

Heliotropine / Piperonal - Independent Comment on Classification Following a Review of the Reproductive and Developmental Toxicity Studies and Comments on the CLH Report of the HSA Ireland (published 06 June 2023)

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This independent opinion was developed following a detailed review of the available reproductive and developmental toxicity studies on Piperonal, also known as Heliotropine. Following the publication on 06 June 2023 of the CLH report prepared by the HSA of Ireland and dated April 2023, the CLH report was reviewed and comments on that report are included below.

CLH Report V2, piperonal; 1,3-benzodioxole-5-carbaldehyde, Ireland HSA

In its review of the Combined Repeated Dose Toxicity Study with Reproduction/Development Toxicity Screening Test the HSA concludes on the basis of the bodyweight gain of one female with an abnormal pregnancy that the effect of the high dose may be an intrauterine effect rather than maternal toxicity. The HSA stated *“However, this individual dam had a higher body weight on LD 1 than the mean control value (284g compared to 267g in the control). Although the data is limited, it provides some indication that the effect on maternal body weight may be an intrauterine effect, rather than maternal toxicity.”* This seems to be a highly speculative conclusion especially as four of 10 animals in the control group had LD 1 bodyweights ≥ 282 g. In addition, the individual animal in question (female 79) gave birth to a single pup and in this case may be expected to have a higher post-natal bodyweight than animals carrying a normal pregnancy because it has to invest less energy into a single pup than the control animals carrying 7 to 16 pups. So, the relatively slightly higher bodyweight of one female provides no evidence that the effect on reproduction or development was directly on the pups rather than secondary to effects on the maternal physiology.

The HSA supports their conclusion with the observations on food consumption, claiming that sporadic statistically significant increases in relative food consumption at 300 and 1000 mg/kg/day support their view that the effects seen at 1000 mg/kg/bw were likely to be intrauterine rather than secondary to maternal toxicity. However, there was a similar trend to increased relative food consumption in the females during the 71-day pre-mating period (82, 84, 86, 93 g/kg/day for groups 1 to 4 respectively); an effect also observed in the male animals (75, 73, 76, 78 g/kg/days on days 1 to 71 and 62, 59, 65, 70 g/kg/day during the mating period). In this case, the trend observed in females post-coitum (78, 80, 85, 90 g/kg/day) seems to be more a general effect on the metabolism of the adult animals of both sexes rather than anything specific to pregnancy. It should also be noted that in this study there was no record of feed scatter behaviour by the animals. The observations of clinical signs indicated that the two highest dose levels induced salivation related to the taste of the test item. It is commonly observed that animals tend to scatter more food in studies with strong tasting test items (both in dietary and gavage studies) and in this case the food consumption data may over-estimate the food consumed. This idea is supported by the fact that bodyweight gain was statistically significantly reduced in the males at practically every timepoint even though their apparent food consumption was the same or greater than controls. The Wistar (Han) rat strain used in these studies is recognised to

consume less food than Sprague-Dawley rats and to only consume the food that it requires (Hayakawa et al, 2013), so the increase in food consumption is unusual and points to either an adverse toxic effect, or more likely is evidence of an increase in feed scatter behaviour that was not recorded.

The HSA concludes that no significant maternal toxicity was observed and thus considers that the effects observed in the study were not secondary non-specific consequences of other toxic effects. However, the HSA ignore the marked effects on the thyroid organ and trabecular bone, the latter being more marked in females than in males and was considered by the study pathologist to be adverse. In this case it seems difficult to understand why the HSA determines that there were no adverse effects in the mothers. Whilst the mechanism involved in these observations is not obvious it raises a doubt about the relevance to man and strongly suggests that the effects on pregnancy may be secondary to these observations.

The HSA also reviewed the available OECD 414 developmental toxicity study in rats, and in this study, there were a number of effects observed both on the dams and on the foetuses. However, the HSA concludes that these effects occurred in the absence of significant maternal toxicity, a conclusion that is contradicted by the study director and the OECD guidance document on mammalian reproductive toxicity testing (OECD 43, 2008). The females of the 1000 mg/kg/day dose group had a 17% reduction in bodyweight gain at the end of the treatment period, described as adverse by the study director. In addition, there were marked reductions in the food consumption in the females, which was particularly marked in the critical period of foetal development between days 6 to 9, where food consumption was reduced on average by 22%, and typically a reduction in food consumption of greater than 10% is considered to be adverse and equivalent to dietary restriction. OECD 43 states *“There is a high degree of correlation between maternal condition and the status of the litter, which is particularly obvious at very toxic dose levels.”* Indeed, there was a direct relationship between individual animal food consumption and developmental outcome (described in detail later in the document) that should not be ignored, and it should be concluded that the effects seen in the foetuses was a direct consequence of the reduced maternal food consumption, particularly during the critical 6–9-day window of gestation.

