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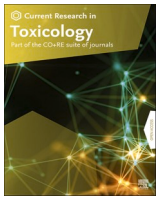

Research Paper

Evaluation of the endocrine disrupting potential of Di-isononyl phthalate

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ABSTRACT

Low molecular weight *ortho*-phthalate compounds have been implicated in disruption of androgen pathways when exposure occurs during the masculinization programming window. Di-isononyl phthalate (DINP) is a high molecular weight phthalate and a high production volume chemical. To understand the potential for DINP and its metabolites to disrupt endocrine pathways, a weight of evidence assessment was conducted according to the European Chemicals Agency (ECHA)/ European Food Safety Authority (EFSA) Endocrine Disruptor Guidance (2018). Toxicological data related to estrogen (E), androgen (A), thyroid (T), or steroidogenesis (S) pathways was assessed. Literature searches returned 110 articles from which data were extracted and assessed in conjunction with 105 high-throughput assays. An *in-silico* assessment of the EATS activity for DINP metabolites also was conducted. Based on the available evidence, DINP did not elicit thyroid- or estrogen-related apical outcomes *in vivo*. There were no studies evaluating thyroid hormone levels *in vivo* which, according to the ECHA/EFSA guidance, constitutes a data gap and prevents a conclusion being drawn on the T-pathway. The E, A, and S-pathways were sufficiently assessed to conclude on the endocrine disrupting potential of DINP. Based on the lack of apical outcomes, DINP did not disrupt the E-pathway. For the A and S-pathways, there was limited evidence to support adverse apical outcomes, so a mode of action assessment using a structured adverse outcome pathway (AOP) framework was performed. No biologically plausible link could be established between the key events in the hypothesized AOP that lead to adverse outcomes. Further, no dose or temporal concordance for A- and S-mediated findings were identified. Therefore, DINP does not meet the ECHA/EFSA criteria to be considered an endocrine disruptor.

Abbreviations: DINP, di-isononyl phthalate; WoE, weight of evidence (WoE); ECHA, European Chemicals Agency (ECHA); EFSA, European Food Safety Authority; E, estrogen; A, androgen; T, thyroid; S, steroidogenesis; AOP, adverse outcome pathway; ED, endocrine disruptor; CLP, Classification, Labelling and Packaging; OECD, Organisation for Economic Co-operation and Development; EDC, endocrine disrupting chemicals; MPW, masculinization programming window; MoA, mode of action; DEHP, diethyl hexyl phthalate; DBP, dibutyl phthalate; CEP, Food Contact Materials, Enzymes and Processing Aids; NOAEL, no observed adverse effect level; NOEL, no observed effect level; MNGs, multinucleated germ cells; MiNP, mono-isononyl phthalate; MOiNP, mono-oxoisononyl phthalate; MCiOP, mono-carboxyisooctyl phthalate; MHiNP, mono-hydroxyisononyl phthalate; EPA, US Environmental Protection Agency; HERO, Health and Environmental Research Online; HT, high throughput; ToxCast, Toxicity Forecaster; TiAB, title and abstract; PECO, Population, Exposure, Comparator and Outcome; MIE, molecular initiating event; KE, key events; QC, quality control; RoB, risk of bias; OHAT, Office of Health Assessment and Translation; AGD, anogenital distance; ER, estrogen receptor; AR, androgen receptor; NIS, sodium iodide symporter; TPO, thyroperoxidase; TR, thyroid hormone receptor; SMILES, simplified molecular-input-line-entry system; QSAR, quantitative structure-activity relationships; PPAR, peroxisome proliferator activated receptor; COUP-TFII, chicken ovalbumin upstream promotor transcription factor 2; EOGRTS, Extended One-Generation Reproductive Toxicity Study; TG, test guideline; LH, luteinizing hormone; FSH, follicle-stimulating hormone; CERAPP, Collaborative Estrogen Receptor Activity Prediction Project; TSHR, thyroid-stimulating hormone receptor; TRHR, thyrotropin releasing hormone receptor; PXR, pregnane X receptor; LABC, levator ani/bulbocavernosus muscle; TP, testosterone propionate; T4, thyroxine; T3, triiodothyronine; TSH, thyroid stimulating hormone; CAR, constitutive androstane receptor; LO(A)EL, lowest observed (adverse) effect level; (F)LC, (fetal) Leydig cell; AO, adverse outcome; StAR, steroidogenic acute regulatory protein; C_{max}, maximum concentration; AUC_{inf}, area under the curve; MEHP, mono (2-ethylhexyl) phthalate; MBP, monobutyl phthalate; WHO, World Health Organization; IPCS, International Programme on Chemical Safety.

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Introduction

In 2023 the European Commission amended the Classification, Labelling and Packaging (CLP) Regulation ((EC) No 1272/2008) to include endocrine disruption as a new hazard class. An endocrine disruptor (ED) is an exogenous substance or mixture that alters function of the endocrine system and consequently causes adverse health effects in an intact organism, its progeny, or subpopulations (WHO/IPCS, 2002). The CLP regulation requires all manufacturers, importers or downstream users of substances or mixtures to classify, label and package hazardous chemicals appropriately before placing them on the market. The European Chemicals Agency (ECHA) is currently preparing an update of the Guidance on Application of the CLP criteria to chemicals including guidance on the new hazard classes. Until then, the Agency has directed reliance on the European Food Safety Authority (EFSA)/ECHA Guidance for biocidal products and plant protection products (ECHA/EFSA, 2018).

In 1998, the Organisation for Economic Co-operation and Development (OECD) began identifying test guidelines for the screening and testing of endocrine disrupting chemicals (EDCs). A conceptual framework for testing and assessment of chemicals as EDs is described in the Revised OECD Guidance Document 150 (OECD, 2018). This framework provides a way to organize data to help determine whether a chemical is acting as an ED. Guidance developed by the EFSA and the ECHA relies heavily on the OECD conceptual framework in the development of additional guidance on how to gather, consider, and analyze data to comply with obligations under the Biocidal Products Regulation or the Plant Protection Products Regulation to identify EDs (ECHA/EFSA, 2018). The framework can also be used to assess endocrine disruption in non-pesticides or biocides.

Phthalates are a group of synthetic compounds that are used in many industrial and consumer products to make plastics more flexible and durable. They are dialkyl or aryl/alkyl diesters of phthalic acid with varying length linear or branched chains. As a chemical class, *ortho*-phthalates have been widely studied as potential male reproductive toxicants. Studies have shown that for some low molecular weight *ortho*-phthalates, exposure of rats during the prenatal period, particularly during the masculinization programming window (MPW), results in a group of effects referred to as rat phthalate syndrome. This syndrome has been well studied with phthalates such as diethyl hexyl phthalate (DEHP) or dibutyl phthalate (DBP) and is characterized by effects such as cryptorchidism, hypospadias, impaired spermatogenesis/infertility, and focal testicular dysgenesis (variably characterized as focal morphological abnormalities of the testis and seminiferous tubules including change such as partially formed testicular cords, Leydig cell hyperplasia and aggregation, or ectopic Sertoli cells) (Conley, 2018; Fisher, 2003; van den Driesche, 2017). Effects are thought to be linked to the length of the carbon chain backbone, with C3-C6 chains associated with reproductive toxicity (Li, 2019).

Di-isononyl phthalate (DINP) is a complex mixture of diesters available as two types: 1,2-benzenedicarboxylic acid esterified with branched alcohols consisting of C8-C10 (C9 rich) alkyl side chains (primarily C7 carbon backbone) or esterified with isononyl alcohols. In 2018, the ECHA Risk Assessment Committee performed an evaluation of the reproductive and developmental toxicity database for DINP and concluded DINP does not induce effects warranting classification according to the CLP Regulation (ECHA, 2018). In 2019, the EFSA Food Contact Materials, Enzymes and Processing Aids (CEP) Panel reviewed the toxicological data for DINP and established a no-observed-adverse-effect level (NOAEL) of 15 mg DINP/kg-bw/day for increased incidence of spongiosis hepatis in liver, increased liver enzyme levels, and increased absolute and relative liver and kidney weights from a study in Fisher 344 rats (EFSA, 2019; Lington, 1997). Regarding reproductive and developmental effects, the EFSA Panel identified a no-observed-effect level (NOEL) of 50 mg DINP/kg-bw/day based on decreased fetal testosterone production and histopathological changes in testis (i.e., multinucleated germ cells, MNGs) (EFSA, 2019; Clewell and Edwards, 2013). They concluded DINP has effects on both the reproductive system and the liver, with the liver as the more sensitive target organ (EFSA, 2019).

In view of the addition of endocrine disruption to the CLP regulation, a comprehensive review of the toxicological data for the endocrine effects of DINP exposure occurring through perturbation of the E, A, T, or S-pathways was performed. The stepwise methodological approach, described in the ECHA/EFSA ED guidance document (2018), was applied, and subsequently a mode of action (MoA) assessment performed.

Methods

The ED potential of DINP (CASRN 28553–12-0, CASRN 68515–48-0) was assessed by applying the ECHA/EFSA guidance (2018) for identification of effects on EATS-pathways using the workflow provided in Supplemental Data (A – ED Assessment Strategy).

Data identification

Per ECHA/EFSA guidance (2018), an assessment of endocrine disrupting properties should be based on all available relevant scientific data to include both data generated in accordance with internationally accepted study protocols and other scientific data applying systematic review methodology. Therefore, structured searches of primary literature were performed for DINP (February 29, 2024) and DINP metabolites: mono-isononyl-phthalate (MiNP), mono-oxoisononyl phthalate (MOiNP), mono-carboxyisooctyl phthalate (MCiOP), mono-hydroxyisononyl phthalate (MHiNP) (February 8, 2024). Searches were not date limited and were conducted in PubMed, Embase, and the U.S. Environmental Protection Agency (EPA) Health and Environmental Research Online (HERO) literature databases using the search syntax provided in Supplemental Data (B – Literature Search Syntax). In addition, citation mining and hand searching of authoritative assessments including EPA (US EPA, 2010), Australia's National Industrial Chemicals Notification and Assessment Scheme (NICNAS 2012), ECHA (ECHA, 1907), California Environmental Protection Agency's Office of Environmental Health Hazard Assessment (OEHHA, 2013), EFSA (EFSA, 2019), and Health Canada (Health Canada, 2020) were performed (March 2024) to identify unpublished (industry) toxicology study reports describing DINP exposures. These unpublished reports were included in the assessment only when cited in publicly available sources. Information from high-throughput (HT) assays available from the EPA Toxicity Forecaster (ToxCast) program (Kavlock and Dix, 2010) was queried for DINP, MiNP, and secondary metabolites MOiNP, MCiOP, and MHiNP (Silva, 2006). Assay data, including activity calls, cytotoxic concentrations, and assay information, were downloaded from the EPA's Tox21/ToxCast downloadable data[1] and reviewed for activity in a battery of assays relevant to each of the EATS-pathways. Concentration-response curves and data quality flags for all active assays were evaluated as part of this assessment.

Relevant studies were identified by title and abstract (TiAb) screening. If study relevance could not be determined at the TiAb level, full text was examined. Inclusion/exclusion criteria were defined by Population (P), Exposure (E), Comparator (C) and Outcome (O) (PECO) as follows: P: mammalian or non-mammalian species at any life stage; E: DINP or DINP metabolites; C: humans exposed to the lowest level of DINP identified in the study or experimental animal receiving vehicle-only; O: any EATS-pathway endpoints for *in vivo*, *in vitro*, *in silico*, or epidemiological studies. Studies considered eligible were moved forward to data extraction.

[1] https://www.epa.gov/comptox-tools/exploring-toxcast-data#Download (invitroDBv4.1, downloaded May 2024).

Following the initial assessment of data, targeted literature searches were performed in the PubMed literature database for molecular initiating events (MIEs) and key events (KEs) relevant to the MoA assessment (April 9, 2024; see Supplemental Data B – Literature Search Syntax). The identified studies were reviewed for relevance to the MoA assessment by TiAb screening.

Data extraction

Data were extracted from *in vivo* toxicology studies (using various study designs), epidemiological studies, *in vitro* assays in the peer reviewed literature, and HT assays that mapped to the E, A, T, or S-pathways. All assays/studies were used to identify apical effects and if these effects occurred through a mechanism that involved perturbation of an endocrine pathway. Data associated with endocrine-related and general toxicity endpoints were reviewed and extracted from identified studies into the Excel template provided as Appendix E of the ECHA/EFSA guidance document (https://efsa.onlinelibrary.wiley.com/doi/full/10.2903/j.efsa.2018.5311). Study metadata (e.g., study principle, test substance purity and data for each endpoint) were captured regardless of the direction or level of the reported change. When not provided by study authors, the approximate relationship between parts per million (ppm) in the diet and dosage in the animals (mg/kg-bw/day) was based on average food consumption in young and old rats with 1 ppm DINP in the diet approximately equivalent to 0.075 mg/kg-bw/day (Lehman, 1954; JECFA, 2000). Quality control (QC) review for each study was performed by a second reviewer to ensure the accuracy of the data extraction. Data relevant to the MoA assessment only were reviewed and summarized independent of the ECHA/EFSA Excel template.

Study quality assessments

For each *in vivo* and *in vitro* study, a study reliability assessment was made for all studies by one reviewer. Evaluations were performed based on the Klimisch scoring approach. This approach assigns scores of reliable without restriction (1), reliable with restriction (2), not reliable (3), and not assignable (4) (Klimisch et al., 1997). If studies were scored as either 1 or 2, they were considered reliable and incorporated into the evaluation. If they were scored as a 3 (not reliable) or 4 (not assignable), they were not incorporated in the evaluation. As such, only studies that were considered reliable with/or without restriction were used in the line of evidence assessments. Excluded studies and reasons for exclusion are provided in Supplemental Data (C – Reason for Exclusion).

Critical appraisal was performed by risk of bias (RoB) assessment on all studies evaluated as part of the MoA assessment. RoB assessments were performed by two scientists with expertise in toxicology, reproductive toxicity, experimental animal studies, and/or *in vitro* studies. The scientists performing RoB assessments were not involved with the EATS data extraction (described in Section 2.2) or evaluation (described in Section 2.4 below). RoB was evaluated for endpoints within studies relevant to the MoA assessment using the Office of Health Assessment and Translation (OHAT) RoB tool (NTP-OHAT, 2019). Endpoints relevant to the MoA assessment were assigned one of the following ratings: 1) definitely low RoB, 2) probably low RoB, 3) probably high RoB or not reported, and 4) definitely high RoB. The lower the RoB, the higher the methodological quality of the study endpoint (NTP-OHAT, 2019). Each critical appraisal underwent QC to ensure a consistent approach was taken across all studies and endpoints.

Evaluation and assessment of lines of evidence

In accordance with the ECHA/EFSA guidance document, lines of evidence were assembled using the data extracted from the literature (ECHA/EFSA, 2018). Lines of evidence describe groups of endpoints that together can be used to assess potential endocrine activity and endocrine mediated apical and potentially adverse effects (EFSA Scientific Committee, 2017). 'Adversity' and 'adverse' are used throughout the work presented herein as defined by ECHA/EFSA as a "change in the morphology, physiology, growth, development, reproduction or life span of an organism, system or (sub)population that results in an impairment of functional capacity, an impairment of the capacity to compensate for additional stress or an increase in susceptibility to other influences" (ECHA/EFSA, 2018).

Endocrine activity was described by two lines of evidence including 1) *in vitro* mechanistic, and 2) *in vivo* mechanistic data. Lines of evidence that identify apical effects were extracted from *in vivo* studies. Adverse effects associated with disruption in EATS-pathways were identified in the ECHA/EFSA template as: 1) EATS-mediated, and 2) EATS-sensitive endpoints; along with generalized effects as associated with systemic toxicity endpoints. Examples of EATS-mediated endpoints include reproductive organ weight and histopathology, as well as developmental markers such as anogenital distance (AGD) and vaginal opening (ECHA/EFSA, 2018). EATS-sensitive endpoints are considered as potentially sensitive to, but not diagnostic of, EATS-pathways (e.g., reproductive outcomes such as gestation length, litter size, and viability). Systemic toxicity data include endpoints such as body weight changes, as well as liver and kidney weight and histopathology, to support and contextualize the distinction between a direct effect of the chemical causing general toxicity versus a perturbation in any of the EATS-pathways. Epidemiological and *in silico* data were assessed as a line of supportive evidence for signals of effects for EATS-mediated endpoints associated with exposure to DINP (ECHA/EFSA, 2018).

