Weight-of-the-evidence evaluation of 2,4-D potential for interactions with the estrogen, androgen and thyroid pathways and steroidogenesis

This review supports the previously established conclusion that 2,4-D is unlikely to disrupt hormone functions under real-world exposure conditions. It provides a weight-of-the-evidence evaluation of a large body of research on the subject.

A weight-of-the-evidence approach involves the analyses of a body of evidence to determine what the majority of evidence tells us. In this case, the evidence is comprised of dozens of published scientific studies, as well as results from studies conducted to meet regulatory needs. Studies were included in the evaluation if they directly assessed the effects of 2,4-D on hormone functions or if they had relevant data. These studies evaluated 2,4-D both in lab settings and in studies conducted on animals, at various concentrations and doses. Epidemiological studies examining real world exposures to 2,4-D were also considered.

The weight-of-the-evidence evaluation analysed the strengths and weaknesses of all the studies, and weighted them according to their quality. Studies that followed regulatory guidelines and adhered to Good Laboratory Practices were weighted more heavily, while studies that used unreliable methods or provided insufficient documentation were weighted less. The results of this evaluation was that the weight-of-the-evidence shows 2,4-D does not disrupt hormone pathways.

This study complements the broad array of evidence showing that 2,4-D is not an endocrine disruptor. This aligns with the U.S. EPA’s 2015 determination that 2,4-D demonstrates no convincing evidence of potential interaction with the estrogen, androgen or thyroid pathways.

Reference:
Weight-of-the-evidence evaluation of 2,4-D potential for interactions with the estrogen, androgen and thyroid pathways and steroidogenesis


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Weight-of-the-evidence evaluation of 2,4-D potential for interactions with the estrogen, androgen and thyroid pathways and steroidogenesis

B. H. Neal®, J. Bus®, M. S. Marty®, K. Coady®, A. Williams®, J. Staveley® and J. C. Lamb®

ABSTRACT
A comprehensive weight-of-the-evidence evaluation of 2,4-dichlorophenoxyacetic acid (2,4-D) was conducted for potential interactions with the estrogen, androgen and thyroid pathways and with steroidogenesis. This assessment was based on an extensive database of high quality in vitro, in vivo ecotoxicological and in vivo mammalian toxicological studies. Epidemiological studies were also considered. Toxicokinetic data provided the basis for determining rational cutoffs above which exposures were considered irrelevant to humans based on exceeding thresholds for saturation of renal clearance (TSRC); extensive human exposure and biomonitoring data support that these boundaries far exceed human exposures and provide ample margins of exposure. 2,4-D showed no evidence of interacting with the estrogen or androgen pathways. 2,4-D interacts with the thyroid axis in rats through displacement of thyroxine from plasma binding sites only at high doses exceeding the TSRC in mammals. 2,4-D effects on steroidogenesis parameters are likely related to high-dose specific systemic toxicity at doses exceeding the TSRC and are not likely to be endocrine mediated. No studies, including high quality studies in the published literature, predict significant endocrine-related toxicity or functional decrements in any species at environmentally relevant concentrations; or, in mammals, at doses below the TSRC that are relevant for human hazard and risk assessment. Overall, there is no basis for concern regarding potential interactions of 2,4-D with endocrine pathways or axes (estrogen, androgen, steroidogenesis or thyroid), and thus 2,4-D is unlikely to pose a threat from endocrine disruption to wildlife or humans under conditions of real-world exposures.

Table of contents
Introduction .................................................. 2
Selection of regulatory toxicology studies for inclusion in the WoE .................................................. 2
Literture search and selection of published studies for inclusion in the WoE evaluation .......................... 3
Evaluation of study quality ................................ 3
Identification of relevant endpoints for each potential endocrine pathway interaction and ranking of endpoints for sensitivity and specificity .......................................................... 4
Differentiating potential endocrine modes of action based on endpoints affected .................................................. 5
Impact of toxicokinetic data for 2,4-D on study design, data interpretation and risk assessment .............. 7
Organization of the WoE evaluation .................... 8
In vitro studies of 2,4-D relevant to assessment of potential endocrine pathway interactions .................. 9
EDSP tier I in vitro studies .................................. 9
Published in vitro studies .................................. 9
ToxCast® assays of 2,4-D .................................... 9
Ecotoxicological studies of 2,4-D relevant to assessment of potential endocrine pathway interactions ...... 14
Marino et al. 2010 (published in Coady et al. 2013) .......................................................... 14
Mitchell et al. 2000 ............................................. 14
Review of studies for study quality ...................... 15
Mammalian toxicological studies of 2,4-D relevant to assessment of potential endocrine pathway interactions .......................................................................................... 16
Selection of regulatory mammalian toxicology studies for inclusion in review ................................. 16
Rodwell and Brown, 1985 .................................... 20
Occupational and epidemiological investigations .......... 20
Male reproductive health .................................... 20
Lerda and Rizzi, 1991 ........................................... 20
Garry et al. 2001 .................................................. 22
Swan et al. 2003 .................................................. 22
Thyroid ............................................................. 22
Knopp, 1994 ....................................................... 22
Goldner et al. 2010 and Goldner et al. 2013 .............. 23
WoE for potential endocrine pathway interactions .................................................................................. 26
WoE evaluation for the estrogen hormonal pathway .......................................................... 33
In vitro studies .................................................. 34
Ecotoxicological studies .................................... 34
Mammalian studies .......................................... 35

