Committee for Risk Assessment

RAC

Opinion

proposing harmonised classification and labelling
at EU level of

piperonal; 1,3-benzodioxole-5-carbaldehyde

EC Number: 204-409-7
CAS Number: 120-57-0

CLH-O-0000007447-67-01/F

Adopted

6 June 2024

6 June 2024

CLH-O-0000007447-67-01/F

OPINION OF THE COMMITTEE FOR RISK ASSESSMENT ON A DOSSIER PROPOSING HARMONISED CLASSIFICATION AND LABELLING AT EU LEVEL

In accordance with Article 37 (4) of Regulation (EC) No 1272/2008, the Classification, Labelling and Packaging (CLP) Regulation, the Committee for Risk Assessment (RAC) has adopted on **6 June 2024** by **consensus** an opinion on the proposal for harmonised classification and labelling (CLH) of:

Chemical name: **piperonal; 1,3-benzodioxole-5-carbaldehyde**

EC Number: **204-409-7**

CAS Number: **120-57-0**

Rapporteur, appointed by RAC: **Karine Angeli**

Administrative information on the opinion

Ireland has submitted on **25 April 2023** a CLH dossier containing a proposal together with the justification and background information documented in a CLH report.

The CLH report was made publicly available in accordance with the requirements of the CLP Regulation at ***http://echa.europa.eu/harmonised-classification-and-labelling-consultation/*** on **12 June 2023**.

Concerned parties and Member State Competent Authorities (MSCA) were invited to submit comments and contributions by **11 August 2023**.

The opinion takes into account the comments provided by MSCAs and concerned parties in accordance with Article 37(4) of the CLP Regulation and the comments received are compiled in Annex 2.

The following table provides a summary of the Current Annex VI entry, Dossier submitter proposal, RAC opinion and potential Annex VI entry if agreed by the Commission.

Classification and labelling in accordance with the CLP Regulation (Regulation (EC) 1272/2008)

	Index No	Chemical name	EC No	CAS No	Classification		Labelling			Specific Conc. Limits, M-factors and ATE	Notes
					Hazard Class and Category Code(s)	Hazard statement Code(s)	Pictogram, Signal Word Code(s)	Hazard statement Code(s)	Suppl. Hazard statement Code(s)		
Current Annex VI entry	No current Annex VI entry										
Dossier submitters proposal	TBD	piperonal; 1,3-benzodioxole-5-carbaldehyde	204-409-7	120-57-0	Repr. 1B Skin Sens. 1	H360FD H317	GHS08 GHS07 Dgr	H360FD H317			
RAC opinion	TBD	piperonal; 1,3-benzodioxole-5-carbaldehyde	204-409-7	120-57-0	Repr. 1B Skin Sens. 1	H360FD H317	GHS08 GHS07 Dgr	H360FD H317			
Resulting Annex VI entry if agreed by COM	TBD	piperonal; 1,3-benzodioxole-5-carbaldehyde	204-409-7	120-57-0	Repr. 1B Skin Sens. 1	H360FD H317	GHS08 GHS07 Dgr	H360FD H317			

GROUNDS FOR ADOPTION OF THE OPINION

RAC general comment

Piperonal (1,3-benzodioxole-5-carbaldehyde, also known as helioptropin) is a solid crystalline product used in the formulation of fragrances and end-products, formulation of tobacco flavours, in industrial washing and cleaning products and as a chemical intermediate.

Toxicokinetics

Two non-guideline *in vivo* studies investigating metabolism and elimination of piperonal in mice (Kamienski, 1970) and rats (Klungsoyr, 1984) are summarised in the REACH registration dossier and considered of low reliability due to insufficient documentation (publications not available).

Radiolabelled piperonal administrated to rats (150 mg/kg bw) and mice (0.75 mg/kg) via gavage was extensively absorbed and rapidly excreted in urine. Similar metabolic fate was described in the two species with piperonyl glycine and piperonylic acid being the major urinary metabolites.

At the 48-h time point, the identified urinary metabolites and their quantities (as a percentage of the administered dose) were as follows: piperonyl glycine (72 %), piperonylic acid (20 %), piperonyl alcohol (0.9 %), and three others (0.1-0.5 %). No unchanged compound was excreted in the urine, and no metabolites were detected in the urine more than 48-h after dosing.

After the consultation of the CLH report, a trade association proposed a read-across approach from toxicokinetic data of benzoate compounds (i.e. benzoic acid, benzoate salts, benzaldehyde, benzyl acetate, benzyl alcohol and benzyl benzoate) to piperonal. In their submitted position paper, they argued that piperonal is mainly excreted via the Hippurate pathway, that a glycine-dependent saturation of the Hippurate pathway takes place, and that this is not relevant to humans. In their view, this would support that the effects on fertility and development observed in piperonal studies at high dose levels are only due to glycine depletion and consequently high free piperonylic acid concentration. Considering that human exposure to piperonal would clearly be below this point of saturation, the trade association concluded that there was doubt about human relevance for effects on fertility and development observed at the limit dose. Therefore, they considered that Category 2 would be more appropriate than Category 1B.

RAC considers that the read-across from benzoate compounds is not justified since piperonal is not a precursor of benzoic acid. Further, the hippurate pathway is not appropriate since piperonylic acid does not give rise to hippuric acid after conjugation with glycine. RAC also notes that among the reports on benzoate compounds (WHO, 2005; EFSA, 2016 and JECFA, 2022) cited by the trade association, none includes piperonal in their grouping.

From the very limited compound-specific toxicokinetic data, RAC considers that no conclusion can be drawn on the saturation of glycine-conjugation in rats exposed to high doses of piperonal since a single dose of 150 mg/kg bw was tested in Klungsoyr (1984) and alternative metabolism pathways (e.g. glucuronidation) at higher dose levels cannot be excluded.

RAC further notes that reproductive effects were also observed at lower dose levels and concludes that the hypothesis put forward by the trade association that the effects in reproductive toxicity studies with piperonal are mainly due to glycine depletion and not relevant to humans is not supported by the available data.