Each line of evidence was evaluated for each of the EATS-pathways by applying a WoE assessment. Where data were available, if none of the EATS mediated endpoints suggested an adverse outcome occurred via E-, A-, T-, or S-pathways, DINP was considered to have no evidence of an endocrine effect. Each line of evidence was categorized based on available data as having 'high', 'medium', or 'low' confidence in the findings, based on the categories described by Escriva et al. (2021) which include: 1) High – outcomes (positive or negative) observed in one or more studies of high reliability (performed according to standardized test guidelines) with no conflicting results; 2) Medium – outcomes (positive or negative) observed in one or more studies or assays of partial reliability (non-guideline), with no conflicting results; effects observed in one or more studies of high or partial reliability with conflicting results (e.g., no change versus decreased testosterone levels) that could be explained by differences in study design; Low – outcomes (positive or negative) were observed in one or more studies of high or partial reliability with conflicting results that could not be explained by differences in study design. Systemic toxicity was assessed in parallel with the EATS endpoints and used for study interpretation but was not used to diminish the confidence in the observed effects.

Based on ECHA/EFSA guidance, an assessment was performed of whether the available DINP information was sufficient to support a conclusion on EATS-mediated adversity in humans and mammals (ECHA/EFSA, 2018). To perform this assessment, the DINP dataset was compared to the available test guidelines for mammals described in Table 14 of the ECHA/EFSA Guidance (ECHA/EFSA, 2018). For assessment of EAS-mediated adversity, the endpoints foreseen to be investigated in an extended one-generation reproductive toxicity study (OECD TG 443) or a two-generation reproductive toxicity study (OECD TG 416) were compared to the endpoints measured in the available DINP studies. For T-mediated adversity, the thyroid endpoints foreseen to be investigated in the following guidelines studies were compared to those measured in the DINP studies: OECD TGs 407, 408, 416, 443 and 451–3. DINP guideline and non-guideline studies were used to determine data sufficiency. Following this analysis, and according to the ECHA/EFSA guidance, if 'EATS-mediated' adversity was not sufficiently investigated and no 'EATS-mediated' adversity was observed, EATS-related endocrine activity was assessed. This was performed by determining whether the following information was available for DINP:

ToxCast Estrogen Receptor (ER) Bioactivity Model output[2] or uterotrophic bioassay (E), Hershberger bioassay (A), and H295R steroidogenesis assay and the 'aromatase assay (human recombinant)' (S). Owing to the lack of T *in vitro* mechanistic assays, endocrine activity for the T-pathway was considered sufficiently investigated if the T endpoints in the *in vivo* assays described above were investigated. The impact of missing data or a lack of evidence were considered when assessing the confidence in the findings. Missing data can either reduce the level of confidence in the findings or, when the data gap is filled by other lines of evidence, have no effect on the overall level of confidence.

Mode of action assessment

Based on the guidance provided by ECHA/EFSA (ECHA/EFSA, 2018), a MoA assessment establishes the biological plausibility of a link between endocrine activity and endocrine related adverse effects using a WoE approach. Because Adverse Outcome Pathways (AOPs) provide a structured framework that can be used to integrate evidence to support MIEs and KEs (ECHA/EFSA, 2018), a hypothesized AOP that describes a phthalate antiandrogenic Leydig cell MoA was used as the framework for a MoA assessment of A- and S-pathways, along with conducting an integration of dose and temporal concordance of any observed effects (Li and Spade, 2021).

Quantitative structure-activity relationship (QSAR) assessment of DINP and metabolites

DINP metabolites, MiNP, MOiNP, MCiOP, and MHiNP, were evaluated by means of the full endocrine activity suite within Leadscope Model Applier (v2022.0.2–3). The suite includes the following endpoints, all of which were considered to address potential for endocrine disruption activity:

- Androgen receptor (AR) binding
- AR transactivation antagonist
- Aromatase inhibition
- ER bioactivity
- Sodium iodide symporter (NIS) inhibition
- Thyroperoxidase (TPO) inhibition
- Thyroid hormone receptor (TR) binding and transactivation

Chemical structures of DINP metabolites were searched in the EPA CompTox Chemicals Dashboard (v 2.2.1) to identify simplified molecular-input-line-entry system (SMILES) codes associated with each compound of interest. These SMILES codes and those representing the product in commerce were then input into the Leadscope interface for QSAR assessment. Descriptions of models used are provided in Supplemental Data (D – QSAR Model Predictions).

Results

Evidence identification and extraction

The literature searches for DINP and DINP metabolites identified 4248 articles (de-duplicated) from PubMed, Embase, and EPA HERO and six additional primary peer reviewed articles from review of authoritative sources (Fig. 1). A total of 102 primary peer reviewed publications met the inclusion criteria and were therefore considered relevant and included in the evaluation. Fourteen unpublished (industry) toxicology study reports containing endocrine endpoints relevant to the EATS assessment were also identified for DINP; therefore, a total of 116 studies were reviewed at full text (Fig. 1). The results for both formulations of DINP (CASRN 28553-12-0 and CASRN 68515-48-0) were combined in this analysis based on the findings of a study testing the effects of the two formations of DINP (CASRN 28553-12-0 and 68515-48-0) on fetal rat testosterone production and testicular gene expression following *in utero* exposure and showing statistically indistinguishable results (Hannas, 2011). In addition, HT assays that mapped to EATS endpoints were identified in the EPA ToxCast database for both formulations of DINP.[3] Data for a total of 99 Tox21/ToxCast HT assays were downloaded and evaluated; no HT data were available for DINP metabolites.

All studies that met the inclusion criteria were evaluated. When study relevance was confirmed and study reliability considered, six studies were scored as 'not reliable' and were removed from the endocrine WoE assessment leaving 110 articles in the initial data extraction and assessment (Fig. 1). Studies excluded based on either relevance and/or reliability of data were excluded for reasons such as no dose levels were provided or insufficient information available in the study report to have confidence in its reliability. After the preliminary data evaluation, a MoA assessment was conducted based on a hypothesized AOP relevant to phthalate toxicity using 19 articles from the preliminary evaluation and 4 articles identified in targeted literature searches for MIEs for this hypothesized AOP (PPAR binding/ antagonism, arachidonic acid release, and chicken ovalbumin upstream promotor (COUP)-transcription factor 2 (TFII) expression).

The OECD Conceptual Framework lists OECD test guidelines and standardized test methods that can be used to assess chemicals for ED potential and organizes these test methods across different levels of biological organization (OECD, 2018). OECD guideline, as well as non-guideline or guideline-like studies that are published in the peer reviewed literature are integrated into the framework (Table 1) to provide an assessment of the sufficiency of the data available and to determine if additional studies may be required.

The DINP evidence base included 26 epidemiology studies and 10 *in silico* models at Level 1; 99 HT assays that mapped to EATS-pathways and 26 *in vitro* assays considered Level 2 methods; four mechanistic studies at Level 3; 45 *in vivo* repeat administration studies at Level 4; and one study at Level 5 (Fig. 2). Nine of the studies were conducted according to OECD test guidelines, but given the age of some of the studies and as the guidelines are periodically updated, it was not assumed that these studies contained sufficient data to meet the current guideline standards. OECD states that the list of test methods provided in the conceptual framework is not exhaustive and assays other than those described in the framework can be used to assess endocrine disruption. All studies (those conducted according to standard test guidelines and those that were not) were evaluated, and data extracted into the ECHA/EFSA Excel template.

After reliability assessment, and as described in the ECHA/EFSA guidance (2018), the studies conducted using *in vitro* and *in vivo* mechanistic methods (Levels 2 and 3, respectively) and the endpoints measured using these test methods were evaluated to provide information on E, A, T, or S endocrine pathway activity. In addition, studies conducted using *in vivo* test methods providing data on effects on endocrine-relevant endpoints (Levels 4 and 5) and the endpoints measured by these studies were evaluated to support the identification of effects associated with endocrine pathway perturbations. The evidence base for endocrine activity included 20 unique endpoints

[2] The ToxCast ER pathway model for bioactivity uses integrated data from 17 of the 18 high throughput assays to measure agonist and antagonist responses in an unweighted manner with subtraction of background and other nonspecific assay interferences including cytotoxicity. The model output includes area under the curve (AUC) scores for agonist and antagonist activity as described by Browne et al. (2015).

[3] US EPA ORD, Center for Computational Toxicology (2018): ToxCast Database: invitrodb version 3.5. The United States Environmental Protection Agency's Center for Computational Toxicology and Exposure. Dataset. https://doi.org/10.23645/epacomptox.6062623.v10.

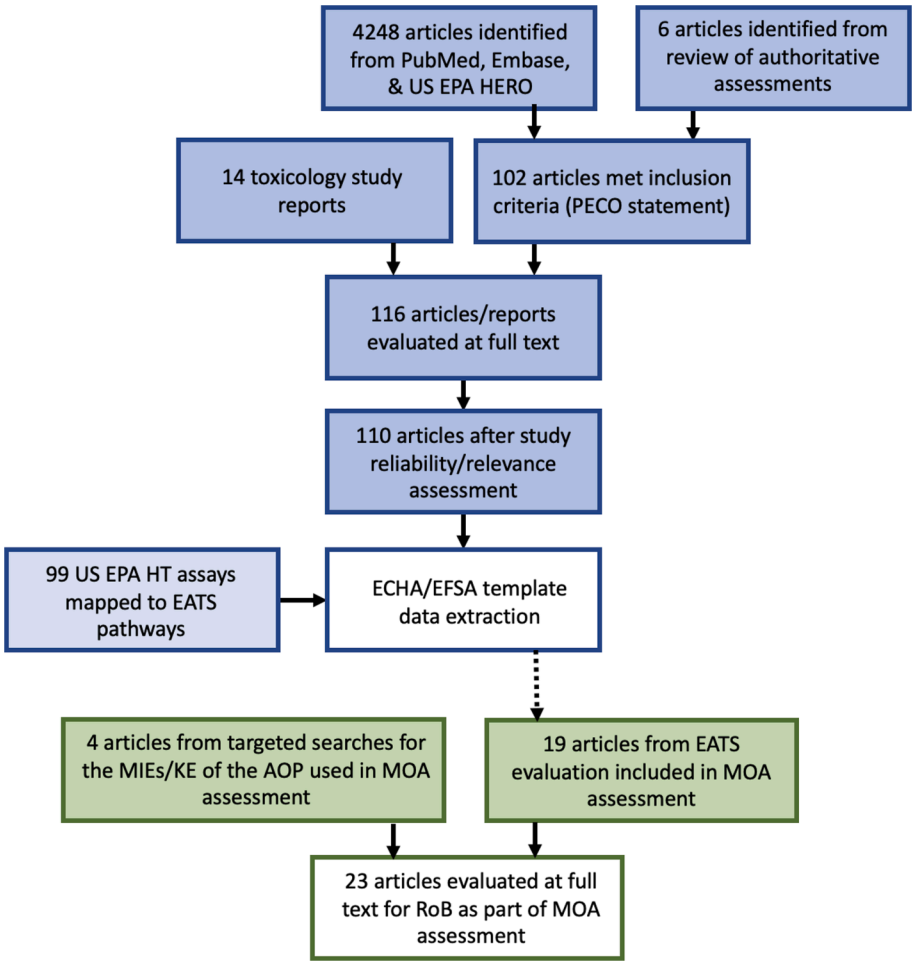

Fig. 1. Total number of articles identified and evaluated for the EATS and MoA assessments of DINP. Data were extracted into the ECHA/EFSA template from a total of 110 articles (lowest and darker blue box) and 99 HT assays (light blue box) that mapped to EATS-pathways. After the preliminary data evaluation, a MoA assessment was conducted based on a hypothesized AOP relevant to phthalate toxicity (Li and Spade, 2021) using 19 articles from the preliminary evaluation and four identified in literature searches for MIEs that were not previously mapped to the androgen pathway (green boxes). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.) Key: US EPA HERO: United States Environmental Protection Agency Health and Envionmental Research Online; PECO: population, exposure, comparator, outcome; HT: high-throughput; ECHA: European Chemicals Agency; EFSA: European Food Safety Authority; MIE: molecular initiating event; KE: key event; AOP: adverse outcome pathway; EATS: estrogen, androgen, thyroid, steroidogenesis; MoA: mode of action; RoB:throug risk of bias.

identified as providing *in vitro* mechanistic data and 15 unique endpoints describing *in vivo* mechanistic data. The evidence base for endpoints identified as endocrine-related included 32 unique EATS-mediated endpoints and 24 unique endpoints classified as sensitive to, but not diagnostic of perturbations in EATS-pathways. The available data and lines of evidence are summarized in Fig. 2.

Identification of data and integration of lines of evidence

Each line of evidence was organized by E-, A-, T-, or S-pathway (Supplemental Data E – Lines of Evidence). Where available, general adversity or systemic toxicity data were considered in conjunction with the EATS-mediated endpoints (OECD, 2018). *In silico* and epidemiological data were considered as a supportive line of evidence only (ECHA/EFSA, 2018).

Analysis of evidence: estrogen pathway

In the assessment of DINP endocrine activity mediated by E, there was evidence from two different sources: *in vitro* assays and *in vivo* mechanistic assays (Table 2). The *in vitro* assays (ToxCast HT and peer reviewed literature) were not metabolically competent and lacked the capability to activate the DINP diester to the monoester forms. The evidence available for DINP metabolites (MiNP, MOiNP, MCiOP, and MHiNP) came from *in silico* predictions for ER binding and agonist and antagonist activity and a single *in vitro* study of MiNP, MOiNP, and MHiNP that showed no evidence of activity (agonist or antagonist effects) at 100 μM using human ERα or ERβ reporter gene constructs (Table 2). A summary of the evaluated evidence for DINP E-mediated effects is provided in Table 2; detailed information for each study is provided in Supplemental Data (F – All EATS Data) and in Supplemental DINP Evidence Analysis.

Overall, the WoE (Table 1) showed DINP only caused effects at high dose levels that were also associated with general toxicity. This finding was supported by studies that showed a lack of association between estradiol, follicle-stimulating hormone (FSH), luteinizing hormone (LH) levels, age at puberty, and DINP metabolite concentrations in epidemiology studies, negative predictions in for DINP metabolites in *in silico* models, as well as the overall lack of activity in *in vitro* assays for ER antagonist/antagonism activity and *in vivo* mechanistic studies (Levels 1, 2, and 3 methods) that showed DINP did not elicit activity in the estrogen pathway. Detailed information for each study is provided in Supplemental Data (F – All EATS Data) and in Supplemental DINP Evidence Analysis.

Table 1
OECD Conceptual Framework for Assessment of Endocrine Disrupting Chemicals used to Group Studies in the eEvaluation of DINP.

OECD Conceptual Framework [Test Method] Level[1]	Description of Test Methods
1	Non-test information, e.g., epidemiology studies, read-across approaches, and *in silico* models (e.g., quantitative structure activity relationships [QSAR])
2	*In vitro* assays providing data for endocrine mechanism(s) or pathway(s), e.g., H295R Steroidogenesis Assay (OECD TG 456); HT assays from Tox21/ToxCast or *in vitro* assays published in the peer reviewed literature
3	*In vivo* assays providing data for endocrine mechanism(s) or pathway(s), e.g., Hershberger (OECD TG 441) or uterotrophic assays (OECD TG 440)
4	*In vivo* studies providing data for adverse effects on endocrine-relevant endpoints, e.g., repeated dose 90-day study (OECD TG 408), prenatal developmental toxicity studies (OECD TG 414), reproduction/developmental toxicity tests (OECD TG 421, 422)
5	*In vivo* assays providing data on adverse effects on endocrine-relevant endpoints over multiple life stages, e.g., extended one-generation reproductive toxicity study (EOGRTS; OECD TG 443), two-generation reproductive toxicity study (OECD TG 416 recent update)

Key: OECD: Organisation for Economic Co-operation and Development; TG: test guideline; HT: high-throughput; EOGRTS: extended one-generation reproductive toxicity study.

