxicity can be found on page 11 and 12 of Annex 1.

In the OECD 414 study an increased incidence of foetuses with malformations was noted for dams administered at 200 mg/kg/day, compared with controls. Malformations noted included supernumerary digit/polydactyly, fused ribs, diaphragmatic cyst, and short humerus. Foetal findings often observed in females administered a maternally toxic dose level include bent scapula and increased incidence of incomplete ossification and unossified bones and these were also noted at this dose level. However, the number of foetuses showing malformations at the top dose were increased when compared with controls. Other abnormalities and malformations were either considered unrelated to treatment or unlikely to be related to treatment. They were noted at low dose levels or there were small number of foetuses or similar findings were observed in the controls or historical control data. More detailed analysis can be found on page 12 and 13 of Annex 1.

The pattern of malformations also varied greatly. In the many affected high dose foetuses, the observations were random and did not repeat the complex skull malformations observed in the 2 low dose foetuses. Therefore, a treatment-related origin of this complex development abnormality is unlikely. Concerning non-malformation anomalies, the increased incidence of foetal variations in the high dose animals can, on the most part, be set in relation to a slower foetal development which is supported by statistically lower foetal weights in male and female foetuses (page 12 and 13 of Annex 1). Furthermore, several of the variation incidences were study-wide outside the HCD, including in control foetuses (e.g. abnormal iliac alignment in the pelvic girdle, unossified hindlimb metatarsals, wavy/nodulated ribs) and this should not be misinterpreted as a flaw in the study but rather underlines the meticulous work of the study's foetal pathologist. Mitchard and Stewart (Reproductive Toxicology, Volume 45, 2014, Pages 39-44) investigated "chondrodystrophy" syndrome manifested as e.g. characteristic skeletal abnormalities such as bent and/or short long bones in the Han Wistar rat fetus and PND 21 offspring and concluded that these defects observed in fetuses resolve after birth and should be considered as "minor malformations".

In summary, no dose-relationship was seen in the incidence and pattern of observed malformations. Based on the higher incidence (based on affected foetuses AND litters), a teratogenic potential in the high-dose animals cannot be excluded but relevance for human exposure is unclear and neither functional deficits nor malformations were observed in the subsequent OECD 443 study where high dose animals were exposed to half the dose applied in the teratogenicity assay.

In the OECD 443 study, no test item related mortality occurred in the F0 generation or test item related effects on body weights or food consumption. However, there were test item effects seen in all treatment group and both sexes. This included effects on haemoglobin, red blood cell count, and packed cell volume for males from all test item treated groups, compared with controls. Red blood cell count was also lower in females administered at the top 2 does, compared with controls and haemoglobin levels were also marginally lower for females administered at the top dose, compared with controls. Effects on other parameters such as mean reticulocytes, prothrombin time, platelet counts, platelet crit, alanine aminotransferase activity, total protein and globulin levels and albumin:globulin (A:G) were also observed. Microscopic changes were noted in the liver, spleen, kidneys, and prostate. No effect was seen on libido or fertility of the males, therefore, no functional consequences resulted from the observation noted in the prostate. A

more detailed analysis can be found on page 3 and 4 of Annex 1. F1 generation findings mirrored the changes seen in the parental animals by dose distribution and severity and no increased sensitivity was observed in the offspring generation after *in utero* and life-long exposure. Test article-related increases in liver weights (absolute and body weight ratios) were observed for females at the top two doses and decreased seminal vesicle weights (absolute and body weight ratios) were recorded for males administered at the top two doses. Test article-related microscopic changes were noted in the liver, spleen, kidneys and seminal vesicles. A more detailed analysis can be found on page 3 and 4 of Annex 1.

In summary, as outlined in detail in the comprehensive “Reproductive Toxicological Evaluation of 3,5-Dimethyl pyrazole” by an external toxicologist, classification as reproductive toxicant Cat. 1b is not appropriate; classification as reproductive toxicant Cat. 2 (suspected human reproductive toxicant) is considered justified as a precautional measurement. With missing data on a possible mode of action and obvious parental toxicity, it is our view that this categorization takes into account a worst-case scenario.

Yours sincerely,

LANXESS Solutions UK Ltd.

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Annex 1: Reproduction toxicological Evaluation of 3,5-Dimethyl pyrazole, Preclinical Science, 9th October 2023

PRECLINICAL SCIENCE

Reproduction Toxicological Evaluation of 3,5-Dimethyl pyrazole

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October 9, 2023

The aim of this dossier is the evaluation of reproductive and developmental toxicity of 3,5-Dimethyl pyrazole (DMP, CAS No. 67-51-6), based on data from 3 recent GLP-DART studies (OECD 422, 414 and 443) and their impact on Classification and Labelling.

The substance is registered under the REACH Regulation and is manufactured in and / or imported to the European Economic Area, at ≥ 100 to < 1 000 tonnes per annum. DMP is currently recognized as hepatotoxicant and irritant to skin and eyes [ECHA].

The 3 DART studies were performed in close timely proximity (within 5 years, based on start of in-life phase) and used the same rat strain, administration route and vehicle and are therefore suitable for direct comparison of obtained results (table 1).

Table 1: Study specifications

	OECD 422	OECD 414	OECD 443
Laboratory	WIL Research Europe B.V. (now Charles River Laboratories)	Covance Laboratories Ltd (now Labcorp)	Covance Laboratories Ltd (now Labcorp)
Start in-life phase	Jun 2012	Aug 2017	Oct 2017
Report Date	Jun 2013	Apr 2018	May 2020
Rat Strain	Wistar (Han)	Wistar (Han)	Wistar (Han)
Administration Route	Oral gavage	Oral gavage	Oral gavage
Vehicle	Propylene glycol	Propylene glycol	Propylene glycol
Dosing Volume	5 ml/kg	5 ml/kg	5 ml/kg
Dose Groups	0, 20, 60, 200 mg/kg/d	0, 20, 60, 200 mg/kg/d	0, 20, 50, 100 mg/kg/d
F0 Animal No.	10/sex/group	22 F/group, pre-mated	25/sex/group
Study specifics	2-week pre-mating dosing, up to PND 4	dosing GD 6-20, Caesarean section GD 20	full spectrum F1 cohorts (1A+B, 2A+B, 3)

The evaluation will be based majorly on the results of the OECD 443 study results, as the most complex study design with the highest number of animals and parameters assessed and therefore the highest statistical power. The results of the OECD 414 teratogenicity examinations will of course be taken into consideration for Classification & Labelling.

Justification of Dose Selection

The dose selection in the OECD 443 study was based on limited data from 2 previous DART studies (OECD 422 (only n=10/sex/group) and OECD 414 (providing no data on impact on fertility or postnatal survival). In both previous studies, dose levels of 20, 60, and 200 mg/kg/d were applied and resulted in reduced female food consumption and body weight gain (BWG) during gestation (OECD 414, 422), reduction in foetal/pup BW (OECD 414, 422), and an increased incidence of foetuses with malformations (OECD 414) in the 200 mg/kg/d group. Furthermore, there were treatment-related effects on pup mortality (postnatal loss) at 200 mg/kg/d (OECD 422). Parental findings pointed to toxicologically relevant liver, and adverse effects on male reproductive organs seen along with lower fertility and conception indices at 60 and 200 mg/kg/d (OECD 422). Therefore, dose selection for the OECD 443 was set at 20, 50, and 100 mg/kg/d in order to still cover test item doses that previously caused parental toxicity but could be assumed to ensure sufficient reproductive success to allocate enough F1 pups in the dose groups for all 5 F1 cohorts (n=70/sex/group). Any higher dosing might have jeopardised the validity of the study. In the opinion of the expert, dose setting was completely justified and carefully chosen in terms of animal welfare reasoning and ensuring full spectrum offspring assessments in the OECD 443 study.

