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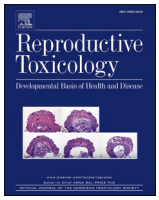

Statistical and experimental approaches to discriminate between substance-specific reproductive effects and those mediated by maternal undernutrition – A case study with Rosin and derivatives

Theo Le Godec *, Larry G. Higgins, Michael G. Penman, Michael McMahon

Penman Consulting bvba, Rue du Trône 108, Brussels 1050, Belgium

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ABSTRACT

It is well-accepted that undernutrition lowers fertility of rodents and retards growth of their offspring. However, accounting for undernutrition's confounding effects in reproductive toxicology studies remains challenging. Rosin (EC 232–475–7), a substance derived from pine trees, and fourteen of its derivatives were tested in OECD test guideline 422 studies. Dietary exposure to several of the substances lowered ovulation rate and retarded offspring growth. However, the substances proved unpalatable and rats consumed lower amounts of diet. The resulting undernutrition was suspected to be the cause of the reproductive effects. Consistent with this suspicion, the extent of undernutrition correlated with size of impact on reproduction. Furthermore, after statistically controlling for undernutrition, in general there was no evidence for a direct effect of the substances on offspring growth. A pair-fed feed restriction study confirmed the hypothesis that undernutrition was the sole cause of the developmental effect of dietary Rosin. Unexpectedly, dietary Rosin did not lower ovulation rate during this study and, so, the hypothesis that undernourishment is the sole cause of this reproductive effect remains unproven. However, results from a second investigative study suggest that reduced ovulation rate is a transient response to undernutrition. It is concluded that the reproductive effects of Rosin and derivatives are generally caused by undernutrition. The approaches reported here might be of use to other workers in the field and we suggest some possible improvements to the pair-fed restriction study design.

1. Introduction

That undernutrition affects reproduction is a settled fact. Indeed, nutritional status is the most important environmental factor governing the reproductive capacity of mammals [1–6]. Of course, whilst the *general* principle is beyond question, it might reasonably be asked whether undernutrition is the cause of reproductive effects seen in *specific* instances, such as those occurring during particular regulatory study designs, or with certain substances. Perhaps for this reason, the extent to which undernutrition and, especially, maternal undernutrition, confounds reproductive endpoints in the specific context of regulatory study designs remains a contentious issue that continues to elicit vigorous debate between regulators and toxicologists [7–9].

For developmental endpoints, the fact that the debate continues is particularly puzzling; feed restriction studies, amongst other approaches, have repeatedly confirmed the developmental impact of undernutrition in studies based on the OECD 414 test guideline or similar study design [7,10–19]. However, for sexual function and fertility, the impact of undernutrition has been little explored in the regulatory arena.

A few previous studies have reported the effect of undernutrition on fertility in circumstances relevant to regulatory toxicology. First, Chapin and colleagues used feed-restriction to model "non-specific toxicity" and study its impact on reproductive performance in adult Sprague Dawley rats [20]. They reported lower *Corpora lutea* numbers in feed-restricted animals and a transient prolongation of estrogenic cycle length.

Second, Terry and coworkers conducted a fertility and early embryonic development study in which Sprague Dawley rats were subjected to graded levels of feed restriction throughout a 2-week premating period, mating, and up to gestational day 7 [21]. Although mating behavior was unaffected, a subtle prolongation of estrogenic cycle length was observed in females offered approximately 75% of the food consumed by control animals. More obvious was a significantly lower mean *Corpora lutea* counts in these animals, indicating a lower

* Corresponding author.
E-mail address: h4r@penmanconsulting.com (T. Le Godec).

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ovulation rate.

Finally, although they did not report *Corpora lutea per se*, Ramirez-Lopez *et al*. reported that reducing caloric intake of females by only 20% during a two-week pre-pairing period sufficed to lower litter size [22].

Rosin (EC 232–475–7) is a bio-based Unknown or Variable composition, Complex reaction products or of Biological materials (UVCB) substance derived from pine trees. Comprised primarily of unsaturated resin acids, it is amenable to a few particular chemical reaction types, including esterification and Diels-Alder condensation reactions. As depicted in Fig. 1, these chemistries and others allow a diverse range of chemically related substances, falling into four categories, to be synthesized for use in various applications.

Since the early 2000's, the Hydrocarbon Resins, Rosin Resins and Pine Chemicals Materials REACH (H4R) Consortium (https://h4rconsortium.com/), which manages REACH compliance for these substances, alongside the Pine Chemicals Association (PCA, https://www.pinechemicals.org/), have commissioned sixteen GLP-compliant OECD 422 studies on fifteen related Rosin and derivatives substances. During these regulatory studies, dietary exposure to several, but not all, of these substances lowered female rat fertility (as shown by reduction of *Corpora lutea* numbers, Implantation Site counts, and Total Pups Born) and/or mildly retarded growth of pups. Here we report new analyses of these regulatory data, alongside more recent mechanistic studies, that collectively point to undernutrition as the cause of the reproductive effects.

Other endpoints beyond those relating to ovulation and offspring growth were evaluated during these studies; however, these are beyond the scope of the present paper.

2. Material and method

2.1. Overview of studies

The sixteen regulatory studies reported in this paper were designed to be compatible with the Organization for Economic and Co-operation and Development (OECD) test guideline No. 422 "Combined Repeated Dose Toxicity Study with the Reproduction / Developmental Toxicity Screening Test" (adopted 22nd March 1996) [23].

The two additional mechanistic studies conducted to investigate some of the findings of the original set of regulatory studies, a transiency/reversibility study and a pair-fed feed restriction study, were based on the OECD test guideline No. 421 "Reproduction / Developmental Toxicity Screening Test" (adopted 28th of July 2015) [24].

The regulatory studies were conducted to meet REACH data requirements over a considerable time frame and at three different test sites. The first two studies, reported in 2003/2004, were conducted at Inveresk (Edinburgh, UK) and used Sprague Dawley rats. The remaining fourteen studies reported between 2010 and 2017 were conducted at two separate test sites and used Han Wistar rats. The first of these two test sites, Harlan Laboratory (Itingen, Switzerland) conducted seven studies, all on H4R Category 1 or 3 substances. The remaining seven studies, all involving H4R Category 2 or 4 substances, were conducted at Envigo (Shardlow, UK).

The two additional mechanistic studies were conducted at Sequani (Ledbury, United Kingdom) and Charles River Laboratory (Ashland, United States) for the transiency/reversibility and the pair-fed feed restriction study, respectively.

All studies are listed in Table 1.

2.2. GLP compliance

Studies conducted in United Kingdom (UK) were performed in compliance with Good Laboratory Practice Regulations 1999, SI 1999 No. 3106, as amended by SI 2004 No. 994 [43]. Studies conducted in Switzerland were performed in compliance with the OECD Good Laboratory Practice regulations, ENV/MC/CHEM (98) 17 as specified in France as "article Annexe II à l'article D523–8 du Code de l'Environnement du 16 octobre 2007". Finally, the pair-fed feed restriction study, was conducted in accordance with the United States Code of Federal Regulations, Title 40, Part 792: Good Laboratory Practice Standards Environmental Protection Agency [44].

2.3. Animal welfare

Studies in the UK were designed and conducted to cause the minimum suffering or distress to the animals used and in accordance with UK policy on animal welfare and the requirements of the United Kingdom's Animals (Scientific Procedure) Act 1986. Those conducted in Switzerland complied with the Swiss Animal Protection Law. The pair-fed restriction study conducted in the USA was designed to comply with all applicable sections of the Final Rules of the Animal Welfare Act regulations (Code of Federal Regulations, Title 9), the *Public Health Service Policy on Humane Care and Use of Laboratory Animals* from the Office of Laboratory Animal Welfare, and the *Guide for the Care and Use of Laboratory Animals* from the National Research Council.

2.4. Chemicals and dosing

All test materials were obtained directly from the manufacturer. A full list of test material details are provided in Table 1.

Exposure to test material was achieved through dietary exposure in all the studies, apart from the OECD 422 performed with Resin acids and Rosin acids, fumarated, esters with pentaerythritol [39], which used oral gavage as a route of administration.

Dose levels were chosen based on the contemporaneous data available for each substance. Dose levels used for each OECD 422 study, and the single dose used for the mechanistic studies, are detailed in Table 1.

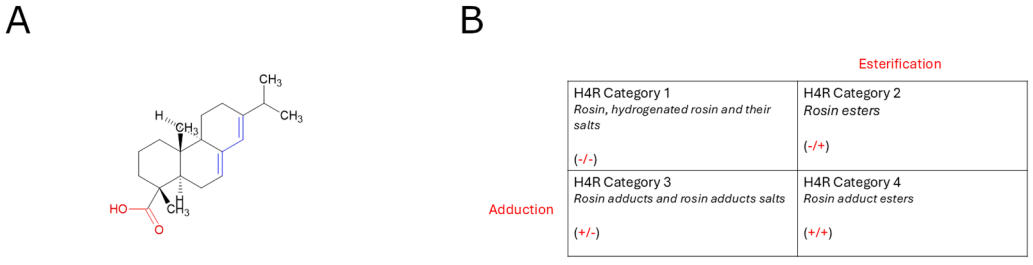

Fig. 1. Basic Structure of Rosin and Derivatives. Panel A depicts the structure of abietic acid, the prototypic Resin acid. Two reactive moieties are present, a carboxylic group (shown in red) and a conjugated diene (shown in blue). The carboxylic group can be esterified with various alcohols to produce Rosin esters, whereas the conjugated diene group can engage in Diels-Alder reactions with maleic acid anhydride or fumaric acid to produce what are known as adducted Rosins. Both types of reaction can occur in isolation or sequentially to produce the four broad categories of Rosin derivatives shown in panel B.

Table 1
Studies information.

Substance	EC#	H4R Category	Study design	Laboratory	Dose level	Reference
Rosin (Gum)	232–475–7	1	OECD 422	Harlan Laboratories	2500, 5000, 10,000 ppm (196.4, 346.8 and 635.9 mg/kg bw/day)	[25]
Rosin (Tall-oil)	232–475–7	1	OECD 422	Harlan Laboratories	2500, 5000, 10,000 ppm (204.3, 435.4 and 854.3 mg/kg bw/day)	[26]
Rosin, hydrogenated	266–041–3	1	OECD 422	Harlan Laboratories	1000, 3000, 10,000 ppm (82.7, 250.2, 788.8 mg/kg bw/day)	[27]
Rosin, oligomers	500–163–2	1	OECD 422	Harlan Laboratories	3000, 7500, 15,000 ppm (243.4, 578.4, 986.7 mg/kg bw/day)	[28]
Rosin, reaction products with formaldehyde	293–659–0	1	OECD 422	Harlan Laboratories	1000, 3000, 10,000 ppm (79.0, 226.5, 785.6 mg/kg bw/day)	[29]
Resin acids and Rosin acids, hydrogenated, Me esters[d]	232–476–2	2	OECD 422	Inveresk Research	5000, 10,000, 20,000 ppm (476.3, 914.9, 1617 mg/kg bw/day)	[30]
Resin acids and Rosin acids, esters with ethylene glycol	270–986–7	2	OECD 422	Envigo Research Limited	3000, 7500, 18,000/15,000 ppm (263.1, 717.2,1339 mg/kg bw/day)[a]	[31]
Resin acids and Rosin acids, esters with diethylene glycol	268–884–2	2	OECD 422	Harlan Laboratories	3000, 7500, 18,000/15,000 ppm (253.4, 382.5, 1675.6 mg/kg bw/day)[a]	[32]
Resin acids and Rosin acids, hydrogenated, esters with triethylene glycol	271–996–4	2	OECD 422	Envigo Research Limited	3000, 7500, 18,000/15,000 ppm (261.5, 680.9, 1485.2 mg/kg bw/day)[a]	[33]
Resin acids and Rosin acids, esters with glycerol	232–482–5	2	OECD 422	Harlan Laboratories	3000, 7500, 18,000/15,000 ppm (274.9, 637.9, 1376.8 mg/kg bw/day)[a]	[34]
Rosin, fumarated[d]	266–040–8	3	OECD 422	Inveresk Research	1000, 3000, 10,000 ppm (93.4, 260.2, 676.4 mg/kg bw/day)	[35]
Rosin, maleated	232–480–4	3	OECD 422	Harlan Laboratories	1750, 3500, 7000 ppm (135.5, 280.9, 299 mg/kg bw/day)[b]	[36]
Fatty acids, tall oil, oligomeric reaction products with maleic anhydride and rosin, calcium magnesium zinc salts	500–451–8	3	OECD 422	Harlan Laboratories	1000, 5000, 15,000 ppm (82.4, 434.2,1102 mg/kg bw/day)	[37]
Resin acids and Rosin acids, fumarated, esters with glycerol	307–051–0	4	OECD 422	Harlan Laboratories	3000, 7500, 18,000/15,000 ppm (245, 375.5, 1187.4 mg/kg bw/day)[a]	[38]
Resin acids and Rosin acids, fumarated, esters with pentaerythritol	305–514–1	4	OECD 422	Harlan Laboratories	30, 300, 1000 mg/kg bw/day by gavage	[39]
Resin acids and Rosin acids, maleated, esters with pentaerythritol	305–516–2	4	OECD 422	Envigo Research Limited	3000, 7500, 18,000/15,000 ppm (254.3, 570.3,1069.8 mg/kg bw/day)[a]	[40]
Rosin	232–475–7	1	Transiency/ Recovery Based on OECD 421	Sequani	10,000/5000 ppm (816.1, 729.3, 568.0 mg/kg bw/day for cohort 1, 2 and 3, respectively)[c]	[41]
Rosin	232–475–7	1	Pair-fed restriction Based on OECD 421	Charles River Laboratories	10,000/5000 ppm (599.4 mg/kg bw/day)[e]	[42]

Doses between brackets (expressed in mg/kg bw/day) are mean doses achieved for females throughout the study (including pre-pairing, gestation and lactation).
[a] The dietary concentration given to the high dose females during gestation and lactation was decreased to 15,000 ppm.
[b] In the high dose group (7000 ppm), four females were found dead during the pre-pairing period. Remaining females in this group were terminated on day 14 of the pre-pairing period due to significant reduction in food consumption, body weight loss and clinical signs indicating bad condition.
[c] Dietary concentration was halved from Day 20 of gestation and throughout lactation for females of cohort 1 and 2.
[d] This study did not collect *Corpora lutea* counts.
[e] Dietary concentration was halved from Lactation Day 1 until euthanasia for animals exposed to Rosin.