[1] OECD (2018), Revised Guidance Document 150 on Standardised Test Guidelines for Evaluating Chemicals for Endocrine Disruption, OECD Series on Testing and Assessment, No. 150, OECD Publishing, Paris, https://doi.org/10.1787/9789264304741-en.

Analysis of evidence: thyroid pathway

In the assessment of endocrine activity mediated by T, the evidence for DINP comes from two different sources: *in vitro* data and *in vitro* mechanistic assays (Table 3). The *in vitro* assays (ToxCast HT and peer reviewed literature) were not metabolically competent and lacked the capability to activate the DINP diester to the monoester forms. The evidence available for DINP metabolites (MiNP, MOiNP, MCiOP, and MHiNP) came from *in silico* predictions for NIS and TPO inhibition as well as TR binding and activation (Table 3). *In vitro* mechanistic data for the T-pathway showed DINP had no effect on TR, thyroid-stimulating hormone receptor (TSHR), or thyrotropin releasing hormone receptor (TRHR) in 16 out of 16 assays (Tox21/ToxCast HT assay data). In HT assays of liver nuclear receptors relevant to thyroid pathways (conjugation and elimination of the thyroid hormone (TH)) (Noyes, 2019), DINP had no effect in constitutive androstane receptor [CAR] binding assays. In two (out of five) assays of the pregnane X receptor [PXR], DINP was active with an AC50 between 1.63 and 54.33 μM, suggesting that DINP may induce drug metabolism enzymes. *In vitro* mechanistic data for the T-pathway showed increased iodide uptake in a single *in vitro* assay conducted in FRTL-5 thyroid follicular cells at 10^{-3} M DINP (for 72 h). Thyroid-mediated adversity was assessed in a total of 16 studies and no changes in thyroid weight or histopathology were observed (Table 3). A summary of the evaluated evidence for DINP mediated effects on the T-pathway are provided in Table 3. Detailed information for each study is provided in Supplemental Data (F – All EATS Data) and in Supplemental DINP Evidence Analysis.

Overall, DINP did not produce T-mediated apical effects even at high dose levels that were also associated with general toxicity. This finding was supported by epidemiology studies that showed a lack of an association in hormone changes and concentration of DINP as well as *in silico* models that showed an overall lack of NIS and TPO inhibition and TR binding. One *in vitro* mechanistic study showed DINP had the potential to change iodide uptake by NIS.

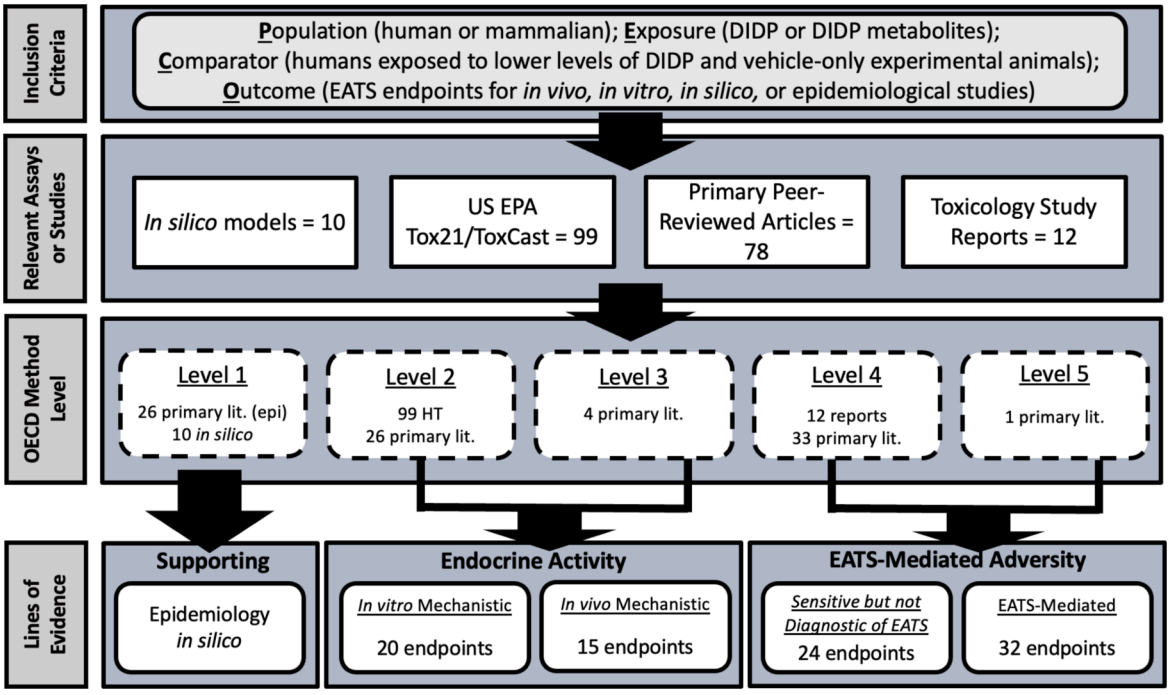

Fig. 2. Available DINP Studies and Integrated Lines of Evidence for Outcomes and Endocrine Activity for E, A, T or S Pathways. The number of mammalian studies or HT assays from which data were extracted providing data for each OECD test method level is shown (Relevant Assays or Studies). Six studies excluded from the data assessment and two with a study principle that was not in the ECHA/EFSA list are not included in this count. The number of studies or assays at each OECD method level is provided (each study may be counted at more than one method level). The lines of evidence show the number of unique endpoints that provide information on E, A, T, or S endocrine pathway activity or adversity. Key: lit: literature; epi: epidemiology.

Table 2
Integration and Assessment of Lines of Evidence for DINP and DINP Metabolite Perturbation of the E-pathway. Key: HT: high-throughput; ER: estrogen receptor; DINP: di-isononyl phthalate; MiNP: mono-isononyl phthalate; MOiNP: mono-oxoisononyl phthalate; MCiOP: mono-carboxyisooctyl phthalate; MHiNP: mono-hydroxyisononyl phthalate; AGD: anogenital distance; FSH: follicle-stimulating hormone; LH: luteinizing hormone; QSAR: quantitative structure-activity relationship; SMILES: simplified molecular-input-line-entry system; CERAPP: Collaborative Estrogen Receptor Activity Prediction Project.

	Line of Evidence	Observed Effects (Confidence in the Line of Evidence) [1,2]	Integrated Lines of Evidence[3]
Lines of evidence for endocrine activity	ER binding/ transactivation	• No activity in HT assays[4] (Medium) • Weak or no binding to ER (Akahori, 2005; Akahori, 2008; Lee, 2019; Zacharewski, 1998) (Medium) • ER agonist effect in ZR-75 cells at 10^{-5} M (11-day exposure) (Harris, 1997); no ER agonist effects in HEK293, CHO-K1, MCF-7, MVLN, and HepG2 cells or in the recombinant yeast screen (Harris, 1997; Ghisari and Bonefeld-Jorgensen, 2009; Lv, 2022; Moche, 2021; Engel, 2017; Takeuchi, 2005) (Low) • No ER antagonist effects in HEK293, CHO-K1, MCF-7, and HepG2 cells (Lv, 2022; Moche, 2021; Engel, 2017; Takeuchi, 2005) (Medium) • DINP metabolites (MiNP, MOiNP, and MHiNP) had no ER agonist or antagonist effects (Engel, 2017) (Medium)	**Overall negative for endocrine activity across the E-pathway.**
	Uterotrophic (estrogenic / anti-estrogenic)	• No increase in uterine weight (Akahori, 2008; Zacharewski, 1998; Sedha, 2015) (High) • No decrease in uterine weight when DINP co-administered with 17a-ethynylestradiol (Akahori, 2008) (High)	
	Hormone levels	• No dose-related changes in serum estradiol in adult mice or monkeys (Chiang, 2020; Chiu, 2020; Hall, 1999; Lee et al., 2006; Bhurke, 2023); decreased serum estradiol in adult mice (Chen, 2022) (Low)	
Lines of evidence for E-mediated adversity (adult exposure)	Organ weight	• Decreased uterus weight in rats exposed to 20,000 ppm DINP (~1,500 mg/kg-bw/day) (Hazleton Laboratories Inc, 1991b). Non-dose dependent decrease in absolute uterus weight in mice exposed with up to 8,000 ppm DINP (~600 mg/kg-bw/day) (Aristech Chemical Corporation, 1998a) (Low) • Decreased absolute ovary weight in mice exposed to 20,000 ppm (~1,500 mg/kg-bw/day) DINP (Midwest Research Institute, 1981) (Low) • Increased relative uterus weight in mice at 2, 20, and 200 mg/kg-bw/day (Hwang, 2017) (Low) • No change in ovary or uterus weight up to 2,500 mg/kg-bw/day DINP (Lington, 1997; Hall, 1999; Aristech Chemical Corporation, 1998b; Chiang and Flaws, 2019) (Low)	**Overall negative for E-mediated adversity (adult exposure to DINP).** Lines of evidence show evidence for a lack of adversity in endocrine mediated endpoints. Consistent findings were observed at dose levels associated with general toxicity.
	Organ histopathology	• No change in ovary, uterus, or vagina at up to 40,000 ppm (~3,080 mg/kg-bw/day) DINP (Hall, 1999; Aristech Chemical Corporation, 1998a; Hwang, 2017; Aristech Chemical Corporation, 1982a; Hazleton Laboratories Inc, 1971a; Hazleton Laboratories Inc, 1971b) (Medium) • Increased in the disorderly arrangement of granulosa cells, ovarian atrophy and endometrial hyperplasia in mice or rats exposed up to 25,000 ppm (~1,875 mg/kg-bw/day) DINP (Chen, 2022; Bio/dynamics Inc, 1987; Hazleton Laboratories Inc, 1991a) (Low)	
	Estrus cyclicity	• Change in estrus cyclicity at 3- and 9-months post-dose (dose dependency not established); mice exposed to dose levels up to 200 mg/kg-bw/day DINP for 10 days (Chiang and Flaws, 2019) (Low) • Change in estrus cyclicity at 3 and 7 months of dosing (dose dependency not established); no change at 1, 5, and 11 months of dosing; mice exposed to dose levels up to ~ 240 mg/kg-bw/day for 11 months) (Laws, 2023) (Low)	
	Pubertal (female)	• No increase in uterine weight or effect on age of vaginal opening (Sedha, 2015) (Medium)	
Evidence of general toxicity (adult exposure)	Body weight	• Decrease in body weight or body weight gain (Hazleton Laboratories Inc, 1991b; Aristech Chemical Corporation, 1998a; Hazleton Laboratories Inc, 1991a) (Medium)	
Lines of evidence for E-mediated adversity (*in utero* exposure)	Organ weight	• Decreased ovary and/or uterus weight up to 20,000 ppm DINP (~1,540 mg/kg-bw/day) in parental females and F1 offspring (Masutomi, 2003; Waterman, 2000) (Medium) • No change in uterus and/or ovary weight at up to 1,000 mg/kg-bw/day in parental or F1 adults (Boberg, 2011; Hellwig et al., 1997; Waterman, 1999) (Medium)	**Overall negative for E-mediated adversity (developmental exposure to DINP)** Lines of evidence show an overall lack of evidence for endocrine adversity. Findings were observed at dose levels also producing evidence of general toxicity.
	Organ histopathology	• No change in ovary, uterus, cervix, and/or vagina at up to 20,000 ppm (~1,540 mg/kg-bw/day) DINP in adult P or F1 females (Hazleton Laboratories Inc, 1991b; Midwest Research Institute, 1981; Bio/dynamics Inc, 1987; Masutomi, 2003; Waterman, 2000) (Medium)	
	Estrus cyclicity	• No effect on estrus cyclicity in F1 adults at up to 20,000 ppm (~1,540 mg/kg-bw/day) DINP (Lee et al., 2006; Masutomi, 2003) (Medium)	
	Developmental markers	• No change in age at vaginal opening or AGD in females at up to 20,000 ppm DINP (~1,540 mg/kg-bw/day) (Lee et al., 2006; Masutomi, 2003; Gray, 2023; Gray, 2000) (Medium)	
Evidence of general toxicity (*in utero* exposure)	Body weight (repro)	• Decreased body weight observed (Hazleton Laboratories Inc, 1991a; Masutomi, 2003; Waterman, 2000)	
Supporting lines of evidence	Epidemiology	• Estradiol: no association between the molar sum of DINP metabolite concentrations and estradiol levels (Frederiksen, 2012; Hart, 2018; Henrotin, 2020; Joensen, 2012; Lenters, 2015; Specht, 2014) • FSH: no association between the molar sum of DINP metabolite concentrations and FSH levels (Frederiksen, 2012; Henrotin, 2020; Lenters, 2015; Specht,	These epidemiology lines of evidence suggest a lack of E-mediated adversity. The ER model and QSAR evidence provide

(*continued on next page*)

Table 2 (*continued*)

Line of Evidence	Observed Effects (Confidence in the Line of Evidence) [1,2]	Integrated Lines of Evidence[3]
	2014); MiNP concentration negatively associated with FSH levels (Joensen, 2012) • LH: no association between the molar sum of DINP metabolite concentrations and LH levels (Frederiksen, 2012; Hart, 2018; Henrotin, 2020; Joensen, 2012; Lenters, 2015; Specht, 2014) • No association between the molar sum of DINP metabolites and age at puberty in girls (Frederiksen, 2012)	overall support for a lack of ER activity (agonist or antagonist).
In silico predictions	• MiNP, MOiNP, MCiOP, and MHiNP (DINP metabolites) negative QSAR prediction for ER agonism or antagonism (CompTox Chemicals Dashboard DINP SMILES)[5] • MiNP, MOiNP, MCiOP, and MHiNP positive QSAR prediction for ER agonism or antagonism (commercial DINP SMILES)[5] • DINP (parent) negative for CERAPP potency consensus ER model[6] • DINP (parent) negative ER agonist and/or antagonist prediction in ToxCast ER Pathway Model[7]	

[1] Endpoints that are 'sensitive to but not diagnostic of EATS' are reported in Supplemental Data (F – All EATS data).
[2] High: effects observed in one or more guideline study with no conflicting results; Medium: effects observed in one or more non-guideline studies with no conflicting results; effects observed in one or more guideline or non-guideline studies with conflicting that could be explained by differences in study design; Low: effects were observed in one or more studies of guideline or non-guideline studies with conflicting results that could not be explained by differences in study design.
[3] Assessment of whether a sufficient dataset was available to support a conclusion on E activity in Section 3.8: Data Sufficiency and Identification of Data Gaps.
[4] https://www.epa.gov/comptox-tools/exploring-toxcast-data#Download (invitroDBv4.1, downloaded May 2024).
[5] Leadscope Model Applier (v2022.0.2–3); QSAR results provided in Supplemental Data (D – QSAR Model Predictions).
[6] CERAPP (Collaborative Estrogen Receptor Activity Prediction Project) describes binding, agonist, or antagonist activity (Mansouri et al., 2016).
[7] ToxCast ER pathway model describes agonist or antagonist activity (Browne, 2015).

Analysis of evidence: steroidogenesis pathway

In the assessment of endocrine activity mediated by S, the evidence for DINP came from two sources: *in vitro* and *in vivo* mechanistic assays (Table 4). The *in vitro* assays (ToxCast HT and peer reviewed literature) were not metabolically competent and lacked the capability to activate the DINP diester to the monoester forms. The evidence available for DINP metabolites (MiNP, MOiNP, MCiOP, and MHiNP) came from supporting *in silico* predictions for aromatase inhibition activity (Table 4). No *in vitro* studies of DINP metabolites were available. *In vitro* mechanistic data for the S-pathway showed DINP had no effect in an HT aromatase assay (Table 4). No DINP related effects were observed in the HT H295R steroidogenesis assay in which 11 steroid hormones were evaluated (17α-hydroxypregnenolone, progesterone, 17α-hydroxyprogesterone, 11-deoxycorticosterone, androstenedione, deoxycortisol, cortisol, corticosterone, testosterone, estrone, and estradiol). In other *in vitro* (peer reviewed literature) H295R assays, a statistically significant increase in estradiol level was detected at all concentrations of DINP (0.03–30 μM) (Moche, 2021) and a weak increase in estradiol levels that Lee and colleagues interpreted to be of low relevance (Lee, 2019). Overall, *in vivo* mechanistic studies supported the lack of effects of DINP on serum estradiol levels. A single study showed a decrease in serum estradiol at 20 and 200 mg/kg-bw/day in adult mice administered 0, 2, 20, and 200 mg/kg-bw/day for 14 days (Chen, 2022) (Table 4). No assessment of systemic toxicity was performed in this study but in other mouse studies conducted using similar doses, toxicity was not observed (Table 4). In two other studies in which adult mice were administered up to 200 mg/kg-bw/day DINP for 10–14 days, there were no changes in serum estradiol levels (Chiang, 2020; Chiu, 2020). Similarly in adult marmosets, no change in serum estradiol was observed following administration of 100, 500, or 2,500 mg/kg-bw/day DINP for 13 weeks (Hall, 1999). Further, no dose-related changes in serum estradiol were observed in two studies of rats and mice exposed *in utero* to DINP up to 20,000 ppm (~1,500 mg/kg-bw/day) (Lee et al., 2006; Bhurke, 2023) (Table 4).

