Public consultation for the DART Technical Report on the Modified One-Generation Study of 2-Hydroxy-4-methoxybenzophenone (CAS #131-57-7)

Comments provided by the Danish EPA, September 29th 2021

We find the study to be well designed and relatively thoroughly conducted and reported. Generally, we agree with the provided conclusions, but we have identified a few inconsistencies in the report, which we have summarized below. Some are related to the reporting of certain endpoints, while others are related to the methods used in the assessment of specific endpoints during the conduct of the study.

Reporting:

There seems to be a discrepancy in relation to how the **sperm quality** findings are reported. On page 61, chapter;"F1 reproductive Performance Cohort Andrology" it is stated that; "*Males in the 30,000 ppm 2H4MBP group displayed lower cauda epididymal sperm counts (approximately 14%), relative to control males*". When looking in the supplementary tables in appendix D, there is indeed an asterisk, indicating a statistically significant change in this dose group.

However, throughout all of the other parts of the report, the results on sperm quality are reported as being unaffected by the exposure. In the abstract (page xviii) it is stated that: "*Sperm and spermatid counts were not affected by 2H4MBP exposure*". On page 82, the results are summarized in the following way; "*No exposure-related changes in sperm motility, sperm concentration, or testicular sperm head concentration were found*", and on page 95 it is stated that: "*2H4MBP did not result in any significant effects on… in sperm parameters at concentrations up to 30,000 ppm*".

Some previous studies have shown effects on sperm quality in rodents, after exposure to 2H4MBP. In a 90-day study both rats and mice exposed to 50,000 ppm displayed lower sperm density than control animals (27% in both species)(NTP 1992). In the range-finding study performed prior to this full MOG study, spermatocyte development in the 50,000 ppm males was also impaired (Nakamura *et al.* 2015). Thus, sperm quality after 2H4MBP exposure seems to be an endpoint which should be evaluated extra carefully, and we find that reporting of the sperm quality results would benefit from a revision, to avoid discrepancies within the report.

A similar issue was identified for **vaginal cytology**. On Page 61 it is stated that: "*Rats in the 10,000 and 30,000 ppm 2H4MBP groups of both cohorts displayed a higher probability of extended estrus and spent approximately 5% more time in estrus than did the control group. Analysis of estrous cyclicity using the continuous-time Markov model resulted in an increase in the stage length of estrus in the 10,000 and 3,000 ppm groups (approximately 5 hours), but only attained significance relative to the control group in the 10,000 ppm group".* Yet, the paragraph is concluded in the following way:"*These minimal estimated changes in stage length likely represent normal biological variability and are not considered biologically adverse"*.

Based on this conclusion, the results showing changes in estrous cyclicity are not included as a treatment-related finding anywhere else in the report. Like with the sperm quality results, this endpoint has previously been shown to be affected by 2H4MBP exposure. In a 90-day study, female rats displayed a slight increase in estrous cycle length (>1 day) in the 12,500 and 10 50,000 ppm groups, and female mice in the 50,000 ppm group displayed a slight increase in estrous cycle length relative to control animals (>0.5 days) (NPT 1992). While it is possible that the identified changes in estrous cyclicity in the high dose group were indeed caused by biological variability, we find that it is also possible that this was a treatment-related effect – especially when bearing in mind that similar effects have previously been reported. We therefore find that the vaginal cytology findings should be included in the conclusions of the study.

The last point we have identified in relation to reporting, deals with **thyroid gland** investigations. When looking in the method description, it is clear that thyroid gland were excised, weighed and that samples were prepared for histopathological examination.

*Prenatal Cohort: After positive evidence of mating, male sires were weighed, euthanized, and necropsied, and the following **organ weights recorded**: adrenal glands (paired), testes (left and right), epididymides (left and right), kidneys, liver, dorsolateral and ventral prostate gland, seminal vesicles with coagulating glands, **thyroid gland (fixed**), LABC muscle, Cowper's glands (paired), and preputial glands.*

*Reproductive Performance Cohort: Terminal body weights and the following **organ weights** were recorded: adrenal glands (paired), liver, kidneys (left and right), ovaries (left and right), uterus, cervix, vagina, testes (left and right), epididymides (left and right), cauda epididymis, dorsolateral and ventral prostate gland, seminal vesicles with coagulating glands, **thyroid gland (fixed**), LABC muscle, Cowper's glands (paired), and preputial gland.*