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Introduction

2,4-Dichlorophenoxyacetic acid (2,4-D) was the first synthetic herbicide introduced into commerce in 1947, and as a result, 2,4-D is also among the best understood and most thoroughly researched herbicides in the world. Many studies have been conducted on 2,4-D including in vitro, ecotoxicological and mammalian toxicological studies. The influence of dose-dependent 2,4-D toxicokinetics (TK) as an important determinant for expression of toxicity has also been characterized in multiple species, including humans. 2,4-D has been the subject of several epidemiological studies and comprehensive reviews as well as multiple studies to characterize potential human and environmental exposure levels (reviewed in Munro et al. 1992; USDA 1998; Garabrant & Philbert 2002; US EPA 2005; Bus & Hammond 2007; Aylward et al. 2010; Burns & Swaen 2012).

2,4-D is one of the most widely used herbicides worldwide and the third most widely used herbicide in the USA and Canada. Its major uses in agriculture are on wheat and small grains, sorghum, corn, rice, sugar cane, low-till soybeans, rangeland and pasture. It is also used on rights-of-way, roadsides, non-crop areas, forestry, lawn and turf and aquatic weeds. Despite its widespread use, urinary levels of 2,4-D are largely undetectable in the general population in Canada and in the USA (CDC 2005; Health Canada 2010).

In recent years, increased attention has been paid to the potential endocrine-modulating effects of environmental and occupational exposures to pesticides and chemicals. Based on its production volume, 2,4-D was recently screened using the US Environmental Protection Agency (US EPA) Tier I Endocrine Disruptor Screening Program (EDSP) assays for potential interactions with the estrogen, androgen, thyroid (EAT) pathways or with steroidogenesis (Coady et al. 2013, 2014). The endocrine activity of 2,4-D also has been comprehensively evaluated in a state-of-art apical in vivo extended one-generation reproduction study (EOGRT; Marty et al. 2013). Overall, the toxicological, epidemiological, exposure and biomonitoring information available for 2,4-D represents a useful and comprehensive dataset to further assess the potential for endocrine interactions. Consideration of exposure information is critical for assessing the human relevance of effects restricted to high in vitro concentrations or high in vivo dosing, and in determining the likelihood and appropriate level of concern for any given effect.

This assessment of potential endocrine pathway interactions reviews the relevant toxicological and ecotoxicological databases including both regulatory toxicological studies (studies required or developed to support US registration) and published literature. The objective of this review was to construct a weight of the evidence (WoE) assessment of potential endocrine pathway interactions and implications for adverse effects on human health and environmental species, with a particular focus on potential for interactions with EAT pathways and with steroidogenesis.

The WoE approach included:

- identifying and reviewing studies of 2,4-D conducted for regulatory purposes and selection of studies with endpoints potentially relevant to EAT and steroidogenesis;
- identifying and reviewing published in vitro, in vivo ecotoxicological, in vivo mammalian, epidemiological and mechanistic studies of 2,4-D with endpoints potentially relevant to EAT and steroidogenesis;
- evaluating study quality for both regulatory and published studies;
- identifying relevant endpoints for each potential endocrine pathway interaction (EAT or steroidogenesis) within each study;
- ranking endpoints for specificity and sensitivity in the context of the specific studies;
- identifying potential confounding factors;
- evaluating the consistency and coherence of the reported findings suggesting potential pathway interactions, i.e. testing the hypothesis that the compound may act as an estrogen agonist or antagonist, an androgen agonist or antagonist, a thyroid agonist or antagonist or a modulator of steroidogenesis;
- assessing the completeness of the available data and developing conclusions on the likelihood of compound-related impacts for each potential EAT or steroidogenesis endocrine pathway interaction.