DINP had weak or no effect on testosterone synthesis as evaluated in the H295R steroidogenesis assay (Lee, 2019; Moche, 2021). *In vivo* assessment of testosterone levels in males, was evaluated in 12 *in utero* exposure and three adult exposure studies. No changes in serum testosterone levels were observed in adult exposure studies in mice or monkeys. In five (of six) studies with DINP exposure (up to 1,500 mg/kg-bw/day) conducted during the masculinization programming window (MPW; approximately gestational day [GD] 15.5–18.5 in rats), *ex vivo* rat testis testosterone production in GD 18–21.5 animals was reduced (Hannas, 2011; Borch, 2004; Furr, 2014; Gray, 2021; Li, 2015). When fetal intratesticular testosterone content (testicular homogenates or extracts) was measured, the majority of studies (four of six) showed no change following DINP exposure between GD 17.5–21 despite exposure to high levels of DINP (up to 900 mg/kg-bw/day) (Clewell and Edwards, 2013; Boberg, 2011; Adamsson, 2009; van den Driesche, 2020). In one study in which a reduction of fetal intratesticular testosterone was observed, testosterone content was measured at both 2- and 24-h post-dose. At 2 h post-dose, testosterone level was reduced, but no change was detected at 24 h (compared to concurrent controls), suggesting the change in testosterone level may be a transient effect (Clewell and Edwards, 2013). No effects were observed in serum testosterone levels in rats exposed to DINP (up to 20,000 ppm [~1,400 mg/kg-bw/day]) during the MPW in five of five studies (Table 4). DINP showed inconsistent effects in transcriptomic assays evaluating steroidogenic gene/protein expression, providing some support for S-mediated endocrine activity (Table 4). A summary of the evaluated evidence for DINP mediated effects on the S-pathway are provided in Table 4. Detailed information for each study is provided in Supplemental Data (F – All EATS Data).

Overall, the WoE showed some evidence in DINP-mediated effects on the S-pathway leading to changes in steroidogenic gene or protein expression and hormone levels (estradiol and testosterone). In epidemiology studies, limited studies showed a lack of an association between changes in hormones and DINP metabolite concentrations. *In silico* models showed no effects on steroidogenesis.

Analysis of evidence: androgen pathway

In the assessment of endocrine activity mediated by A, the evidence for DINP came from two different sources: *in vitro* and *in vivo* mechanistic assays (Table 5). The *in vitro* assays (ToxCast HT and in peer reviewed literature) were not metabolically competent and lacked the capability to activate the DINP diester to the monoester forms. The evidence available for DINP metabolites came from supporting *in silico* predictions for AR binding, agonist, and antagonist activity (Table 5) and a single *in vitro* study evaluating AR activity for MiNP, MOiNP, and MHiNP that showed no agonist or antagonist activity at 100 μM using

Table 3
Integration and Assessment of Lines of Evidence for DINP and DINP Metabolite Perturbation of the T-pathway. Key: TR: thyroid hormone receptor; HT: high-throughput; TSHR: thyroid-stimulating hormone receptor; TRHR: thyrotropin release hormone receptor; NIS: sodium-iodide transporter; KE: key event; CAR: constitutive androstane receptor; PXR: pregnane X receptor; DINP: di-isononyl phthalate; TSH: thyroid-stimulating hormone; T3: triiodothyronine; T4: thyroxine; QSAR: quantitative structure-activity relationship; TPO: thyroperoxidase; MiNP: mono-isononyl phthalate; MOiNP: mono-oxoisononyl phthalate; MCiOP: mono-carboxyisooctyl phthalate; MHiNP: mono-hydroxyisononyl phthalate; SMILES: simplified molecular-input-line-entry system.

	Line of Evidence	Observed Effects (Confidence in the Line of Evidence) [1,2]	Integrated Lines of Evidence[3]
Lines of evidence for endocrine activity	Receptor binding	• No activity in TR binding (HT assays)[4] (Medium) • No activity in TSHR binding (HT assays) (Medium) • No activity in TRHR binding (HT assays) (Medium)	**Evidence suggests DINP induces Phase I metabolism enzymes and potentially stimulates iodide uptake in the thyroid.** Lines of evidence show evidence for lack of endocrine activity across KEs in the thyroid pathway network (i.e., TR, TSH receptor and CAR binding). One non-guideline study shows potential changes in iodide uptake.
	Iodide uptake	• Increased uptake of iodide in sodium-iodide symporter (NIS) uptake assay conduced in FTRL-5 cells (Wenzel et al., 2005) (Medium) • No effect on luciferase expression from NIS promoter or rat endogenous NIS gene expression in PCCL3 cells (Breous et al., 2005) (Medium)	
	Cell proliferation	• Slight but significant effect on thyroid hormone mediated pituitary cell proliferation (Ghisari & Bonefeld-Jorgensen, 2009) (Medium)	
	Nuclear receptor induction	• No activity in hepatic CAR binding HT assays (Medium) • Activity detected in 2 of 5 assays of PXR binding HT assays (Medium)	
Lines of evidence for T-mediated adversity (adult exposure)	Organ weight	• No change in thyroid weight at dose levels up to 40,000 ppm DINP in dogs or 900 mg/kg-bw/day DINP in rats (Lington, 1997; Hall, 1999; Hazleton Laboratories Inc, 1971a; Hazleton Laboratories Inc, 1971b; Boberg, 2011; Kwack, 2010; Kwack, 2009; Pugh, 2000) (Medium)	**Overall negative for T-mediated adversity (adult exposure to DINP).** Lines of evidence strongly support the lack of adversity mediated by perturbation in the thyroid pathway. A consistent lack of effects was observed even at dose levels that produced general toxicity.
	Histopathology	• No change in thyroid histopathology at dose levels up to 1,888 mg/kg-bw/day (Lington, 1997; Hall, 1999; Hazleton Laboratories Inc, 1991b; Aristech Chemical Corporation, 1998a; Aristech Chemical Corporation, 1982a; Hazleton Laboratories Inc, 1971a; Hazleton Laboratories Inc, 1971b; Bio/dynamics Inc, 1987; Hazleton Laboratories Inc, 1991a) (Medium) • Dose related increase in vacuolation in follicular epithelial cells in autoimmune thyroid disease model at dose levels up to 15 mg/kg-bw/day (Duan, 2019) (Low)	
Evidence of general toxicity	Body weight	• Decreased body weight in high-dose groups (Lington, 1997; Aristech Chemical Corporation, 1998a; Aristech Chemical Corporation, 1998b; Hazleton Laboratories Inc, 1971a)	
Lines of evidence for T-mediated adversity (*in utero* exposure)	Histopathology	• No change in thyroid histopathology in rats exposed to dose levels up to 20,000 ppm (~1,500 mg/kg-bw/day) (Masutomi, 2003; Boberg, 2011) (Medium)	**Overall negative for T-mediated adversity (developmental exposure to DINP)**
Supporting lines of evidence	Epidemiology	• TSH: no association between changes in TSH serum levels and concentration of DINP metabolites (Boas, 2010; Choi, 2021; Derakhshan, 2021; Rodriguez-Carrillo, 2023; Villanger, 2020); serum TSH levels were negatively associated with daily intake of foods contaminated with DEHP and DINP; at 6 month follow-up serum T3 levels were reduced in exposed children compared to baseline measurement at study start (Wu, 2013) • T4: no association between changes in T4 free or total serum levels and DINP metabolites (Boas, 2010; Choi, 2021; Derakhshan, 2021; Villanger, 2020; Wu, 2013); urinary DINP metabolites associated with higher (% difference) free T4 levels (Rodriguez-Carrillo, 2023); lower serum total T4 associated with urinary DINP metabolite concentration (Derakhshan, 2021) • T3: no association between changes in free or total T3 levels and concentration of DINP metabolites (Boas, 2010; Derakhshan, 2021; Rodriguez-Carrillo, 2023; Wu, 2013); DINP metabolite concentration negatively associated with total and free T3 levels (Choi, 2021; Villanger, 2020)	Overall, the epidemiological data provides weak evidence for a lack of T-mediated adversity based on the lack of an association in hormone changes and concentration of DINP. The QSAR evidence provides support for a lack of NIS and TPO inhibition as well as TR binding and activation for DINP metabolites.
	In silico prediction	• DINP metabolites (MiNP, MOiNP, MCiOP, and MHiNP) negative QSAR prediction for NIS and TPO inhibition or TR binding and activation (commercial and CompTox Chemicals Dashboard DINP SMILES)[5]	

[1] Endpoints that are 'sensitive to but not diagnostic of EATS' are reported in Appendix E – All EATS data.
[2] High: effects observed in one or more guideline study with no conflicting results; Medium: effects observed in one or more non-guideline studies with no conflicting results; effects observed in one or more guideline or non-guideline studies with conflicting that could be explained by differences in study design; Low: effects were observed in one or more studies of guideline or non-guideline studies with conflicting results that could not be explained by differences in study design.
[3] Assessment of whether a sufficient dataset was available to support a conclusion on T activity in Section 3.8: Data Sufficiency and Identification of Data Gaps.
[4] https://www.epa.gov/comptox-tools/exploring-toxcast-data#Download (invitroDBv4.1, downloaded May 2024).
[5] Leadscope Model Applier (v2022.0.2–3).

Table 4
Integration and Assessment of Lines of Evidence for DINP and DINP Metabolite Perturbation of the S-pathway. Key: HT: high-throughput; DINP: di-isononyl phthalate; FSH: follicular-stimulating hormone; GD: gestational day; insl3: insulin-like peptide 3; Cyp: cytochrome P450; StAR; steroidogenic acute regulatory protein; 3b-HSD; 3-beta hydroxysteroid dehydrogenase; SF-1: steroidogenic factor 1; Hsd17b3: 17-beta hydroxysteroid dehydrogenase 3; Scarb1: scavenger receptor class B member 1; Dhcr7: 7-dehydrocholesterol reductase; PND: postnatal day; PNW: postnatal week; 7cx-MMeHP: mono(4-methyl-7-carboxy heptyl) phthalate; QSAR: quantitative structure-activity relationship; SMILES: simplified molecular-input-line-entry system.

	Line of Evidence	Observed Effects (Confidence in the Line of Evidence) [1,2]	Integrated Lines of Evidence[3]
Lines of evidence for endocrine activity	Aromatase activity *In vitro* assays	• No effect in an aromatase inhibition assay (HT assay)[4] (Medium) • No effect in H295R steroidogenesis assays (HT assay) (Medium) • H295R steroidogenesis assays: increased estradiol biosynthesis observed (0.03 to 30 μM DINP) (Moche, 2021); weak increase in estradiol synthesis at 20 mg/L (Lee, 2019) (High) • Decreased estradiol and no change in progesterone synthesis in FSH-stimulated porcine granulosa cells at 10^{-4} M DINP for 72 h (Mlynarcikova et al., 2007) (Low) • H295R steroidogenesis assays: no change or weak, non-dose dependent increase in testosterone biosynthesis (Lee, 2019; Moche, 2021) (High)	**Evidence for endocrine activity based on inconsistent changes in gene expression and estradiol synthesis.** Lines of evidence showed no change in testosterone synthesis. Effects of DINP on estradiol synthesis in H295R assays were inconsistent with increased synthesis shown in one assay and no or weak 'inconsequential' effects observed in others. Supporting transcriptomic assays provide inconsistent evidence of changes in steroidogenic gene/protein expression.
	Gene/protein changes	• No change in expression of *StAR*, *Cyp11a1*, and *Cyp17a1* genes in fetal rats (GD 17.5 or 21.5) (van den Driesche, 2020) (Low) • Increased expression of *Insl3* gene; no change in *StAR*, *3b-HSD*, or *SF-1* testicular mRNA or proteins levels in fetal rat testis (GD 19.5) (Adamsson, 2009) (Low) • Decreased expression of *Cyp11a1*, *Hsd3b1*, *StAR*, *Cyp17a1*, and *Hsd17b3* genes in GD 21.5 rat testis; non-dose dependent decrease in *HSD3B1* protein; no change in *CYP11A1* (Li, 2015) (Low) • Decreased expression of steroidogenesis genes: *StAR*, *Cyp11a1*, *Cyp11b2*, *Hsd3b*, *Cyp17a1*, *Scarb1*, *Dhcr7*, and *Cyp11b1* in GD 18 or 21 fetal rat testis (Hannas, 2011; Gray, 2021; Hannas, 2012) (Low)	
Lines of evidence for *in vivo* effects mediated by S-pathway (adult exposure)	Hormone levels	• No dose-related or consistent changes in serum estradiol in monkeys or mice (Chiang, 2020; Chiu, 2020; Hall, 1999); decreased serum estradiol at 20 and 200 mg/kg-bw/day DINP (Chen, 2022) (Low) • No changes in testosterone levels in adult monkeys or mice administered up to 2500 mg/kg-bw/day DINP (Chiang, 2020; Chiu, 2020; Hall, 1999) (Medium) • No change in progesterone serum levels in adult mice administered up to 200 mg/kg-bw/day DINP (Chiang, 2020) (Medium)	**Overall negative for S-mediated effects on serum testosterone (adult exposure).** **Evidence of change in serum estradiol levels based on inconsistent changes (adult exposure).** Lines of evidence consistently show no changes in testosterone levels in adult animals. Some evidence for change in estradiol level in adult animals.
Lines of evidence for *in vivo* effects mediated by S-pathway (*in utero* exposure)	Hormone levels	• No dose-related changes in serum estradiol (Lee et al., 2006; Bhurke, 2023) (Medium) • Fetus: reduction in *ex vivo* rat testis testosterone production (GD 18–21.5 (Hannas, 2011; Borch, 2004; Furr, 2014; Gray, 2021; Li, 2015); no dose dependent effects *ex vivo* rat testis testosterone production (GD 21) (Boberg, 2011) (Low) • Fetus: reduction in intratesticular testosterone content (GD 19 or 21) (Clewell and Edwards, 2013; Borch, 2004); no change in intratesticular testosterone content (GD 17.5–21) (Clewell and Edwards, 2013; Boberg, 2011; Adamsson, 2009; van den Driesche, 2020) (Low) • F1 offspring: DINP had no effect on intratesticular testosterone content (PND 49–50) (Clewell et al., 2013) (Low) • F1 offspring/adult: DINP had no effect on serum testosterone level (GD 21, PND 7, PND 90, PNW 20, or 3–7 months of age) (Lee et al., 2006; Boberg, 2011; Gray, 2000; Borch, 2004; van den Driesche, 2020) (Medium)	**S-mediated effects based on changes in fetal testosterone levels and no evidence of change in serum estradiol (developmental exposure).** Lines of evidence show changes in testosterone levels in fetuses and F1 offspring. No evidence for changes in estradiol levels in F1 offspring.
Evidence of general toxicity	Body weight	• Decreased body weight of F1 offspring (PND 1) (Lee et al., 2006) • No change in body weight in adult, parental, or F1 adult males or fetuses when animals were exposed to dose levels up to 1,500 mg/kg-bw/day DINP (Boberg, 2011; Adamsson, 2009) • Decreased body weight in F1 offspring when dams exposed to dietary concentrations up to 1,500 mg/kg-bw/day DINP (Lee et al., 2006; Boberg, 2011)	
Supporting lines of evidence	Epidemiology	• No consistent association between changes in levels of progesterone, cortisol, androstenedione, or 17-OH-progesterone in amniotic fluid at ~ 16 week's gestation with the presence of the DINP metabolite 7cx-MMeHP (Jensen, 2015)	Epidemiological data shows no evidence for S-mediated effects based a single study reporting the lack of an association between changes in hormone levels in amniotic fluid and the concentration of DINP metabolites.