HUMAN HEALTH HAZARD EVALUATION

RAC evaluation of skin sensitisation

Summary of the Dossier Submitter's proposal

The dossier submitter (DS) proposed to classify piperonal as skin sensitiser (Skin Sens. 1; H317) based on a positive response in a Guinea pig maximisation test (GPMT) with 40 % responding at 1.5 % intradermal induction dose. Since it cannot be excluded that a lower (i.e., ≤ 1 %) intradermal induction dose would have led to positive skin reactions, the DS considered that sub-categorisation is not appropriate.

Comments received during consultation

Two Member State Competent Authorities (MSCAs) supported the proposed classification as Skin Sens. 1; H317 without sub-categorisation.

Additional key elements

While limited, some human and *in chemico*/*in vitro* data related to the skin sensitising potential of piperonal have been identified in the open literature. These data have been considered by RAC in addition to animal studies reported in the CLH report in a weight-of-evidence approach.

In chemico/in vitro data

In a publication of the Research Institute for Fragrance Materials (RIFM) (Lee et al., 2022), dedicated to the assessment of the skin sensitisation potential of 67 fragrance ingredients using the U-SENS™ assay, piperonal is reported to be:

- Positive in a direct peptide reactivity assay (DPRA) addressing key event 1 "Covalent interaction with skin proteins",
- Positive in the KeratinoSens™ assay addressing key event 2 ''Keratinocyte responses",
- Positive in the human cell line activation test (h-CLAT) while negative in the U-SENS™ assay, both addressing key event 3 ''Dendritic cell responses'' of the adverse outcome pathway (AOP) for skin sensitisation.

While only the results are reported, hampering a full assessment of these *in chemico*/*in vitro* assays (considered of non-assignable reliability), RAC notes that applying the "2 out of 3" Defined Approach (OECD TG 497) criterium, piperonal is predicted to be a skin sensitizer.

Assessment and comparison with the classification criteria

Animal data

In a GPMT conducted with piperonal (Anonymous, 1978, considered reliable with restrictions), the tested concentrations were 1.5 % for the intradermal induction, 80 % (in acetone maximum possible concentration, non-irritant) for topical induction and challenge (day 14), and 80 % (day 21, day 28 and day 42) or 20 % (day 42) for re-challenges. Piperonal induced positive reactions in the same 4 out of 10 animals (40 %) on days 14, 21 and 28 with 80 % test substance and on day 42 with 20 % test substance. Among the limitations of this pre-guideline GPMT that could lead to an underestimation of the skin sensitising potential of piperonal, is that the selected

concentration of 1.5 % for intradermal induction was not clearly justified. In the preliminary study for the selection of the intradermal induction dose, positive skin reactions described as 'faint pink' were already observed at lower concentrations. Furthermore, for topical induction, sodium lauryl sulphate should have been applied in order to create a local irritation since piperonal 80 % in acetone was not irritant in the preliminary test (OECD TG 406).

In a published screening of 32 fragrance ingredients for skin sensitisation using four different methods in Guinea pigs (Klecak et al., 1977), piperonal was found to be positive in two tests (Open Epicutaneous test and Maximisation test) and negative in the two other tests (Draize test and Freuds Complete Adjuvant test). Despite the limited reporting of these non-guideline studies, RAC considers that the positive Open Epicutaneous test and the Maximisation tests provide some supporting evidence for the skin sensitising potential of piperonal.

In the OECD Toolbox, an EC3 of 25 % in a Local Lymph Node Assay from the RIFM database is reported which would support a moderate potency. However, this assay is not published nor submitted in the REACH dossier and therefore its reliability cannot be independently verified.

Human data

Human Repeated Insult Patch Test (HRIPT) & Human Maximisation Test (HMT)

An HMT with piperonal 6 % in petrolatum (3 724 µg/cm^2) induced no reaction in 25 subjects (Greif et al., 1967 reported in the database of human predictive patch test data for skin sensitisation from NICEATM and BfR; Strickland et al., 2023).

In a HRIPT (unpublished) performed by the RIFM to confirm a no-effect level obtained from other non-human tests, piperonal in ethanol and diethyl phthalate (1:3) was negative in 112 human volunteers (2953 µg/cm^2) as reported by Na et al. (2021) and Lee et al. (2022).

Diagnostic patch tests

Piperonal was tested among other fragrances in 1 606 consecutive patients of contact dermatitis clinics at 6 European dermatology departments. Piperonal induced 2 (0.1 %) and 6 (0.4 %) positive reactions at concentrations of 1 % and 5 % in petrolatum, respectively. The 6 patients had no history of adverse reactions to scented products (Frosch, 2002; SCCS, 2012).

In a review on fragrance allergy (de Groot, 2020), the reported frequency of occurrence of skin sensitisation in routine testing for piperonal ranged from 0.4 % to 1.0 % (no reference available), and a frequency of 5 % (1/20) in selected patients (contact allergy related to cosmetics) is mentioned in a single study (Larsen, 1977).

Larsen (1975) described a 62-year-old woman with a perfume dermatitis on the face. When tested with 94 liquid fragrance components, she reacted positively to 11 of them including piperonal.

RAC notes that relatively low/moderate to high frequency of occurrences of skin sensitisation are observed in the available human data. However, in view of their limited number and in the absence of data to estimate exposure, the classification and sub-categorisation are primarily based on the available animal studies.

Comparison with the criteria

Human, animal and *in vitro* data provide consistent evidence that piperonal is a skin sensitizer.

According to section 3.4.2.2.1.1 of Annex I of the CLP Regulation, '*skin sensitisers should be classified in Category 1, where data are not sufficient for sub-categorisation'*.

In the GPMT (Anonymous, 1978), 40 % animals responded at 1.5 % intradermal induction concentration which fulfils the CLP criteria for Category 1B. However, due to the uncertainties linked to the selected induction concentration, RAC considers that Category 1A cannot be excluded and Category 1 should be applied instead of Category 1B in accordance with the CLP guidance (2024).