Selection of regulatory toxicology studies for inclusion in the WoE

Toxicology studies of pesticides in the USA conducted for regulatory purposes (referred to as "regulatory toxicology
studies”) are conducted to support product registration. The goal of these studies, many of which are required by the US EPA under contemporaneous detailed study guidelines, is to develop adequate data so that the US EPA can have high confidence in the toxicological endpoints serving as the basis for its short, intermediate or long-term risk assessments to protect worker health, other potentially exposed populations, e.g. exposed through ingestion of dietary residues and the environment. In addition to guideline compliance, the studies are required to be conducted under Good Laboratory Practice (GLP) regulations.

The EDSP Tier 1 testing performed for 2,4-D included five mechanistic in vitro assays: estrogen receptor (ER) binding and transactivation, androgen receptor (AR) binding, aromatase inhibition and steroidogenesis. Additionally, an amphibian metamorphosis assay (AMA), which focuses on potential thyroid effects, and a fish short-term reproductive assay (FSTRA), which has endpoints sensitive to estrogen and androgen pathway interactions, were performed for 2,4-D. At the time the Tier 1 EDSP requirements were promulgated, 2,4-D had already been tested in mammals in an EDSP Tier 2 equivalent EOGRT study. This study had multiple endocrine system-related endpoints that were specifically added in consultation with the US EPA and the Pest Management Regulatory Agency of Health Canada (PMRA). This battery of EDSP studies constitute a robust core body of information for the WoE evaluation of the potential of 2,4-D for EAT and steroidogenesis pathway interactions.

Fourteen other regulatory studies with the most relevant and comprehensive endocrine pathway-related endpoints were selected for this WoE evaluation. The majority of the selected regulatory studies were mammalian toxicology studies, including: an EPA guideline two-generation reproductive toxicity study, developmental toxicity studies in rats and rabbits, subchronic toxicity studies in rats, mice and dogs and chronic toxicity studies in rats, mice and dogs. This extensive mammalian regulatory toxicological data-base provides an opportunity to evaluate consistency of responses across species and strains, and also across exposure durations. A single relevant ecotoxicological study, a one-generation quail reproductive toxicity study, was identified. Other ecotoxicological regulatory studies included too few relevant endpoints to be useful in an endocrine WoE evaluation.

Many of the regulatory toxicity studies of 2,4-D (including EDSP studies) have been published. We cite the publications as well as the laboratory study reports. For all regulatory studies with 2,4-D, the laboratory study reports were used to evaluate study quality and derive results to include in the WoE. The reports provide methodological details including protocol, amendments and protocol deviations, supporting data on compound identity, purity and dose confirmation analyses and comprehensive results, including both summary and individual animal results, generally not available in published articles.

The guidelines for regulatory toxicology studies have undergone significant changes over time. Many parameters have been added which more completely characterize potential endocrine pathway-related effects. Thus more recent regulatory studies were prioritized for inclusion in the WoE while some older studies were omitted in that the results of these studies are largely supplanted by the findings derived from higher quality and more comprehensive protocols.

**Literature search and selection of published studies for inclusion in the WoE evaluation**

Databases searched included: BHCPLUS, MEDLINE, AGRICOLA, CABA, BIOSIS, ESIROBASE, EMBASE, TOXCENTER, PASCAL, POSCITECH and SCISEARCH. The search included studies conducted on all forms of 2,4-D including the acid, salt and ester forms. Terms used in the search strategy are included in Supplementary Appendix I. This search covered studies published from 2009 to mid-2013 (depending on the database); further PubMed searches were done to ensure capture of relevant late 2013–early 2014 relevant studies. A similar literature search was conducted in 2009, and coverage of literature extended to the early 1960s in PubMed. Retrieval was limited to English language articles.

Multiple in vitro, ecotoxicological and mammalian toxicological studies on 2,4-D were identified from the published literature with potentially relevant endpoints.

**Evaluation of study quality**

All studies were evaluated for quality prior to inclusion in the assessment, and perceived weaknesses or gaps in available information were tabulated. Although regulatory toxicology studies are typically conducted under GLP, which ensures an a priori protocol, a record of any protocol amendments and deviations, and accuracy of data collection and reporting, GLP compliance alone does not guarantee that the studies are of high scientific quality or are the most relevant for evaluation of endocrine pathway modulation. A detailed evaluation of both the regulatory and published studies cited in this article was conducted, and study deficiencies identified where they were found.