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Table 4 (*continued*)

	Line of Evidence	Observed Effects (Confidence in the Line of Evidence) [1,2]	Integrated Lines of Evidence[3]
		• DINP metabolites were associated with lower serum progesterone levels in adult females. No association with changes in serum pregnenolone, allopregnanolone, and pregnanolone levels (Jacobson, 2021)	The QSAR evidence shows no evidence for DINP metabolite effects on S-pathway.
	In silico predictions	• MiNP, MOiNP, MCiOP, and MHiNP negative QSAR prediction for aromatase inhibition (commercial and CompTox Chemicals Dashboard DINP SMILES)[5]	

[1] Endpoints that are 'sensitive to but not diagnostic of EATS' are reported in Appendix E – All EATS data.
[2] High: effects observed in one or more guideline study with no conflicting results; Medium: effects observed in one or more non-guideline studies with no conflicting results; effects observed in one or more guideline or non-guideline studies with conflicting that could be explained by differences in study design; Low: effects were observed in one or more studies of guideline or non-guideline studies with conflicting results that could not be explained by differences in study design.
[3] Assessment of whether a sufficient dataset was available to support a conclusion on S activity in Section 3.8: Data Sufficiency and Identification of Data Gaps.
[4] https://www.epa.gov/comptox-tools/exploring-toxcast-data#Download (invitroDBv4.1, downloaded May 2024).
[5] Leadscope Model Applier (v2022.0.2–3).

human AR reporter gene constructs (Engel, 2017). *In vitro* mechanistic data for the A-pathway showed DINP was active in a single AR antagonist Tox21/ToxCast HT assay (AC_{50} = 7.16 μM). A total of 17 Tox21/ToxCast HT assays were evaluated as part of this assessment. No activity was observed in 16 of these assays. No DINP-related effects were observed in four *in vitro* assays for AR agonist or antagonist activity (Moche, 2021; Engel, 2017; Takeuchi, 2005; Kruger et al., 2008). The Leadscope AR QSAR model prediction showed no AR activity predictions for the monoester forms (MiNP, MOiNP, MCiOP, and MHiNP) (Table 5). The ToxCast Pathway Model, which integrates all AR HT assay data, predicted DINP to be negative for AR-related activity (binding, agonism, or antagonism).

In vivo mechanistic studies evaluating testosterone levels are discussed in Section 3.5, Analysis of Evidence: Steroidogenesis Pathway. In a Hershberger assay, designed to evaluate the antiandrogenic effects of DINP (0, 20, 100, or 500 mg/kg-bw/day for 10 days) in 4-week-old castrated male rats, decreased weight in two of five androgen sensitive tissues was observed: levator ani/bulbocavernosus muscle (LABC) weight in the high dose group ($p < 0.05$) and seminal vesicle weight observed in all dose groups compared to testosterone propionate (TP) alone (Lee and Koo, 2007). There was no significant change in weight, and no indication of reduced growth, of the Cowper's glands, glans penis, or prostate; no change in liver weight was observed. Significantly decreased serum testosterone levels and increased LH levels were observed in the high dose group. No evidence of general toxicity was observed in these animals, with no changes in mortality or body weight. Evidence for DINP related endocrine activity for the A-pathway is summarized in Table 5.

Lines of evidence assessing A-mediated adversity that resulted in changes in apical endpoints were evaluated in animals exposed either during gestation or as adults and showed limited evidence for changes in androgen-mediated end points (AGD, nipple retention, genital abnormalities, reproductive tract weights, and malformation incidence). Results of these studies are summarized in Table 5, are discussed in Section 3.7. Mode of Action Assessment and described in detail in Supplemental Data F – All EATS Data and Supplemental DINP Evidence Analysis.

Overall, the WoE showed inconsistent-evidence for DINP-mediated effects on the A-pathway including changes in fetal testosterone production, and decreased testis weight, testicular dysgenesis (variably characterized but broadly included abnormal testicular histopathology, agenesis and/or atrophy), genital abnormalities (e.g., cryptorchidism, hypospadias), decreased AGD and sperm motility, and increased nipple retention. Epidemiology studies showed some evidence for a A-mediated adversity with changes in testosterone levels associated with DINP metabolites levels but no evidence of AR agonism or antagonism in *in vitro* mechanistic assays.

Analysis of evidence: non-mammalian data

Eighteen non-mammalian studies were evaluated as part of this assessment. Two of the eighteen were excluded (Supplemental Data C – Reasons for Exclusion) and data extracted from the remaining sixteen studies (Supplemental Data F – All EATS Data). Data were available for fish (*Danio rerio*, *Oryzias melastigma, Oryzias latipes, Sparus aurata*) and amphibian (*Xenopus laevis*) species as well as *Daphnia magna, Drosophila melanogaster,* and earthworm (*Eisenia fetida)*. One Level 4 (OECD TG 222[4]) and one Level 5 assay (OECD TG 240[5]) were conducted according to OECD test guidelines to assess endpoints relevant to E-, A-, or S-pathways; none of the studies assessed the T-pathway (Patyna, 2006; ExxonMobil Biomedical Sciences, 2010). The Level 4 study was an earthworm reproduction test in which adult worms were exposed to the limit dose of 1,000 mg DINP/kg soil (nominal concentration; mean measured concentration 982.4 mg DINP/kg soil) for 56 days. No significant changes in mortality or fecundity (number of juveniles produced) occurred (ExxonMobil Biomedical Sciences, 2010). The Level 5 study was an extended one generation assessment of dietary exposure of Medaka fish (*Oryzias latipes*) to 20 mg DINP/kg diet (nominal concentration; mean measured concentration 21.9 mg DINP/kg diet). There were no effects on survival, development, growth, nor reproduction after 280 days of exposure (Patyna, 2006) The authors conclude that DINP had no estrogenic/antiestrogenic or androgenic/antiandrogenic effects based on the lack of changes. Data for all studies are provided in Supplemental Data F – All EATS Data and summarized in Supplemental Data G – Summary Non-mammalian Data.

Data sufficiency and identification of data gaps

According to the ECHA/EFSA guidance, an assessment of the sufficiency of the dataset is required to support a conclusion on the absence of ED potential. Firstly, a determination of whether a dataset is sufficient to support a conclusion on the absence of EATS-mediated adversity is made. According to the assessment strategy, if 'EATS-mediated'

[4] OECD (2016), *Test No. 222: Earthworm Reproduction Test (Eisenia fetida/Eisenia andrei)*, OECD Guidelines for the Testing of Chemicals, Section 2, OECD Publishing, Paris, https://doi.org/10.1787/9789264264496-en.
[5] OECD (2023), *Test No. 240: Medaka Extended One Generation Reproduction Test (MEOGRT)*, OECD Guidelines for the Testing of Chemicals, Section 2, OECD Publishing, Paris, https://doi.org/10.1787/9789264242258-en.

adversity was not sufficiently investigated (i.e., the dataset was insufficient) and no 'EATS-mediated' adversity was observed, then EATS-related endocrine activity should be considered to support a conclusion on the absence of ED potential (ECHA/EFSA, 2018). To perform the data sufficiency assessment, endpoints measured in all DINP studies (guideline and non-guideline) were compared to the endpoints included in regulatory test guidelines based on the ECHA/EFSA recommendation to consider all available information (Supplemental Data L – Data Sufficiency).

To assess the sufficiency of the DINP dataset to conclude on EATS-mediated adversity, the endpoints measured in the DINP guideline two-generation reproductive toxicity study (OPPTS 870.3800, related to OECD TG 416) (Waterman, 2000) were compared to the ECHA/EFSA recommended extended one- or two-generation reproduction toxicity studies (OECD TG 416 or 443, OECD Level 5 test methods). Overall 25 out of the 47 recommended E, A, T, or S endpoints in OECD TG 416 (Table 14; (OECD, 2018) were measured in the two-generation reproductive toxicity study by Waterman et al. (2000; Supplemental Data L – Data Sufficiency). Notably, this two-generation reproductive toxicity study (OECD TG 416) does not include assessment of thyroid hormones. Given the absence of 22 of the 47 recommended E, A, T, and S endpoints, guideline and non-guideline Repeated Dose or Reproductive Toxicity studies for DINP (OECD Level 4 test methods) were next considered to determine whether these remaining recommended endpoints had been investigated for DINP. This assessment provided information for an additional 16 endpoints, including age at balanopreputial separation and vaginal opening, AGD, estrus cyclicity, sperm parameters, and thyroid and uterus weights. Overall, the following EATS-mediated endpoints were not evaluated for DINP: coagulating gland weights, sperm morphology, and vaginal smears. In addition, TH level (T3, T4) or TSH levels were not evaluated. This analysis performed according to the sufficiency criteria outlined in ECHA/EFSA guidance (2018) showed that the dataset for E-, A-, and S-pathways were borderline insufficient and for the T-pathway was insufficient.

As the E-, A-, T-, and S-datasets all showed some data gaps, an assessment of whether 'EATS-mediated' adversity was observed was performed next. Regarding the A- and S-pathways, studies showed some evidence of DINP-mediated adversity (Table 4 and 5) with changes in expression of steroidogenic genes and proteins, decreases in fetal testosterone, testicular dysgenesis, and genital abnormalities. As the potential for adversity was observed, no assessment of related endocrine activity was required for A and S, and a MoA assessment was triggered (Section 3.9. Mode of Action Assessment). For E and T-pathways, there was very minimal evidence of endocrine mediated adversity. No apical effects except for those occurring at dose levels that elicited general toxicity were observed for the E- and T-pathways except for a single study showing a change in estrus cyclicity at three-months following administration of up to 200 mg/kg-bw/day DINP in adult mice for 10 days (Chiang and Flaws, 2019). Overall, the WoE suggests no E- or T-mediated adversity.

According to the ECHA/EFSA criteria, the insufficiency of the E and T datasets and the lack of endocrine-mediated adversity requires an assessment of endocrine activity. According to this criteria, endocrine activity can be assessed using *in vitro* and *in vivo* mechanistic endpoints. To consider E-related endocrine activity to be sufficiently investigated, information from the ToxCast ER Bioactivity Model or uterotrophic bioassay in rodents (OECD TG 440; E-pathway) need to be available to support a conclusion on the absence of EAS-related endocrine activity (ECHA/EFSA, 2018). The endocrine activity of DINP across the E-pathway was investigated using these methods. Information was available from three uterotrophic studies and the ToxCast ER Bioactivity Model for DINP; therefore, the dataset to support an evaluation of the E-pathway was sufficient to support a determination on endocrine activity. DINP had no estrogenic activity in three uterotrophic assays (Akahori, 2008; Zacharewski, 1998; Sedha, 2015) and was predicted to be negative for ER binding in the ToxCast Bioactivity Model that integrated the findings from data from 17 of the 18 HT assays to measure ER agonist and antagonist responses. Overall, the sufficiency of the dataset based on the ECHA/EFSA sufficiency guidance (2018), and lack of findings demonstrated DINP had no endocrine activity for the E-pathway.

In vitro mechanistic test guidelines and *in vivo* mechanistic tests are not available for the T-pathway in mammals; therefore, to consider T-related endocrine activity to be sufficiently investigated, information for *in vivo* mechanistic endpoints from Repeated Dose studies (OECD TG 407, 408, 409, 416, and 451-3) (OECD, 2018) are required. No measurements of thyroid hormones (T3, T4, and TSH), or changes in thyroid histology such as thyroid colloid area were performed following exposure to DINP. Thyroid follicular cell height was assessed in a single repeated dose 90-day feed study in dogs exposed to up to 40,000 ppm (~3,000 mg/kg-bw/day) DINP (Hazleton Laboratories Inc, 1971a). Overall, there was insufficient data to conclude on T-related endocrine activity.

As DINP is rapidly and completely metabolized after oral administration in mammals, the potential endocrine activity of DINP metabolites should be considered when assessing data sufficiency. Metabolism is integral to the *in vivo* studies conducted to assess 'EATS-mediated' adversity endpoints (e.g., OECD TG 416); therefore, the data sufficiency assessment conducted for DINP includes the metabolites. The WoE suggests no E- or T-mediated adversity, however there are some data gaps. As such, an assessment of EATS-related endocrine activity is required for DINP metabolites. *In vivo* mechanistic evidence for DINP is inclusive of DINP metabolites; therefore, uterotrophic bioassays in rodents (E-pathway) provide sufficient evidence to support a conclusion on E-related endocrine activity. There is an overall data gap for DINP (parent) to evaluate T-related endocrine activity, as such there is a data gap for DINP metabolites as well, and therefore, according to ECHA/EFSA (2018) sufficiency criteria, there are insufficient data to make a conclusion on T-related endocrine activity or adversity. For A- and S-pathways, as the potential for EATS-mediated adversity was observed, a MoA assessment is required.

Mode of action (MoA) assessment

While AOPs are chemical-agnostic, a hypothesized AOP that describes a phthalate antiandrogenic Leydig cell MoA has been proposed by Li and Spade (Li and Spade, 2021) and was used as the framework for a MoA assessment of A- and S-pathways, along with integration of dose and temporal concordance of observed effects. This AOP was selected as it is one of the most recent publications describing an AOP related to phthalate *in utero* effects on fetal testis development and function and also includes events aligned with previously published AOPs (Conley, 2018; Conley, 2021; Howdeshell, 2015; Kortenkamp, 2020; Schwartz, 2021) and the AOP-wiki.

The WoE assessment identified a small number of studies that described changes potentially mediated through the A-pathway following *in utero* exposure to DINP during the MPW. Observed effects included an increased incidence of MNGs in F1 testes (Rogers et al., 2025), decreased AGD, reduction in fetal testicular testosterone levels, fetal Leydig cell (FLC) clustering, and changes in spermatogenesis in male rodents.

All data from 23 publications (19 identified from the EATS evaluation and 4 identified from targeted literature reviews (see Fig. 1)) describing potential effects mediated by the A-pathway were mapped to MIEs, KEs, and adverse outcomes (AOs) in the hypothesized Leydig cell AOP in which reduced testosterone biosynthesis in FLCs leads to reproductive tract malformations (Li and Spade, 2021). Critical appraisal of the mapped data showed a medium RoB (appraised as Tier 2) for 3 endpoints in three studies: binding of DINP to PPARγ via surface plasmon resonance spectroscopy (Schaffert et al. 2022), testosterone biosynthesis in the H295R steroidogenesis assay (Moche, 2021), and accessory sex organ weight in F1 rats following exposure to DINP from GD 12-PND 14 (Clewell et al., 2013) (Supplemental Data H – RoB

Assessment); however, the majority of endpoints incorporated into the MoA had a high RoB (appraised Tier 3) and no endpoints had a low RoB (appraised Tier 1). The most common sources of bias in the studies evaluated were the lack of test agent characterization, use of inappropriate statistical methods, use of small numbers of animals per dose group, and assessment of endpoints at time points that are not considered the most sensitive to capture the changes in the endpoint being measured, e.g., evaluation of AGD in animals older than PND 4 when this measure is inherently more variable. To ensure a comprehensive assessment of hazard was conducted, all studies, regardless of appraised RoB tier, were included in the MoA assessment. The evidence for each event in the mapped hypothesized AOP is summarized in Table 6 and illustrated in Fig. 3 and available in full in Supplemental Data (I – MoA Data).