General Toxicity

In the OECD 443 study, no test item-related mortality occurred in the F0 generation. Notable clinical, postdose, and weekly detailed observations were confined to instances of salivation noted in all 3 DMP-treated groups and both sexes. No test ITEM-related effects on body weights or food consumption were noted.

Still, test item-related effects were seen in all treatment group and both sexes. Haemoglobin (HGB), red blood cell count (RBC), and packed cell volume were lower for males from all DMP-treated groups, compared with controls. Mean cell volume (MCV) was increased for males administered 100 or 50 mg/kg/day. RBC was also lower in females administered 100 or 50 mg/kg/day, compared with controls. Haemoglobin levels were also marginally lower for females administered 100 mg/kg/day, compared with controls. Mean reticulocytes were increased for males administered 100 or 50 mg/kg/day, compared with controls. This can be seen as physiological feedback mechanism to the observed anaemia with increased bone marrow haematopoiesis. Males administered 100 mg/kg/day showed increased prothrombin time (PT), platelet counts (PLT), and platelet crit (PCT), compared with controls, and PCT was also increased for males administered 50 mg/kg/day. Similar increases in PLT and PCT were only observed for females administered 100 mg/kg/day, compared with controls. No such test article-related changes were noted for females administered 20 mg/kg/day. The white blood cell count and its cell type distribution were not affected, neither in F0 males nor females.

Alanine aminotransferase (ALT) activity was increased, total protein and globulin levels were reduced, and albumin:globulin (A:G) ratio was increased for males administered 100 mg/kg/day, compared with controls. Reduced globulin and increased A:G ratio were also observed for males administered 50 mg/kg/day, compared with controls. This was not evident for males administered 20 mg/kg/day or for females at any dose level.
No DMP-related changes in thyroxine (T4) or thyroid stimulating hormone (TSH) and for the urine parameters investigated were noted at any dose level, compared with controls.

Microscopic changes were noted in the liver, spleen, kidneys, and prostate. In the liver, centrilobular hepatocyte hypertrophy/degeneration was recorded for males administered 20 mg/kg/day and both sexes administered 50 or 100 mg/kg/day. Hepatocellular karyomegaly/multinucleation was recorded for females administered 100 mg/kg/day. In the spleen, increased pigment was recorded for animals administered 20, 50, or 100 mg/kg/day, compared with controls.

In the F1 generation, no test item-related clinical observations were noted after pups were directly dosed from PND 14. Lower mean body weights were noted from PND 4 onwards for both sexes exposed to 50 or 100 mg/kg/day. By the end of the pre-weaning period, only marginal differences were observed and therefore no indication of increased juvenile toxicity.
No test article-related thyroid hormone level changes, macroscopic findings, or organ weight changes were noted for the unselected offspring on PND 22.
No test item-related mortality occurred in the different F1 cohorts and clinical observations were limited to instances of salivation after dosing.

As in the F0 generation, RBC was reduced and MCV was elevated for males administered 100 mg/kg/day, compared with controls, with reticulocyte increases for males administered 100 or 50 mg/kg/day. Similar changes in red cell parameters were observed for females. Red blood cells and MCV were reduced for females administered 100 or 50 mg/kg/day. Reticulocytes were elevated for females administered 100 mg/kg/day. Platelets counts and PCT were elevated for males from all test article-treated groups and for females administered 50 or 100 mg/kg/day. In addition, platelet distribution width was lower for females administered 100 mg/kg/day, with elevated activated partial thromboplastin time (APTS), compared with controls. For males administered 100 mg/kg/day, PT was

elevated. The white blood cell count and its cell type distribution were not affected, neither in F1 males nor females.
Males administered 100 mg/kg/day were noted with increases in aspartate aminotransferase and ALT activities, with a reduction in total protein. For females administered 100 mg/kg/day, the only test article-related clinical chemistry change was an increase in cholesterol.
No test article-related changes were noted in the urine parameters or thyroid hormone levels assessed.
Test article-related increases in liver weights (absolute and body weight ratios) were recorded for females administered 50 or 100 mg/kg/day, and decreased seminal vesicle weights (absolute and body weight ratios) were recorded for males administered 50 or 100 mg/kg/day. No test article-related macroscopic findings were recorded. Test article-related microscopic changes were noted in the liver, spleen, kidneys, and seminal vesicles. In the liver, centrilobular hepatocyte degeneration was recorded for animals administered 20, 50, or 100 mg/kg/day. In the spleen, increased pigment was recorded for animals from all dose groups, compared with controls.

In summary, F1 generation findings mirrored the changes seen in the parental animals by dose distribution and severity and no increased sensitivity was observed in the offspring generation after *in utero* and life-long exposure. Based on the described findings, neither a no observed effect level (NOEL) nor a no observed adverse effect level (NOAEL) could be established for systemic toxicity for males and a NOAEL for systemic toxicity for females was established as 20 mg/kg/day. Reversibility of these findings was not investigated as part of this study.

<u>Reproductive and Developmental Toxicity</u>

Reproductive Success/Reproductive Indices

In the previous OECD 422 screening study, the reproductive success in the HD group, treated with 200 mg/kg/d DMP, was exceedingly low. There were 8, 9, 9 and 6 pregnant females in the control, 20, 60 and 200 mg/kg treatment groups, respectively. In the HD group, only 6 females were pregnant, all of which had live litters at PND 1. Still, the mating index, precoital time and numbers of corpora lutea and implantation sites were unaffected by treatment up to 200 mg/kg.
The live litter size in the HD animals was lower than in controls at first litter check (11.6 ± 1.4, 12.3 ± 2.3, 12.3 ± 2.6 and 9.8 ± 3.1 live pups in control, 20, 60 and 200 mg/kg treatment groups, respectively). Furthermore, postnatal pup mortality in the HD animals affected 3/6 litters (10 pups in total) and summed up to 16.9 % of the HD pups. These values raised concern and led to the decision to lower the dose range in the subsequently performed OECD 443 study in order to lower the risk of not being able to provide sufficient pup numbers to be allocated to all 5 F1 cohorts (n=70/sex/dose group).

As the OECD 443 F0 parental animals were not sexually mature at study start, sexual maturation was assessed from the start of dosing and was found not to be affected by test article administration (PND 48 ± 2.2 d in HD vs. PND 47 ± 1.9 d in CON males; 32 ± 1.0 d in HD vs. 31 ± 1.3 d in CON females). The values for female sexual maturation tended to be earlier than in the subsequent F1 generation across all study groups (compare to values presented in figures 10 and 11).

No effects on mating, fertility, or fecundity were noted, as comparison for both generation mating phases displays in figures 1 and 2.