2.5. Animals

2.5.1. Regulatory OECD 422 studies

Sprague Dawley or Han Wistar rats were used in the OECD 422 studies. The source of the animals was recorded in the associated study reports. The animals were acclimatized, grouped and housed according to each laboratory's internal Standard Operating Procedures. All diets and water were available *ad libitum* throughout the study. Specific housing conditions and animal details are available in each study report and are also available in Robust Study Summarizes published at ECHACHEM. (https://chem.echa.europa.eu/).

2.5.2. Mechanistic studies

2.5.2.1. Transiency study. Males and females Crl:WI(Han) rats were supplied by Charles River (UK) Limited (Margate, Kent, CT9 4LT, England). On arrival, males were 7–8 weeks of age, and the females were 9–10 weeks old. All animals were found to be healthy during the examination conducted at reception. Males and females were provided at different ages on arrival to ensure sibling pairings were avoided in the mating period. After at least 19 days of acclimatization, all animals were re-examined and confirmed to be suitable for use in the study. On the first day of dosing, the males weighed 314–410 g, and the females weighed 195–261 g.

The animals were housed in groups, by sex, until pairing and for males post-pairing, in solid floor cages with appropriate bedding provided. Bedding was changed as often as was necessary to maintain hygiene. For pairing, one male and one female were housed together in grid-floor cages suspended over paper-lined trays. On confirmation of mating, the males were returned to the group cages, and the females were housed individually and with their litter in solid floor cages. Non-mated females were housed individually in solid floor cages.

The study room was illuminated to give a cycle of 12 h light and 12 h dark and was air-conditioned. The target ranges for temperature and humidity were 20 °C to 24 °C and 40–70%, respectively. Recorded values were within, or only marginally outside, these limits.

A fine ground rodent diet, VRF1, manufactured by Safe or sniff, supplied by Charles River (UK) Limited, (Margate, Kent, CT9 4LT, England) or sniff Spezialdiaten GmbH, (Ferdinan-Gabriel-Weg 16, DE-59494 SOEST, Germany), and mains tap water (in bottles) were freely available.

2.5.2.2. Pair-fed feed restriction study. Males and females Crl:WI(Han) rats were supplied from Charles River Laboratories Inc. (Raleigh, NC, USA). On arrival, animals were approximately 11–12 weeks old. All animals were found to be healthy during the examination conducted at reception. After at least 7 days of acclimatization, all animals were re-examined and confirmed to be suitable for use in the study. On the first day of dosing, the males weighed between 300 and 357 g, and females weighed between 148 and 193 g at the initiation of exposure.

Individual housing was required to adequately monitor individual food consumption (particularly for restricted-fed animals). Solid-bottom cages containing appropriate bedding material (Bed-O-Cobs® or other suitable material) were used. Non-test substance-treated animals' cages were spatially separated on the rack from the test substance-treated groups, on a separate rack.

The study room was illuminated to give a cycle of 12 h light and 12 h dark and was air-conditioned. The target ranges for temperature and humidity were 20 °C to 26 °C and 30–70%, respectively.

A PMI Nutrition International, LLC Certified Rodent LabDiet® 5002 was offered during acclimation and was used to prepare the test and control (basal) diets throughout the study. All groups received *ad libidum* food during the acclimatation period. Group 1 (Control group) and 2 (Test-item treated group) received *ad libidum* food during dosing period. Group 3 animals (feed restricted group) from Study Day 0 were offered diet daily based on the mean food consumption values noted for Group 2 on the same Study Day. Municipal tap water suitable for consumption was available *ad libidum* for all animals in all groups throughout the study.

2.6. Study design and parameters evaluated

2.6.1. OECD 422

The sixteen regulatory studies reported in this paper were conducted according to the OECD test guideline No. 422 (adopted 22nd March 1996) [23]. Note that in accordance with this now obsolete version of the guideline, pups and mothers were euthanized on Lactation Day (LD) 4, rather than on LD13 as recommended by the current version (adopted 25th June 2025) [45].

Each experimental group (Control, Low, Mid and High dose) started with at least 12 males and 12 females. Following the acclimatation period, both sexes were dosed for 2 weeks prior to mating. Dosing was continued in both sexes during the mating period (up to 17 days). Males were dosed after the mating period until at least 28 days of exposure. Females were dosed throughout gestation and at least up to day 4 post-partum.

The following were investigated in the parental (P) generation animals: clinical observations, body weight, food and water intake, clinical hematology and biochemistry parameters, fertility and mating performance, gestation and parturition, *Corpora lutea* (except for studies conducted at Inveresk laboratory), gestation and littering, organ weights, macroscopic and microscopic examination. The following parameters were investigated in pups (F1): external examination at first litter check and during lactation, sex ratios, righting reflex, clinical observations, and body weight.

2.6.2. Mechanistic studies

The study designs were based on the contemporaneous OECD 421 test guideline (adopted 28th July 2015) [24] with several modifications, as indicated below. Notably, pups and mothers were euthanized on LD4, rather than LD13, to align with the earlier regulatory study designs and permit *Corpora lutea* associated with gestation to be counted.

Both mechanistic studies used a single 10,000 ppm dietary dose of Rosin, the same dosage as the High-dose level for the earlier regulatory studies with this substance. The same batch of Rosin was used for both mechanistic studies.

2.6.2.1. Transiency study. Animals were allocated to five groups of 10 males and 10 females following a stratified body weight randomization procedure based on individual body weights recorded on arrival.

The animals were split into three cohorts as follows (Fig. 2):

- Cohort 1 (consisting of one control group and one treatment group): Animals were paired for mating two weeks after commencement of dosing (replicating the dosing regimen in the earlier regulatory studies). Males and females were dosed for 14 days before pairing, throughout pairing and until necropsy (males) or Day 4 of lactation (females). One group was dosed with 10,000 ppm Rosin continuously via the diet, the remaining group received basal diet only. From Day 20 of gestation (approximately exposure day 34) and throughout the lactation periods of the study, the dietary concentration of Rosin was halved for females to account for the increased food intake of lactating females and to achieve a more consistent achieved dose in terms of mg/kg/day over the duration of the study.
- Cohort 2 (consisting of one control group and one treatment group): To assess transiency of fertility effect, animals were paired for mating four weeks, rather than two weeks, after commencement of dosing for females (mating delayed allowing more time for undernourishment to resolve). Males and females were dosed for 14 or 28 days,

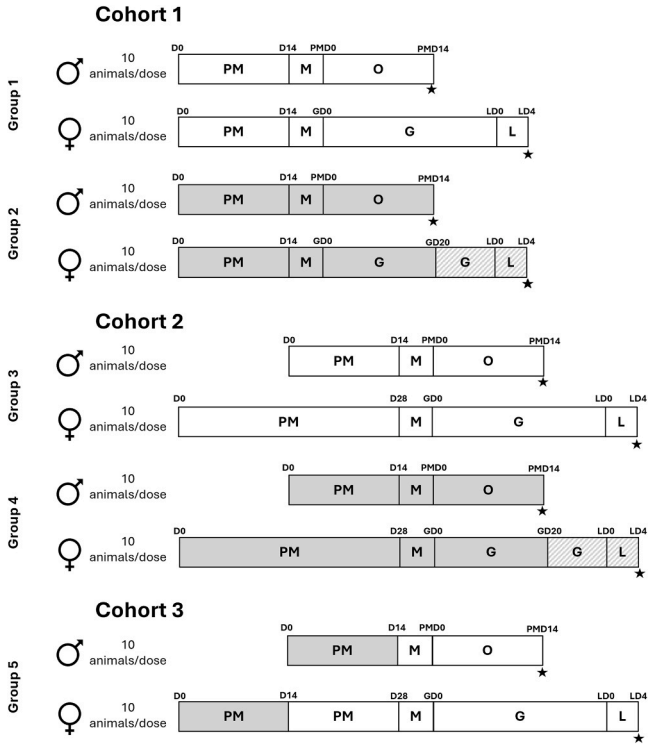

Fig. 2. Transiency Study Design. PM= pre-mating; M=Mating; O=Observation; L=Lactation; G=Gestation. *=Termination; D=Day; GD= Gestation Day; PMD= post-mating day; LD= Lactation Day. Gray= exposure to Rosin; Gray diagonal stripes = exposure to Rosin halved.

respectively, during the pre-pairing period, throughout pairing and until necropsy (males) or Day 4 of lactation (females). One group was dosed with 10,000 ppm Rosin continuously via the diet, the remaining group received basal diet only. From Day 20 of gestation and throughout the lactation periods of the study, the dietary concentration of Rosin was halved for females to account for the increased food intake of lactating females and to achieve a more consistent achieved dose in terms of mg/kg/day over the duration of the study.

- Cohort 3 (consisting of a single group): To assess reversibility of fertility effect, females were dosed for only the first two weeks of the four-week pre-pairing period and males were dosed for a two-week pre-pairing period before both males and females were switched to basal diet for the remainder of the study.

The following were investigated in the parental (P) generation animals: clinical observations, body weight, food intake, estrogenic cycles, fertility and mating performance, gestation and parturition, *Corpora lutea*, gestation and littering, organ weights, thyroid hormone assessment, macroscopic and microscopic examination, and ovarian follicle quantification. The following parameters were investigated in pups (F1): clinical observations, litter viability, sex ratio, body weight, anogenital distance and thyroid hormone assessment.

2.6.2.2. Pair-fed feed restriction study. Animals were assigned to three groups of 12 males and 12 females following a stratified body weight randomization procedure based on individual body weights designed to achieve similar group mean body weights.

Males and females in Group 1 were provided access to food *ad libitum*. Males in Group 2 were exposed to 10,000 ppm Rosin continuously via the diet for 14 days prior to mating and continuing throughout mating until euthanasia. Females in Group 2 were exposed to 10,000 ppm Rosin continuously via the diet for 14 days prior to mating and continuing throughout mating and gestation. From Lactation Day 1 until euthanasia, the dietary concentration of Rosin was halved for females to account for the increased food intake of lactating females and to achieve a more consistent achieved dose in terms of mg/kg/day over the duration of the study. Males and females in Group 3 were offered a restricted amount of basal diet, based on daily mean food consumption values for Group 2 (Fig. 3).

Study Day 0 for Group 3 was approximately 2 weeks following the start of dosing for Groups 1 and 2. Diet was offered daily, based on the daily mean food consumption values noted for Group 2 on the equivalent Study Day. During the breeding period, when males and females were housed in the same cage, the Group 3 animals were offered the combined amount consumed by Group 2 males and females during the interval prior to breeding and this amount was offered daily until evidence of mating was observed.

The following parameters and endpoints were evaluated in this study: mortality, clinical signs, body weights, body weight gains, food consumption, estrogenic cycles, reproductive performance, parturition, litter viability and survival, thyroid hormones, organ weights, and macroscopic and microscopic examinations.

2.7. Statistical analysis

Raw data, as provided in the final study reports appendices, were (re-)analysed by the authors of this paper.