***Histopathology** was performed on the following organs (predominantly reproductive tissues): adrenal glands, liver, kidneys, pituitary gland, **thyroid gland,** ovaries, testes,*

epididymides, dorsolateral and ventral prostate gland, seminal vesicles, coagulating glands, LABC muscle, Cowper's glands, preputial glands, and gross lesions

Yet, no thyroid gland results are presented anywhere in the study report or in the supplementary data (at least we did not find them). Since this MOG study also aims at clarifying the endocrine disrupting potential of 2H4MPB, showing the results from the investigation of the thyroid gland would improve the study report and its usefulness for assessing potential endocrine disrupting effects on the thyroid hormone system.

Assessment methods, which we suggest to revise, in order to improve their accuracy

Nipple retention

In the method description, it is stated that; "*F1 and F2 male pups were evaluated for retention of areolae/nipples on PND 13*", and in the results it is stated that; "*2H4MBP did not affect areola/nipple retention at the tested concentrations*". However, when looking in more detail at the single animal data for areolae/nipple retention in the supplementary tables (appendix E), it becomes clear that in all investigated male offspring (873 pups to be exact) – not a single areola was identified. To us this indicates that this endpoint was not assessed in an optimal way, since a low background incidence (some males with 1 or 2 areola) should have been seen in all dose group.

The MOG study was performed at the RTI laboratory, and we do not know if observation of 0 nipples/areola is normal in the developmental toxicity assessments performed there. We do however know that some of the authors/evaluators of the MOG 2H4MBP study report, including Dr. BS McIntyre and Dr. PM Foster have previously published several papers where a low background incidence of areolae was present in the control group (for instance McIntyre et al 2001, McIntyre et al 2002, and Carruthers & Foster (2005). We therefore find that a critical review of the methods used to assess nipple/areolae retention at the RTI laboratory would be recommended, and we are pleased to see that experienced researchers with the needed expertise are already involved in the present project. We would therefore recommend that this issue is investigated further.

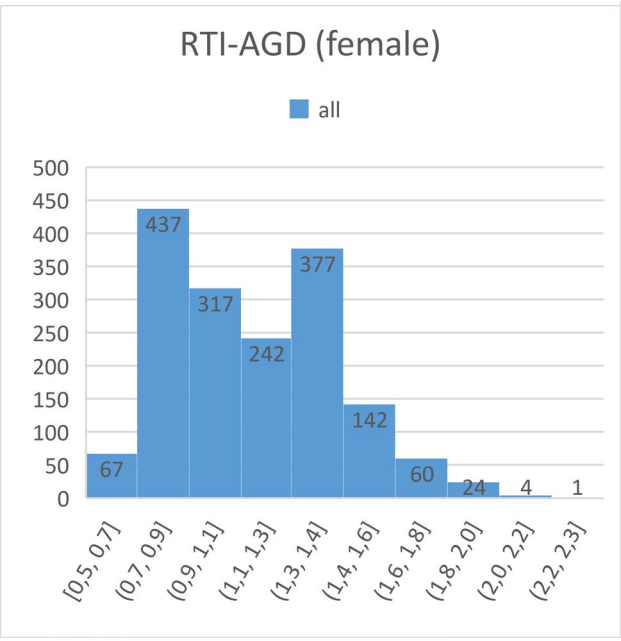
Anogenital distance (AGD)

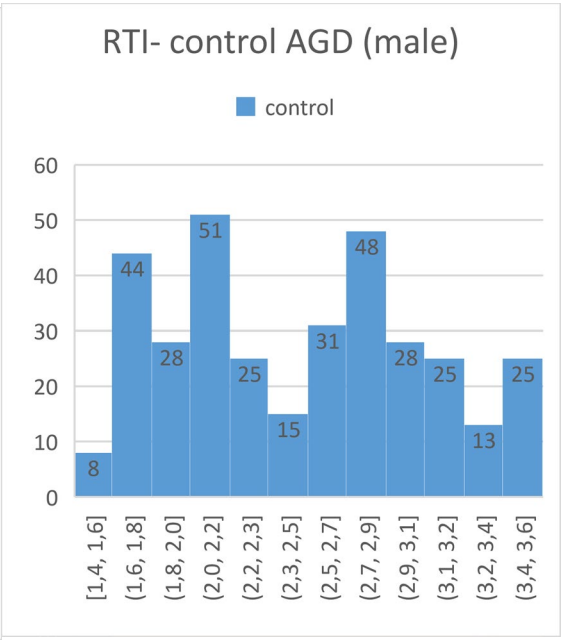
On a related matter, measurement of AGD in the male offspring also seems to have been measured with low accuracy at the RTI facility. We base this statement on the fact that the variation in male AGD measurements was very high.