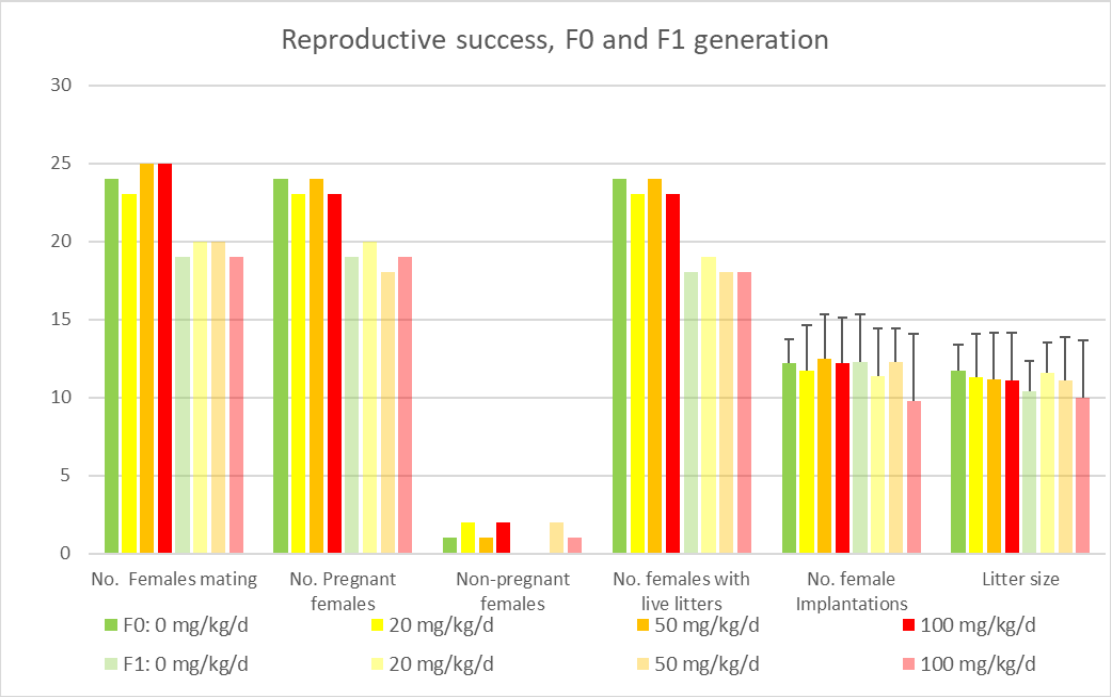

Figure 1: Comparison of reproductive success in F0 generation and F1 1B cohort
Note: In the F0 generation n=25/sex/group were cohabitated, in the F1 1B cohort n=20/sex/group, therefore, group mean value variations (SD values) are expected to be higher in the F1 1B cohort due to lower statistical power

The different reproductive indices varied within normal physiological ranges across the study groups but in no instance dropped below 90 % and assured enough live pups for subsequent evaluations. No consistent dose-dependency was seen in comparison of F0 and F1 results and reproductive success in the F1 generation was comparable or higher than in the parental generation (fertility index, live birth index, pup viability) as seen in figure 2. Therefore, life-long exposure to DMP, starting *in utero*, did not seem to impair female reproductive capacity.

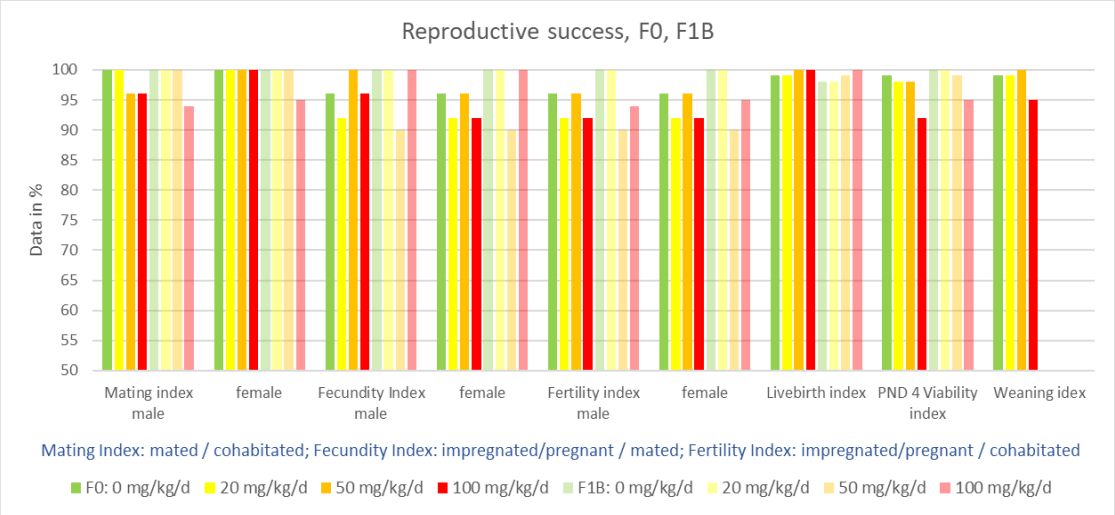

Figure 2: Comparison of reproductive indices in F0 generation and F1 1B cohort

Following parturition, 24, 23, 24, or 23 females administered 0, 20, 50, or 100 mg/kg/day, respectively, produced a live litter. Possible test article-related effects were noted in the group administered 100 mg/kg/day. Viability indices on PND 4 and weaning indices were marginally lower for litters of the

group administered 100 mg/kg/day (F0: 92 % vs. 99 % in CON; F1: 95 % vs. 100 % in CON). Still, early pup survival was on a high level. The effects on pup survival and development will be discussed below.

Semiology and Ovarian Function

No test article-related changes in sperm count, sperm motility, velocity, or morphology were noted following DNP administration of any dose level, as displayed in figure 3 for the males in the cohort 1A. The percentage of abnormal sperm ranged between 0.6 % (20 mg/kg/d) and 1.3 % (100 mg/kg/d), altogether on a very low incidence level.

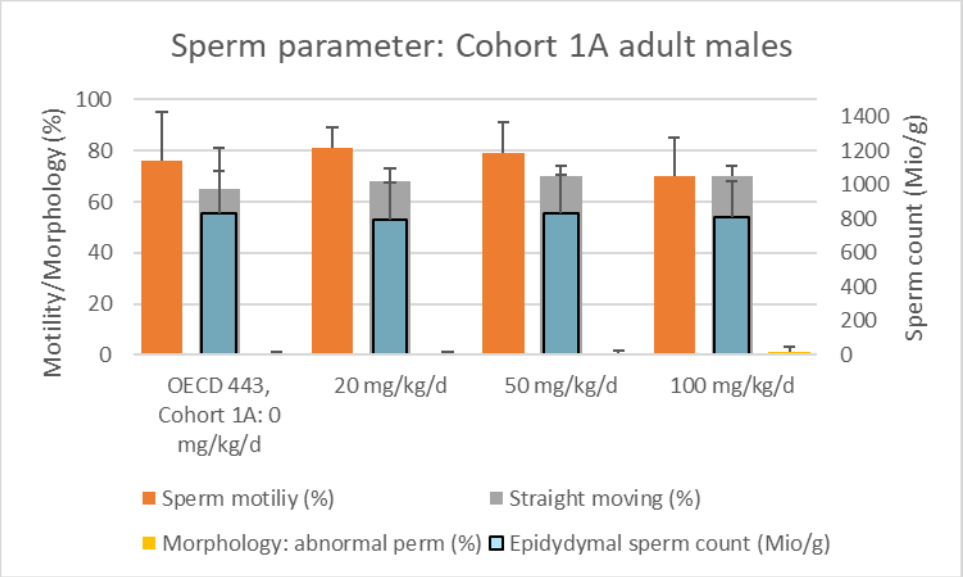

Figure 3: Epididymal sperm parameter in F1 cohort 1A

Furthermore, oestrous cycles were unaffected by the test article at any dose level, as depicted for F0 females and both 1A and 1B cohort females in figure 4. The mean oestrus cycle length in all groups was with the normal physiological values of either 4 or 5 days.