2.7.1. Inter-group comparisons

For experiments with a single categorical variable, inter-group comparisons were conducted using ordinary one-way ANOVA with a Dunnett's multiple comparison post-test; or by Kruskal-Wallis with a Dunn's post-test. One-way ANOVA was used if the data in each experimental group were normally distributed, as evaluated by at least three of the following four tests (D'Agostino & Pearson; Anderson-Darling; Shapiro-Wilk; Kolmogorov-Smirnov); and if the ratio of the minimum to maximum standard deviation across all experimental groups was less than four. Otherwise, Kruskal-Wallis was used.

For experiments with two categorical variables, a full two-way ANOVA model was conducted to examine the effect of each categorical variable and the interaction between the two. Simple main effects of treatment were examined with Sidak's multiple comparison test.

All analyses were conducted using Graphpad Prism v10 (www.graphpad.com) with a Type I error rate (false positive – α) of 0.05.

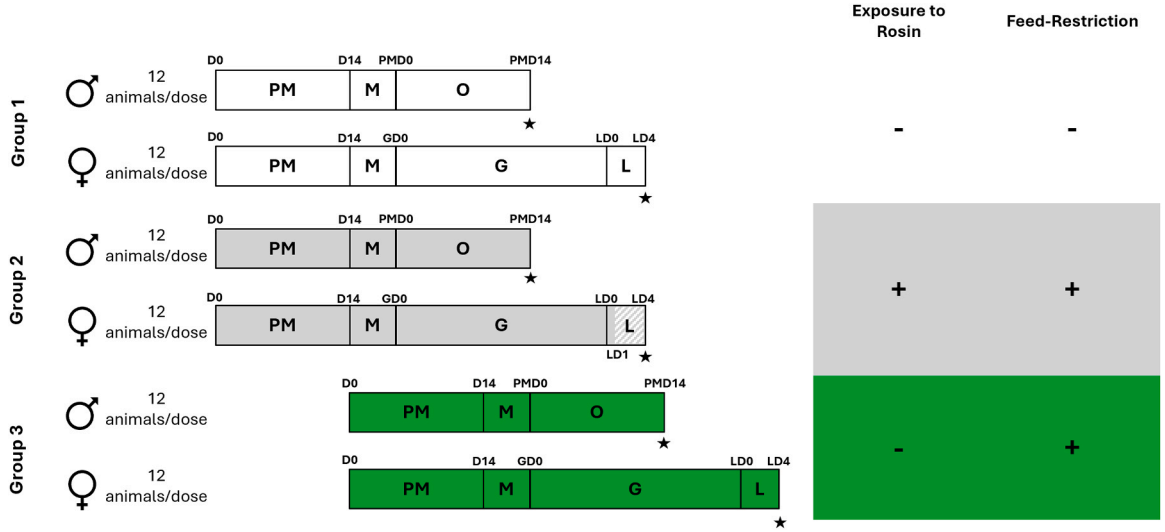

Fig. 3. Fed-Restriction Study Design. PM= pre-mating; M=Mating; O=Observation; L=Lactation; G=Gestation. *=Termination; D=Day; GD= Gestation Day; PMD= post-mating day; LD= Lactation Day. Gray= exposure to Rosin; Green= Restricted amounts of diet offered daily, based on the daily mean food consumption values noted for Group 2 on the equivalent Study Day. Gray diagonal stripes = exposure to Rosin halved from Lactation Day 1 to euthanasia.

2.7.2. Correlation analysis (study means) - fertility

Where possible, for each OECD 422 study listed in Table 1, the value for each of the following two metrics was calculated:

Undernutrition (U):

$$U = 100 * \frac{\overline{BWGPP}_{HD}}{\overline{BWGPP}_{CON}} \quad (1)$$

Fertility (F):

$$F = 100 * \frac{\overline{CL}_{HD}}{\overline{CL}_{CON}} \quad (2)$$

Where:

$BWG = Bodyweight_{Prepairing\ Day\ 15} - Bodyweight_{Prepairing\ Day\ 1}$

$\overline{BWGPP}_{HD}$ = mean BWG during prepairing period of females in High Dose group

$\overline{BWGPP}_{CON}$ = mean BWG during prepairing period of females in Control group

$\overline{CL}_{HD}$ = mean number of Corpora Lutea in High Dose group

$\overline{CL}_{CON}$ = mean number of Corpora Lutea in Control group

Bodyweight gain (BWG) values for individual animals were calculated using mathematical functions in Microsoft Excel. Mean values were calculated using GraphPad Prism v10 software. The Pearson r correlation between the resulting two datasets (Fertility[F] versus Undernutrition[U]) was calculated, alongside a 2-tailed *P*-value, using GraphPad Prism v10 software. The best-fit line, alongside 95% Confidence Bands, was calculated by Least Squares Regression as implemented in GraphPad Prism v10 software.

Pre-pairing Day 1 refers to the first Day of treatment. Pre-pairing Day 15 refers to the first day female rats were paired with male rats for mating. However, female bodyweights were not always available on this day. For this reason, the BWG values were calculated using the last day of Pre-pairing for which BW data were available. This was pre-pairing Day 13, 14, or 15, depending on the study.

2.7.3. Correlation analysis (study means) – offspring growth

Where possible, for each OECD 422 study listed in Table 1, the value for each of the following five metrics was calculated:

BWG values for individual female parental animals were calculated using mathematical functions in Microsoft Excel. Pup BWG values for individual litters were calculated using mathematical functions in Microsoft Excel. Mean values were calculated using GraphPad Prism v10 software. The Pearson r correlation between the following pairs of datasets were calculated, alongside a 2-tailed *P*-value, using GraphPad Prism v10 software.

- PW_{LD1} *versus* U_{LD1}
- PW_{LD4} *versus* U_{LD4}
- PG *versus* U_{LD4}

The best-fit line, alongside 95% Confidence Bands, was calculated by Least Squares Regression as implemented in GraphPad Prism v10 software.

2.7.4. Multiple linear regression analyses

Multiple Linear Regression analysis was used to investigate correlations between reproductive parameters and undernutrition at the level of individual dams. Multiple Linear Regression modeling was performed with the *lm()* function from the "*stats*" package in R [46]. All references to pup BWG or pup bodyweight (BW) on LD1 below refer to "litter mean" values.

Model assumptions were checked by examining:

- Linear Relationship Between Dependent Variable and Each Continuous Covariate
- Homogeneity of Regression
- Normal distribution of residuals
- Homogeneity of residual variance

R code used for modeling is reproduced in Supplementary Information.

$Undernutrition_{LD1}$ (U_{LD1})

$$U_{LD1} = 100 * \frac{\overline{BWG\ to\ LD1}_{HD}}{\overline{BWG\ to\ LD1}_{CON}} \quad (3)$$

$Pup\ Weight_{LD1}$(PW_{LD1})

$$PW_{LD1} = 100 * \frac{\overline{Litter\ mean\ PW\ LD1}_{HD}}{\overline{Litter\ mean\ PW\ LD1}_{CON}} \quad (4)$$

$Undernutrition_{LD4}$ (U_{LD4})

$$U_{LD4} = 100 * \frac{\overline{BWG\ to\ LD4}_{HD}}{\overline{BWG\ to\ LD4}_{CON}} \quad (5)$$

Pup Weight $_{LD4}$ (PW_{LD4})

$$PW_{LD4} = 100 * \frac{\overline{Litter\ mean\ PW\ LD4}_{HD}}{\overline{Litter\ mean\ PW\ LD4}_{CON}} \quad (6)$$

Pup Growth (PG)

$$PG = 100 * \frac{\overline{pupBWG}_{HD}}{\overline{pupBWG}_{CON}} \quad (7)$$

Where:

$\overline{BWG\ to\ LDX}_{HD}$ = mean Bodyweight gain (BWG) from treatment start to LDX of females in High Dose group

$\overline{BWG\ to\ LDX}_{CON}$ = mean BWG from treatment start to LDX of females in Control group

$\overline{Litter\ mean\ PW\ LDX}_{HD}$ = mean litter mean pup bodyweight on LDX in High Dose group

$\overline{Litter\ mean\ PW\ LDX}_{CON}$ = mean litter mean pup bodyweight on LDX in Control group

$\overline{BWG\ LD1\ to\ LD4}_{HD}$ = mean BWG from LD1 to LD4 of mothers in High Dose group

$\overline{BWG\ LD1\ to\ LD4}_{CON}$ = mean BWG from LD1 to LD4 of mothers in Control group

$\overline{pupBWG}_{HD}$ = mean litter mean pup BWG from LD1 to LD4 in High Dose group

$\overline{pupBWG}_{CON}$ = mean litter mean pup BWG from LD1 to LD4 in Control group

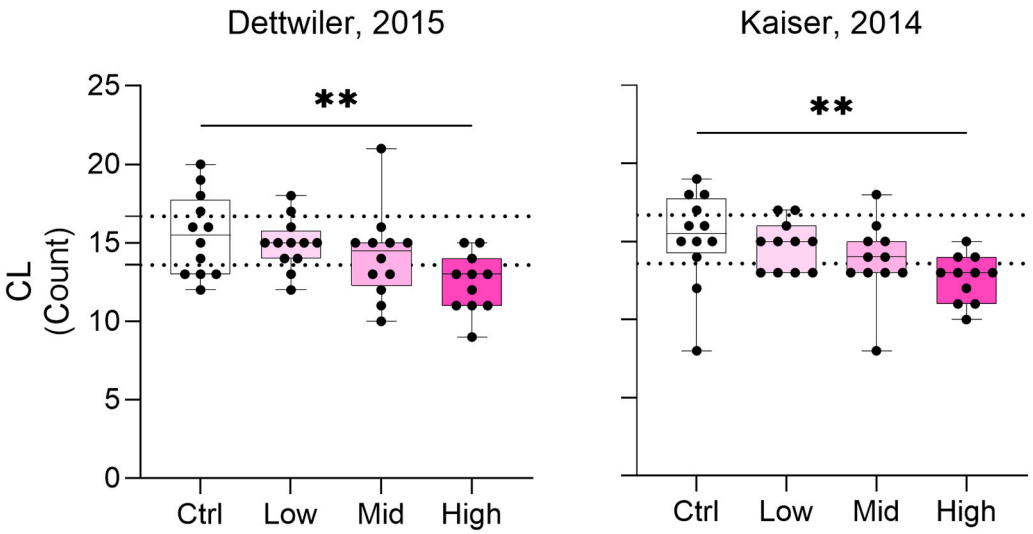

Fig. 4. *Corpora lutea* Counts in Rats Exposed to Dietary Rosin. For each of the two regulatory studies conducted with Rosin [25,26], *Corpora lutea* (CL) counts are plotted as a function of Control, Low-dose, Mid-dose, and High-dose. Data are presented as Tukey Box & Whisker plots with each individual datum point shown. The upper and lower bounds of the HCD range (min to max) for mean *Corpora lutea* counts are shown as horizontal dotted lines. Statistically significant effects of treatment by ordinary one-way ANOVA with a Dunnett's multiple comparison test [25] or Kruskal-Wallis test with a Dunn's multiple comparison test [26] are depicted. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

2.7.4.1. Pup BW on LD1. The following model was used:

$$\text{Pup BW on LD1} = \beta_0 + \beta_1 \text{Mat BWS} + \beta_2 \text{Mat BWG} + \beta_3 \text{Treatment} + \varepsilon \quad (8)$$

Where:

β_0 = the intercept

β_1 = the unknown slope of the linear relationship between pup BW on LD1 and maternal bodyweight at treatment start (mat BWS)

β_2 = the unknown slope of the linear relationship between pup BW on LD1 and maternal Bodyweight Gain from treatment start to Lactation Day 1 (Mat BWG)

β_3 = the unknown mean effect of treatment on pup BW on LD1 (β_3 has a different value for each level of treatment)

ε = residual (the difference between fitted and actual values of pup BW on LD1, and representing effects of unknown important covariates and/or random experimental error

Although animals were randomized, mat BWS remains an uncontrolled variable and was included as a covariate to improve precision of model estimates.

2.7.4.2. Pup BWG. The following model was used:

$$\text{Pup BWG} = \beta_0 + \beta_1 \text{Mat BWG} + \beta_2 \text{Treatment} + \varepsilon \quad (9)$$

Where:

β_0 = the intercept

β_1 = the unknown slope of the linear relationship between pup BWG and mat BWG (maternal BWG from treatment start to Lactation Day 1)

β_2 = the unknown mean effect of treatment on pup BWG (β_2 has a different value for each level of treatment)

ε = residual (the difference between fitted and actual values of pup BWG, and representing effects of unknown important covariates and/or random experimental error

Mat BWS was not included in the final model as it did not appreciably improve the precision of estimates.