Modified Klimisch criteria (Klimisch et al. 1997) were used for scoring study quality and included several additional

<table>
<thead>
<tr>
<th>Score</th>
<th>Description</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Reliable without restriction</td>
<td>Generally guideline and GLP compliant studies using validated methodology; study is transparently reported and report internally consistent.</td>
</tr>
<tr>
<td>2</td>
<td>Reliable with restriction</td>
<td>Generally studies from the published literature, often not GLP compliant but sufficient to accept the data and “scientifically acceptable,” or GLP studies that do not follow a specific guideline or cover limited components of a guideline.</td>
</tr>
<tr>
<td>3</td>
<td>Not reliable</td>
<td>Studies generated by a method that is not acceptable, insufficiently documented, or not convincing using expert judgment.</td>
</tr>
<tr>
<td>4</td>
<td>Not assignable</td>
<td>Studies reported only in abstracts or as part of book chapters with insufficient detail to evaluate study quality.</td>
</tr>
</tbody>
</table>
factors suggested by Schneider et al. 2009, in their discussion of a Tox-R-Tool for study quality evaluation. The scores are summarized in Table 1.

Study quality factors evaluated in in vitro studies are

- identification of compound name and purity;
- description of test method;
- identification of dose concentrations;
- rationale (if any) for dose selection;
- use of positive control;
- use of appropriate vehicle or solvent control;
- cytotoxicity evaluations (when appropriate);
- internal consistency and, when tested, reproducibility of reported results;
- biological plausibility of reported results and
- compliance with regulatory guideline or otherwise validated and scientifically appropriate methodology.

There was an exception to application of Klimisch scoring to in vitro assays for the assays from EPA’s in vitro ToxCast™ Program (US EPA 2010). There is insufficient information available on the methodology of the majority of the proprietary ToxCast™ assays to develop a Klimisch score; typically, these would be scored “4” for lack of information. However, data derived from ToxCast™ include assays specifically designed to elucidate potential endocrine-active mechanisms, and currently are being considered by EPA for use in priority setting for the screening of chemicals under EDSP. The selected studies from this program used for the 2,4-D WoE include those most similar in design to the EDSP Tier 1 in vitro assays and are therefore considered relevant to the WoE.

Study quality factors evaluated for in vivo ecotoxicological and mammalian studies are

- identification of compound and purity;
- description of test method;
- identification of dose concentrations;
- rationale (if any) for dose selection;
- adequacy of method or limitations;
- parameters evaluated and methods used for evaluation;
- completeness of data including identification of source, age and strain of test species;
- number of animals and dose groups tested;
- use of appropriate statistical methods;
- information on analytical dose confirmation, homogeneity and stability of dosing formulations;
- appropriate randomization procedures including accounting for potential litter effects in developmental, reproductive or perinatal studies;
- internal consistency of reported results;
- presence or absence of dose response;
- biological plausibility of reported results and
- compliance with regulatory guideline or otherwise validated and scientifically appropriate methodology.

In general, studies with a Klimisch criteria score of 1 or 2 are included in the WoE; however all studies were reviewed for potentially relevant information.

No attempt was made to score epidemiological or occupational health studies; study limitations are generally discussed. Additionally, non-guideline mechanistic studies were not scored because these studies often use single dose levels and/or unconventional routes of exposure. Klimisch et al. (1997) assign this type of study a “5”, outside of the scoring criteria.

Published mammalian toxicological studies of 2,4-D salts and esters (Charles et al. 1996a, 1996b, 2001) were the primary source of information regarding activity of the salts and esters and were reviewed to determine only whether any of these forms presented a unique hazard of endocrine-related toxicity compared to the acid form; therefore, the original study reports were not reviewed in depth.

Identification of relevant endpoints for each potential endocrine pathway interaction and ranking of endpoints for sensitivity and specificity