Other available animal, human and *in vitro* data do not allow to propose any sub-categorisation.

Therefore, based on a weight of evidence approach and in line with the DS proposal, **RAC concluded that classification as Skin Sens. 1; H317 (May cause an allergic skin reaction) without sub-categorisation, is warranted.**

The generic concentration limit (1 %) for Category 1 with no sub-categorisation should apply.

RAC evaluation of reproductive toxicity

Summary of the Dossier Submitter's proposal

The DS considered that classification in Category 1B is warranted for adverse effects on sexual function and fertility based on a significant decrease in the fertility index, mean implantation sites and gestation index observed at 1 000 mg/kg bw/d in a Combined Repeated Dose Toxicity Study with the Reproduction/Developmental Toxicity Screening Test conducted according to OECD TG 422 (Anonymous, 2020a). At the same dose level, a statistically significant decrease in prostate and epididymides weights was noted as well as a statistically significant increase in ovary and uterus weights, both in the absence of significant systemic toxicity.

For adverse effects on development, the DS proposed classification in Category 1B based on the following findings: increased post-implantation loss in both the OECD TG 422 study (Anonymous, 2020a) and a prenatal developmental toxicity (PNDT) rat study conducted according to OECD TG 414 (Anonymous, 2020b), as well as increased incidences of skeletal malformations and other anomalies observed in the absence of significant maternal toxicity in the PNDT study (Anonymous, 2020b).

The DS did not propose classification for adverse effects on or via lactation due to absence of relevant data.

Comments received during consultation

Two MSCAs supported to classify piperonal as a reproductive toxicant Category 1B for both adverse effects on sexual function and fertility and on development.

One company did not support Repr. 1B classification for the following reasons:

- in the PNDT study (Anonymous, 2020b), they considered that the effects on the foetuses can be clearly linked to the marked reduction in food consumption and body weight gain of the females and that classification is not warranted based on this study.
- in the OECD TG 422 study (Anonymous, 2020a), they considered that the adverse effects on both the parents and offspring were likely linked to the same mechanism, which has yet to be identified.

They considered that whilst it is highly likely that this mechanism is not relevant to humans, this conclusion cannot be made with absolute certainty, but that there is sufficient doubt about human relevance to classify in Category 2 instead of Category 1B.

One trade association did not support Repr. 1B classification as they considered that no definitive conclusion can be drawn on whether the effects on fertility and development seen in the OECD TG 422 study (Anonymous, 2020a) are direct reproductive toxicity effects, or whether they are secondary to systemic toxicity in the parents. They argued that 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. They also considered that there is doubt about the relevance to humans in the absence of an obvious mechanism of action and considered that human relevance of the effects is highly questionable.

The DS in their response to the company/importer and the trade association considered that the maternal toxicity in the high dose group of the PNDT study (Anonymous, 2020b) was slight and pointed out that developmental effects were already observed from the low dose level.
Regarding the OECD TG 422 study (Anonymous, 2020a), the DS replied that there were no effects on maternal body weight nor any evidence of severe maternal toxicity, while there was a decrease in post-implantation survival which was outside the historical control data (HCD) range and concluded that this supports that the effects were specific intrauterine effects rather than secondary to maternal systemic toxicity.

RAC agrees with the DS responses. RAC also notes that there is no biological plausibility nor empirical support to substantiate that the fertility and developmental outcomes in the OECD TG 422 study (Anonymous, 2020a) are secondary to a specific mechanism and no data that raises doubt about the human relevance of those effects are available (refer to the section of this opinion Supplemental information - In depth analyses by RAC).
RAC is of the opinion that the OECD TG 422 study provides clear evidence of adverse effects on fertility in the absence of marked systemic toxicity. Therefore, the fertility outcomes are not considered to be non-specific consequences of the parental toxic effects. There are no mechanistic data to support that the effects on sexual function and fertility are not relevant to humans. The detailed assessment is provided under "Assessment and comparison with the classification criteria", below.

Assessment and comparison with the classification criteria

One GLP-compliant OECD TG 422 (Combined repeated dose toxicity study with the reproduction/developmental toxicity screening test) study (Anonymous, 2020a) and one GLP-compliant OECD TG 414 (PNDT) study (Anonymous, 2020b) both performed in Wistar rats are available. A summary of a non-guideline study (Vollmuth, 1990), where SD rat females were exposed via gavage to up to 1 000 mg/kg bw/d piperonal from 7 days before mating to lactation day 4, is not considered reliable due to the poor reporting of the methods and results.

Adverse effects on sexual function and fertility

In the OECD TG 422 study (Anonymous, 2020a), 10 Wistar CrLl. WI (Han) rats per sex per dose were administered 0, 100, 300 or 1 000 mg/kg bw/d of piperonal for a minimum of 13 weeks including a 10-week premating period. Females were treated during the 10-week pre-mating period and throughout the 2-week mating period, the gestation period and the lactation periods, until post-natal day (PND) 13. Males were treated during the 10-week pre-mating period and 2-week mating period.

Parental systemic toxicity

There were no test substance related deaths, and no significant clinical signs recorded for either sex.

At 1 000 mg/kg bw/d, absolute body weights in males were slightly decreased from day 22 onwards reaching statistically significance from day 43 (-9 % and -12 % compared with controls at the end of the premating period and at sacrifice, respectively) while food consumption was not affected.

In high-dose females, no effects on body weight, body weight gain and food consumption were noted during the pre-mating and the mating periods. From post-coitum day 14 onwards, a lower mean body weight and body weight gain were observed in high-dose females. However, at this dose level, the four pregnant females had abnormal pregnancies (only one female bearing a single foetus), which compromises direct comparison to concurrent controls.