Note that in contrast to pup BW on LD1, initial exploratory data analyses indicated that the identity of the test site had a significant effect on pup BWG. Therefore, the model was applied independently to two datasets, one dataset comprising studies conducted with the Han Wistar strain of rat (Harlan and Envigo test sites); and a second dataset comprising the earlier studies with Sprague Dawley rats conducted at the Inveresk site.

2.8. Statistical control for effects of undernutrition

Undernutrition was not experimentally controlled during the regulatory studies. Moreover, it is difficult to see how it could have been controlled, as the undernutrition was an inevitable consequence of dietary exposure to these substances. In the absence of experimental control, an attempt was made to statistically control for this factor.

For the purposes of statistical control, residuals (ε) were calculated by applying the *augment()* function from the *'Broom'* R package to the *lm ()* model output. Thereafter, adjusted pup bodyweight and bodyweight gain values were calculated as the sum of $\beta_0 + \varepsilon$.

3. Results

3.1. Rosin

To exemplify the findings seen with several Rosin and derivatives, detailed data from the two OECD 422 studies conducted with dietary Rosin (EC# 232–475–7) are presented below.

3.1.1. Exposure to dietary rosin reduces fertility of female rats and retards offspring growth

Mean *Corpora lutea* counts, indicative of ovulation rate, declined in a dose-dependent manner in both studies (Fig. 4). The effect was statistically significant in the High-dose group and the mean value fell below the lower bound of the Historical Control Data (HCD) range. Relatedly, Implantation Site counts and the number of Total Pups Born were also reduced (data not shown).

In addition to reduced ovulation rate, mean pup bodyweight on both LD1, LD4, and bodyweight gain in the interval were dose-dependently and statistically significantly reduced in one of the two studies (Fig. 5). In the second study with Rosin, where maternal undernutrition (as assessed by maternal BWG) was less pronounced than in the first study (see Supplementary Table 2), the mean value for each of the three offspring growth parameters was lower in the High-dose group than in the concurrent Control group, even if the differences were not deemed statistically significant.

3.1.2. Effects of dietary rosin on food consumption and bodyweight gain

The food consumption (FC) trajectory of female rats exposed to

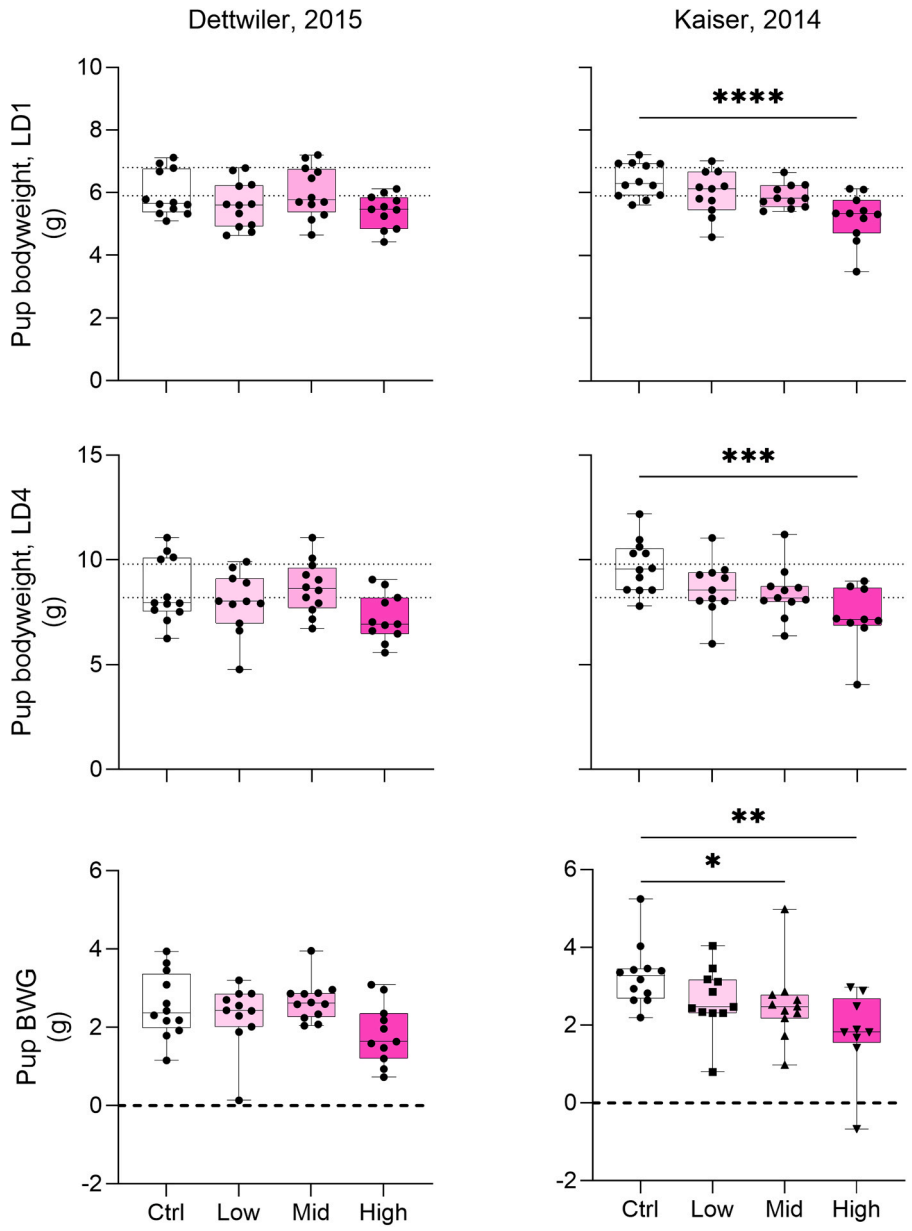

Fig. 5. Offspring Growth in Dams Exposed to Dietary Rosin. For each of the two regulatory studies conducted with Rosin [25,26], pup bodyweight on LD1, LD4, and bodyweight gain in the interval are plotted as a function of Control, Low-dose, Mid-dose, and High-dose. Data are presented as Tukey Box & Whisker plots with each individual datum point shown. The upper and lower bounds (min to max) of the HCD ranges are shown as horizontal dotted lines, where data are available. Statistically significant effects of treatment by ordinary one-way ANOVA with a Dunnett's multiple comparison test (panels B & E) or Kruskal-Wallis test with a Dunn's multiple comparison test (panels A, C, D, & F) are depicted. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

dietary Rosin or Control diet are presented in Fig. 6 & Supplementary Figure 1. Dietary Rosin appears to be unpalatable to rats and elicited aversive behavior, as indicated clearly by the initial sharp decrease in FC by High-dose animals during the first days of pre-pairing. Thereafter, FC starts to recover but never recovers fully to levels seen in concurrent Control animals.

The related findings of reduced FC & cumulative BWG were not restricted to the High-dose group but were dose-dependent, occurring to a lesser degree in the Mid-dose group.

Consistent with their reduced FC, High-dose females lost weight during the first days of treatment before starting to gain weight once more as FC recovered (Fig. 6 & Supplementary Figure 1). However, at the point of pairing for mating, High-dose female rats had, on average, only just recovered to approximately the same bodyweight as at treatment start. These animals remained underweight compared to concurrent Control throughout gestation and lactation.

3.2. *Reproductive effects of rosin and derivatives correlate with degree of undernutrition*

Amongst the tested substances, undernutrition (as evaluated by reduction in maternal BWG) was not restricted to Rosin but occurred to a greater or lesser extent with several related derivatives. To investigate whether the degree of undernutrition was correlated with the size of the reproductive effects, a series of regression analyses was conducted.

3.2.1. *Corpora lutea counts*

For each regulatory OECD 422 study conducted with Rosin and derivatives, fertility was quantified by expressing the mean *Corpora lutea* count in the High-dose group as a percentage of the mean Control value.

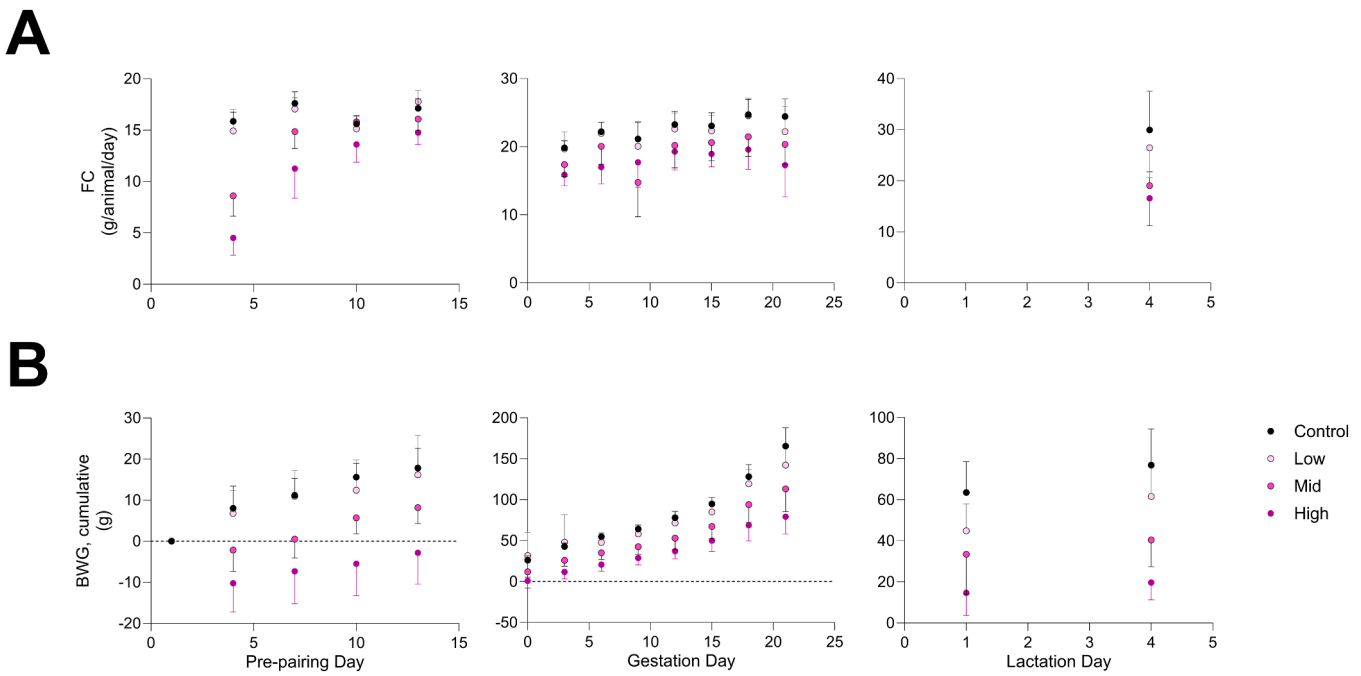

Fig. 6. Female Bodyweight Gain and Food Consumption [25]. For females on one of the two regulatory studies conducted with Rosin, food consumption (FC) (A) and cumulative bodyweight gain (BWG) from treatment start (B) are plotted versus time as a function of Control, Low-dose, Mid-dose, and High-dose. With one exception, data are presented as mean ± SD (n = 6–12 animals). The exception is the FC data during the pre-pairing period for which data are presented as mean ± SD, (n = 4 cages).

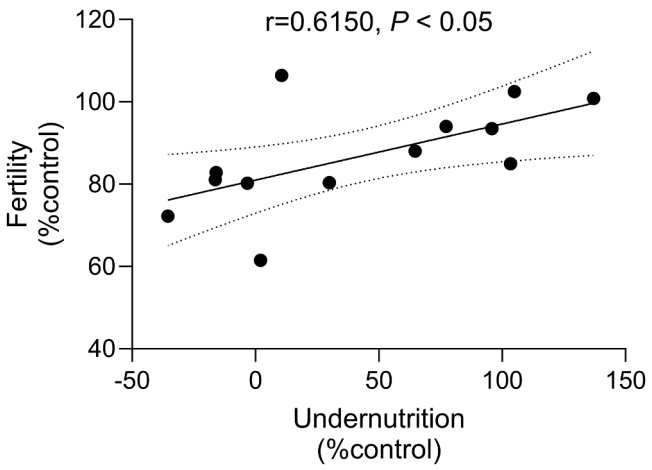

Fig. 7. Fertility Correlates with Undernutrition. For the 13 of 16 OECD 422 studies where *Corpora lutea* data were available in the High-dose group, fertility is plotted as a function of undernutrition. The Pearson r and associated *P* value are shown. The 95% Confidence Band is shown by dashed curves. Note that neither of the two Inveresk studies reported *Corpora lutea* count; and these data are also not available for the High-dose group in the Rosin, maleated study, as the group was euthanized early due to excessive toxicity.