Each study design was examined to determine endpoints potentially relevant to specific endocrine pathway interactions. The endpoints selected will be reviewed in the WoE discussion. For mammals, endpoints include: developmental landmarks (anogenital distance (AGD), nipple retention in males, vaginal opening and balano-preputial separation); estrous cyclicity; reproductive organ weight and histopathology; mammary gland histopathology; sperm parameters; ovarian follicular counts; thyroid hormones, weight and histopathology; adrenal weight and histopathology; and pituitary weight and histopathology. The EOGRT and two-generation reproductive toxicity studies provide the majority of relevant mammalian endpoints, particularly in the absence of the EDSP Tier 1 mammalian screening studies which were not required for 2,4-D because of the availability of the comprehensive EDSP Tier 2-equivalent EOGRT study. Further, we consider the most robust data to be derived from studies which have internal checks for consistency because of evaluation of similar endpoints across life-stages. For example, there were sporadic findings of testicular atrophy in the EOGRT study parental generation. These findings were of low incidence and within historical control range, but more importantly, were not seen in the F1 generation adults, even after a longer duration of exposure to 2,4-D. (Study results were also examined to confirm there were no increases in implantation loss or fetal deaths that could have signified potential culling of a sensitive sub-population.) Thus, the F1-generation results provide additional confidence that the findings in the parental generation were not exposure related.

Subchronic and chronic toxicity studies, however, often have information on reproductive organ weight and histopathology, and sometimes hormone data (e.g. thyroid hormones T4 and thyroid-stimulating hormone (TSH)). Oncogenicity studies may shed light on potential endocrine-mediated toxicity by increases or decreases in certain tumor types. Subchronic and chronic studies may also help identify differences due to route of administration or varied responses due to species or strain differences. Therefore, these studies were also included in the WoE.
As noted previously, the AMA focuses primarily on thyroid-related endpoints as development of the tadpole is highly dependent on thyroid hormone economy. The FSTRA and the quail one-generation study provide additional apical ecotoxicological studies, providing information on potential estrogen, androgen or steroidogenesis pathway interactions. Endpoints in the FSTRA include vitellogenin (VTG) measurements, presence or absence of nuptial tubercles and gonadal histopathology; in the quail, endpoints include fertilization, eggshell thickness and hatching.

In a recent paper, Borgert et al. 2014 proposed the following ranking scheme for evaluating the endpoints assessed in the EDSP Tier 1 Tests for relevance, sensitivity and specificity to testing hypothesized endocrine pathway interactions:

“Rank 1 was assigned to in vivo endpoints that characterize the fundamental physiological actions for androgen, estrogen and thyroid activities. Rank 1 endpoints are specific and sensitive for the hypothesis, interpretable without ancillary data, and rarely confounded by artifacts or non-specific activity. Rank 2 endpoints are specific and interpretable for the hypothesis but less informative than Rank 1, often due to oversensitivity, inclusion of narrowly context-dependent components of the hormonal system (e.g. in vitro endpoints) or confounding by non-specific activity. Rank 3 endpoints are relevant for the hypothesis but only corroborative of Ranks 1 and 2 endpoints.”

Note that these rankings are made for each relevant endpoint, not for each assay as a whole. Ranking of endpoints is preset depending on the assay type and relevance to the hypothesis being tested, i.e. whether estrogenicity, anti-estrogenicity, androgenicity, anti-androgenicity or impact on steroidogenesis or on the hypothalamic–pituitary–thyroid (HPT) axis. It should be noted that there is currently, to our knowledge, no agreed-upon quantitative weighting system for specific potentially endocrine-related parameters for studies outside of the EDSP screening studies.

Three other factors were considered when scoring the individual assay parameters. The first is the context of the parameter and how or if potential confounding factors are eliminated or controlled. For example, a relatively high degree of confidence for assessing potential estrogenicity can be placed on uterine weights in uterotrophic assay study animals, which are either ovariectomized and hence not cycling or for uterine weights in immature females. Uterine weights in reproductive toxicity study or subchronic toxicity study females, if they are cycling, however, are not reliable endpoints because the uterine weight varies markedly with the stage of the estrous cycle at the time of necropsy (Stoker & Zorrilla 2010). In the latter case, higher confidence in a potential endocrine interaction would be made if other correlating endpoints, particularly in the same study, also showed a response suggesting estrogenic activity. For example, if uterine weights were increased, and if the females showed persistent estrus, this would provide a much more robust signal of potential estrogenic activity.

Second, the magnitude of responses needs to be evaluated carefully. In the context of evaluating potential estrogenicity in rat pubertal development, slight advancement of the time of vaginal opening, e.g. 0.5 day, is not strong evidence of potential estrogenicity, whereas a three-day advance would be (Edwards & Kay 1985). Evaluation of response magnitude requires an appreciation of the variability inherent to the parameter in control test systems or species. Historical control data (HCD) are particularly useful in evaluating whether a statistically significant change is also biologically significant. One of the strengths of the regulatory database generally lacking in other studies in the published literature (and still being developed for many relatively newer endpoints in the EDSP data set, particularly in fish and frogs) is the availability of HCD to help interpret the biological significance of responses, and to determine if the control population is behaving normally.