The mean and standard deviation in AGD in control males was 2.56 ± 0.59, leading to a coefficient of variation (CoV) of 23 %. This is a very high variation compared for instance to the CoV in the studies used for assessing the feasibility of including measurements of AGD in several recently updated OECD test guidelines. Here the mean CoV for both AGD and AGD index in fetuses and pups was around 4-5% (OECD, 2018).

To illustrate further, how imprecise the AGD measures in the MOG study were, we have compiled two figures based on the data provided in the supplementary tables of the MOG study report. The first figure show AGD in all female pups in the study, whereas the second figure shows the distribution of AGD in control males. The data are not depicted within litter, but as separate measurements – each pup is represented by a single value.

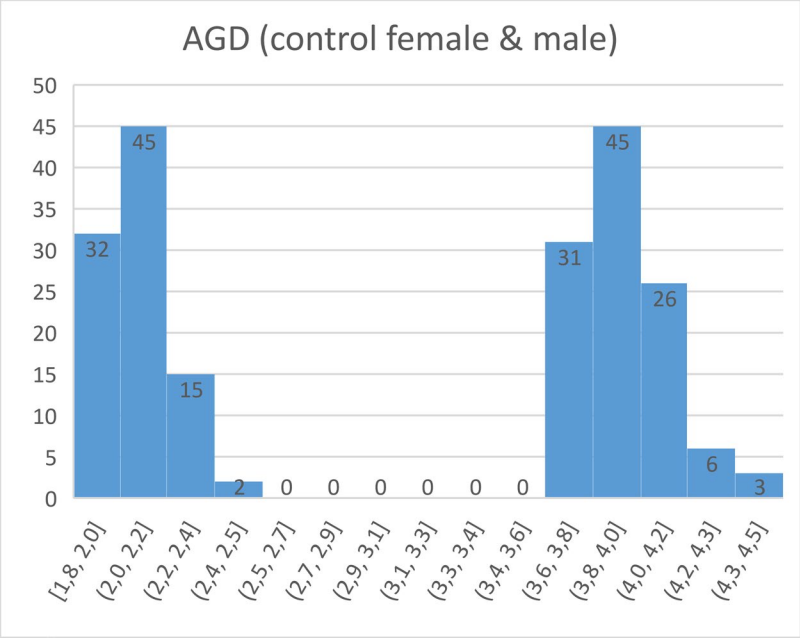
It is worth noting that there is a substantial overlap between the AGD measurements in control males and in females (values between 1.4-2.3 mm are shared by “long females” and “short males”). This large overlap is unexpected, and should definitely not be so large, as male AGDs are typically twice as long as in females. They only come close to each other if the males have been exposed to anti-androgenic chemicals during fetal development, and to some degree if the females have been exposed to androgens. This is however not the case with 2H4MBP, and this data therefore illustrates how inaccurately the male AGD measurement must have been assessed in the study.





When variation in the control group is high, it is almost impossible to find effects related to the test compound that is being investigated. Thus, we find it problematic that the AGD measurements were so imprecisely measured.

For comparison AGD data from female and male control pups (from a recent publication by Johansson *et al.* 2021) are presented in the figure below, depicted in a similar way as the results from the MOG study.



The same x-axis is used as in the figures above, and even though both male and female AGDs differ somewhat between the two studies, the figure clearly illustrates how control data are distributed in a laboratory with much expertise in measurement of this endpoint.

Like for the endpoint “nipple retention”, there are scientist with great expertise in assessing AGD within the MOG study review panel, and we hope that they will chose to pursue this issue, so that precision in AGD measurements can be improved in future studies at the RTI testing facility.

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