Similarly, as a measure of undernutrition at the point of pairing for mating, the mean BWG in the High-dose group during the pre-pairing period was expressed as a percentage of the mean Control value (see Section 2.7.2 for more detail). The resulting fertility and undernutrition metrics, which are plotted in Fig. 7 and tabulated in Supplementary Table 1, were significantly correlated with each other (Pearson r of 0.615, $P < 0.05$).

Next, the relationship between *Corpora lutea* counts and undernutrition at the level of individual animals was examined. We pooled all available *Corpora lutea* and undernutrition data from the 14 studies for which such data were available and fitted a linear regression model separately at each level of treatment (Control, Low, Mid, High). Once more, female BWG during pre-pairing was used as a proxy measure of undernutrition. Note that because the studies were conducted across two separate test sites (Envigo and Harlan), test facility was included as a factor in the analysis. The results are presented in Fig. 8. There is no statistically significant correlation between *Corpora lutea* counts and undernutrition under control conditions or in animals in the Low-dose groups at either test site. Nor is there a statistically significant effect of undernutrition on *Corpora lutea* counts of animals in the Mid-dose group, although visual inspection suggests a relationship is beginning to emerge for the Mid-dose animals from studies conducted at Harlan. However, there is a statistically significant correlation between *Corpora lutea* counts and undernourishment for High-dose animals from studies conducted at Harlan.

The lack of correlation between *Corpora lutea* counts and undernutrition in the High-dose animals of studies run at Envigo is not surprising as the test substances investigated at this site, with only one exception, did not affect mean *Corpora lutea* counts.

3.2.2. *Pup Bodyweight / growth retardation*

Analogous to the approach taken for fertility, offspring growth and maternal undernutrition metrics were quantified for each OECD 422 study, as described in Section 2.7.3. All three growth metrics (pup bodyweight on LD1; pup bodyweight on LD4; and pup BWG in the interval) were highly significantly correlated with maternal undernutrition (Fig. 9; data tabulated in Supplementary Table 2).

Subsequently, the relationship between pup growth and undernutrition was investigated at the level of individual dams. Both pup bodyweight on Lactation Day 1 (pup BW on LD1) and pup bodyweight gain in the interval from LD1 to LD4 (pup BWG) were considered. Multiple Linear Regression models were built and validated for this purpose (see Section 2.7.4 for details). The models confirmed that there was a positive correlation between undernutrition and either pup BW on LD1 (Fig. 10) or pup BWG (Fig. 11).

Finally, an attempt was made to statistically control for

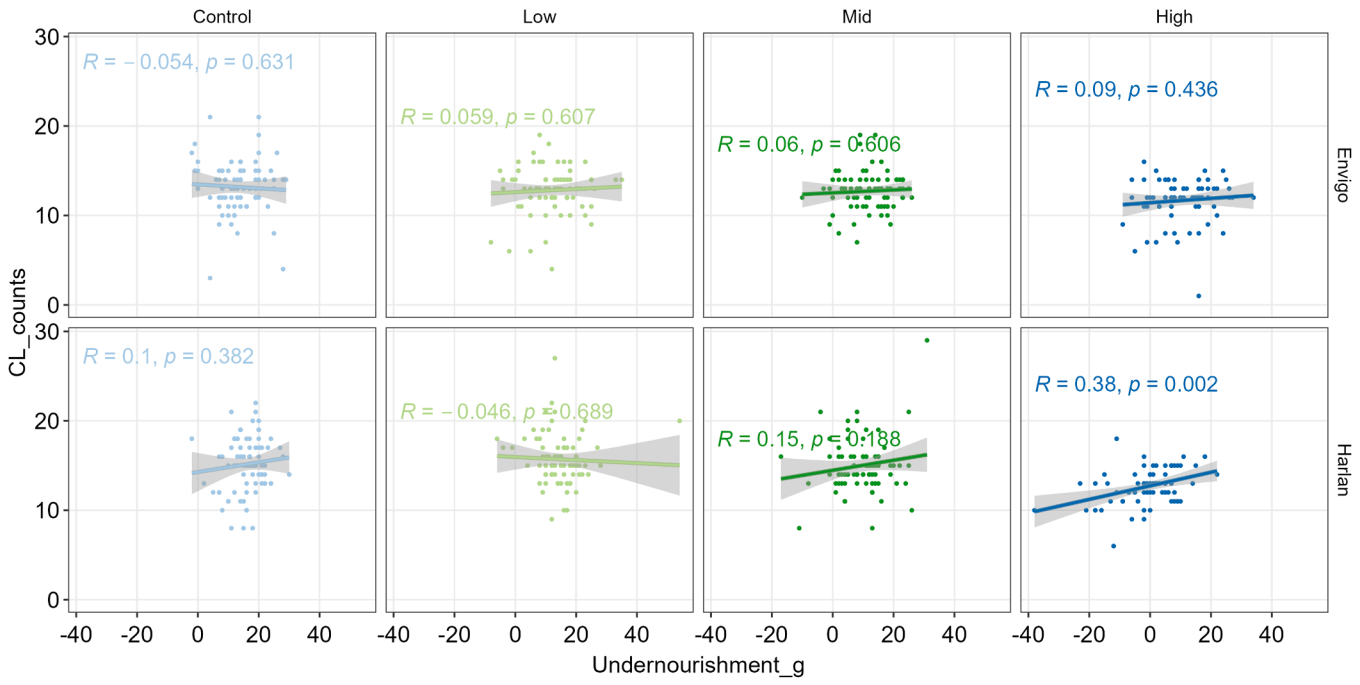

Fig. 8. Correlation of *Corpora lutea* counts and Undernourishment. Across all 14 OECD 422 studies where the data were reported, *Corpora lutea* counts ("CL_counts") are plotted versus maternal bodyweight gain during pre-pairing ("Undernourishment_g") as a function of treatment and test site ("Envigo" or "Harlan"). The Pearson r and its associated *P* value are presented. The 95% Confidence Band is shown in gray. Regression analysis was conducted using the stat_smooth(method = "lm") function from the "ggplot2" package in R and the data were plotted using the "ggplot2" R package.

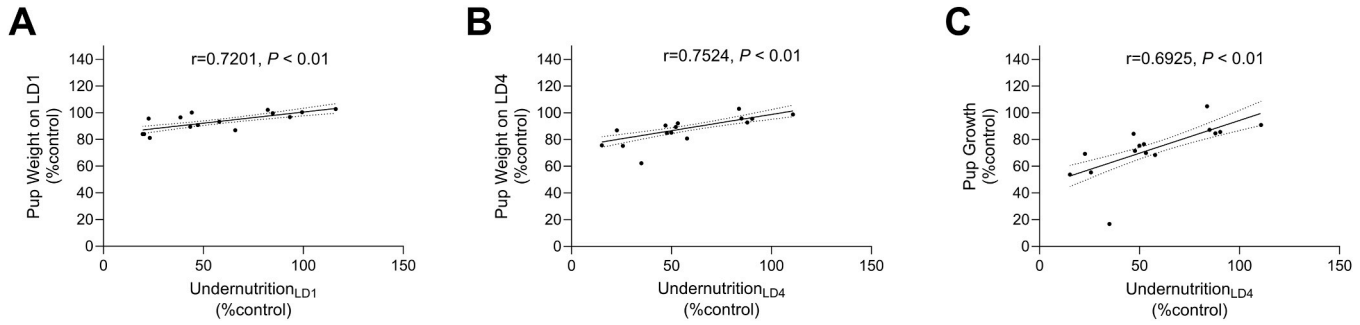

Fig. 9. Correlation of Pup Growth and Undernutrition. For the 15 of 16 OECD 422 studies where pups were born to dams in the High-dose group, various measures of pup growth are plotted as a function of undernutrition. The Pearson r and associated *P* values are presented in the graphs. The 95% Confidence Bands are shown by dashed curves. Note that these data are not available for the High-dose group in the Rosin, maleated study, as the group was euthanized early due to excessive toxicity.

undernutrition to isolate and quantify the direct effect, if any, of Rosin and derivatives on each of the two pup growth metrics. To this end, the Multiple Linear Regression models were used to adjust the growth data based on maternal BWG. The absolute and adjusted pup BW on LD1 data (for adjusted pup BW definition, please refer to Section 2.8) are plotted in Fig. 12 (Category 1 substances), Supplementary Figure 2 – 4 (Category 2, 3 and 4 substances, respectively) and tabulated in Supplementary Table 3. Similarly, the absolute and adjusted pup BWG values (for adjusted pup BWG definition, please refer to Section 2.8) are plotted in Fig. 13 (Category 1 substances), Supplementary Figure 5 – 7 (Category 2, 3 and 4 substances, respectively) and tabulated in Supplementary Table 4. None of the substances had a dose-dependent statistically significant effect on the adjusted growth metrics except for Rosin, maleated.

3.3. Fertility effect of rosin is reversible

Stimulated by the observation that undernourishment (as indicated by cumulative bodyweight gain) in response to dietary administration of Rosin apparently reached a maximum during pre-pairing before ostensibly ameliorating over time (Fig. 6 & Supplementary Figure 1), a mechanistic study was commissioned to investigate whether the fertility effect might prove transient. The study used a single dose of Rosin that was the same as the High-dose level of the substance in the earlier regulatory studies (see Section 2.6.2.1 for experimental design).

The FC and maternal BWG data from the transiency study are presented in Supplementary Figure 8. The overall trajectory for both parameters was reminiscent of that seen in the earlier regulatory studies with Rosin. One notable difference was that the rate of bodyweight accrual by female rats was somewhat slower in this study than anticipated based on the earlier studies. Consequently, the mean bodyweight of female rats exposed to a High-dose of dietary Rosin remained below that recorded on Study Day 0 during the pre-pairing period, even when this period was extended to 4-weeks. A second unexplained finding was that treated animals had apparently elevated mean FC during the gestation phase of the study compared to concurrent Control; the

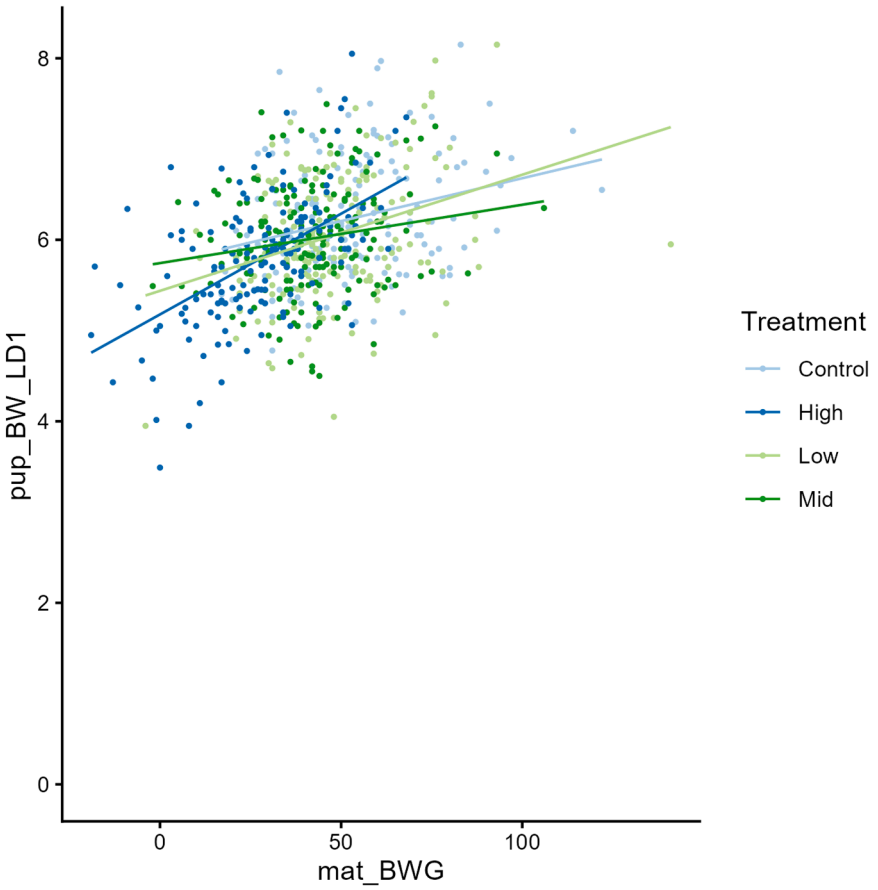

Fig. 10. Pup BW on LD1 Correlates with Maternal Undernutrition. Scatterplot of pup BW on LD1 (g) versus mat BWG (g). A best-fit linear regression model was fitted independently at each level of treatment. The best-fit regression lines are displayed. The slope of the regression lines are statistically different from each other – but no dose-dependent trend was evident. Regression analysis was conducted using the lm() function from the "stats" package in R and the data were plotted using the "ggplot2" R package.

variance of FC by these animals was also markedly increased. The reason for this finding remains unclear. However, intriguingly and unexpectedly, offspring growth was not impaired by dietary exposure to Rosin during this study (data not shown).