The overall conclusion of the HSA, that the available data warrant a classification of reproductive toxicant category 1B, is not supported by the data. There were clear and significant toxic effects on the female animals in both studies that were incorrectly interpreted by the HSA as not adverse. There is a doubt about both the mechanistic relevance of the observed effects to man and the link between the effects and maternal toxicity. In this case it is considered that classification as a reproductive toxicant category 2 for effects on sexual function and fertility and effects on development is warranted.

[Prenatal Developmental Toxicity Study of Piperonal or Heliotropine by Oral Gavage in Rats – Charles River Study No. 20172127 – Final Report 26 March 2021](#)

The final report of the OECD 414 study on Piperonal/Heliotropine was reviewed as described below.

Food Consumption and Bodyweights.

Food consumption of the females was reduced by ~22% between post-coitum Days 6 to 9 (0.78x of control mean) at 1000 mg/kg/day. Although mean food intake recovered to control levels as treatment progressed, several animals at 1000 mg/kg/day also showed a notable reduction in food

intake over post-coitum Days 15-21. Bodyweight gain was reduced by ~17% at 1000 mg/kg bw/day (0.83x of the control mean at the end of the treatment period). No effects on food consumption or bodyweight gain were observed at 100 or 300 mg/kg/day. The effects at 1000 mg/kg/day were considered to be adverse by the Study Director, which is considered to be a valid conclusion because effects on food consumption and bodyweight gain are considered to be evidence of maternal toxicity (OECD 43, 2008). It is logical to conclude that the reductions in maternal bodyweight gain were a direct consequence of the reduced food intake and were evidence of moderate/severe maternal toxicity effects.

Organ weights and thyroid hormone levels.

Mean total Triiodothyronine (T3) and total Thyroxine (T4) levels showed an apparent dose related trend towards a reduction across the dose groups, and mean Thyroid-Stimulating hormone (TSH) levels showed an apparent dose-related trend towards an increase. At 1000 mg/kg/day, the decrease in mean T3 and T4 was statistically significant (0.65x and 0.73x of control mean for total T3 and T4, respectively). The mean total T3 was below the historical control data range at 100, 300, and 1000 mg/kg/day; the possible adversity of this change could not be established within this study. Both mean total T4 and TSH remained within the historical control data range at these dose levels, and as such these changes were considered not to represent an adverse effect. In particular, there were no test item-related correlative alterations in thyroid gland weights or thyroid histopathology. The liver weight of the dams was not recorded, which may have provided definitive evidence of the likely induction of liver metabolic enzymes. However, based on the evidence of the 10-day range-finding study, where increases in liver weight were observed at 500 and 1000 mg/kg bw/day, it may be assumed that liver hypertrophy was induced at 1000 mg/kg bw /day and probably also at 300 mg/kg bw/day in this study. The probable effects on liver weight can explain the effects on thyroid hormone levels because liver hypertrophy is associated with the induction of enzyme levels and the spectrum of induced enzymes can include UDP, which metabolizes thyroid hormones and is considered to be a non-adverse and readily reversible response to an overload of an organic xenobiotic chemical (Janke et al., 2004).

Pregnancy Data.

Two females at 1000 mg/kg bw/day showed 100% fetal loss (post-implantation deaths) and one female in the 300 mg/kg bw/day dose group also showed 100% fetal loss. The Study Director indicates that occurrences of 100% fetal loss are rare in the Historical Control Database (HCD) and therefore considers the single occurrence at 300 mg/kg bw/day as an adverse event, similar to the two occurrences in the high dose group. However, the animal with 100% fetal loss at 300 mg/kg bw/day was the subject of a deviation, described in Appendix 8; *“For one animal at 300 mg/kg/day (A052), the number of implantation sites and uterine content were not documented at necropsy. It was recorded that this animal had implantation sites but that it had no fetuses. Therefore, the animal was recorded as gravid, and was concluded to have a 100% implantation loss. Although it could not be confirmed if this was due to early and/or late resorptions, it was considered that sufficient data was available for adequate evaluation of the study results.”* However, the possibility exists that the error was to record the animal as having implantation sites where there were none, that is, that the female was a non-pregnant animal. In this case, the treatment cannot be the cause of absence of fetuses.