Among other parental effects were:

an increase of trabecular bone (i.e. hyperostosis in sternum and femur) observed in 300 mg/kg bw/d females (2/10 up to slight, considered non-adverse in the full study report) and in 1 000 mg/kg bw/d rats (9/10 males and all females, up to moderate severity; considered adverse in the full study report), and thymus effects in high-dose males (minimal lymphoid atrophy and decreased weight; considered non-adverse in the full study report) and in high-dose females (epithelial hyperplasia, up to moderate in severity, and increased weight; considered non-adverse in the full study report).

(for other effects, please also refer to "Supplemental information - In depth analyses by RAC")

Adverse effects on sexual function and fertility

In the high-dose group, two males (No. 34 and 38) failed to mate after 14 days of pairing (male mating index 80 %). Each non-mated female was re-cohabited with a male of proven fertility of the same group and were proven to be mated (female mating index 100 %).

The number of pregnant females was 10, 9, 9 and 4 leading to fertility indices of 100, 90, 90 and 40 % for the control, 100, 300 and 1 000 mg/kg bw/d groups, respectively.

The mean number of implantation sites was severely decreased at 1 000 mg/kg bw/d (2.3 vs 12.4 in controls) since all the 4 pregnant females in this group had a very low number of implantation sites (1, 1, 2 and 5).

There was no effect on testis weights or indication of abnormal spermatogenesis at microscopic examination and sperm parameters were not investigated (not required in OECD TG 422). However, at 1 000 mg/kg bw/d, absolute prostate weights (0.743 g vs. 0.956 g in controls; -22 %) and epididymides weights (0.959 g vs. 1.205 g in controls; -20 %) were statistically significantly decreased. The relative weights of these organs were also decreased without reaching statistically significance. Prostate and epididymis weights are considered sensitive endpoints for detecting adverse effects of chemicals on male fertility (Mangelsdorf, 2002). One of the two males, which failed to mate, exhibited bilateral germ cell debris and bilateral germ cell degeneration in the testis. The other had bilateral sperm granuloma in the epididymides. A third male had bilateral germ cell debris in the epididymal lumen. There are very few sloughed germ cells and cell debris in the normal adult rat epididymis (OECD GD 106 part 2), making this endpoint a sensitive indicator of testicular toxicity (Foley, 2001).

While oestrous cyclicity was not affected by treatment, at 1 000 mg/kg bw/d, there were statistically significant increases in absolute (+55 %) and relative (+87 %) ovary weights and absolute (+164 %) and relative (+208 %) uterus weights. No associated histopathology changes were recorded. It is noteworthy that high-dose females were in different stages of the oestrous cycle at sacrifice while the control females were all in lactating dioestrous stage. The increased ovary and uterus weights in high-dose females could therefore reflect the difference in physiological status. However, in the absence of HCD for non-lactating dams, a treatment related cannot be totally excluded.

Table: *Selected reproductive parameters in Anonymous, 2020a*

	0 mg/kg bw/d	**100 mg/kg bw/d**	**300 mg/kg bw/d**	**1 000 mg/kg bw/d**
Females/Males paired	10	10	10	10
Females/Males mated	10	10	10	10
Pregnant females	10	**9**	**9**	**4**
Females with implantations only	0	1	0	3
Females with total litter loss on day 1	0	0	0	1
Females with living pups on day 1	10	8	9	0
Male mating index (%) (Males mated / Males paired) * 100	100	100	100	**80**
Female mating index (%) (Females mated / Females paired) * 100	100	100	100	100
Fertility index (%) (Pregnant females / Females mated)	100	**90**	**90**	**40**
Gestation index (%) (Females with living pups on day 1 / Pregnant females) * 100	100	89	100	**0**
Mean ± SD duration of gestation No. dams	21.8±0.4 10	21.3±0.5 8	21.7±0.7 9	22.0±-- 1
Mean ± SD implantation sites No. dams	12.4±2.8 10	12.2±1.9 9	12.3±1.9 9	**2.3±1.9** **4**

No statistics were applied for indices and mean number of implantation sites

In the non-guideline study (Vollmuth, 1990), a decrease in the fertility index was reported at 1 000 mg/kg bw/d while systemic toxicity (decreased body weight gain and clinical signs) was noted from 500 mg/kg bw/d. RAC, in accordance with the DS, considers this study as non-reliable due to the very poor reporting and the unknown purity of the tested material.

Comparison with the criteria

There are no human data to support classification in Category 1A.

In accordance with Annex I to the CLP Regulation, the classification of a substance as Category 1B reproductive toxicant '… *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'*.

RAC considers that the OECD TG 422 study (Anonymous, 2020a) provides clear evidence of adverse effects on sexual function and fertility. Piperonal exposure resulted in a severe decrease of the fertility index (40 % in the high-dose group vs 100 % in controls) as well as a severe decrease of the number of implantations in the 4 pregnant females of the high-dose group (2.3 vs 12.4 in controls). As a result, none of the high-dose couples was able to produce healthy offspring.

The other altered reproductive endpoints at the high-dose level consisted of decreased prostate and epididymis weights as well as bilateral germ cell debris or sperm granuloma in three high-dose males, two of them failing to mate, while in high-dose females, increased ovary and uterus weights were noted. RAC notes that from the available data it is not possible to determine whether the decreased fertility index and the decreased number of implantation sites are due to

effects on the females, the males or both (i.e. decreased number of oocytes ovulated and/or failed fertilisation of those oocytes due to sperm alteration and/or altered implantation). RAC also notes that identifying whether the decreased fertility it is due to effects in females, males or both is not needed in order to conclude on the classification.

The effects on fertility occurred in the presence of slight systemic toxicity in males (9 % decreased body weight at the end of the premating period) while the mean bodyweight of females was not altered. Regarding the effects observed in bones (increased trabecular bones in males and females) and in the thymus (epithelial hypertrophy in females), RAC considers that there is no biological plausibility nor empirical support to substantiate that such effects caused the fertility outcomes. On the contrary, the bone and thymus effects may be secondary to reproductive system alteration (oestrogenic toxicity) (Refer to "Supplemental information - In depth analyses by RAC").