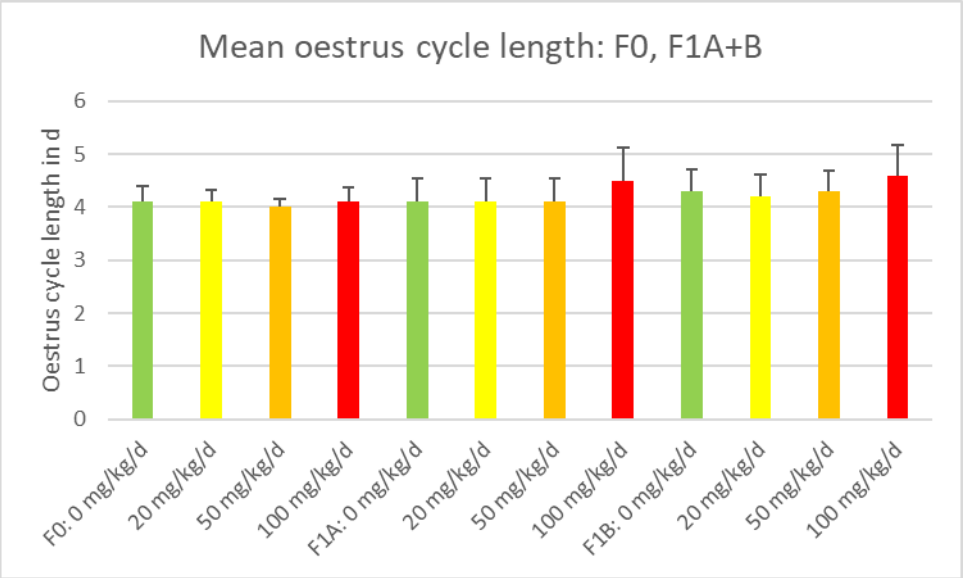

Figure 4: Comparison of mean oestrus cycle length in F0 and F1 cohort 1A+B females

Detailed ovarian staging was performed in the F1 cohort 1A females following necropsy. Here a slight but statistically significant increase in primordial follicles was observed in the 100 mg/kg/d group which resulted also in a statistically significant increase in total follicle numbers (figure 5). As reproductive performance was unaffected in the F1 generation, the increased primordial follicle numbers in the HD group should not be misinterpreted as effect on follicle maturation but seen as incidental finding.

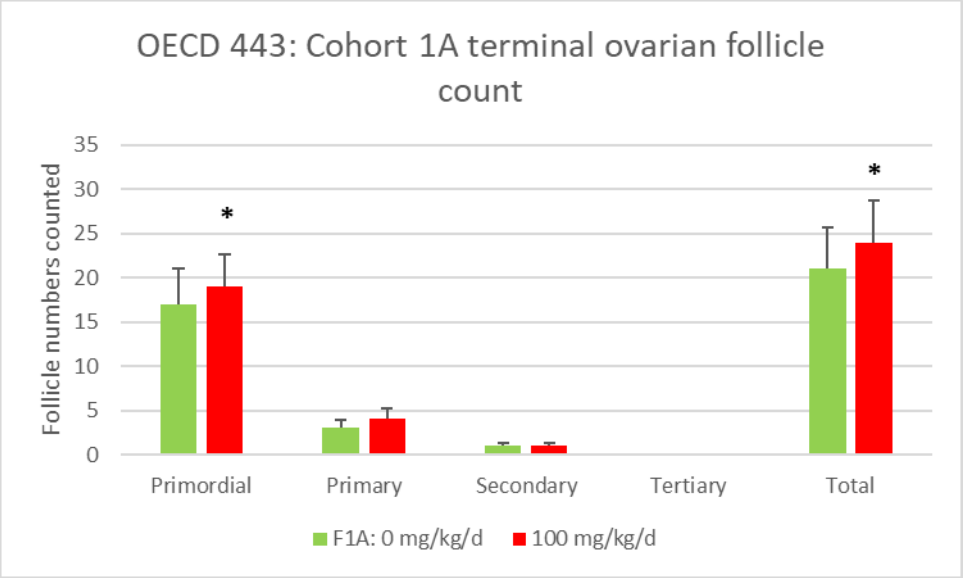

Figure 5: Ovarian staging in cohort 1A females, * p<0.05 vs. CON

Early Life Mortality, Pup Development to Weaning

The results of the previous OECD 422 Screening study indicated a higher early postnatal mortality in the F1 pups (postnatal loss of 16.9 % in the 200 mg/kg/d HD animals) (see data in figure 6). This raised concern and contributed to the decision for lower dose settings in the subsequent OECD 443 study.