The third factor that may influence the scoring is the presence of significant systemic toxicity that may confound the ability to accurately ascribe changes to endocrine modulation. For example, decreases in VTG levels in female fish may be due to other toxicity, such as hepatic toxicity, rather than to potential anti-estrogenicity, whereas a substantial increase in VTG in male fish appears to be closely associated with estrogenicity. Decreased weight gain or weight loss may lead to a decreased incidence of mammary tumors or cell proliferation in chronic studies, delays in sexual maturation, and, particularly in immature animals, decreased testis weight and testicular atrophy.

**Differentiating potential endocrine modes of action based on endpoints affected**

There is overlap between changes in endpoints that may be relevant for either estrogenic or anti-androgenic modes of action. For example, relatively potent estrogens may affect testicular histopathology in ways congruent with anti-androgens. Interactions with ERs or ARs may help delineate modes of action. Endpoints relevant to steroidogenesis or the hypothalamic–pituitary–gonadal (HPG) axis may overlap with either of these mechanisms. Examples of potential indicators of endocrine pathway interactions in mammalian systems are shown in Table 2.

To limit extensive redundancy in the WoE of potential estrogen pathway interactions in mammals we have taken the approach of limiting tabulated endpoints and discussions to female-specific endpoints; for evaluation of potential androgen pathway interactions we focus on male-specific endpoints. Endpoints from the opposite sex are potentially relevant (as can be seen in Table 2) and will be mentioned in each case but not discussed in detail. For fish, the most sensitive indicators of potential endocrine modulation in the current EDSP Tier 1 assay appear to be found in the opposite sex, e.g. increased VTG in male fish is a sensitive endpoint for estrogenicity and the appearance of nuptial tubercles in female fathead minnows is sensitive for androgenicity. Therefore, data from both sexes from the FSTRA are considered for each pathway hypothesis.

Changes in both male and female endpoints may also be indicators of a potential interaction with steroidogenesis or the HPG axis; for this evaluation we have tabulated relevant endpoints in both sexes for both mammals and fish, and provided briefer discussions of any specific study endpoints already discussed in the WoE for estrogen-pathway-related or androgen-pathway-related endpoints.
The WoE generally follows the approach used by de Peyster and Mihaich (2014), in that potentially relevant studies are identified, studies are evaluated for quality, relevant endpoints for each hypothesis tested were ranked for sensitivity and specificity, other factors or confounders potentially influencing each endpoint were evaluated, and, most importantly, the consistency of responses of relevant endpoints is assessed. The goal in this evaluation has been to use the most transparent methods for evaluation possible, recognizing that as more information on adverse outcome pathways are developed, some of the relative rankings of endpoints may change accordingly.

The Weight of Evidence Guidance for the Tier 1 EDSP studies developed by the US EPA (US EPA 2011) was also considered when developing this evaluation. This document indicates:

“...the value of each individual assay cannot be considered in isolation from other assays in the battery, as they have been combined in a manner such that limitations of one assay are complemented by the strengths of another” (quote in EPA document from EDSTAC, 1998).

Although EPA’s approach was developed specifically for the Tier 1 EDSP data set, the same principles were followed for the current evaluation of 2,4-D. The WoE reflects an assessment of whether results might signal a specific endocrine pathway interaction, the relative weight or rank placed on that parameter for specifically and sensitively flagging a potential interaction, and whether a finding (if any) was made only at a systemically toxic or otherwise excessive dose, as discussed above. The WoE tables developed for each pathway provide a visual representation that assists in identifying patterns of findings within or across studies that may indicate a potential endocrine pathway interaction; the