In conclusion, RAC notes the clear, severe adverse effects on sexual function and fertility observed in rats after exposure to piperonal, which were not secondary to paternal toxicity. There are no compound-specific mechanistic data to support that the effects on sexual function and fertility are not relevant to humans. Regarding toxicokinetic considerations, RAC considered that the putative involvement of glycine depletion as the unique cause of the observed effects has not been substantiated (see for further details in the section RAC general comment at the start of this opinion).

Therefore, in line with the DS proposal, **RAC concludes that classification in Category 1B is warranted for adverse effects on sexual function and fertility.**

Adverse effects on development

In the PNDT study (Anonymous, 2020b), 22 Wistr CrLl. WI (Han) female rats per dose were administered 0, 100, 300 or 1 000 mg/kg bw/d of piperonal via oral gavage from gestation day (GD) 6 to GD20; no deaths occurred at any dose level. Clinical signs consisted of salivation from GD1 onwards, piloerection from GD7 (2/22 and 15/22 in mid- and high-dose groups, respectively) and hunched posture from GD13-17 (2/22 animals in mid- and high-dose groups).

At 1 000 mg/kg bw/d, there was a statistically significant decrease in mean body weight gain throughout the treatment period (-17 % compared to controls on GD21, see Table 16 of the CLH report). However, there were no effects on the final mean maternal body weight or on the mean body weight gain corrected for gravid uterus. At 1 000 mg/kg bw/d, there was a statistically significant decrease in food consumption during GD6-9 (22 % below control values). From GD9, mean absolute food intake recovered and was above control values up to termination.

Table: *Dam body weight in Anonymous, 2020b*

	0 mg/kg bw/d	**100 mg/kg bw/d**	**300 mg/kg bw/d**	**1 000 mg/kg bw/d**
No. females with foetuses	21	20	20	20
Final body weight (g) Mean ± SD	319±15.5	318±23.7	320±23.5	307±34.9
Mean gravid weight (g) Mean ± SD	75.3±9.0	72.6±18	74.0±10.3	**61.6±18.5**
Corrected body weight gain (g) Mean ± SD	28.6±6.0	26.4±7.8	29.6±7.6	26.5±10.6

Two high-dose females and one mid-dose females had total resorption (implantation sites only), considered as treatment-related in view of rare occurrence of a 100 % resorption of implantations in HCD (4 out of 1 094 litters). The mean percentage of post-implantation loss increased in a dose-related manner from the mid-dose reaching toxicological significance at the high-dose (3.4-fold higher than controls). This was mainly driven by a significant increase of early resorption at this dose level (Table 17 of the CLH report). Five out of 22 high-dose females had an early

resorption rate that was higher than the concurrent control. Consequently, a lower mean litter size was recorded (9.4 vs 10.6 in controls) at this dose level.

Table: *Post-implantation loss in Anonymous, 2020b*

	0 mg/kg bw/d	**100 mg/kg bw/d**	**300 mg/kg bw/d**	**1 000 mg/kg bw/d**
No. pregnant females	21	20	21#	22
Females with implantations only	0	0	**1**#	**2**
Post-implantation loss % Mean ± SD No. dams	4.3±4.6 21	3.6±4.6 20	8.5±21.6# 21	**14.9±28.7** 22

Note: data in this table includes female A052 with 100 % implantation loss (number of implantations not documented). The study author did not include this female in the calculation leading to a value of 3.9±5.25, N = 20.

No statistics were applied for data on early and late resorptions and post-implantation loss.

HCD of the test laboratory (2014-2018): post-implantation loss (mean; 5.1 %, percentile range=P5-P95 range; 1.9 %-10.1 %).

From 100 mg/kg bw/d, there was a dose-related trend towards lower foetal weights. At 1 000 mg/kg bw/d, the mean foetal body weight was markedly and statistically significantly decreased (3.9 g vs. 5.3 g in the control group).

At 1 000 mg/kg bw/d, a statistically significant increase of total skeletal malformations was noted (15.0 % per litter vs. 1.6 % in the control group), which were localised in the thoracic region. The malformations consisted of vertebral anomaly with or without associated rib anomaly (i.e. rib anomaly, vertebral centra anomaly, sternoschisis and costal cartilage anomaly). In addition, several types of variations (considered as grey zone anomalies in DevTox.org database) also affecting the thoracic region were recorded, some of them increased in incidence from 300 mg/kg bw/d (see table below showing a continuum of thoracic anomalies. The incidences of these variations were statistically significantly above those of the concurrent controls and/or exceeded the HCD range. Finally, increased incidences of variations indicating delayed skeletal ossification were noted from 100 mg/kg bw/d for unossified metacarpal and/or metatarsal bones, from 300 mg/kg bw/d for reduced ossification of the skull and at 1 000 mg/kg bw/d for reduced ossification of sternebrae and vertebral centra and arches.

Regarding visceral abnormalities, the only visceral malformations were observed in a single foetus at 1 000 mg/kg bw/d (malpositioned testis and small kidney). Treatment-related variations affecting the urinary tract occurred in one foetus at 300 mg/kg bw/d (dilated and convoluted ureter) and 4 foetuses in 3 litters at 1000 mg/kg bw/d (dilated and/or convoluted ureters, absent or small renal papilla).