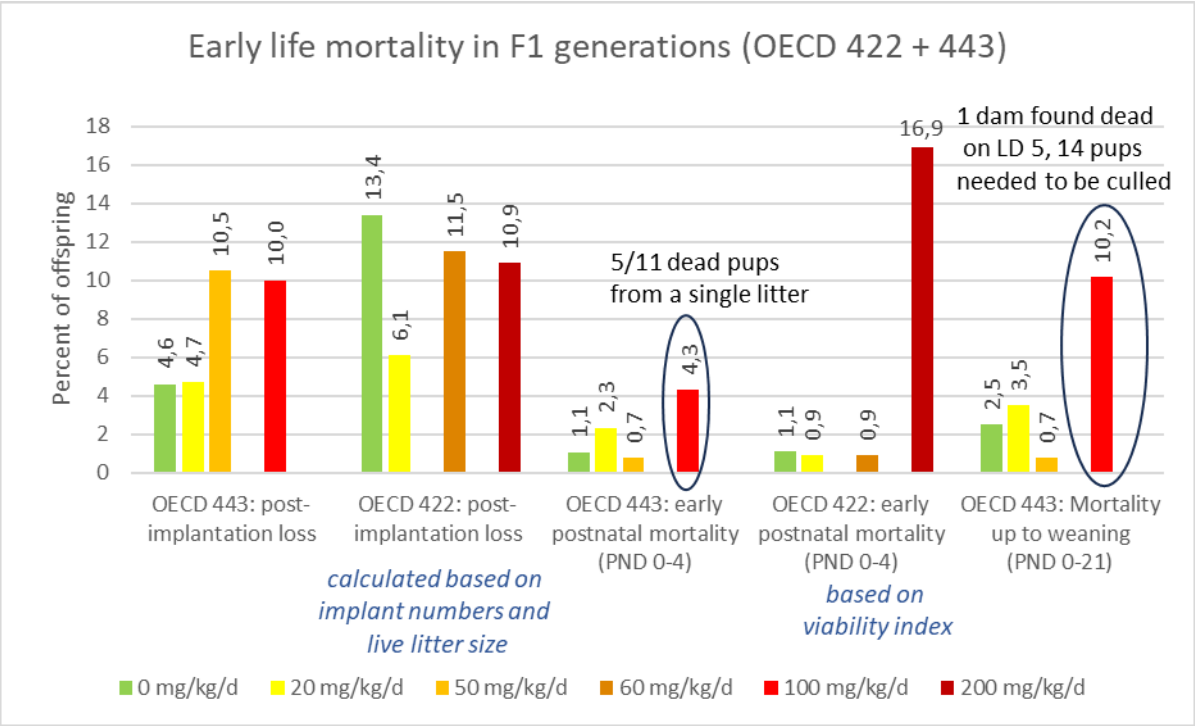

Figure 6: Comparison of early life mortality in OECD 443 and OECD 422 studies

The results of the F0 reproductive success varied obviously from the data seen in the previous OECD 422 study. Maternal mean implant numbers (12.2, 11.7, 12.5, 12.2 in CON, LD, MD, HD) were comparable and mean live litter size was also unaffected by treatment (11.6, 11.1, 11.2, 11.1, respectively). Stillborn pups were only observed in the Con and 20 mg/kg/d groups but not in the 50 or 100 mg/kg/d groups, therefore a treatment-related effect was excluded. Still, the nominal values for the post-implantation loss were (statistically not significantly) higher in the 50 and 100 mg/kg/d groups (about 2fold compared to control animals). Pup mortality PND 0-4 was low in control, LD and MD litters but accounted to 11/255 HD pups. Five of these dead pups originated from a single litter on PND 1 (dam 0585, 12 liveborn on PND 0). The case of mortality in this litter could not identified, the remaining 7 littermates grew up normally. The cumulative mortality to weaning increased to 10 % in the 100 mg/kg/d group but the number is misleading as the increase from PND 4 to PND 21 mortality was majorly based on a single litter where the dam died on lactational day 5 and the entire litter of 14 remaining pups had to be sacrificed as they were too young for independent survival (refer to figure 6). Without this loss, the mortality up to weaning in the 100 mg/kg/d group would be reduced to 4.7 %, much lower but still higher than in the lower dose groups.

In comparison, the postimplantation loss in the cohort 1B/F2 pups was much lower (< 2%) and the lowest value was seen in the HD animals (figure 7). Live litter size was comparable but again, the 100 mg/kg/d animals displayed a higher *post partum* pup mortality (5 %) than the other dose groups or control animals. In general, pup mortality under un-treated conditions is highest within the first 4 days of life and single digit values are commonly seen in control groups. Therefore, the observed range in the OECD 443 study is not unusual but still this does not explain the higher values in the HD groups in the OECD 442 study and 2 generations in the OECD 443 study.

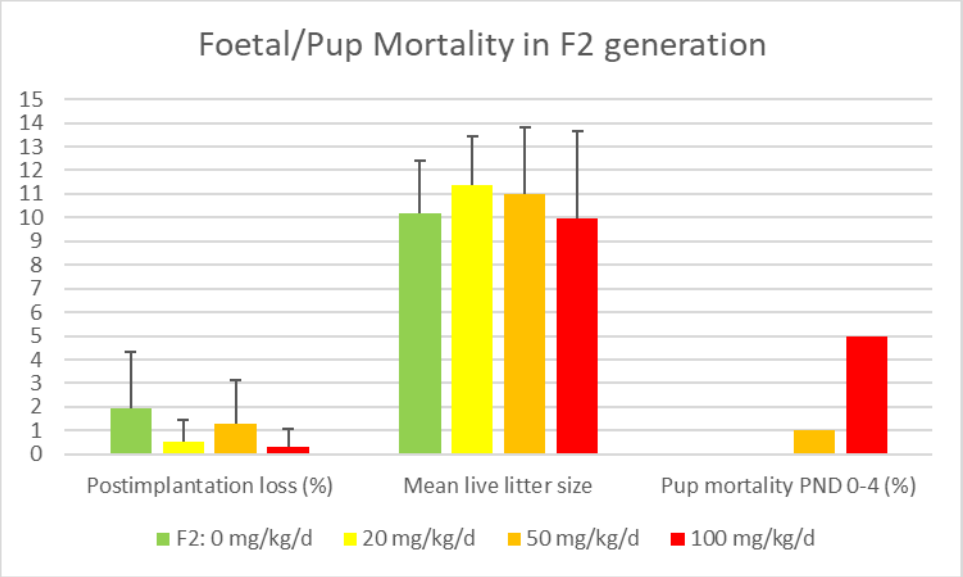

Figure 7: Early life mortality in the OECD 443 F2 generation

Pup development in terms of clinical symptoms and BWG was uneventful (figure 8). Over the first 3 postnatal weeks, pup body weights in the F1 and F2 generations compared to control animals fluctuated within a 6 % range around control BW. At 2 timepoints (PND 7+14), female pup BWs in the F1 50 and 100 mg/kg/d groups were transiently statistically significantly lower than control weights but still within the 6 % range and statistical significance was not reached in male pups (figure 9). Direct pup dosing by oral gavage was initiated at PND 14 in F1 animals and did not show detrimental effects in the pups. Postweaning BW remained comparable to control animals.

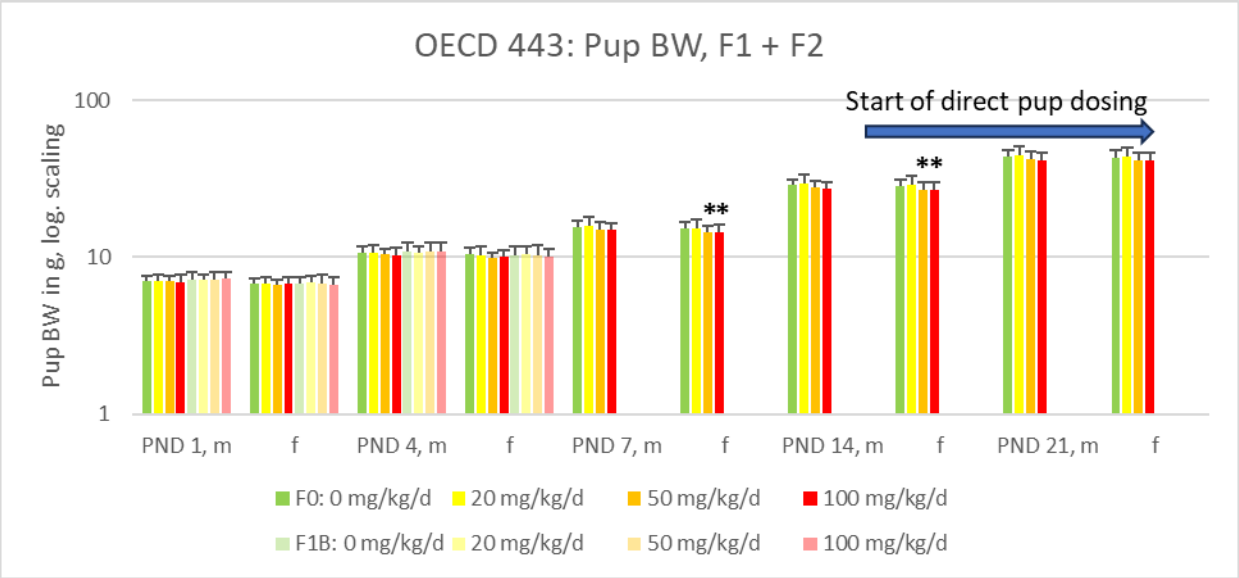

Figure 8: Comparison of BW development in pre-weaning F1 and F2 pups, * p<0.05 vs. CON

In summary, mean live litter size was not affected in the F1 generation by treatment with DMP, even when the calculated postimplantation loss was higher. Early postnatal mortality (PND 0-4) was higher in the HD group, mainly triggered by a single litter and the cause of death in these pups could not be determined. Therefore, a treatment-related effect cannot be excluded. Later postnatal mortality up to weaning (PND 5-21) was numerically higher but attributed by maternal death and subsequent sacrifice of an entire litter at PND 5. Therefore, mortality after the initial direct *post partum* period (the so-called viability index) was not increased by treatment by DMP, neither by maternal transfer nor by direct dosing of the pups starting at PND 14. The high mortality of nearly 17 % observed in the OECD 422 study under 200 mg/kg/d was not seen under treatment with 100 mg/kg/d. This could either be related to the reduced HD concentrations or to aberrant values in the previous study.