The *Corpora lutea* counts, Implantation Site numbers, and Total Pups Born data are presented in Fig. 14. The data of relevance to the question of transiency are to be found in the *top row*. For all three metrics, the effect of Rosin on fertility was moderated by delaying pairing for mating until 4-weeks after start of treatment. Moreover, two-way ANOVA revealed a significant interaction ($P < 0.05$) between the two factors "diet" and "duration of pre-pairing" for both the Implantation Site (IS) and Total Pups Born (but not the *Corpora lutea*) datasets: whereas Rosin statistically significantly lowered mean fertility at two weeks, it did not do so when the pre-pairing period was extended to four weeks, despite greater cumulative exposure to Rosin.

The data of relevance to the assessment of the reversibility of fertility effect are shown in the *bottom row* of Fig. 14. Reversibility could not be assessed using the IS counts or Total Pups Born datasets, as the effects on these parameters were already transient. However, for the *Corpora lutea* counts, to the extent that these data do not unambiguously support transiency of effects, they do at least indicate that the effect is reversible.

Overall, although the three datasets are not entirely congruous, they do broadly support transiency of fertility effect. It is possible that a more definitive conclusion would have been reached had pairing for mating been delayed until 6-weeks or later after treatment start.

3.4. Retarded growth of offspring of rosin-exposed dams is caused by undernutrition

The correlation analyses presented in Section 3.2, alongside the literature cited in the Introduction, provide strong support for the hypothesis that the reproductive effects seen with Rosin and derivatives substances were caused by undernutrition. To formally test this hypothesis, a pair-fed feed restriction study was conducted, as explained in detail in Section 2.6.2.2.

The FC and maternal BWG data from this study are presented in Supplementary Figure 9. Consistent with all the other studies with dietary Rosin, there was a sharp self-imposed restriction in FC by female rats when the Rosin diet was first offered to them, followed by a gradual and partial recovery. The maternal BWG trajectory was commensurate with the pattern of FC. Importantly, in the pair-fed group the engineered restriction in FC, and the accompanying pattern of maternal BWG, mirrored that seen in the animals exposed to dietary Rosin.

Unexpectedly, the mean number of *Corpora lutea* in females administered dietary Rosin was not significantly lower than in Control females (Fig. 15). Consistent with this finding, Rosin-treatment had no significant effect on mean number of Implantation Sites or mean number of Total Pups Born (Fig. 15). Additionally, pair-feeding had no biologically or statistically significant effect on any of the three afore-mentioned fertility metrics.

Whilst it failed to reproduce the lower fertility seen in the previous studies with Rosin, the pair-feeding study did reproduce the retardation of offspring growth seen with this substance. Offspring of Rosin-treated female rats were statistically significantly lighter on average than

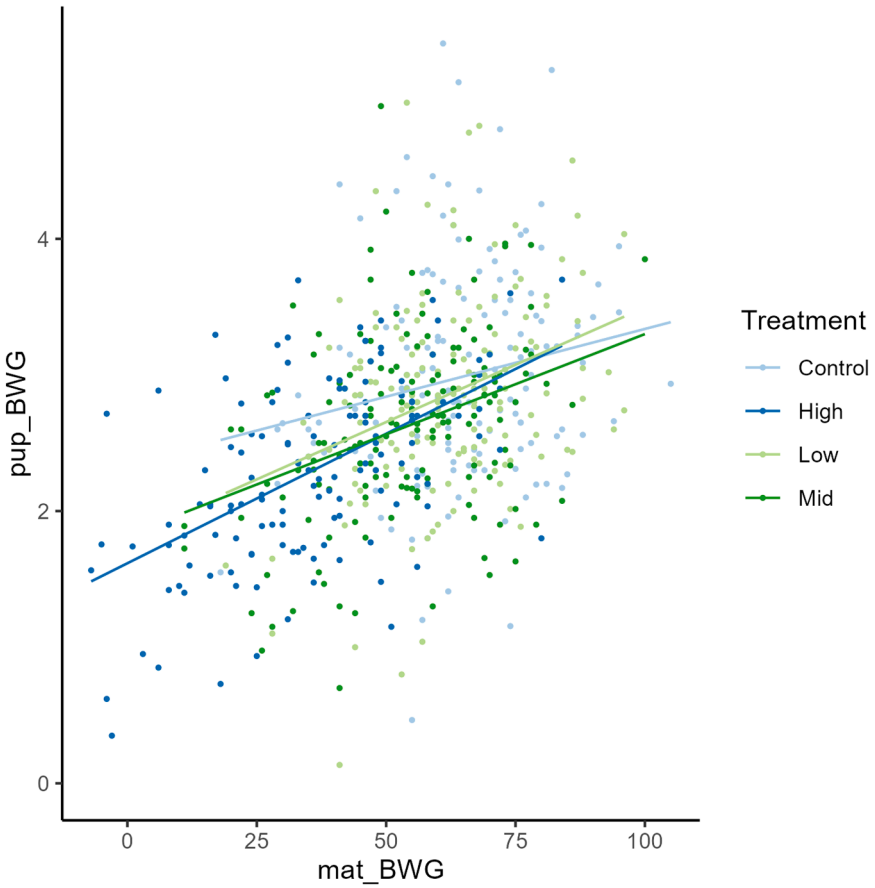

Fig. 11. Pup BWG Correlates with Maternal Undernutrition. Scatterplot of pup BWG (g) versus mat BWG (g). A best-fit linear regression model was fitted independently at each level of treatment. The best-fit regression lines are displayed. The slope of the regression lines are not statistically different from each other. Regression analysis was conducted using the lm() function from the "stats" package in R and the data were plotted using the "ggplot2" R package. The data are from the Harlan and Envigo test sites.

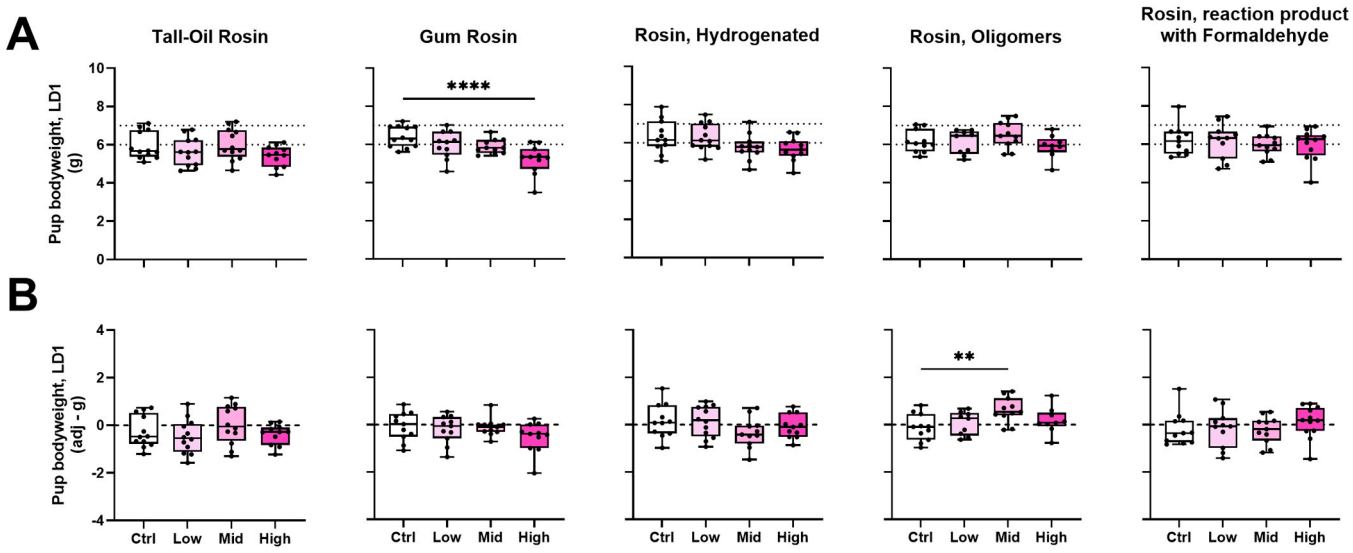

Fig. 12. Category 1: Absolute and Adjusted Pup BW on LD1. Pup BW on LD1, absolute values (A) or adjusted for mat BWG and mat BWS (B), are presented as a function of Control, Low-dose, Mid-dose, and High-dose for each of the five OECD 422 studies conducted with H4R Category 1 test substances. Data are presented as Tukey Box & Whisker plots with each individual datum point shown. The upper and lower bounds (min to max) of the HCD ranges for mean absolute pup bodyweights on LD1 are shown as horizontal dotted lines. Statistically significant effects of treatment are depicted. * $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$ **** $P < 0.0001$.

offspring of control female rats (Fig. 16). Their average weight gain from LD 1 to LD4 was also significantly lower than that of the offspring of control female rats (Fig. 16). Critically, the litter mean bodyweights on LD1 and LD4, and bodyweight gain in the interval, of offspring of feed-restricted females were biologically and statistically indistinguishable from the offspring of Rosin-treated females (Fig. 16).

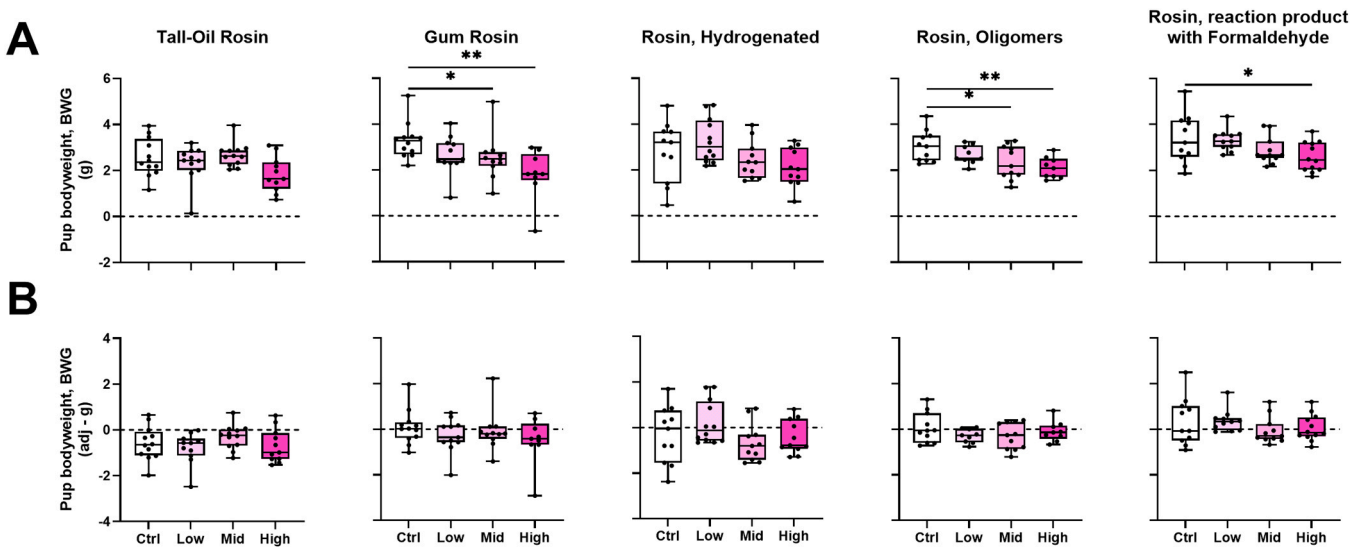

Fig. 13. Category 1: Absolute and Adjusted Pup BWG. Pup BWG, absolute values (A) or adjusted for mat BWG (B), are presented as a function of Control, Low-dose, Mid-dose, and High-dose for each of the five OECD 422 studies conducted with H4R Category 1 test substances. Data are presented as Tukey Box & Whisker plots with each individual datum point shown. Statistically significant effects of treatment are depicted. * $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$ **** $P < 0.0001$.