<table>
<thead>
<tr>
<th>Mechanism</th>
<th>Potential effects in males</th>
<th>Potential effects in females</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estrogenicity</td>
<td>• Delayed preputial separation (marked, or in absence of significant body weight decreases)</td>
<td>• Accelerated vaginal opening</td>
</tr>
<tr>
<td></td>
<td>• ↓ sperm counts</td>
<td>• Persistent estrus</td>
</tr>
<tr>
<td></td>
<td>• ↑ fertility</td>
<td>• ↑ time to mating</td>
</tr>
<tr>
<td></td>
<td>• Presence of hypospadias/epispadias</td>
<td>• ↓ gestation duration</td>
</tr>
<tr>
<td></td>
<td>• ↓ reproductively organ weight (particularly prostate, seminal vesicles)</td>
<td>• ↓ uterine weights (particularly indicative in immature or ovariectomized animals)</td>
</tr>
<tr>
<td></td>
<td>• Histopathological findings of the testes (e.g. leydig cell proliferation and/or tumors)</td>
<td>• Histopathological findings of the female reproductive organs (e.g. vaginal cornification, uterine hypertrophy and hyperplasia)</td>
</tr>
<tr>
<td></td>
<td>• ↑ incidence, growth of pituitary tumors</td>
<td>• ↓ mammary tumors</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• ↑ incidence, growth of pituitary tumors</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Ovarian, uterine and vaginal tumors in female offspring</td>
</tr>
<tr>
<td>Anti-estrogenicity</td>
<td>• ↑ Height of epithelium in testicular tubules</td>
<td>• Delayed vaginal opening (VO)</td>
</tr>
<tr>
<td></td>
<td>• ↑ testicular weight (short term)</td>
<td>• Delayed start of estrous cycling</td>
</tr>
<tr>
<td></td>
<td>• Testicular atrophy (long term)</td>
<td>• Irregular or absent estrous cyclicity</td>
</tr>
<tr>
<td></td>
<td>• Infertility and testicular atrophy (moderate term)</td>
<td>• ↓ fertility</td>
</tr>
<tr>
<td>Androgenicity</td>
<td>• ↑ or ↓ male reproductive organ weights</td>
<td>• ↓ corpora lutea, implantations</td>
</tr>
<tr>
<td></td>
<td>• ↓ sperm counts</td>
<td>• ↓ female reproductive organ weights</td>
</tr>
<tr>
<td></td>
<td>• Testicular atrophy</td>
<td>• ↓ mammary tumor incidence</td>
</tr>
<tr>
<td>Anti-androgenicity</td>
<td>• Delayed preputial separation</td>
<td>• ↑ Anogenital distance</td>
</tr>
<tr>
<td></td>
<td>• ↓ anogenital distance</td>
<td>• Accelerated vaginal opening</td>
</tr>
<tr>
<td></td>
<td>• Ectopic testes (pre-natal exposure)</td>
<td>• ↓ fertility</td>
</tr>
<tr>
<td></td>
<td>• Hypospadias/epispadias (pre-natal exposure)</td>
<td>• Altered differential follicle count</td>
</tr>
<tr>
<td></td>
<td>• ↓ fertility</td>
<td>• Histopathological findings of the female reproductive organs (e.g. vaginal agenesis)</td>
</tr>
<tr>
<td></td>
<td>• ↓ reproductive organ weight (particularly prostate, seminal vesicles)</td>
<td>• Induced male sex accessory tissues</td>
</tr>
<tr>
<td></td>
<td>• Retained nipples/areolas in male pups</td>
<td>• Altered pup sex ratios between external and internal sexing</td>
</tr>
<tr>
<td></td>
<td>• Histopathological findings of the reproductive organs (e.g. epididymal agenesis, testicular tumors)</td>
<td></td>
</tr>
<tr>
<td>Aromatase inhibition</td>
<td>• ↑ time to mating</td>
<td>• ↑ Body weight</td>
</tr>
<tr>
<td></td>
<td>• ↑ male mounting behavior</td>
<td>• ↑ uterine weight</td>
</tr>
<tr>
<td></td>
<td>• ↓ body weight (chronic)</td>
<td>• ↓ ovary size</td>
</tr>
<tr>
<td></td>
<td>• ↑ testis weight (chronic)</td>
<td>• Polycystic ovaries</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Stromal hyperplasia in ovary (chronic)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Hyalinization in ovary (chronic)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• ↑ ureter and bladder infection</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• ↓ mammary tumor incidence (S-D rats)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Similar to anti-estrogenicity/aromatase inhibition</td>
</tr>
<tr>
<td>Reduced steroid biosynthesis</td>
<td>• Similar to anti-androgenicity</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Possible increased serum cholesterol levels</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• ↑ incidence Leydig cell tumors</td>
<td></td>
</tr>
</tbody>
</table>

↑ Increase
↓ Decrease
subsequent discussions evaluate potentially confounding or other factors that need to be considered to evaluate the likelihood of an endocrine pathway interaction.