Three MIEs have been proposed for the phthalate toxicity pathways although these should be considered as hypothesized only as there is no known MIE for phthalate toxicity in the developing male reproductive system (Li and Spade, 2021). No studies were available assessing the effects of DINP exposure on arachidonic acid release or prolonged expression of the nuclear receptor COUP-TFII in Leydig cells. In this

Table 5
Integration and Assessment of Lines of Evidence for DINP and DINP Metabolite Perturbation of the A-pathway. Key: DINP: di-isononyl phthalate; AR: androgen receptor; HT: high-throughput MiNP: mono-isononyl phthalate; MOiNP: mono-oxoisononyl phthalate; MCiOP: mono-carboxyisooctyl phthalate; MHiNP: mono-hydroxisooctyl phthalate; LH: luteinizing hormone; SC: Sertoli cell; LC: Leydig cell; LABC: levator ani-bulbocavernous muscle; GD: gestational day; PND: postnatal day; PNW: postnatal week; AGD: anogenital distance.

	Line of Evidence	Observed Effects (Confidence in the Line of Evidence)[1,2]	Integrated Lines of Evidence[3]
Lines of evidence for endocrine activity	*In vitro* assays: AR binding / transactivation	• No activity (DINP parent) in AR binding (HT assays)[4] (Medium) • No agonist or antagonist activity for DINP (parent) in *in vitro* AR transactivation assays (Moche, 2021; Engel, 2017; Takeuchi, 2005; Kruger et al., 2008) (High) • DINP metabolites (MiNP, MOiNP, and MHiNP) had no agonist or antagonist activity *in vitro* (Engel, 2017) (Medium)	**Evidence of A-mediated endocrine activity based on transient reduction in fetal testicular testosterone levels (developmental exposure).**
	In vitro assays: other	• No change in basal and LH-stimulated testosterone secretion from 15.5-day-old rat fetal testes cultured for 3 days with 10 μM DINP (Tardif, 2023) (Medium) • No effect on germ cell, SC or LC density; no change in SC or LC proliferation at 10 μM DINP (Tardif, 2023) (Medium)	Lines of evidence showed some evidence for endocrine activity through a transient reduction in fetal testicular testosterone at high levels of DINP. No changes were observed in adult animals.
	Hershberger (antiandrogen effects in castrated immature rats)	• Decreased LABC and seminal vesicle weight. No change in liver weight. No significant weight change or indication of reduced growth of glans penis, Cowper's gland or prostate or adrenal gland. Decrease in exogenously administered testosterone levels; increased serum LH levels (Lee and Koo, 2007) (High)	In the Hershberger mechanistic assay, DINP showed no evidence of antiandrogenic effects. Weights of 2/5 androgen responsive tissues were decreased but the reduction of exogenously administered testosterone suggests increased metabolism and clearance of testosterone led to decreased tissue weight.
	Hormone levels (adult exposure)	• Adult: no changes in testosterone levels in adult monkeys or mice administered up to 2500 mg/kg-bw/day DINP (Chiang, 2020; Chiu, 2020; Hall, 1999) (Medium)	
	Hormone levels (*in utero* exposure)	• Fetus: reduction in *ex vivo* rat testis testosterone production (GD 18–21.5) (Hannas, 2011; Borch, 2004; Furr, 2014; Gray, 2021; Li, 2015); no dose dependent effects *ex vivo* rat testis testosterone production (GD 21) (Boberg, 2011) (Low) • Fetus: reduction in intratesticular testosterone content (GD 19 or 21) (Clewell and Edwards, 2013; Borch, 2004); no change in intratesticular testosterone content (GD 17.5–21) (Clewell and Edwards, 2013; Boberg, 2011; Adamsson, 2009; van den Driesche, 2020) (Low) • F1 offspring: DINP had no effect on intratesticular testosterone content (PND 49–50) (Clewell et al., 2013) (Low) • F1 offspring/adult: DINP had no effect on serum testosterone level (GD 21, PND 7, PND 90, PNW 20, or 3–7 months of age) (Lee et al., 2006; Boberg, 2011; Gray, 2000; Borch, 2004; van den Driesche, 2020) (Medium)	
Lines of evidence for A-mediated adversity (adult exposure)	Organ weight	• No change in testis, epididymis, prostate, and/or seminal vesicle weight following exposure up to dose levels of 40,000 ppm (~3,080 mg/kg-bw/day) (Lington, 1997; Hall, 1999; Hazleton Laboratories Inc, 1991b; Aristech Chemical Corporation, 1998a; Midwest Research Institute, 1981; Aristech Chemical Corporation, 1982a; Hazleton Laboratories Inc, 1971a; Hazleton Laboratories Inc, 1971b; Kwack, 2010; Kwack, 2009; Pugh, 2000; Bio/dynamics Inc, 1982b; BIBRA, 1986; Bio/dynamics Inc, 1982c) (Medium) • Decrease in mouse testis with epididymis weight following exposure to 6,000, 12,500 and 25,000 ppm DINP (~450 – 1,875 mg/kg-bw/day) (Hazleton Laboratories Inc, 1991a) (Low)	**Overall negative for A-mediated adversity (adult exposure).** Lines of evidence show weak evidence for A-mediated adversity following DINP exposure of adults with decrease in sperm parameters and increased incidence of testicular interstitial hyperplasia.
	Histopathology	• No change in testis, prostate, epididymis, and/or seminal vesicle histopathology at dose concentrations up to 40,000 ppm (~3,080 mg/kg-bw/day) (Lington, 1997; Hall, 1999; Hazleton Laboratories Inc, 1991b; Aristech Chemical Corporation, 1998a; Midwest Research Institute, 1981; Aristech Chemical Corporation, 1982a; Hazleton Laboratories Inc, 1971a; Hazleton Laboratories Inc, 1971b; Bio/dynamics Inc, 1987; Hazleton Laboratories Inc, 1991a; Pugh, 2000; BIBRA, 1986; Bio/dynamics Inc, 1982c) (Medium) • No change in the number or percent of apoptotic cells in the testis or changes in testis morphology at 3, 6, 12, 16, or 24 h after administration of a single dose DINP (1,200 mg/kg) (Bhattacharya, 2005) (Medium) • Minimally increased cellular debris in mouse epididymis (25,000 ppm (~1,875 mg/kg-bw/day) (Hazleton Laboratories Inc, 1991a) and increased incidence of rat testicular interstitial cell hyperplasia (672 mg/kg-bw/day) (Bio/dynamics Inc, 1987) (Low)	
	Reproductive endpoints	• Decreased sperm count and curvilinear velocity but no change in overall motility (Kwack, 2009) (Medium)	

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Table 5 (*continued*)

	Line of Evidence	Observed Effects (Confidence in the Line of Evidence)[1,2]	Integrated Lines of Evidence[3]
Lines of evidence for A-mediated adversity (*in utero* exposure)	Organ weight	• No change in prostate, Cowper's gland, or LABC weights in parental, F1 offspring, or F1 rats (Hall, 1999; Masutomi, 2003; Waterman, 2000; Boberg, 2011; Gray, 2023; Gray, 2000; van den Driesche, 2020; Clewell et al., 2013) (Medium) • No effect on testis weight in adult (parental or F1) animals, F1 offspring or fetuses up to doses of ~ 1,500 mg/kg-bw/day (Masutomi, 2003; Waterman, 2000; Boberg, 2011; Gray, 2023; Gray, 2000; van den Driesche, 2020; Clewell et al., 2013). Increased testis weight in adult (parental) animals when exposed to dietary concentrations of ~ 1,100 mg/kg-bw/day in one generation study (Waterman, 2000). Decreased absolute testis weight in PND 27 F1 animals (no dose response shown; occurred with decreased body weight) (Masutomi, 2003) (Low) • No change in epididymis weight in adult (parental or F1) animals when exposed to dietary concentrations up to 1,500 mg/kg-bw/day (Waterman, 2000; Gray, 2023; Gray, 2000). Increased right (not left) epididymis weight in adult (parental) animals when exposed to dietary concentrations of ~ 1,100 mg/kg-bw/day in one generation study (no dose response established) (Waterman, 2000) (Medium) • No change in seminal vesicle or glans penis weight in adult (parental or F1) animals or F1 offspring when exposed to dietary concentrations up to 1,500 mg/kg-bw/day (Waterman, 2000; van den Driesche, 2020; Clewell et al., 2013); decreased absolute seminal vesicle and glans penis weights in F1 adults at 1,500 mg/kg-bw/day DINP (no dose relationship established) (Gray, 2023) (Low)	**Evidence for A-mediated adversity in F1 males based on histopathological changes in testis and epididymis (developmental exposure).** Lines of evidence show some evidence for adversity associated with endocrine perturbation resulting from *in utero* exposure during the rat MPW with histopathological changes in testis and epididymis in F1 males after *in utero* exposure to DINP. Some studies showed effects on developmental markers (nipple retention and AGD) but these findings appeared to be transient with no evidence for changes in adult animals.
	Histopathology	• No change in testis, prostate, epididymis, seminal vesicle, and/or coagulating gland histopathology in parental, fetal, or F1 generation animals when exposed at dietary concentrations up to ~ 1540 mg/kg-bw/day DINP (Masutomi, 2003; Waterman, 2000; Boberg, 2011; Adamsson, 2009; van den Driesche, 2020) (Low) • Testicular dysgenesis, presence of MNGs, hypospermatogenesis/atrophy, unilateral testis and gubernacular testicular agenesis, and LC aggregates in fetuses and F1 males exposed to dietary concentrations of up to ~ 1,500 mg/kg-bw/day (Clewell and Edwards, 2013; Boberg, 2011; Gray, 2023; Li, 2015; Adamsson, 2009; Clewell et al., 2013) (Low)	
	Developmental markers	• No change in age at balanopreputial separation, AGD, or nipple development in fetuses, F1 or F2 male offspring (Clewell and Edwards, 2013; Masutomi, 2003; Boberg, 2011; Gray, 2023; Gray, 2000; Li, 2015; van den Driesche, 2020; Clewell et al., 2013) (Low) • Increased incidence of genital abnormalities in F1 offspring at dose levels up to 1,500 mg/kg-bw/day DINP (Gray, 2023; Gray, 2000) (Low) • No genital abnormalities at dose levels up to 750 mg/kg-bw/day DINP (van den Driesche, 2020; Clewell et al., 2013) (Low) • Decrease in AGD in F1 offspring exposed to dietary concentrations of 1,500 mg/kg-bw/day DINP (Lee et al., 2006; Boberg, 2011; Clewell et al., 2013) (Low) • Increased nipple development in F1 offspring and adults exposed to dietary concentrations of up to 1,500 mg/kg-bw/day (Boberg, 2011; Gray, 2023) (Low)	
	Reproductive endpoints	• Decrease in sperm motility in F1 adults (PND 90) exposed to dietary concentrations of 900 mg/kg-bw/day DINP (Boberg, 2011) (Low) • No change in sperm motility and head count in F1 adults (PND 90) exposed to dietary concentrations of 750 mg/kg-bw/day DINP (van den Driesche, 2020) (Low)	
Evidence of general toxicity	Body weight	• No change in body weight in non-parental, parental, or F1 adult males or fetuses when animals were exposed to dose levels up to 1,500 mg/kg-bw/day DINP (Clewell and Edwards, 2013; Hall, 1999; Hazleton Laboratories Inc, 1971b; Masutomi, 2003; Waterman, 2000; Boberg, 2011; Gray, 2023; Gray, 2000; Adamsson, 2009) • Decreased adult, parental, or F1 adult male body weight when exposed to dietary concentrations up to 3,000 mg/kg-bw/day DINP (Aristech Chemical Corporation, 1998a; Aristech Chemical Corporation, 1982a; Hazleton Laboratories Inc, 1971a; Waterman, 2000; Kwack, 2010; Kwack, 2009; Bio/dynamics Inc, 1982b) • Decreased body weight in male F1 or F2 offspring when exposed to dietary concentrations up to 1,500 mg/kg-bw/day DINP (Lee et al., 2006; Masutomi, 2003; Waterman, 2000; Boberg, 2011; Gray, 2023; Li, 2015; Clewell et al., 2013)	

(*continued on next page*)

Table 5 (*continued*)

	Line of Evidence	Observed Effects (Confidence in the Line of Evidence)[1,2]	Integrated Lines of Evidence[3]
Supporting lines of evidence	Epidemiology	• Change (increase or decrease) in testosterone levels and/or free androgen index associated with increase in the molar sum of DINP metabolite (Hart, 2018; Henrotin, 2020; Joensen, 2012; Lenters, 2015; Specht, 2014; Mieritz, 2012)	
		• No association between concentration of DINP metabolites and testosterone level, free androgen index, sperm endpoints, cryptorchidism, and/or changes AGD in male children (Frederiksen, 2012; Joensen, 2012; Lenters, 2015; Specht, 2014; Jensen, 2015; Jensen, 2016; Minguez-Alarcon, 2022; Rana, 2020)	Epidemiological data provides some evidence for a A-mediated adversity with changes in testosterone levels associated with DINP metabolites levels.
		• Decreased association between sperm motility, sperm count, testicular volume, semen volume and/or reduced AGD and molar sum of DINP metabolites (Hart, 2018; Minguez-Alarcon, 2022; Axelsson, 2015; Bornehag, 2015)	The QSAR evidence shows a lack of AR binding for DINP metabolites.
		• No association between the molar sum of DINP metabolite concentrations and age at puberty in boys (Mieritz, 2012; Burns, 2023; Freire, 2022)	
		• Urinary DINP metabolite (MHiNP, MOiNP, MCiOP) concentration associated with later pubertal onset in boys (Burns, 2022)	
	In silico predictions	• MiNP, MOiNP, MCiOP, and MHiNP negative QSAR prediction for AR binding and/or antagonism (commercial and CompTox Chemicals Dashboard DINP SMILES)[5]	
		• Negative AR agonism or antagonism prediction for DINP (parent) in ToxCast-pathway model[6]	

[1] Endpoints that are 'sensitive to but not diagnostic of EATS' are reported in Appendix E – All EATS data.

[2] High: effects observed in one or more guideline study with no conflicting results; Medium: effects observed in one or more non-guideline studies with no conflicting results; effects observed in one or more guideline or non-guideline studies with conflicting that could be explained by differences in study design; Low: effects were observed in one or more studies of guideline or non-guideline studies with conflicting results that could not be explained by differences in study design.

[3] Assessment of whether a sufficient dataset was available to support a conclusion on A-pathway activity in Section 3.8: Data Sufficiency and Identification of Data Gaps.

[4] https://www.epa.gov/comptox-tools/exploring-toxcast-data#Download (invitroDBv4.1, downloaded May 2024).

[5] Leadscope Model Applier (v2022.0.2-3).

[6] ToxCast AR pathway model describes agonist or antagonist activity (Kleinstreuer, 2017).

analysis, there was some evidence that showed DINP monoesters (MiNP and MHiNP) bound to and activated human PPARs (PPARα, PPARβ and PPARγ) in immortalized cell lines with a lowest NO(A)EC of 3 μM (Laurenzana, 2016; Schaffert, 2022; Bility, 2004). There was less evidence to show DINP activation of PPARs with only one of four studies showing activation of human PPARγ with an NO(A)EC of 1 μM (Pomatto, 2018) (Supplemental Data I – MOA Data). While phthalates are known to bind and activate PPARs in the liver, available evidence suggests PPAR activation in the fetal testis is not causally related to phthalate-driven, androgen-related effects in the developing male rat, (i.e., changes in fetal testosterone production following exposure to phthalates during the MPW were not coincident with changes in expression of PPARα target genes; also, exposure to known PPARα activators during the MPW produced changes in PPARα target genes but no changes in fetal testosterone production) (Furr, 2014; Gray, 2021; Hannas, 2012; Boberg, 2008).