Table: *Incidence of skeletal abnormalities in Anonymous, 2020b*

		0 mg/kg bw/d	**100 mg/kg bw/d**	**300 mg/kg bw/d**	**1 000 mg/kg bw/d**	**HCD % per litter basis** Mean (P5-P95)
M Skeletal malformations	No. Foetus/Litter % per Litter	2/2 1.6±5.01	2/2 1.8±5.67	2/2 1.8±5.67	**20/7** **15.0±25.17***	-
M Vertebral anomaly (with or without rib anomaly)	No. Foetus/Litter % per Litter	0/0 0±0	0/0 0±0	0/0 0±0	**5/3** **5.1±14.50**	0.3 (0.0-1.6)
M Vertebral centra anomaly	No. Foetus/Litter % per Litter	0/0 0±0	0/0 0±0	0/0 0±0	**4/2** **3.7±12.12**	0.0 (0.0-0.4)

M Rib anomaly	No. Foetus/Litter % per Litter	0/0 0±0	0/0 0±0	0/0 0±0	**5/5** **4.7±8.19***	0.1 (0.0-1.0)
M Sternoschisis	No. Foetus/Litter % per Litter	0/0 0±0	0/0 0±0	0/0 0±0	**3/3** **3.0±7.11**	0.1 (0.0-0.8)
M Costal cartilage anomaly	No. Foetus/Litter % per Litter	0/0 0±0	0/0 0±0	0/0 0±0	**1/1** **1.1±4.59**	0.1 (0.0-0.5)
V Bent ribs ***GZ***	No. Foetus/Litter % per Litter	32/13 27.6±28.1	31/14 28.5±26.7	**66/17** **61.3±36.1****	**54/14** **48.4±36.8**	13.7 (2.1-25.8)
V 7th cervical full rib ***GZ***	No. Foetus/Litter % per Litter	2/2 1.9±6.02	0 0±0	1/1 1.0±4.47	**21/8** **16.2±27.7***	0.4 (0.0-1.7)
V 7th cervical ossification site ***GZ***	No. Foetus/Litter % per Litter	6/4 5.4±14.0	5/5 4.7±8.3	**13/9** **11.4±17.2**	**35/16** **32.8±25.6****	3.8 (0.0-8.7)
V 14th full rib ***GZ***	No. Foetus/Litter % per Litter	8/4 8.3±20.1	13/7 12.7±20.5	6/5 5.5±10.8	**20/9** **21.0±27.9**	6.3 (0.7-12.1)
V Pelvic girdle (Caudal shift) ***GZ***	No. Foetus/Litter % per Litter	8/4 7.3±18.0	9/5 8.9±17.4	7/4 5.9±15.6	**29/15** **28.1±23.6****	5.9 (1.9-12.3)
V Malaligned sternebrae ***GZ***	No. Foetus/Litter % per Litter	26/17 22.9±16.0	22/15 24.5±22.7	27/16 25.3±17.3	**41/17** **39.8±26.1**	7.9 (#)
Reduced ossification						
V Reduced ossification of the skull ***GZ***	No. Foetus/Litter % per Litter	28/13 23.5	25/9 21.8	**60/17** **46.8***	**64/19** **57.3****	8.5 (0.0-18.8)
V Metacarpal and/or Metatarsal, unossified ***GZ***	No. Foetus/Litter % per Litter	1/1 1.0	**6/6** **5.5***	**16/9** **13.0****	**97/19** **87.3****	3.3 (0.0-12.4)
V Vertebral centra	No. Foetus/Litter % per Litter	1/1 0.8	0/0 0.0	2/1 1.4	**32/15** **31.4****	0.8 (0.0-3.2)
V Vertebral arches	No. Foetus/Litter % per Litter	0/0 0.0	1/1 1.0	0/0 0.0	**14/7** **12.6****	0.1 (0.0-1.1)
V Sternebrae 1, 2, 3 and/or 4	No. Foetus/Litter % per Litter	0/0 0.0	0/0 0.0	0/0 0.0	**8/7** **6.9****	0.1 (0.0-0.8)
V Sternebrae 4 and/or 6	No. Foetus/Litter % per Litter	0/0 0.0	0/0 0.0	0/0 0.0	**51/16** **44.8****	0.4 (0.0-2.5)

M: malformations, **V**: variations according to the study author, ***GZ:*** *Grey Zone in the DevTox.org database (i.e. no consensus on whether they should be considered as variations or malformations)*

*p < 0.05, **p < 0.01; Percentile range = P5-P95; HCD; Rat Crl: WI (Han) on GD21, Study Date Range: 2014 – 2018
Number (No.) of foetuses/litters examined=6219; Number of studies = 49
insufficient historical control data available

In the OECD TG 422 (Anonymous, 2020a), among the four pregnant females at 1 000 mg/kg bw/d, only one female delivered one pup, found dead at PND1. Consequently, developmental data from PND1 is only available for females in the control, low- (100 mg/kg bw/d) and mid-dose (300 mg/kg bw/d) groups.

Post-implantation survival index (total number of offspring born as percentage of total number of implantation sites) was decreased in a dose-related manner (94, 85, 81 and 11 % for the control, low-, mid- and high-dose groups, respectively). The values at the mid- and high-dose levels were outside the HCD range of the laboratory (mean = 92; P5–P95 = 83–98). Consequently, the mean litter size on PND1 in mid-dose females was lower than that of controls (-17 %), see table below.

Postnatal survival at the low- and mid-dose levels was not affected by treatment.

At PND1, mean pup weight was decreased (-10 %) at low- and mid-dose levels.

There were no significant differences across groups as regards to pup's body weights (PND4 or PND13), T4 levels, anogenital distance or areola/nipple retention.

Table: *Selected developmental endpoints in Anonymous, 2020a*

	0 mg/kg bw/d	**100 mg/kg bw/d**	**300 mg/kg bw/d**	**1 000 mg/kg bw/d**
No. females with implantations	10	9	9	4
No. females with pups	10	8	9	**1**
Total number of implantation sites	124	110	111	**9**
Total number of offspring born	117	93	**90**	**1**
Number of live offspring on day 1 after littering	117	93	**87**	**0**
Post-implantation survival (%)	94	85	**81**	**11**
Post-implantation loss, pups born (%) #	6.6±8.9	16.6±32.1	**17.7±21.3**	**95±10**
Post-implantation loss, live pups on PND1 (%) ##	6.6±8.9	16.6±32.1	**20.1±24.5**	**100**
Mean litter size at PND1	11.7±2.3	11.6±1.1	**9.7±2.7**	**0**
PND1 mean BW Males	6.6±0.4	6.1±0.5	6.1±0.6	**-**
PND1 mean BW Females	6.5±0.5	5.8±0.4*	5.9±0.6*	**-**
PND1 mean BW Males + Females	6.5±0.4	6.0±0.4*	6.0±0.6	**-**

*p < 0.05; No statistics were applied for data on post-implantation loss calculated as mean % of (number of implantation sites - number of live foetuses)/ number of implantation sites × 100

#Calculated from individual data considering number of born pups.
Post-implantation loss = (No. of implant sites – No. of born pups) × 100 /No. of implant sites
##Calculated from individual data considering number of live pups at first check (i.e. PND1)
Post-implantation loss = (No. of implant sites – No. of live pups) × 100 /No. of implant sites

Comparison with the criteria

There are no human data to support classification in Category 1A.