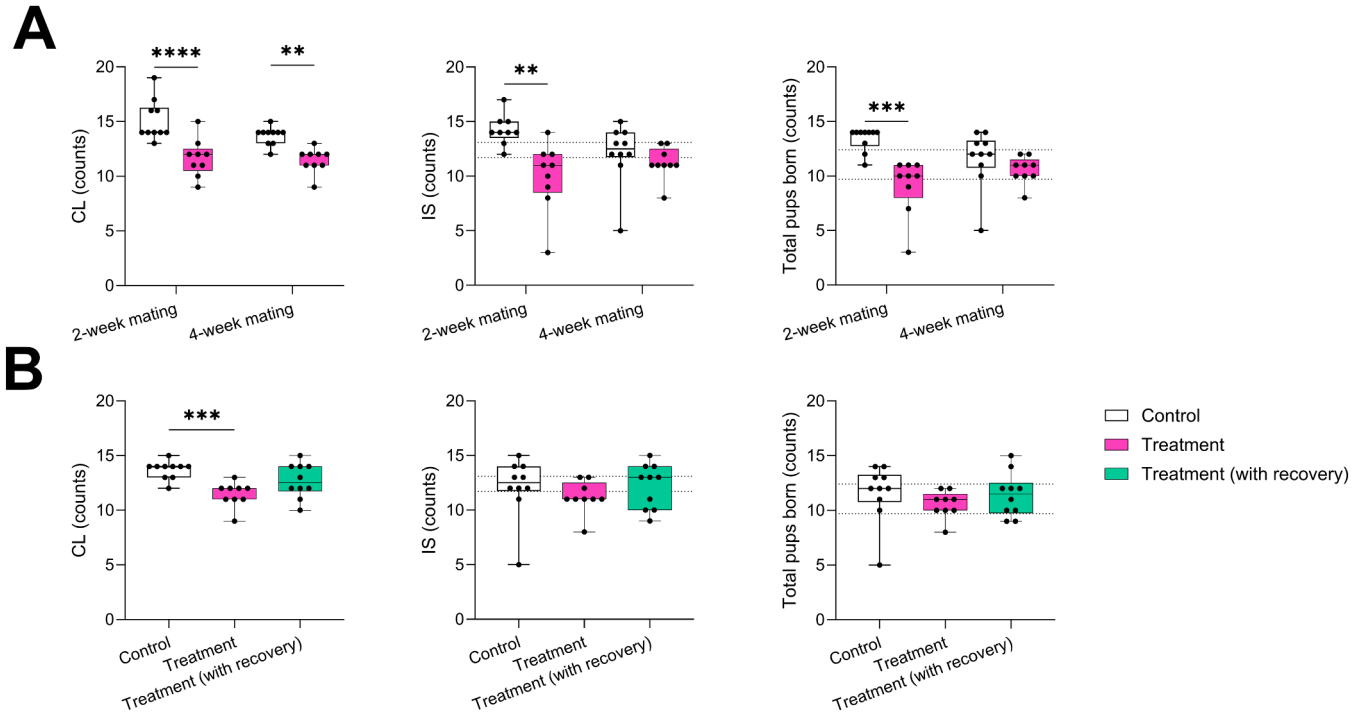

Fig. 14. Transiency and Reversibility of Effect of Rosin on Fertility. (A) (to evaluate transiency): Various fertility metrics (*Corpora lutea* (CL) counts, Implantation Site (IS) numbers, or Total Pups Born) are shown as a function of two factors, "diet" (control or treatment) and "duration of pre-pairing period" (2-week (i.e., Cohort 1 animals); or 4-week (i.e., Cohort 2 animals). Data are presented as Tukey Box & Whisker plots with each individual datum point shown. There was a significant interaction between the two factors ($P < 0.05$) by two-way ANOVA, except for the CL dataset. Simple main effects of diet are shown. ** $P < 0.01$; **** $P < 0.0001$. (B) (to evaluate reversibility): CL counts, IS counts, or Total Pups Born are plotted as a function of three experimental groups (Cohort 2 animals, "Control" & "Treatment", and Cohort 3 animals "Treatment with Recovery"). Statistically significant differences from the Control group by one-way ANOVA are reported. ** $P < 0.01$; **** $P < 0.0001$. Where available, the upper and lower bounds of the HCD range (min to max) are shown as dashed, horizontal lines.

4. Discussion

We report that dietary rosin and derivatives elicit food aversion in rats to a greater or lesser extent. Although the substances clearly elicit aversive behavior in rats, it remains unclear whether the suppression in food intake represents an *innate* response to some gustatory property of the substances or represents a *learned* response to, for example, gastrointestinal malaise. We favor the former explanation for several reasons. First resin acids contain carboxylic acid groups, which are sensed by taste receptors in the oral cavity [47]. Second, esterification reactions, which reduce the acid value of the substances, ablated the aversive response (data not shown). Finally, resin acids are produced by pine trees as a defensive response to deter predators. Whether innate or learned, the resulting transient anorectic response appears to cause the

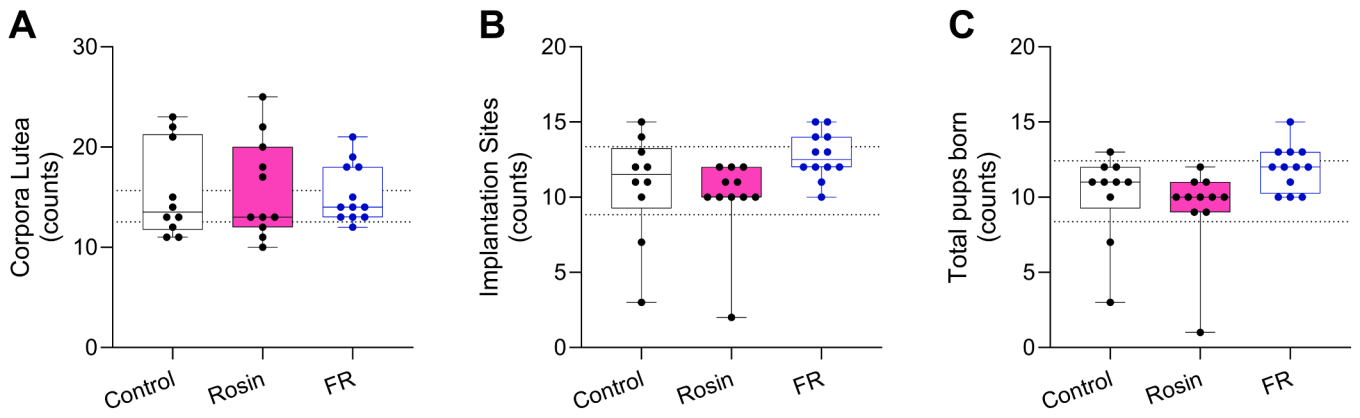

Fig. 15. Fertility Metrics, Female Rats (Feed-restriction Study). (A) *Corpora lutea* (CL) counts, (B) Implantation Site (IS) counts, or (C) "Total Pups Born" counts are presented as a function of experimental group. Data are presented as Tukey Box & Whisker plots with each individual datum point shown. The upper and lower bounds of the HCD range (mean ± 2 SD) are shown as dashed, horizontal lines. There were no statistically significant differences from control, as assessed by Kruskal-Wallis tests. FR: Feed Restriction.

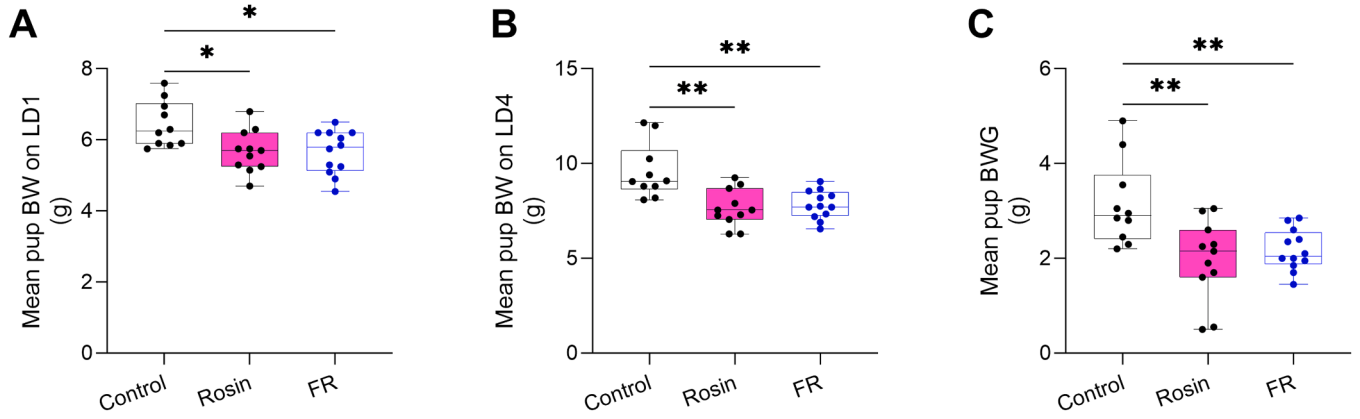

Fig. 16. Pup Bodyweights and Bodyweight Gains (Feed Restriction Study). Litter mean pup bodyweights (BW) on Lactation Day (LD) 1 (A) & LD4 (B); and litter mean pup bodyweight gains (BWG) from LD1 to LD4 (C) are presented as a function of experimental group. Data are presented as Tukey Box & Whisker plots with each individual datum point shown. Statistically significant differences from control were assessed by one-way ANOVA with a Dunnett's multiple comparisons test. * $P < 0.05$, ** $P < 0.01$. FR: Feed Restriction.

reproductive effects seen in studies with these substances.

4.1. *Developmental effect - Pup growth/development*

With the possible exception of one substance, the correlation analyses presented in this paper support the hypothesis that all other dietary Rosin and derivatives substances affect pup growth in the OECD 422 study design solely and indirectly through undernutrition effects. Consistent with this, there were no statistically significant effects of any of these substances on either pup BW on LD1 or pup BWG after statistically controlling for undernutrition effects. Moreover, the pair-feeding study confirms the hypothesis for Rosin. Furthermore, insofar as the correlation analyses support a single common Mode of Action based on undernutrition across all Rosin and derivatives substances, by inference, the result from the pair-feeding study with Rosin would appear to apply also to the other tested substances.

The possible exception is Rosin, maleated. Uniquely amongst the tested substances, it had a statistically significant effect on both pup BW on LD1 and pup BWG even after statistically controlling for undernutrition (see Supplementary Figure 3 and Supplementary Figure 6). For this substance, we cannot exclude the possibility that it might have a mild specific intrinsic effect on offspring growth. The concern around a direct intrinsic property is heighted by the fact that dietary administration of the substance was associated also with a striking number of skeletal variations indicating delayed ossification when administered to pregnant rats (unpublished OECD 414 study data). The number of these skeletal findings, alongside their high incidences, are not obviously explained by the limited degree of undernutrition seen during this study. Nevertheless, a separate pair-feeding OECD 414 study would be required to definitively conclude that the skeletal findings represent an intrinsic property of the substance. Moreover, insofar as delayed skeletal ossification often resolves post-natally [48], it remains unclear whether these effects are adverse.

It is useful to consider prior work on the impact of maternal undernutrition on offspring growth. Carney and colleagues examined the impact on pup growth of feed-restricting Sprague Dawley dams from Gestation Day 7 and throughout lactation [10]. The Control animals were not fed *ad libitum* but fed a constant daily defined amount of food calculated based on the laboratory's historical control food consumption data. In addition to the Control group, a graded series of feed restriction experimental groups were established, from a group with a 10% reduction in the amount of food provided compared to Control (10% group), to a 30% group and a 50% group. Bodyweight gain data were not explicitly quantified, but this metric was statistically significantly lower than Control throughout gestation for the 30- and 50% groups; and between Gestation Days 7–14 for the 10% group. Mean pup bodyweights on Lactation Day 1 were only lower in the 50% group (for example, female pups: 5.5 g *versus* 6.3 g in Control, $P < 0.05$).

Fleeman and colleagues compared mean fetal weight between *ad libitum* control Sprague-Dawley rats and those provided with defined amounts of food (20-, 15-, 10-, or 7.5 g/animal/day) between Gestation Days (GD) 6 & 17, inclusive [11]. As *ad libitum* controls consumed on average 21–26 g/food/day, the animals in each of the 20-, 15-, 10-, and 7.5 g groups can be considered feed-restricted. Caesarian sections were conducted on GD 21. Maternally adjusted bodyweight gains (bodyweight gain from Gestation Day 6–21 minus gravid uterine weight) as a percentage of control can be calculated from the data summarized in Table 1 of their report as 48%, 30%, −11%, and −64% in the feed-restricted groups, from least to highest degree of feed restriction. The equivalent mean fetal weights in the feed restricted groups expressed as a percentage of Control were 95%, 93%, 90%, and 76%, respectively ($P < 0.01$ for each difference from Control).