Integration of evidence to support the hypothesized testosterone-mediated AOP (Fig. 3 and Supplemental Data H – MoA Data) showed some/limited evidence of DINP effects in the KEs leading to apical outcomes. A decrease in *Insl3* gene or INSL3 protein expression in fetal rat testis was observed in 3 of 4 studies suggesting a potential disruption in INSL3 signaling (following *in utero* exposure to DINP), which plays a role in masculinization of the reproductive tract. In one study, these changes were reported at dose levels of 10 mg/kg-bw/day but were coincident with changes in fetal (but not maternal) body weight that could suggest systemic toxicity (Li, 2015). No measurements of fetal body weight were provided in the other two studies reporting deceased *Insl3* expression and using higher dose levels of DINP (500 mg/kg-bw/day), which precluded assessment of toxicity in these studies. There was some evidence (five of six studies) for a reduction in fetal or neonatal testicular testosterone levels when measured *ex vivo*; however, when using testicular homogenates, five of six studies showed no changes in testosterone level despite use of similar exposure periods, dose levels, and sampling ages as the *ex vivo* studies. Supporting an overall lack of effect on testosterone production, there was no evidence of serum testosterone changes in neonatal (GD 21 and PND 7) and older rats (PND 90, PNW 20, and 3–7 months of age) (Supplemental Data J – MoA Supporting Data). Only two studies that provided evidence of changes in testicular testosterone content also provided data on the dam or fetal body weight for assessment of systemic toxicity. In the two studies that provided these data, no change in dam/pup body weights were observed in one study (Clewell and Edwards, 2013), whereas a decrease in pup weights was observed at all doses (LO(A)EL 10 mg/kg-bw/day) suggesting fetal toxicity may factor into the findings of the latter study (Li, 2015).

There was limited evidence for DINP's effects on FLC clustering that may be associated with changes in testosterone biosynthesis. The incidence or size of FLC clusters was increased in three of six studies. In two of these, there was a significant reduction in maternal and/or fetal body weight at doses as low as 10 mg/kg-bw/day in one study, which suggests some degree of systemic toxicity in GD21.5-PND 2 rats (Li, 2015; Clewell et al., 2013). At a later timepoint (PND 14), a reduction in AGD occurred in one study using a similar dose levels (Clewell et al., 2013), but no changes in AGD were noted at even later timepoints (PND 49–50 and 90) (Boberg, 2011; van den Driesche, 2020; Clewell et al., 2013).

When considering the AOs, only one of eight studies showed a dose dependent decrease in AGD (absolute and normalized to the cube root of the body weight) in neonates (defined as PND 0–4 per OECD 443 recommended sampling time), and this change (LO(A)EL 40 ppm (~3 mg/kg-bw/day) at PND 1) was coincident with a reduction in fetal body weight at the same dose levels and sample time; no assessment of maternal toxicity was provided in this study (Lee et al., 2006). Reduced male accessory sex organ weight was observed in one of seven studies using high doses (up to 1,500 mg/kg-bw/day) that resulted in seminal vesicle but not prostate or Cowper's gland weight reduction (Gray, 2023). In other studies, using similar doses, exposure windows, and sampling times, no changes in accessory sex organ weight occurred. This lack of findings was supported by evidence that showed decreased testis, penis, or LABC weights in only two of seven studies (Supplemental Data J – MoA Supporting Data). *In utero* exposure to DINP did not produce evidence for cryptorchidism or hypospadias (four studies) at dose levels up to 1,500 mg/kg-bw/day (Supplemental Data I – MoA Data).

Assessment of dose and temporal concordance showed, in the absence of changes in fetal body weight, DINP exposure at dose levels of

≥ 250 mg/kg-bw/day produced changes in KEs (Supplemental Data K – Temporal + Dose Concordance). However, dose levels of DINP ≥ 250 mg/kg-bw/day also failed to produce changes in KEs or to produce AOs. Overall, there was no dose and temporal concordance between KEs and AOs in this hypothesized AOP, and, in the absence of generalized toxicity, it is therefore concluded that it is unlikely there is a biologically plausible link between the endocrine-related activity and EATS-mediated adversity.

Discussion

Due to the well documented effects of low molecular weight phthalates on male reproductive tract development, and their widespread use, phthalates have been extensively reviewed by multiple regulatory authorities. With the upcoming addition of endocrine disruption to the list of information requirements considered under EU REACH regulations, this comprehensive review of the literature was conducted both to inform the likelihood that the high molecular weight phthalate, DINP, acts as an endocrine disruptor and to identify any limitations or data gaps in the existing body of evidence. One of the limitations identified in the course of this work was that many of the studies for DINP were conducted before test method guidelines were developed or updated and, while they provided valuable and consistent information, the confidence in these methods could not be classified as 'high' given the ECHA/EFSA sufficiency criteria requires data on all endpoints consistent with the most recent test guidelines.

Overall, there were sufficient data to support a conclusion on the potential for EATS-mediated adversity for DINP in E-, A-, and S-pathways. For the T-pathway, a data gap was identified in the form of a lack of *in vivo* mechanistic data (TSH and TH levels), and the lack of thyroid colloid area measurement. For DINP metabolites, there was sufficient evidence to support a conclusion on EATS-mediated adversity for E-, A-, and S-pathways based on *in vivo* data, but the lack of mechanistic data prevented a conclusion on endocrine activity for the T-pathway. ECHA/EFSA requires an assessment of the sufficiency of a dataset to support a conclusion on the absence of EATS-related endocrine activity only if EATS-mediated adversity has not been sufficiently investigated and no EATS-mediated adversity was observed (E- and T-pathways). In the case of DINP and its metabolites, an assessment of EATS-related endocrine activity was triggered by the stringent data sufficiency requirements in the ECHA/EFSA guidance and because there were some, albeit inconsistent, adversity outcomes within the dataset and minimal data gaps. This assessment identified the need for additional testing for the T-pathway; however, while generating data for TSH or TH levels would fill the data gap, changes in these hormones are proposed to drive the apical outcomes that were not observed in *in vivo* DINP studies and therefore these measurements are unlikely to change the outcome of the hazard classification conclusion for endocrine disruption or effect risk assessment. When considering the ED potential of DINP through E- and T-pathways, there is no evidence that DINP mediates endocrine-related effects. The analysis herein relies on the ECHA/EFSA guidance for the identification of endocrine disruptors for biocidal and plant protection products as this is serving as an interim guidance until the Agency releases the guidance on application of CLP criteria for endocrine disruptors. It remains to be understood if the sufficiency criteria and new data generation requirements will be harmonized across regulations.

"Rat phthalate syndrome" describes a group of effects observed in male rats from exposure to low molecular weight phthalates (C3-C6 backbone) during a critical developmental window of male sexual differentiation. These effects include reproductive abnormalities characterized by malformations of the epididymis, vas deferens, seminal vesicles, prostate, external genitalia (hypospadias), and cryptorchidism together with retention of nipples/areolae and demasculinization of the growth of the perineum resulting in a reduced AGD (Foster, 2006). Rat phthalate syndrome can be experimentally induced in rats by exposure to chemicals that block androgen synthesis or action during the MPW (GD 15.5–18.5 in rats) (van den Driesche, 2017; Welsh, 2008). Rat phthalate syndrome can be induced by phthalate esters such as DEHP and dibutyl phthalate (DBP). Inconsistent evidence of effects arising from exposure to DINP during the MPW has led to the conclusion that DINP has a lower antiandrogenic potency than other phthalates (Hannas, 2011).

In this evaluation, there was some evidence that exposure to DINP within the MPW resulted in a limited number of effects that could potentially be mediated through the A- and S-pathways; however, the MoA analysis failed to show evidence that could plausibly link early KEs to AOs of male reproductive tract malformations and fertility through the hypothesized testosterone-dependent phthalate toxicity AOP proposed by Li and Spade (Li and Spade, 2021). This AOP was selected as it is one of the most recent publications describing an AOP related to phthalate *in utero* effects on fetal testis development and function. Numerous AOPs related to 'phthalate syndrome' (decreased AGD, infertility, decreased sperm count, cryptorchidism, and hypospadias) have been proposed, most of which include A-pathway related KEs involving FLC changes and decreased androgen production (Howdeshell, 2015; Kortenkamp, 2020; Arzuaga, 2020; Howdeshell et al., 2017). The Li and Spade AOP includes events that align with these previously published AOPs and therefore was used for in the MoA assessment. There is no known MIE for phthalate toxicity of the developing male reproductive tract, but several have been proposed. Low molecular weight phthalates are associated with increased expression of COUP-TFII in FLCs, which can lead to suppression of Leydig cell steroidogenic factor 1 (SF-1)-regulated gene expression and reduced production of testosterone (van den Driesche, 2012). More recently, inhibition of phospholipase A2 (cPLA2) enzyme leading to arachidonic acid release from intracellular stores and resulting in reduced steroidogenesis gene transcription has been proposed as an MIE (Clewell et al., 2020). No experimental data were available to assess DINP for either of these proposed MIEs. However, this analysis showed that regarding the third proposed MIE, PPAR activation, there is *in vitro* evidence of DINP monoester binding / activation of PPARs, but the relevance of this finding as an MIE for perturbation of the A-pathway is doubtful in the light of evidence suggests that activation of PPAR signaling is not a component of the phthalate toxicity in the fetal rat testis (Furr, 2014; Gray, 2021; Hannas, 2012; Boberg, 2008).

Decreased Leydig cell *Insl3* gene expression plays a role in gubernacular outgrowth and testicular descent (Nef and Parada, 1999; Tomiyama, 2003). While there is little known about the regulation of INSL3, there is evidence that decreased INSL3, and testosterone levels are associated with AOs such as cryptorchid testis. The evidence available for DINP shows a decrease in *Insl3* gene expression in fetal testis following *in utero* exposure, with three (of four) studies providing evidence to support this change and a LO(A)EL of 10 mg/kg-bw/day based on changes in gene expression. Maternal toxicity (assessed by decrease in body weight or body weight gain) was not evident in two of these studies and not measured in the third, but decreased pup body weight was coincident with the observation of decreased *Insl3* expression in one of the three studies (and not measured in the third) (Gray, 2021; Li, 2015; Hannas, 2012).

The assessment described herein showed some evidence of a reduction in fetal testicular testosterone following exposure to DINP. The evidence was stronger when measured using an *ex vivo* culture model of rat testis rather than tissue homogenates or extracts. In other proposed AOPs related to phthalate toxicity, decreased androgen production is associated with decreased steroidogenic biosynthetic protein expression e.g., steroidogenic acute regulatory protein (StAR), CYP11A1, CYP17 (Arzuaga, 2020; Baken, 2019). The loss of testosterone ultimately leads to inhibition of urethral tube closure and hypospadias. Some evidence of changes in steroidogenic gene expression was reported with lower levels of *Cyp11a1 Hsd3b1*, *StAR*, *Cyp17a1 Cyp11b1*, *Cyp11b2*, *Hsd3b*, *Scarb1*, *Dhcr7*, and *Hsd17b3* mRNA in GD 18, 21, or 21.5 rat testis following exposure to DINP (Hannas, 2011; Gray, 2021; Li, 2015; Hannas, 2012).

Table 6
Summary Event Table for Hypothesized Reduced Testosterone Biosynthesis in FLCs Leading to Reproductive Tract Malformations MoA.[1]. Key: MIE: molecular initiating event; PPAR: peroxisome proliferator activated receptor; DINP: di-isononyl phthalate; MiNP: mono-isononyl phthalate; MHiNP: mono-hydroxisooctyl phthalate; FLC: fetal Leydig cell; KE: key event; insl3: insulin-like peptide 3; GD: gestational day; PND: postnatal day; AGD: anogenital distance; AO: adverse outcome".

Event	Description	Lines of evidence[2]
MIE (proposed)[3]	PPAR binding / antagonism	**Evidence that MiNP and MHiNP induce this (proposed) MIE** • DINP metabolites, MiNP and MHiNP, activate PPARα, PPARβ, and/or PPARγ in transfected kidney cell lines (Laurenzana, 2016; Schaffert, 2022) • DINP metabolite MiNP activates PPARα and PPARγ in a transfected adipocyte cell line (Bility et al. 2004) • DINP metabolite MHiNP, but not DINP, bound the recombinant hPPARγ ligand-binding domain (Schaffert et al. 2022) **Very limited evidence that DINP induces this MIE (and no evidence that this MIE is relevant to phthalate reproductive tract malformations)** • Limited evidence that DINP activates PPARs in kidney cell lines; one study showed PPARγ activation by DINP in HepG2 cells (Pomatto, 2018), but other *in vitro* and Tox21/ToxCast assays consistently demonstrated no activity (Laurenzana, 2016; Schaffert, 2022)
KE	Decreased FLC secretion of INSL3	**Some evidence that DINP induces this KE** • Decreased *Insl3* gene expression in fetal testis at GDs 18 and 21.5 using doses of 10 mg/kg-bw/day (Hannas, 2011; Gray, 2021; Li, 2015) • At higher doses (250 mg/kg-bw/day) increased *Insl3* gene expression in fetal testis observed at GD 19.5 despite a similar exposure window (Adamsson, 2009)
KE	Decreased testosterone biosynthesis	**Some evidence that DINP induces this KE** • Reduced fetal testicular testosterone production in five *ex vivo* studies at GD 18 and 21 using ≥ 500 mg/kg-bw/day DINP (Hannas, 2011; Borch, 2004; Furr, 2014; Gray, 2021; Li, 2015) • No changes in testosterone production at GD 21 in one *ex vivo* study using higher doses (≤900 mg/kg-bw/day) (Boberg, 2011) • Reduced fetal testicular testosterone content in 2/6 studies measuring testosterone in extracts or homogenates using ≤ 750 mg/kg-bw/day DINP (Clewell and Edwards, 2013; Borch, 2004)[4] • No changes in fetal testicular testosterone content (GD 19 – 21) in 5/6 studies measuring testosterone in extracts or homogenates using ≤ 900 mg/kg-bw/day DINP [4] • All studies (positive and negative) used similar dose levels, exposure windows, and sampling ages
KE	Increased clustering of FLCs	**Limited evidence that DINP induces this KE** • An increase in the number of animals with FLC clusters or an increase in the size of FLC clusters at GD 20, 21.5, and PND 2 using doses of ≥ 10 mg/kg-bw/day (Clewell and Edwards, 2013; Li, 2015; Clewell et al., 2013) • No evidence of FLC clusters in studies using higher doses and sampled at similar timepoints (≤750 mg/kg-bw/day, GD 19.5 – 21.5) (Boberg, 2011; Adamsson, 2009; van den Driesche, 2020) • Inconsistency between studies may be due to different exposure periods, dose levels, and measuring sensitivity
AO	Decreased AGD	**Limited evidence that DINP induces this AO** • Reduction of AGD was seen in two studies evaluating AGD at PND 1 at doses of 40 ppm (~3 mg/kg-bw/day) and 900 mg/kg-bw/day (Lee et al., 2006; Boberg, 2011) • No changes in AGD in three studies using higher doses on GDs 20–21.5 (Clewell and Edwards, 2013; Li, 2015; van den Driesche, 2020) or four studies evaluating AGD at higher doses and PND 2 (Masutomi, 2003; Gray, 2023; Gray, 2000; Clewell et al., 2013) • Reduction of AGD in one study at PND 14 at a targeted dose of 720 mg/kg-bw/day DINP (Clewell et al., 2013) • No changes in three studies when AGD was measured at later timepoints (PND 49–50 and 90) using doses of ≤ 900 mg/kg-bw/day DINP (Boberg, 2011; van den Driesche, 2020; Clewell et al., 2013) • Inconsistency between studies may be due to different exposure periods, dose levels, and measuring sensitivity
AO	Decreased accessory sex organ weight	**Very limited evidence that DINP induces this AO** • No evidence that DINP affects prostate or Cowper's gland weight in rats evaluated at PND 49–210 and as F1 adults at dose levels ≥ 400 ppm (~30 mg/kg-bw/day) (Masutomi, 2003; Waterman, 2000; Boberg, 2011; Gray, 2023; Gray, 2000; van den Driesche, 2020; Clewell et al., 2013) • Limited indication of DINP effects on seminal vesicle weight at PND 210–240 using 1,000 mg/kg-bw/day DINP (Gray, 2023), but no effects on seminal vesicle weight in other studies with comparable design evaluating animals from PND 49 to F1 adults (Waterman, 2000; Boberg, 2011; Gray, 2000; van den Driesche, 2020; Clewell et al., 2013) • In a mechanistic study, DINP showed no evidence of antiandrogenic effect in a Hershberger study (Lee and Koo, 2007)
AO	Cryptorchidism	**No evidence that DINP induces this AO** • No evidence that DINP produces cryptorchid testis in rats evaluated between PND 49–50 and 220–240 at doses ≤ 1,500 mg/kg-bw/day (Gray, 2023; Gray, 2000; van den Driesche, 2020; Clewell et al., 2013)
AO	Hypospadias	**No evidence that DINP induces this AO** • No evidence that DINP produces hypospadias in rats evaluated at PND 49–50 to PND 220–240 using doses of ≤ 1,500 mg/kg-bw/day (Gray, 2023; Gray, 2000; van den Driesche, 2020; Clewell et al., 2013)

[1] Events and outcomes associated with phthalate toxicity proposed by Li and Spade (Li and Spade, 2021). Events with no study data are not included in the table.
[2] Lines of evidence were categorized as follows: 'Evidence' = all studies provide consistent evidence of an effect; 'Some' = majority, but not all, studies provide consistent evidence of an effect (>50 % and < 100 % of studies); 'Limited' = a few studies provide consistent evidence of an effect (>1 study but ≤ 50 % studies); "Very limited' = evidence of an effect observed in a single study; 'No evidence' = none of the studies provided evidence of an effect.
[3] PPAR activation is one of the three proposed MIEs for this hypothesized AOP by Li and Spade (2022); however, available data suggest that this effect is not causally relevant to androgen-dependent effects on the development male rat.
[4] Testosterone measured at two different timepoints in one study so one study appears twice.