In accordance with Annex I to the CLP Regulation, the classification of a substance as Category 1B reproductive toxicant '*…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'*

According Annex I: 3.7.1.4 of CLP Regulation, the major manifestations of developmental toxicity include (1) death of the developing organism, (2) structural abnormality, (3) altered growth, and (4) functional deficiency.

RAC considers that the available data provide clear evidence of adverse effects on development:

(1) Death of the developing organism: Piperonal exposure induced dose-related increases of post-implantation-loss in the two reliable studies resulting in decreased mean litter sizes from 300 mg/kg bw/d in the OECD TG 422 study (Anonymous, 2020a) and at 1 000 mg/kg bw/d in the PNDT study (Anonymous, 2020b).

(2) Structural abnormality: In the PNDT study (Anonymous, 2020b), exposure to piperonal 1 000 mg/kg bw/d induced a statistically significant increased incidence of skeletal malformations localised in the same thoracic region (each malformation exceeding the HCD range) while the incidence of thoracic skeletal variations was increased from 300 mg/kg bw/d showing a continuum of thoracic anomalies. Increased incidence of variations indicating delayed skeletal ossification were noted from 100 mg/kg bw/d (unossified metacarpal and/or metatarsal), from 300 mg/kg bw/d (reduced ossification of the skull) and at 1 000 mg/kg bw/d only (reduced ossification of sternebrae and vertebral centra and arches).

(3) Altered growth: Dose-related decreased foetal weights and delayed skeletal ossification were noted from 100 mg/kg bw/d onwards in the PNDT study (Anonymous, 2020b) and mean birth weight was decreased (-10 %) at the low- and mid-dose levels in the OECD TG 422 study (Anonymous, 2020a).

Concerning the potential role of maternal toxicity on the effects observed:

- In the PNDT study (Anonymous, 2020b), effects on development of lower concern (the skeletal variations, decreased foetal weight and delayed ossification) were noted in the absence of maternal toxicity. RAC notes that the effects of major concern (i.e. post-implantation loss and specific skeletal malformations) were mainly observed at the highest dose level in the presence of slight maternal toxicity.
- High-dose females in the PDNT study exhibited lower mean food consumption compared to controls on GD6-9 (absolute 22 % and relative 19 % below controls, respectively; s.s.), and a statistically significant increase in relative food consumption on GD12-15 (10 % above controls) and GD15-18 (11 % above controls).
- There was a statistically significantly lower body weight gain in high dose on GD9-21 (48 % in controls vs. 40 % in high dose on GD21, see table 16 in the CLH report) while corrected body weight gain was not affected. RAC, however, notes that feed restriction studies in pregnant rats has shown that from 50 % less corrected maternal body weight gain up to 15 % maternal gestational body weight loss did not cause embryo-lethality or skeletal malformations (Fleeman, 2005; Nitzsche, 2017). Dose-related decreases in maternal T3 (43.3 in control vs. 28.3 ng/dL in high dose) and T4 (2.35 in controls vs. 1.71 µg/dL in high dose). According to the study authors, several of the total T3 values across all dose groups and the total T4 values in the high dose were below LLOQ and reported as LLOQ/2. There was no effect observed on absolute or relative thyroid weight.
- In the OECD TG 422, post-implantation loss was increased in a dose-related manner from the low dose level where no maternal toxicity was seen.

Therefore, RAC considers the developmental effects not to be secondary non-specific consequences of other toxic effects.

In addition, RAC concluded that there is no compound-specific mechanistic data to support that the effects on development are not relevant to humans. Additionally, the putative involvement of glycine depletion at high dose levels as the unique cause of the observed effects, as put forward by the trade association after the consultation, has not been substantiated. Furthermore,

some developmental effects were already observed from the low dose level (see for further details in the section RAC general comment at the start of this opinion).

Therefore, in line with the DS proposal, **RAC concludes that classification in Category 1B for adverse effects on development is warranted.**

Lactation

In the OECD TG 422, there was no indication of impaired pup viability or adverse effect on the offspring up to 300 mg/kg bw/d. Therefore, in line with the DS proposal, **RAC concludes that no classification is warranted for effects on or via lactation in the absence of relevant data.**

Based on the available data, **RAC concludes that classification of piperonal as Repr. 1B; H360FD is warranted**.

Supplemental information - In depth analyses by RAC

Fertility outcomes vs. other toxic effects in the OECD TG 422 study (Anonymous, 2020a).