It is also noted that all four groups, including the control group, had several animals (5/21, 4/20, 5/20, 7/22; control to high dose respectively) with percentages of early resorptions per litter that exceeded the historical control range, so it is possible that the HCD was not fully representative for the batch of animals used in this study. Overall, the effect on early resorptions was considered to be adverse at

1000 mg/kg/day but not adverse at the two lower dose levels. Premature abortion and foetal loss have been reported to occur after diet restriction and reductions in maternal bodyweight gain in rabbits (Cappon, 2005) but not in Sprague-Dawley rats (Fleeman, 2005), although there may be a rat strain differences in this case (Han-Wistar rats used in the current study). A comparison of the individual animal food consumption rates during days 6-9 and the frequency of post-implantation (PI) loss reveals that the two animals (Nos. 80 and 84) with 100% PI loss consumed only 6 or 8 grams per day of food compared to the average of the control group of 18g, meaning only 33-44%. Three of the other animals with relatively high %PI loss (18.2, 28.6, and 15.4 for animal Nos. 75, 79, and 85 respectively) also had markedly low food consumption during this critical 6-9 day period, with only 12, 6, and 14 grams of food consumed per day (67, 33, 78% of the control average respectively). **It can be concluded that there is a direct relationship between the adverse effect on individual maternal food consumption during the critical post-implantation period of 6-9 days post conception and the frequency of PI loss. The higher frequency of PI loss at 1000 mg/kg/day is therefore secondary to maternal toxicity.**

Developmental Findings

Adverse skeletal malformations were observed at 1000 mg/kg bw/day at a higher frequency than in the control group (15% vs 1.6%; 7 litters vs 2 litters affected; 3 fetuses with two malformations each). These malformations were specific in that they were localized to the thoracic region of the body; no external fetal lesions were noted. In addition to these malformations there was an increase in several types of non-adverse variations and reduced ossifications that also affected the thoracic region of the body. Of the affected litters in the high dose group, 3 were from mothers (Nos. 75, 79, and 85) that had showed drastically reduced food consumption (33, 33, 44% of control mean respectively) during the critical 6-9 day period and also higher levels of %PI loss than expected). **It can be concluded that there is a direct relationship between the adverse effect on individual maternal food consumption during the critical post-implantation period of 6-9 days post conception and the frequency of skeletal malformations. The higher frequency of skeletal malformations at 1000 mg/kg/day was therefore secondary to maternal toxicity.**

NOAEL Conclusion

The Study Director concluded that the NOAEL was 300 mg/kg/day for maternal effects (based on lower body weight gain and food intake, and clinical signs at 1000 mg/kg/day) and 300 mg/kg/day for developmental effects ((based on two cases of 100% implantation loss at 1000 mg/kg/day, along with lower fetal body weights and skeletal malformations). The nature and frequency of the adverse developmental effects was closely associated with the adverse maternal effects and there was a direct correlation between individual maternal food consumption during the critical period of Days 6-9. It seems logical to conclude that the developmental effects were secondary to maternal reduced food consumption and bodyweight gain. Furthermore, missing vertebrae have been associated with maternal toxicity caused by reduced maternal food consumption and nutritional deficiency (Lankas, et al., 2004). Reduced ossification and an increase in non-adverse skeletal variations have been reported as being associated with dietary restriction in rats (Fleeman et al., 2005). **In this case, it can be concluded with a high level of confidence that the NOAEL for development is the same as for maternal effects, and furthermore, that the effects in the offspring are concluded to be secondary to the maternal toxic effects.**

Combined Repeated Dose Toxicity Study with the Reproduction/Developmental Toxicity Screening Test of Piperonal or Heliotropine by Oral Gavage in Rats – Charles River Study No. 20172124 – Final Report 26 March 2021

The final report of the combined OECD 408 and OECD 422 study on Piperonal/Heliotropine was reviewed as described below.