Based on the study design reported by Fleeman and colleagues [11], Gotardo and coworkers studied the impact on mean fetal weight of feed restricting dams between GD 6 &17; however, they used Han Wistar rats in place of Sprague Dawley [12]. In addition to an *ad libitum* control group, there were four experimental groups featuring graded levels of feed restriction (16-, 12-, 9-, or 6 g/animal/day). From Table 1 of their report, it appears that the mean amount of food consumed by dams in the 16 g feed restriction group was comparable to the amount consumed by *ad libitum* controls. Therefore, the animals in the 12-, 9-, and 6 g groups can be considered feed-restricted. Unfortunately, maternally adjusted bodyweight gains are not reported and cannot be calculated from the data presented in the paper. However, at Caesarian section on GD21, mean fetal weights in each of these three groups were (from least to most restricted) 4.1-, 4.0-, and 3.9 g compared to 4.5 g in *ad libitum* control ($P > 0.05$ for each difference from Control).

Because the studies cited above are of a very different design from the studies reported in this paper, it is difficult to make reliable conclusions around the coherency of the data. On the whole, however, we tentatively conclude that the effects on pup bodyweights in our studies occurred at levels of maternal undernutrition that are – at face value – less severe than those reported in the papers cited above. If indeed this is the case, it is plausible that the main explanation for this discrepancy is the fact that the prior work cited, unlike ours, did not involve maternal undernutrition during the pre-conception period. It might also be relevant that for two of the three cited studies, the feed restriction was terminated several days before Caesarian section and the dams returned to an *ad libitum* feeding schedule.

4.2. Fertility effects

Mating, gestation, and lactation are energetically demanding processes. Consequently, mammals in general, and rats in particular, adjust their fertility in response to nutrient availability [2,3,49,50]. This response is rapid and periods of fasting or undernourishment lasting as little as 48 h suffice to reduce ovulation and alter estrogenic behavior [2, 51–53]. Mechanistically, this intimate relationship is believed to be orchestrated by peripheral metabolic cues, such as leptin and insulin, that signal to and adjust the Hypothalamus-Pituitary-Ovary (HPO) axis. This response is beneficial and transient, acting to maintain ovarian reserve until environmental conditions improve [6,54–56]. This type of evolutionary adaptation is not restricted to mammals; it is found in birds, reptiles and even in invertebrate animals, such as *C. elegans* [6,57, 58]. That this relationship has been conserved across the phylogenetic spectrum attests to it importance.

Considering the above facts, the lower *Corpora lutea* counts (reduced ovulation) seen after dietary exposure to several Rosins and derivatives was hypothesized to be a reversible, adaptive, and beneficial response to acute maternal energy deficit (undernourishment) during the early pre-pairing period. Unfortunately, the pair-fed feed restriction study to test this hypothesis failed to reach a conclusion on the issue, as neither Rosin-exposed nor pair-fed animals had lower mean *Corpora lutea* counts compared to control. The reason for this unexpected result with Rosin is unclear; we speculate that it might be related to the requirement to use single-housing rather than group-housing to facilitate pair-feeding. Whatever the cause, it does mean that further work is required to provide the final proof of the hypothesis.

Although formal proof remains elusive, there is very strong statistical support for the hypothesis, as undernourishment and fertility are correlated at the level of study means across the OECD 422 studies with Rosin and derivatives substances. Also consistent with this hypothesis is the finding that the effect of Rosin on ovulation appears to be transient and is certainly reversible. Furthermore, pituitary weight, a marker of pituitary activity [59,60], is reduced in animals exposed to dietary Rosin, as required by the hypothesis (unpublished data).

Perhaps most importantly, *Corpora lutea* counts were correlated with undernourishment during pre-pairing at the level of individual animals in the High-dose groups. This provides strong support the hypothesis that undernourishment causes the lower ovulation seen in these animals. But, just as importantly, it contradicts the idea that the lower fertility is caused by an intrinsic property of the substances themselves. This is because in dietary studies, such as these, where the undernourishment is a consequence of test substance unpalatability, the *most undernourished* animals are those *least exposed* to test substance. The strong implication here is that High-dose animals that were most exposed to Rosin or derivatives during pre-pairing actually had the best ovulation rates – the opposite of what one would predict were the substances to directly impair ovulation.

It is also worth comparing the case of Rosin and derivatives with that of semaglutide, a pharmaceutical that is believed to lower fertility through effects on undernutrition. Semaglutide is a Glucagon-like Peptide-1 (GLP-1) receptor agonist that is approved for clinical use in Europe and the USA, amongst other jurisdictions, for several therapeutic indications, including diabetes and obesity. Consistent with its pharmacology, semaglutide suppresses appetite, leading to a reduction in food consumption and bodyweight gain. Of relevance to the Rosin and derivatives case, statistically significant reductions in *Corpora lutea* were observed in CD rats administered semaglutide during a GLP-compliant combined fertility and embryo-fetal toxicity study [61]. Interestingly, the food consumption and bodyweight trajectories of treated rats were very reminiscent of those seen in rats administered dietary Rosin and derivatives substances; an immediate reduction in food consumption and bodyweight gain at treatment start followed by recovery thereafter [61]. Both the FDA and the EMA concluded that the fertility effect of semaglutide was a consequence of the reduced food consumption rather than representing an intrinsic property of the substance itself [61,62].

We conclude that none of the Rosin and derivatives substances tested possess the intrinsic property to lower *Corpora lutea* counts in rats.

It is instructive to attempt to compare the degree of undernourishment seen in our studies with that reported by other workers studying how nutritional status affects fertility. Chapin and colleagues feed-restricted female Sprague Dawley rats for 17-weeks, such that they reached and then maintained either 90-, 80-, or 70% of mean bodyweight of *ad libitum* fed controls [20]. For this purpose, female animals in feed-restricted groups were initially fed 18-, 16-, or 14 g/food/day for 8 (90%) of 16 (80- & 70%) days to reduce bodyweight to the target; thereafter feed levels were varied to maintain group mean bodyweights at the target. Bodyweight gain data were not reported. However, the mean number of *Corpora lutea* in each of the feed-restricted groups was lower than mean control, with the effect being statistically significant for the 70% group (15.7 *versus* 19.8, $P < 0.05$).

Terry and colleagues compared mean *Corpora lutea* between *ad libitum* control Sprague Dawley rats and those provided with defined amounts of food (20-, 15-, 10-, or 7.5 g/animal/day) for two-weeks prior to pairing for mating [21]. As *ad libitum* controls also consumed on average approximately 20 g/food/day, the animals in the 15-, 10-, and 7.5 g groups can be considered feed-restricted. Again, group mean bodyweight gain data are not explicitly reported; however, it is clear from Fig. 1 in their publication that each group of feed-restricted

animals on average lost weight over the two-week pre-pairing period. Although the 7.5 g group could not be maintained to mating, the mean number of *Corpora lutea* in the 15- (13.2) and 10 g (12.0) groups were significantly lower than *ad libitum* controls (15.9, $P < 0.05$ for both differences from Control).

If we take the example of one of the studies with Rosin [25], it appears that the broad thrust of our data are consistent with the prior work cited above; the female animals exposed to the High-dose of dietary Rosin on average lost weight during the two-week pre-pairing period and their decrement in *Corpora lutea* counts (12.6 versus 15.3, $P < 0.01$) resonates with the earlier reported data.

The coherence of our data with those reported earlier is striking, especially when one considers the different experimental designs. Differences include our use of Han Wistar rats *versus* the Sprague Dawley strain used in the prior studies. More importantly, the trajectory of food consumption varies substantially between our studies and those reported earlier. This is especially true when comparing our work with that of Terry and colleagues, who applied an unchanging degree of feed-restriction throughout the pre-pairing period in contrast to the dynamic changes in consumption seen in our studies. Related to this, and although its relevance remains to be determined, the restricted food consumption seen in our studies was self-imposed by the animals themselves whereas feed-restriction was engineered by the experimenters, and imposed upon the animals, in the earlier work cited.

Finally, it is worth noting that as it has been conserved over evolutionary timescales, the relationship between fertility and nutrition must be beneficial and adaptive. The specific explanation offered for why this should be so is based on the requirement for all organisms to allocate finite resources across the four fundamental life processes of growth, reproduction, maintenance, and defense [56]. Given this constraint, deferring or moderating reproduction under conditions of nutrient scarcity might maximize evolutionary fitness by allowing the individual to divert resources from costly reproduction towards somatic maintenance until such time as conditions improve and another opportunity for reproduction presents itself [63]. Consistent with this thesis, whilst the fertility of feed restricted adult rodents at a given point in time might be lower than *ad libitum* controls, their cumulative fertility over their lifespan appears to be maintained as their reproductive lifespan is extended considerably (see for example [54,64]).

4.3. Statistical control undercorrects for effects of undernutrition on Pup bodyweight

Undernutrition broadly refers to an insufficient intake of nutrients and energy for the purposes of maintaining normal biological processes. Although the concept is simple to define and understand, biological responses to undernutrition are complex. Immediate changes include broad and varied alterations in circulating levels of critical signaling molecules, primarily metabolic hormones and metabolic fuels [4,65,66]. It is these signaling molecules that are collectively responsible for orchestrating the subsequent physiological adaptations and responses typically seen in undernourished animals, such as changes in fertility, organ weights, offspring weights etc.

From the foregoing considerations, it will be appreciated that it is not possible to uniquely quantify undernutrition in a meaningful manner. A further problem is that metabolic fuels and hormones are not in any case measured routinely during regulatory studies. Consequently, we had no choice but to use female BWG as an operational proxy measure of undernutrition.

As a cautionary note, this proxy measure appears to undercorrect for the effects on undernutrition on pup bodyweights. This is best illustrated by the example of gum Rosin. Dietary exposure to gum Rosin had one of the largest effects on mean adjusted pup BWG (approximately 0.5 g lower in High-dose offspring versus control, not significant – Supplementary Table 4). Despite this, the pair-feeding study confirmed that maternal undernutrition alone is responsible for the lower pup BWG seen in offspring of animals treated with this substance [42]. It follows from this finding that our statistical model, and specifically its use of maternal BWG as a proxy measure, undercorrects for effects of maternal undernutrition.

As a consequence, the adjusted pup BWG values are best considered as representing an upper bound on the direct effect size of the substances.

4.4. Final remarks

The analytical approaches and experimental designs described in this paper might prove useful to other reproductive toxicologists concerned that undernutrition is confounding their data. We also wish to highlight two possible improvements to the pair-feeding study design. First, it would potentially be beneficial for such studies to be conducted with group-housed animals. Although not straightforward, microchip-operated hoppers for specific rats might be one possible approach to this end. Second, in our study, all animals in the pair-fed group were provided with the same amount of food (the mean amount of food consumed in the dietary group). It might have been better to recreate in the engineered pair-fed group the diversity of self-imposed feed restriction seen in the Rosin-exposed animals; this could be achieved by matching the food consumption trajectory of each animal in the pair-fed group with a particular animal from the treatment group.

Finally, we note that the reported studies are all screening studies with relatively small group sizes (10–12 animals per experimental group at study start). In addition, dams and offspring were euthanised on LD4 in accordance with the contemporaneous OECD 422 test guideline; consequently, pup development was only followed for a brief time. Conducting a more definitive study type, for example an Extended One-Generation Reproductive Toxicity Study, would allow a more thorough and robust evaluation of potential effects of undernutrition on fertility and development.

5. Conclusions

Based on these data and analyses, it can be concluded that:

- The effect on offspring growth of dietary exposure to Rosin and derivatives is a non-specific secondary consequence of undernutrition. Rosin, maleated might be an exception to this rule.
- The effect of dietary Rosin and derivatives on ovulation is an adaptive beneficial response to undernutrition rather than maladaptive, toxic ones. Additional work is required to confirm formally this hypothesis.

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CRediT authorship contribution statement

Larry G. Higgins: Writing – review & editing. **Theo Le Godec:** Writing – review & editing, Writing – original draft, Visualization. **Michael McMahon:** Writing – review & editing, Visualization, Methodology, Investigation, Formal analysis, Conceptualization. **Michael G. Penman:** Writing – review & editing, Conceptualization.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Theo Le Godec reports financial support was provided by Hydrocarbon Resins, Rosin Resins and Pine Chemicals Materials REACH (H4R) Consortium. Larry G. Higgins reports financial support was provided by Hydrocarbon Resins, Rosin Resins and Pine Chemicals Materials REACH (H4R) Consortium. Michael G. Penman reports financial support was provided by Hydrocarbon Resins, Rosin Resins and Pine Chemicals Materials REACH (H4R) Consortium. Michael McMahon reports financial support was provided by Hydrocarbon Resins, Rosin Resins and Pine Chemicals Materials REACH (H4R) Consortium. Theo Le Godec reports financial support was provided by Pine Chemicals Association. Larry G. Higgins reports financial support was provided by Pine Chemicals Association. Michael G. Penman reports financial support was provided by Pine Chemicals Association. Michael McMahon reports financial support was provided by Pine Chemicals Association. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.reprotox.2026.109261.

Data availability

The data that has been used is confidential.

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