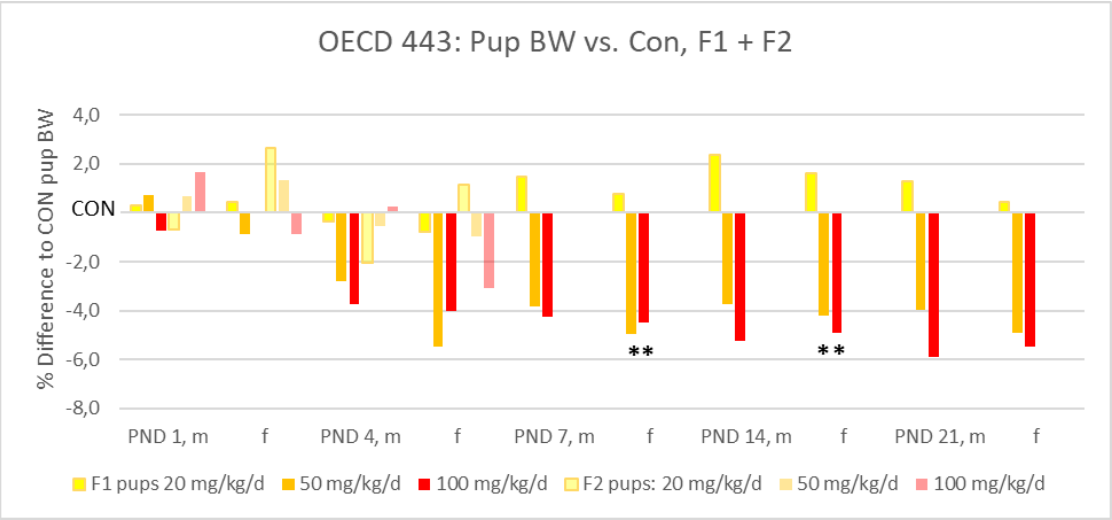

Figure 9: BW development in pre-weaning F1 and F2 pups in direct comparison to their control animals, * p<0.05 vs. CON

Sexual Maturation

Sexual maturation was evaluated in all F1 cohort 1A, 1B, 2A and 3 animals. Therefore, with n=60/sex/group, a reliable statistical power was achieved. In figures 10 and 11, the different cohorts are depicted as correlation of mean BW at time of achievement and the BW, both incl. SD. The range

of values (age and BW) of the control animals are indicated. In one of 4 cohorts (cohort 3, n=10/sex), HD females had a statistically higher BW and age at vaginal opening (mean PND 36 at 115 g) than the respective control group animals. Here it has to be noted that the cohort 3 control females reached vaginal opening much earlier and at a lower BW (mean PND 32 at 99 g) than the other 3 control groups (PND 35, 34, 34 at 113, 110, 109 g in cohorts 1A, 1B, 2A respectively. Therefore, the statistically significant delay in the HD animals simply originated in unusual findings in the control animals. No delay was observed in male pups in any test item-treated dose group.

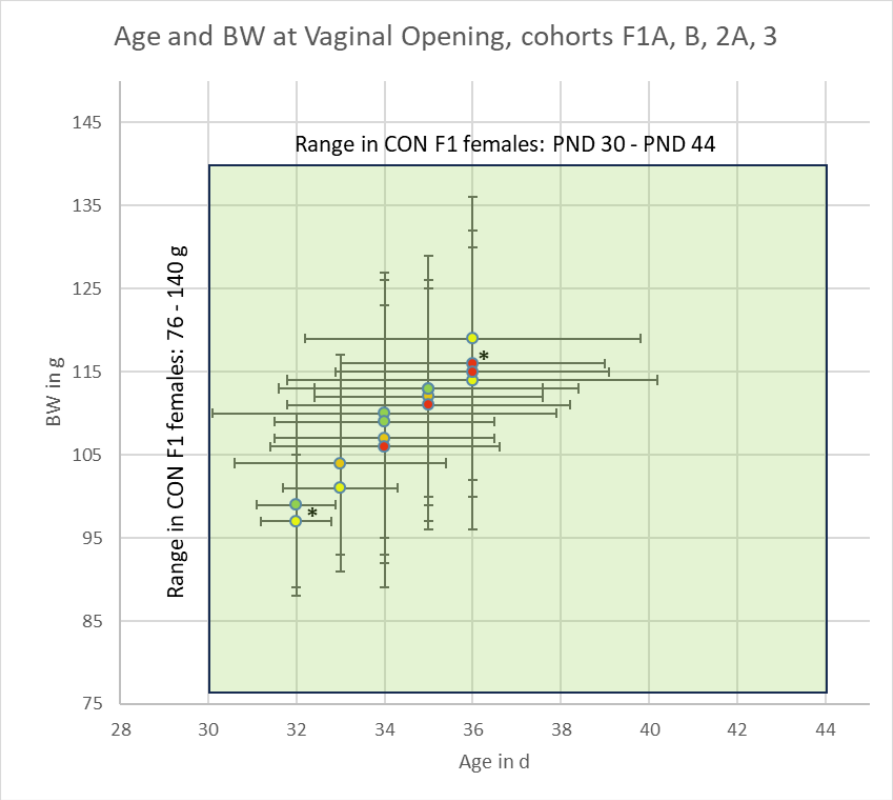

Figure 10: Achievement of female sexual maturity (indicated by vaginal opening) in F1 animals (cohorts 1A+B, 2A, 3), Note: CON, 20 mg/kg/d, 50 mg/kg/d, 100 mg/kg/d, * p< 0.05 vs. CON

Based on the natural variation in the CON groups (as marked in figures 10 and 11), the variation in DMP-treated group male and female pups in terms of age and BW at sexual maturation was comparable. The HD female with the highest age at vaginal opening (animal 842 from cohort 1B: PND 44 at 143 g), developed a regular oestrus cycle after achieving sexual maturation, mated successfully at the first day of cohabitation, became pregnant and had an all-live litter without any post-implantation loss. The litter was small (n=3) but pups were healthy and heavier than the group mean (a physiological adaption due to small litter size). Upon necropsy, the females' reproductive organs were unremarkable.

The oldest HD male pup at achieving preputial separation (animal 339 from cohort 1B: PND 69 at 302 g) also mated successfully at the first cohabitation day and sired a healthy litter of 13 pups. Here again, no post-implantation loss was observed, based on implants/pups. The male had a remarkably high food consumption (approx. 2fold of group mates) but was anatomically unremarkable at necropsy with no anomalies in its reproductive organs.

Therefore, in both pups, even with delayed sexual maturation, their reproductive capacity was not affected and the late achievement should be considered as incidental findings.