Table: *Overview of findings, individual data females*

Dose level (mg/kg bw/d)	Female No	Pregnancy status Implantation sites	Post-implantation loss	Oestrous stage at sacrifice	Thymus epithelial hyperplasia	Trabecular bones	
						Sternum	Femur
100	51	NP		E	-	-	-
	60	10	100 %	P	-	-	-
300	61	10	50 %	Lactating	+	-	-
	66	14	57 %	Lactating	-	-	-
	68	NP		P	++	-	++
	69	16	50 %	Lactating	-	-	-
1 000	71	NP		E	+	++	+++
	72	NP		E	+	+	+++
	73	NP		M	+	++	+++
	74	1	100 %	P	+++	++	+++
	75	NP		P	-	++	++
	76	1	100 %	P	++	++	+++
	77	NP		M	++	++	++
	78	2	100 %	Mu ++++	++	++	+++
	79	5	80 %	Mu +++	++	++	+++
	80	NP		E	+	++	+++

NP: non-pregnant
+: minimal; ++: slight; +++: moderate; ++++: marked
P: prooestrous; E: oestrous; M: metoestrous; Mu: Increased mucification

Table: *Overview of findings, individual data males*

Dose level (mg/kg bw/d)	Male No	Successful mating	Mated females	Epididymis	Thymus lymphoid atrophy	Trabecular bones	
						Sternum	Femur
100	11	Y	51 (NP)	-	-	-	-
	20	Y	60	-	-	-	-
300	21	Y	61	Not examined	-	-	-
	26	Y	66	Not examined	-	-	-
	28	Y	68 (NP)	-	-	-	-
	29	Y	69	Not examined	-	-	-
1 000	31	Y	71 (NP) & 74	-	+	-	-
	32	Y	72 (NP) & 78	-	+	+	+
	33	Y	73 (NP)	-	-	+	++
	34	N		Cell debris + (testis)	+	+	+++
	35	Y	75 (NP)	-	-	-	++
	36	Y	76	-	-	+	++
	37	Y	77 (NP)	-	+	+	+++
	38	N		Sperm granuloma ++	-	+	++
	39	Y	79	-	-	-	++
	40	Y	80 (NP)	Cell debris +	-	+	++

NP: non-pregnant; Y: yes; N: no
+: minimal; ++: slight; +++: moderate; ++++: marked

Trabecular bone

Increased trabecular bone (i.e. hyperostosis in sternum and femur) was observed in 300 mg/kg bw/d females (2/10 up to slight) and in 1 000 mg/kg bw/d rats (9/10 males and all females, up to moderate) slightly diminishing the spaces for the bone marrow. However, there was no impact in haematology parameters or haematopoiesis in other organs and therefore RAC considers the increased trabecular bone of doubtful adversity.

Regarding the causes of such lesions, increased bone results from either decreased resorption of bone by osteoclasts or increased bone formation by osteoblasts. According to the NTP Nonneoplastic Lesion Atlas, increased bone is rarely observed as a treatment-related effect, an increased amount of bone is an occasional background lesion seen in aged F344 rats, with females more commonly affected than males. In rats, oestrogen toxicity results in decreased bone resorption from metaphyseal trabeculae, leading to densely thickened medullary bone. Treatment-related increased trabecular bone has been observed in adult female Wistar rats treated with 17β-oestradiol (Thobias et al., 1991).

RAC notes that fertility impairment was observed in high-dose animals also exhibiting increased trabecular bone. However, the correlation between the incidences of increased trabecular bones with the observed effects on fertility is not a causal proof that these changes are secondary to systemic toxicity. On the opposite, the bone effects may be secondary to reproductive system alteration (oestrogenic toxicity).

Thymus

Table: *Thymus effects in males and females*

	Males				Females			
Dose level (mg/kg bw/d)	0	100	300	1 000	0	100	300	1 000
No. of animals	10	10	10	10	10	10	10	10
Thymus weight difference from controls								
Absolute	-	8	-12	- 32**	-	18	41**	45**
Relative to body weight	-	7	-8	- 20*	-	24	47**	75**
Lymphoid atrophy								
Minimal	-	-	-	4	2	2	-	1
(Cystic) Epithelial hyperplasia								
Minimal	3	1	-	-	3	2	2	4
Slight	-	-	-	-	-	1	1	4
Moderate	-	-	-	-	-	-	-	1

*:P < 0.05, **: P < 0.01

Minimal lymphoid atrophy in the thymus of 4 males was recorded in relation with a lower thymus weight. In view of the low severity, RAC considers those effects as non-adverse. Furthermore, based on the individual data there is no evidence of a relationship between these findings and fertility impairment.

In high-dose females, increased incidence and severity of epithelial hyperplasia in the thymus (9/10 up to moderate compared to 3/10 minimal in controls) were recorded and were associated with increased thymus weights.

Epithelial hyperplasia is often associated with involution and/or atrophy of the thymus and may occur at relatively high incidences in some rat strains such as the Wistar rats (NTP Nonneoplastic Lesion Atlas). A variety of factors modulate the proliferative and secretory activity of medullary thymus epithelial cells (TEC). For example, TECs are stimulated by oestrogen and inhibited by testosterone (goReni, version 3). Treatment-related epithelial hyperplasia has been observed with diethylstilbesterol administration in mice and epithelial cyst formation has been recorded in rats treated with exogenous oestrogen (Pearse, 2006).

RAC notes that fertility impairment was observed in high-dose females also exhibiting increased epithelial hyperplasia in the thymus. Despite the correlation, a causal relationship between thymic effects and the failure of pregnancy is not established. On the contrary, increased incidence and severity of epithelial hyperplasia may be secondary to reproductive system alteration (oestrogenic toxicity) or may reflect the different physiological status of high-dose females (cycling) versus controls (lactating).

Other effects

In high-dose males, absolute and relative liver weights were increased (+21 % and +41 % compared to controls, respectively), which were associated with minimal hepatocellular hypertrophy (7/10) and slight alterations of clinical pathology parameters related to hepatic function. With respect to the low severity of the liver effects, RAC considers them an adaptive and reversible response.

Finally, there was a dose-related reduction in T4 levels observed in males, which is not considered adverse in the absence of significant changes in T3 and TSH levels, thyroid histopathology or thyroid weight.

Overall, RAC considers that the severe fertility impairments observed in the OECD TG 422 study (Anonymous, 2020a) study are not secondary non-specific consequences of other toxic effects.

There is no mechanistic data to support that the effects on sexual function and fertility are not relevant to humans.

Additional references

Additional references not included in the CLH report

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ANNEXES:

Annex 1 The Background Document (BD) gives the detailed scientific grounds for the opinion. The BD is based on the CLH report prepared by the Dossier Submitter.

Annex 2 Comments received on the CLH report, response to comments provided by the Dossier Submitter and RAC (excluding confidential information).