P0 Animals – Effects observed at 1000 mg/kg bw/day

Food Consumption and Bodyweights.

In males there was no statistically significant changes in food consumption at any dose level, although there was a trend towards increased relative food consumption. This may have been evidence of an increase in feed scatter behaviour caused by the strong taste of the test item because the males of the 1000 mg/kg/day dose group were observed to gain significantly less bodyweight than controls throughout the study. At 1000 mg/kg/day, absolute body weights in males were decreased from Day 22 onwards (up to 12% compared with concurrent controls at end of treatment), reaching statistical significance from Day 43 onwards. Moreover, statistically significant reduced body weight gain was recorded for males treated at 1000 mg/kg/day from Day 22 onwards (up to 29% lower than concurrent controls at end of treatment).

Female rats treated at 1000 mg/kg/day showed no statistically significant effects on body weights and body weight gain during the pre-mating and mating period. From post-coitum Day 14 onwards, a lower mean body weight and body weight gain was observed, reaching statistical significance on Day 17 and/or Day 20, respectively. This could be explained by the reduced weight gain and/or weight loss in 4/4 pregnant females (Nos. 74, 76, 78, and 79), which had abnormal pregnancies (three females with implantation sites only and one female with total litter loss on PND 1). At Day 1 of lactation, mean body weight of the one female (No. 79) that delivered one pup (with total litter loss on PND 1) was similar to that of controls. In female rats treated up to 300 mg/kg/day, during lactation from Day 4-7 onwards, relative food consumption was decreased (8% lower than concurrent controls, statistically significant on Days 4-7 and 7-13), which was considered not toxicologically relevant, due to the minimal magnitude of the change, and/or the absence of an effect on body weight. During the post coitum period, a higher absolute and relative food consumption was noted in females treated at 300 mg/kg/day on Days 11-14 (up to 13% higher than concurrent controls). This change was considered to be unrelated to treatment with the test item, due to the minimal magnitude of the change, and/or no clear trend was apparent regarding dose and duration of treatment. Female rats treated at 1000 mg/kg/day showed food consumption, before or after correction for body weight, was increased during pre-mating Day 8-71, although no statistical significance was achieved. In addition, absolute food consumption (of 4 pregnant females only) was increased on post coitum Days 4-7 and relative food consumption was increased from post coitum Days 4-7 onwards, reaching statistical significance on Days 4-7, 14-17 and 17-20. These changes were considered not toxicologically relevant, due to the minimal magnitude and direction of change.

Thymus organ weight and histopathological changes.

In males there were dose-related reductions in relative thymus weights that were significant at 1000 mg/kg bw/day ($p < 0.01$), together with minimal lymphoid atrophy. However, in females the observations were different, in this case there was a dose related increase in thymus organ weight

that was significant ($p < 0.01$) at both 300 and 1000 mg/kg bw/day, together with (cystic) epithelial hyperplasia, the effects at 1000 mg/kg bw/day may be considered as adverse. In this case the relationship of these observations at 1000 mg/kg bw/day with the failure of pregnancy is unclear but the possibility of failure of pregnancy as being secondary to the thymus organ changes cannot be excluded.

Trabecular bone.

Effects were seen in both sexes, although of greater severity in females, judged by the pathologist to be adverse at 1000 mg/kg bw/day. The increased trabecular bone was most obvious in the femur, just below the growth plate in the metaphysis area and to a lesser extent in the femur head. Due to the increase in bone, the spaces for the bone marrow appeared to be slightly diminished. However, this was not expressed in hematology parameters or hematopoiesis/ myelopoiesis in other organs. For the sternum, the bone adjacent to the intervertebral discs was the most affected. The causes and adversity, or consequences, of trabecular bone are difficult to assess and it is possible that it is a transient reversible effect.

Other Effects

In males, the liver weight was observed to be increased in a dose-related fashion reaching 1.4x at 1000 mg/kg bw/day compared to the control. This was considered to be an adaptive and reversible response commonly observed in such studies. A similar effect was not observed in females but may have been confounded by the low frequency of pregnancy in the high dose group. In the males there was a dose-related reduction in T4 levels but not in T3 and no significant increase in TSH levels. In females there were no significant changes in thyroid hormone levels. The reduction in T4 in males is considered to be non-adverse and likely a secondary consequence of liver hypertrophy.