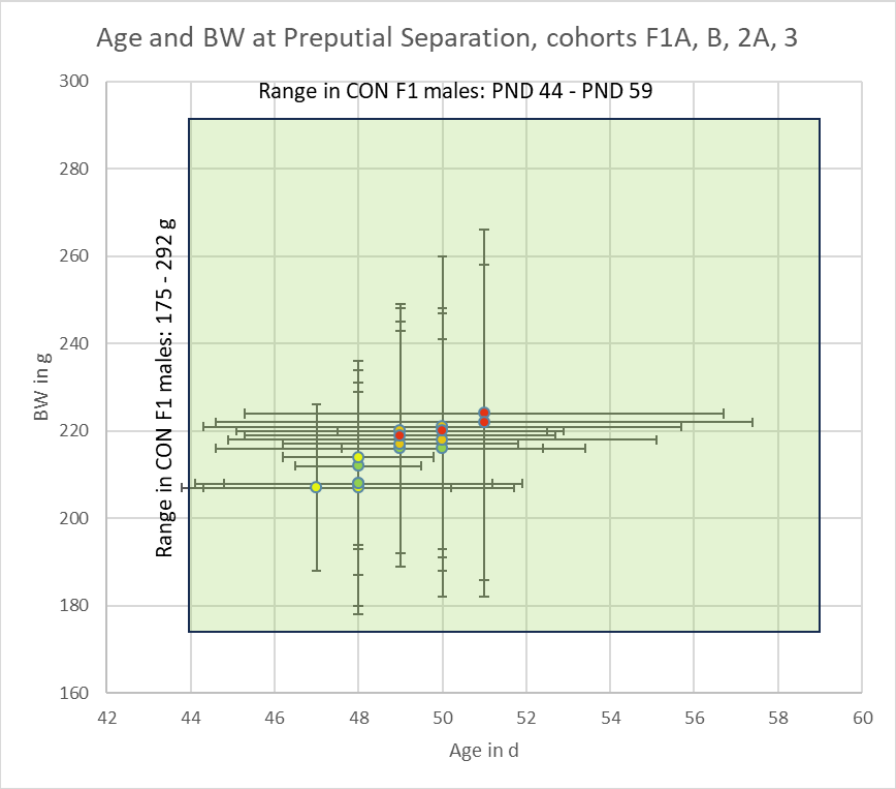

Figure 11: Achievement of male sexual maturity (indicated by preputial separation) in F1 animals (cohorts 1A+B, 2A, 3), Note: CON, 20 mg/kg/d, 50 mg/kg/d, 100 mg/kg/d

Developmental Neurotoxicity (Cohorts 2A + B)

All animals allocated to these cohorts survived to the terminal sacrifice, therefore n=10 animals/sex/group/cohort were available for evaluation. No effect on acoustic startle response, motor activity, or grip strength was noted, and no overt differences were noted following functional observational batteries in cohort 2A animals around weaning or at around 10 weeks of age. No DMP-related changes in brain measurements, macroscopic or neuropathological findings were noted in these animals sacrificed between PND 100-112. In the cohort 2B animals, sacrificed on PND 76, whole body perfusion was performed and they underwent neuropathology evaluation. Here also, no test item-related effects on brain weight, brain measurements, macroscopic or microscopic changes were recorded. Microscopic findings were generally infrequent, of a minor nature, and consistent with the usual pattern of findings in rats of this strain and age.

Developmental Immunotoxicity

All animals allocated to this cohort survived to terminal sacrifice. No test article-related differences in lymphocyte populations were evident within the
spleen.
Immunophenotyping of splenocytes revealed that absolute cell counts and relative percentages for T lymphocytes (CD3+, CD3+/CD4+ and CD3+/CD8+) and B lymphocytes (CD3-/CD35RA+) were comparable across all groups, including controls. The evaluation of natural killer (NK) cells (CD3-/CD161a+) revealed slightly reduced cell counts and relative percentages following administration of 100 mg/kg/day, compared to controls without reaching statistical significance. The average difference in cell counts between group administered control article (vehicle) and those administered 100 mg/kg/day was -31% for males and -34% for females, with similar reductions observed for relative percentages (4.1 ± 1.4 % vs. 6.8 ± 2.8 % in CON males; 4.8 ± 0.5 % vs. 6.8 ± 2.1 % in CON females). These

observations were considered as not adverse. The NK results for all other dose groups were comparable with controls.
Evaluation of the anti-KLH IgM response showed no toxicologically significant effects at any dose level. On average, all groups were noted with a moderate IgM response at both time points after the initial administration of keyhole limpet hemocyanin (KLH) - (PND 59 to 65 and 68 to 72). In response to the second challenge with KLH (PND 70 [±3 days]), a marked rise in the titre of specific IgM was observed on PND 76 through 78. The increase was in the region of 7 to 10 times the values observed at the previous time points. On PND 84/85/87, group mean anti-KLH IgM serum titres in all groups, including controls, had returned to values similar to those observed on PND 59 to 65 and 68 to 72.

No test article-related spleen weight changes were noted. Splenic weight and weight ratio changes were attributed to biological variation and were considered not test article related as they were small in magnitude, not dose dependent, inconsistent between sexes, attributed to normal inter-animal variability, and/or lacked a microscopic correlate.

Additional haematology assessments in F0 parental animals and the additional F1 cohorts did not display changes in white blood cell count (total and differential) that could point towards detrimental effects on immune function after DMP treatment.

<u>Teratogenic Potential</u>

According to the OECD 414 Report: An increased incidence of foetuses with malformations was noted of dams administered 200 mg/kg/day, compared with controls. Test article related malformations including supernumerary digit/polydactyly, fused ribs, diaphragmatic cyst, and short humerus were all noted. Foetal findings often observed in females administered a maternally toxic dose level including; bent scapula, and increased incidence of incomplete ossification and unossified bones were also noted at this dose level. Supernumerary digit/polydactyly was noted in three foetuses from two litters maternally administered 200 mg/kg/day. Diaphragmatic cyst, and short humerus were also noted in foetuses with maternally administered 200 mg/kg/day. Incidences of these observations have not been noted in background data. Incidences of bent scapula blade were noted in foetuses from dams administered 20 or 200 mg/kg/day. This observation was noted in one foetus from one dam administered 20 mg/kg/day and six foetuses from three dams administered 200 mg/kg/day. Incidences of bent scapula in foetuses from dams administered 200 mg/kg/day also exceeded background control ranges of 1.5 % litters / 0.3 % foetuses, compared with 14 % litters / 4.81% foetuses in 200 mg/kg/day litters. In general, the number of foetuses showing malformations from the 200 mg/kg/day litters, compared with controls, was greatly increased (16 foetuses from 9 litters, compared with 4 foetuses from 4 litters in controls). The aetiology of this is unclear.

Other abnormalities, including those not seen in background control data, were considered to be unrelated to treatment. The head abnormalities (jaw, tongue, and palate) were only noted at the low dose level and were not observed in foetuses from higher dose groups. Additionally, while these specific observations were noted, the observations are tied to similar findings observed within historical background control data and presented in small number of foetuses (2 foetuses) that presented with multiple malformations and were therefore considered to be unrelated to treatment.

Other malformations (including those observed in the sternebra, kidney hydronephrosis, ventricular septum defect of the heart and associated major blood vessels) were observed in foetuses from dams administered 20 or 200 mg/kg/day. Similar findings were observed in the control group and/or present in the historical control background data. Therefore, a relationship to test article was considered “unlikely.”

The very detailed and precise description, allocation and reporting of the observed malformations are the signature of a highly skilled foetal pathologist and are therefore not questioned.