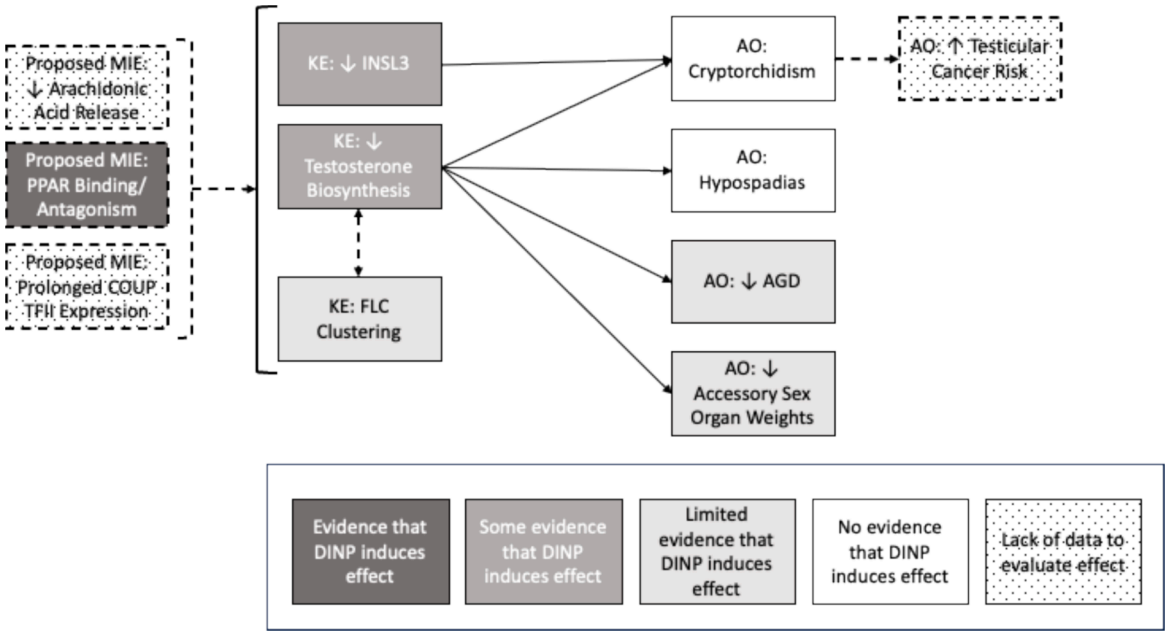

Fig. 3. Evidence for Each MIE, KEs and AOs in the Hypothesized Mapped LC AOP. Level of evidence was determined by the proportion of studies reporting effects following DINP exposure during the MPW. Dashed lines indicate the uncertainty of the MIE and some KE relationships (KERs) for this hypothesized pathway. Key: MIE: molecular initiating event; PPAR: peroxisome proliferator activated receptor; COUP TF II: chicken ovalbumin upstream promote transcription factor 2; KE: key event; INSL3: insulin-like peptide 3; FLC: fetal Leydig cell; AO: adverse outcome; AGD: anogenital distance; DINP: di-isononyl phthalate.

The NO(A)EL for these studies was 10 mg/kg-bw/day and occurred with a loss in male pup body weight but no obvious signs of maternal toxicity. Not all studies reported changes in steroidogenic genes *in vivo* and *in vitro*. H295R steroidogenesis assays showed no effects, although *in vitro* assays do not account for the metabolism of DINP. The inconsistent changes in androgen production may partly be attributed to the different methodologies used for testosterone measurement but may also be the result of differences in the age at which animals were sampled. In one study, fetal testicular testosterone at GD 19 was shown to be reduced at 2 h post-dose, but when GD 20 (24 h post-dose) animals were assessed, the hormone levels had recovered to control levels (Clewell and Edwards, 2013), suggesting the potency of DINP (at dose levels up to 750 mg/kg-bw/day) was not sufficient to support a persistent change to androgen synthesis. Indeed, no changes in serum testosterone levels were observed in rats from PND 1 to adulthood.

In utero exposure to some phthalates leads to FLC changes (such as aggregates and intratubular LCs), immature Sertoli cells, and malformed tubules (Fisher, 2003). While there is no direct connection between the formation of FLC aggregates and reduction in fetal testosterone, FLC cytoplasmic volume tends to mirror steroidogenic activity (Ewing and Zirkin, 1983). Formation of large aggregates as a result of *in utero* exposure to DBP produces FLC aggregates through altered cellular migration, rather than excessive LC proliferation, but also reduces FLC size by reduction in cytoplasmic volume (Mahood, 2005). In this analysis, three studies showed an increase in the incidence of animals with large FLC clusters (Clewell and Edwards, 2013; Li, 2015; Clewell et al., 2013). One of these studies assessed cell size and cytoplasmic volume and showed an increase in both at lower doses of DINP (10 and 100 mg/kg-bw/day). Even at dose levels up to 1,000 mg/kg-bw/day, no decrease in cell or cytoplasmic volume was reported, although concentrations of intratesticular testosterone were significantly reduced at 1,000 mg/kg-bw/day DINP. As FLC clustering and reduction in testosterone occurred at the same time, it is possible that generalized toxicity was a factor in these findings because loss of male pup body weight was observed in this study at all dose levels of DINP (Li, 2015). Similarly, the finding of increased incidence of FLC aggregates reported by Clewell et al. may also have been confounded by systemic toxicity as male pup body weights were reduced at the same dose level as FLC aggregates were reported (750 mg/kg-bw/day) (Clewell et al., 2013). Saturation of DINP metabolism and significant changes of kinetics occur between the DINP dose levels of 250 mg/kg-bw/day and 750 mg/kg-bw/day, where despite a three times increase in dose, maximum concentration (C_{max}) and area under the curve (AUC_{inf}) do not increase (Clewell and Edwards, 2013). The deviation from linearity in metabolism at 750 mg/kg-bw/day may reflect reduced bioavailability at higher dose levels and indicate the maximal biological response to DINP exposure may be reached at a doses of $\leq$ 750 mg/kg-bw/day. In turn, this may have implications for the ability of DINP to manifest the downstream outcomes that occur as a result of fetal androgen reduction.

Markers of FLC function such as AGD and nipple retention, show limited evidence of effect following DINP exposure. AGD in particular, can be considered a marker of androgen levels throughout the whole MPW (McIntyre et al., 2001). In pups aged PND 0 to PND 4 (OECD 433 recommended sampling time), there were two studies showing a reduction in absolute or scaled (to the cube root of body weight) AGD in PND 1 males exposed *in utero* to 40 ppm DINP (~3 mg/kg-bw/day) or 900 mg/kg-bw/day (Lee et al., 2006; Boberg, 2011). In Lee et al. (2006), this occurred in association with a decrease in male pup body weight (on PND 1), which may suggest systemic toxicity. No maternal body weights were provided in this study for an assessment of maternal toxicity. In Boberg et al. (2011), while there was no significant effect on male pup body weight at PND 1, there was a (non-statistically significant) trend for decreased body weight with increasing DINP dose. In addition, there are reported challenges in reproducing the statistical significance of the change in AGD (Chen, 2017), although, in a corrigendum to their article, Boberg et al. published additional details regarding the statistical analysis important to informing on the reliability of the outcomes (Boberg, 2016). Overall, the WoE for changes in AGD in early postnatal life was negative, with eight studies showing no change in AGD before PND 3 despite use of dose levels up to 1,500 mg/kg-bw/day.

In animals PND 14 and older, one study showed evidence of decreased AGD, but this occurred at the same dose as decreased male pup body weight, suggestive of a systemic effect rather than an anti-androgenic effect (Clewell et al., 2013). The decreased AGD occurred on

PND 14 in pups exposed to the highest DINP dose (11,400 ppm; target dose 720 mg/kg-bw/day), but did not occur at PNDs 2 and 49. Given that differences in AGD induced by antiandrogenic influences *in utero* should be apparent at birth, the difference observed at PND 14 without a correlative difference at PND 2 suggests that the PND 14 observation was unlikely due to an anti-androgenic effects. The lack of change in AGD supports the idea that exposure to DINP during the MPW does not persistently alter androgen production in developing fetuses. Another biomarker of antiandrogenic effects is nipple retention. In rat toxicity studies, exposure to dihydrotestosterone during male development induces regression of the nipples (Schwartz, 2021). Exposure to high dose levels of some high molecular weight phthalates e.g., DEHP during the MPW can induce a high number of retained nipples in individual pups (Moore, 2001), although the majority of studies report lower numbers for individual animals and a higher frequency of animals with retained nipples (Schwartz, 2021). In this analysis, while nipple retention is not a KE or AO defined in the hypothesized AOP used for the MoA, this endpoint is a mandatory measurement under several OECD test guidelines (e.g., OECD TG 443) and is typically assessed in combination with AGD to assess antiandrogenic effects. Exposure to high doses of DINP induced nipple retention in PND 13–14 rats in three of four studies (LO(A)EL 750 mg/kg-bw/day) (Boberg, 2011; Gray, 2023; Gray, 2000), although a dose response could be established in only two of the studies. This finding was persistent to adulthood in only one of the four studies, where a statistically significant increase in the mean number of nipples per rat (1.38 nipples/rat) was observed at 1,500 mg/kg-bw/day (Gray, 2023). By comparison, in a review article by Schwartz et al., the number of nipples found in control rats was shown to vary by up to 2/rat (Schwartz, 2021). The distinction between a transient and permanent effect is an important consideration, as the OECD guidance document on mammalian reproductive toxicity testing defines permanent, not transient, nipples as a malformation (OECD, 2008). The WoE suggests no permanent effect on nipple retention as a result of DINP exposure.

A mechanistic assessment of the antiandrogenic effects of DINP in a Hershberger assay has sometimes been cited as evidence of DINP's effects on the A-pathway based on decreased weights for two androgen-dependent tissues (seminal vesicle and LABC) in castrated rats (Lee & Koo, 2007). Compounds that significantly decrease weights of two or more androgen-sensitive tissues (as well as displaying some degree of reduced growth in all other target tissues) in the presence of TP are generally considered positive for anti-androgenicity (Marty and O'Connor, 2014). However, a significant reduction in exogenously administered TP was found at 500 mg/kg-bw/day DINP, and it has been shown that a positive Hershberger result (when it is performed to assess antiandrogens) can be achieved when the test compound induces liver drug metabolizing enzymes (Freyberger et al., 2007; Freyberger & Schladt, 2009). While exposure of castrated rats to DINP did not increase liver weight, the reduction in TP levels suggests increased metabolism and clearance of the exogenously administered TP, rather than DINP acting as an antiandrogen. Induction of liver enzymes is suggested by the observed activity of DINP in PXR HT assays, activation of PPARs by the MiNP and MHiNP, and reports of increased liver weights following DINP exposure in the literature (McKee, 2000). The reduction in TP means less is available to interact with the ARs to maintain androgen-sensitive tissue weight, and as the rats used in this assay were castrated, there was no endogenous testosterone that could compensate for the lower levels of testosterone due to increased metabolism (Marty and O'Connor, 2014; OECD, 2009).

Reductions in androgen dependent organ weights (testis and accessory organs) has been included in some, but not all, proposed AOPs as an AO following phthalate exposure (Li and Spade, 2021; Howdeshell, 2015; Arzuaga, 2020). There was very little evidence that exposure to DINP during the MPW results in reduction of organ weights with observed changes occurring in two studies using high doses of DINP. Decreased absolute penis, LABC, and seminal vesicle weights occurred in adult F1 animals in one study, although the effects occurred only with the use of high dose levels (1,500 mg/kg-bw/day) DINP (Gray, 2023). In the other study showing effects on organ weight (absolute and relative testis weight), the decrease was measured in PND 27 animals exposed to high levels of DINP (up to 20,000 ppm DINP; ~1,500 mg/kg-bw/day) but the authors note that the body weight of male pups was reduced during the exposure period (GD 15-PND 10), returning to control levels only on cessation of exposure. Whether the effects on testis weight were indicative of an antiandrogenic effect or whether they were the result of systemic toxicity is not clear; however, in most studies, no change in androgen dependent organ weights was evident. Moreover, there was no evidence that DINP elicited genital malformations such as cryptorchidism or hypospadias, nor dose related changes in sperm motility or reduced fertility in rats. Taken together, these findings indicate DINP exposure during the MPW may induce KEs in the hypothesized phthalate Leydig cell AOP proposed by Li and Spade (2022), but also show very limited evidence of DINP producing permanent or AOs in the hypothesized AOP and no dose or temporal concordance for observed effects.

The relevance of changes in the rat fetal testosterone pathway following phthalate exposure is of questionable relevance to humans. Although it is well established that for some phthalates e.g., DBP, toxicity in male rats occurs through suppression of testosterone production in FLCs, this MoA is not relevant in all species. In fetal mice, decreased testicular testosterone was not associated with exposure to DBP, mono (2-ethylhexyl) phthalate) (MEHP) or monobutyl phthalate (MBP) (Gaido, 2007). Similarly, studies of fetal human testis xenograft models in mice exposed to DBP have shown normal fetal testis growth and function at dose levels $\leq$ 500 mg/kg-bw/day that suppresses testosterone production in rats (Mitchell, 2012). Coupled with the fact that the limited effects observed in rats following exposure to DINP occurred at doses far exceeding human exposure estimates (mean exposure from food: 0.232–4.27 μg/kg-bw/day) (EFSA, 2019) the results of this ED assessment suggests that DINP does not pose an endocrine disruption hazard to humans.

Conclusion

In conclusion, according to the ECHA/EFSA criteria to evaluate a substance as an ED, as defined by World Health Organization (WHO)/International Programme on Chemical Safety (IPCS), DINP does not show endocrine disruptor activity in the E-pathway and is unlikely to mediate ED effects through the A- or S-pathways. This conclusion is based on a lack of evidence for endocrine activity across these pathways for which a biologically plausible link can be established to EAS-mediated relevant adverse effects noted *in vivo*. DINP does not show T-mediated adverse effects *in vivo*, but insufficient evidence was available to evaluate endocrine activity.

CRediT authorship contribution statement

I.A. Lea: Data curation, Writing – original draft. **A.N. Buerger:** Writing – review & editing, Visualization. **D. Feifarek:** Data curation. **A. Mihalchik:** Investigation. **M.M. Heintz:** Conceptualization, Project administration. **L.C. Haws:** Supervision, Funding acquisition. **H. Nyambego:** Writing – review & editing. **K. Goyak:** Conceptualization, Resources, Writing – review & editing. **C. Palermo:** Conceptualization, Resources, Writing – review & editing. **S.J. Borghoff:** Conceptualization, Writing – review & editing, Supervision.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: ToxStrategies LLC reports financial support and article publishing charges were provided by ExxonMobil Biomedical Sciences Inc. H. Nyambego reports a relationship with ExxonMobil Biomedical Sciences Inc that includes: employment. K. Goyak reports a relationship with ExxonMobil Biomedical Sciences Inc that includes: employment. C. Palermo reports a relationship with ExxonMobil Biomedical Sciences Inc that includes: employment. 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. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crtox.2025.100220.

Data availability

I have shared a Supplemental Data file that contains the data associated with this manuscript

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