Summary.

The Study Director defined the NOAEL as 300 mg/kg bw/day for parental toxicity and also for reproduction and developmental toxicity. For parental toxicity the study directors' conclusion is based solely on the effects in males and females of an increase in 'trabecular bone' at 1000 mg/kg/day. The adversity of the reproductive and fertility toxicity effects is unambiguous because there was 100% absence of healthy offspring at 1000 mg/kg bw/day. However, there is doubt about the relevance to the human because of the absence of an obvious mode of action.

Opinion on Classification

The question is, are the data generated in the OECD 414 study in the rat and the combined 90-day and screening reproductive and developmental toxicity study (OECD 422), sufficient for classification as a reproductive toxicant? The possible classifications for reproductive or developmental toxicity are Cat 1B or Cat 2 and the criteria are as follows:

Cat 1B Presumed human reproductive toxicant. The classification of a substance in this 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.

*Cat 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.***

For both Cat 1B and Cat 2, toxic effects did not occur in the offspring at dose levels where no other adverse effects were observed; the NOAELs for reproduction and development were the same as, or higher, than the NOAEL for parental adverse toxicity. Specifically, in the OECD 414 study there were marked effects on maternal food consumption and body weight gain. In the OECD 408/422 study there were effects on bone development and the thymus, in both males and females.

The question as to whether Cat 2 classification should be applied depends on whether the adverse effects on reproduction and development are considered to be a secondary non-specific consequence of other toxic effects. The ECHA CLP guidance provides a decision logic for the classification of substances in section 3.7.2.7. In this case, it is possible to arrive at no classification decision or a Cat 2 classification decision, depending on how the criteria are applied. The criteria described in the guidance document make it difficult to avoid the application of Cat 2. For example, even if there are doubts about the relevance to humans then the criteria suggest that Cat 2 may be more appropriate than Cat 1B. However, for Cat 2 to apply then reproductive and/or developmental 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.*”

For heliotropin, effects were observed only in the presence of parental toxicity, but it is not clear whether the effects are secondary to the parental toxicity.

In the OECD 414 study then the situation is clear because there were marked reductions in food consumption and clear adverse effects on maternal BW gain. An effect on maternal nutrition is a recognized secondary non-specific cause of developmental effects and may be the cause of the fetal losses and skeletal effects. Furthermore, it can be concluded that a secondary mechanism driven by a reduction in food consumption is not relevant to humans. **Classification is not warranted based on the data of the OECD 414 study.**

For the OECD 422 study then the decision is less obvious. The adverse effects on the males seem not to be associated with the fetal loss because all the males mated and probably all the pairings resulted in fertilization, although this cannot be known for certain. The effects on the thymus in the females were not considered to be adverse by the Study Director and it is difficult to envisage a Mode of Action (MoA) linking the effects on the thymus with the effects on reproduction. Equally, it seems difficult to consider the reproductive effects as secondary to the increased trabecular bone seen in the females (and males). However, it is possible that these effects are associated to a common mechanism, and also therefore the effects on the offspring. Classification for reproductive toxicity (Cat 1B) is dependent on whether there is **doubt** that the effects in the rats may be considered to be caused by

a mechanism that is not relevant to humans. In this case it can be concluded that there is sufficient doubt about human relevance.

Conclusion on classification

The data are not definitive on whether the effects seen in the OECD 422 study are direct reproductive or developmental toxicity, or secondary to effects in the parents. In the absence of an obvious mechanism of action, it may be concluded that there is a doubt about the relevance to humans. In this case, it is recommended that classification for Cat 2 reproductive toxicity is warranted, but not Cat 1B, because the reproductive toxicity occurs in the presence of parental toxicity and the effects are likely to be a non-specific consequence of the parental toxic effects. Furthermore, the human relevance of the effects is highly questionable. In the OECD 414 study the effects on the fetuses can be clearly linked to the marked effect on food consumption and reduced body weight gain of the females. In the OECD 408/422 study, the adverse effects on both the parents and offspring were likely linked to the same mechanism, which has yet to be identified. Whilst it is highly likely that this mechanism is not relevant to humans, this conclusion cannot be made with absolute certainty, and so a Category 2 classification is recommended.

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