Apparently, no dose-relationship was seen in the incidence of observed malformations. The observations were allocated to 4F from 4 L in controls, 3F from 3L in the LD, none in the MD but 16F from 9L in the HD.

The pattern of malformations varied greatly between CON and HD, and between LD and HD.

Within CON foetuses, the observed malformations within each foetus followed a different pattern, as expected in natural variability: 1 foetus with absent eye, 1 foetus with correlated malformations in the urogenital system, 1 foetus with abnormal sternebrae, 1 foetus with correlated anomalies of the cardiac vessels.

In the 20 mg/kg/d 3 foetuses, 2 had complex malformations of the skull and 1 displayed a common abnormal development of the scapula.

In the many affected HD foetuses, the observations were random and did not repeat the complex skull malformations in the 2 LD foetuses. Therefore, a treatment-related origin of this complex development abnormality is extremely unlikely. Still, 3F from 2 litters displayed supernumerary digits in their hind limbs and therefore a treatment-related pattern should be taken into consideration.

Concerning non-malformation anomalies, the increased incidence of foetal variations in the HD animals can majorly be set in relation to a slower foetal development which is underlined by statistically lower foetal weights in male and as well as female foetuses (4.75 g (M)+4.43 g (F) in HD vs. 5.30 g (M)+5.02 g (F) in CON). Furthermore, several of the variation incidences were study-wide outside the HCD, including in control foetuses (e.g. abnormal iliac alignment in the pelvic girdle, unossified hindlimb metatarsals, wavy/nodulated ribs). This should not be misinterpreted as a flaw in the study but rather underlines the meticulous work of the study's foetal pathologist.

In summary, no dose-relationship was seen in the incidence and pattern of observed malformations. Based on the higher incidence (based on affected foetuses AND litters), a teratogenic potential in the high-dose animals cannot be excluded but relevance for human exposure is unclear and neither functional deficits nor malformations were observed in the subsequent OECD 443 study where HD animals were exposed to half the dose applied in the teratogenicity assay.

For the OECD 414 Teratogenicity study, the maternal and foetal No Observed Adverse Effect Levels (NOAELs) is considered to be 60 mg/kg/day. In the opinion of the expert, this decision is justified.

<u>Summary & Conclusion</u>

Once daily oral gavage administration of 20, 50, or 100 mg/kg/day 3,5-Dimethylpyrazole to Han Wistar rats for up to two consecutive generations resulted in test article-related effects at all dose levels in adult animals. Based on these findings, **neither a NOEL nor a NOAEL was established for systemic toxicity for males**. **A NOAEL for systemic toxicity for females is established as 20 mg/kg/day**.

No adverse effects on reproductive organ function, mating performance, fertility, fecundity, gestation, partition, or lactation were noted; as such, the **NOAEL for reproductive toxicity was set at 100 mg/kg/day**.

Based on the lower F0 female pup body weight gain at 50 and 100 mg/kg/day, and marginally higher early pup mortality in F0 generation litters following maternal exposure of 100 mg/kg/day, and increased incidence of offspring mortality for the F1 generation following maternal exposure of 50 or

100 mg/kg/day. As such, the NOEL for offspring growth and development is established as 20 mg/kg/day. In the opinion of the expert, the **NOAEL for developmental toxicity should be established at 50 mg/kg/d**, as increased postnatal mortality was confined to the 100 mg/kg/d group and the lower BW in the 50 mg/kg/d pups was transient and did not affect mortality or further offspring development.

With no adverse effects observed in immunotoxicity and neurotoxicity assessments, the **NOAELs for developmental immunotoxicity and for developmental neurotoxicity were set at 100 mg/kg/day**.

Due to the low pregnancy rate in the previous OECD 422 screening study (n=6), which was not seen in the subsequent OECD 443 study, the informative value of the study is limited and not considered for derivation of a NOAEL for reproductive/developmental toxicity.

For the OECD 414 Teratogenicity study, the **maternal and foetal No Observed Adverse Effect Levels (NOAELs) is considered to be 60 mg/kg/day.**

Recommendation for Classification & Labelling

EU Regulation 1272/2008, Annex III, table 3.7.1(a) clearly defines the hazard categories for reproductive toxicants.

Hazard categories for reproductive toxicants

Categories	Criteria
CATEGORY 1	Known or presumed human reproductive toxicant Substances are classified in Category 1 for reproductive toxicity when they are known to have produced an adverse effect on sexual function and fertility, or on development in humans or when there is evidence from animal studies, possibly supplemented with other information, to provide a strong presumption that the substance has the capacity to interfere with reproduction in humans. The classification of a substance is further distinguished on the basis of whether the evidence for classification is primarily from human data (Category 1A) or from animal data (Category 1B).
Category 1A	Known human reproductive toxicant The classification of a substance in Category 1A is largely based on evidence from humans.
Category 1B	Presumed human reproductive toxicant The classification of a substance in Category 1B is largely based on data from animal studies. Such data shall provide clear evidence of an adverse effect on sexual function and fertility or on development in the absence of other toxic effects, or if occurring together with other toxic effects the adverse effect on reproduction is considered not to be a secondary non-specific consequence of other toxic effects. However, when there is mechanistic information that raises doubt about the relevance of the effect for humans, classification in Category 2 may be more appropriate.
CATEGORY 2	Suspected human reproductive toxicant Substances are classified in Category 2 for reproductive toxicity when there is some evidence from humans or experimental animals, possibly supplemented with other information, of an adverse effect on sexual function and fertility, or on development, and where the evidence is not sufficiently convincing to place the substance in Category 1. If deficiencies in the study make the quality of evidence less convincing, Category 2 could be the more appropriate classification. Such effects shall have been observed in the absence of other toxic effects, or if occurring together with other toxic effects the adverse effect on reproduction is considered not to be a secondary non-specific consequence of the other toxic effects.

Based on the available data from 3 independent DART studies, performed under recent guideline standard and under GLP conditions, and the definitions provided in the EU regulation, no classification in category 1 is justified. 3,5-Dimethyl pyrazole is not a known reproductive toxicant (Cat. 1A) nor are the prerequisites of a presumed human reproductive toxicant provided. Classification as “Presumed

human reproductive toxicant" (Cat. 1B) requires clear evidence of an adverse effect on sexual function and fertility (not seen), or on offspring development in the absence of other toxic effects or proof that the observed other toxic effects did not lead to the effects seen in the offspring. With the 2 effects defining the developmental NOAEL in the OECD 443 study being a transient (PND 7-14) slightly lower pup bodyweight which did not worsen with direct dosing of the pups starting at PND 14 and a not statistically significant increased early postnatal mortality that was at least partially driven by maternal mortality, the prerequisites of classification as reproductive toxicant Cat. 1B are not provided. Furthermore, the observed (not statistically significant) increase in non-specific malformations in the OECD 414 study was confined to the highest dose group of 200 mg/kg/d, which was clearly maternally toxic.

Therefore, classification as reproductive toxicant Cat. 2 (suspected human reproductive toxicant) is justified as a precautional measurement. With missing data on a possible mode of action and obvious parental toxicity, this categorization takes into account a worst-case scenario.

References:

ECHA: https://echa.europa.eu/de/substance-information/-/substanceinfo/100.000.597, last assessed 23.09.2023

REGULATION (EC) No 1272/2008 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 16 December 2008 on classification, labelling and packaging of substances and mixtures, amending and repealing Directives 67/548/EEC and 1999/45/EC, and amending Regulation (EC) No 1907/2006. 31.12.2008

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