**Impact of toxicokinetic data for 2,4-D on study design, data interpretation and risk assessment**

Extensive research has been done to characterize 2,4-D TK. 2,4-D clearly exhibits species-, dose- and sex-dependent non-linear TK in animal test species (Gorzinski et al. 1987; Van Ravenswaay et al. 2003; Timchalk 2004; Saghir et al. 2006; 2013). The non-linear TK is directly and primarily attributable to high-dose saturation of a renal anion transporter, OAT-1, that is responsible for rapid renal clearance of 2,4-D (Hasegawa et al. 2003; Saghir et al. 2013). Non-linear TK, in which metabolism or excretion pathways available at lower blood concentrations are partly or fully saturated with increasing dose, may be a clear confounder both in appropriately designing toxicological studies and evaluating results for hazard and human risk assessment. Use of TK to inform human-relevant dose selection in animal toxicity studies has been affirmed in recent reviews and Organization for Economic Cooperation and Development (OECD) guidance on conduct of the EOGRT study (Barton et al. 2006; Carmichael et al. 2006; Cooper et al. 2006; OECD 443, 2012a). The guidance recommended that top dose level(s) should not exceed the inflection point of onset of TK non-linearity if the inflection point dose was well separated from human exposures, and further concluded that toxicity limited to doses above the onset of non-linear TK behavior was not quantitatively relevant to human risk. Both of these criteria, evidence of non-linear TK in animal test systems and low human exposure levels, are fulfilled for 2,4-D.

Consideration of saturated TK is particularly important for interpretation of the human health relevance of high-dose specific 2,4-D toxicity, including potential endocrine effects. 2,4-D is a structural analog of thyroxine, and has been shown to be weakly active in displacing plasma protein bound thyroxine following administration at a toxicokinetically saturated 80 mg/kg/day dose in rats (Florsheim & Velcoff 1962; Florsheim et al. 1963; Van den Berg et al. 1991). Given the weak competitive binding activity of 2,4-D to thyroid hormone binding sites, any substantial displacement would be unlikely at disproportionately lower plasma concentrations associated with non-saturating 2,4-D doses. In addition, high-dose administration of 2,4-D to mice and rabbits results in increased distribution to and/or retention in brain (Kim et al. 1988). The overall TK data suggest that potential central nervous system (CNS) effects are secondary to two sequential and mechanistically related dose-disproportionate events resulting in increases in 2,4-D brain concentrations: (1) initial saturation of OAT-1 renal clearance leading to dose-disproportionate elevation in plasma 2,4-D plasma concentration allowing for increased organ distribution of non-plasma-protein bound 2,4-D (Timchalk 2004; van Ravenswaay et al. 2003); and (2) followed by augmented non-linear increases in brain concentration associated with high-dose specific saturation of OAT-1 clearance from brain (Kim et al. 1988). Although the quantitative contribution of each of these saturation events to altered distribution of 2,4-D within the brain is unknown, such alterations to and within an endocrine-modulatory organ such as brain have the potential to initiate high-dose specific secondary modes of action. These include reduced clearance of potentially toxic neurotransmitter metabolites such as 5-hydroxy-3-indole acetic acid from brain by the choroid plexus OAT-1 transporter (5-HIAA; Kim et al. 1988; Elo & MacDonald 1989) that ultimately have no quantitative relevance to adverse health outcome potential in humans exposed to far lower, non-saturating, environmental exposures.

Studies in rats have confirmed that 2,4-D TK exhibits non-linear behavior following dietary administration, a route of administration commonly employed in 2,4-D toxicity studies, and have titrated the doses at which the inflection point of onset of non-linear TK begins in both male and female rats (Saghir et al. 2008a, 2008b, 2013). In early work using dietary dose levels of 5 and 100 mg/kg/day, Saghir and coworkers demonstrated saturation of renal clearance and distinctly non-linear TK in male F344 rats fed diet approximating 100 mg/kg/day 2,4-D for 28 days (Saghir et al. 2006). However, to better inform dose selection for the EOGRT study, more comprehensive dietary range finding and TK studies were conducted over multiple life stages in both sexes of CD® rats (Saghir et al. 2008a, 2008b, 2013). These data provided information on plasma concentrations over a wide range of 2,4-D dietary doses and identified inflection points for transition from linear to non-linear TK in both male and female Sprague-Dawley rats.

Following an integrated analysis of the TK information, toxicity and human exposure information, top doses of 600 ppm (30 mg/kg/day, non-pregnant females) and 800 ppm (40 mg/kg/day, males) were selected for the EOGRT study. These doses were anticipated to be either at or slightly above the inflection point for non-linear TK behavior of 2,4-D, considered a threshold for saturation of renal clearance (TSRC) for each respective gender (also referred to as a KMD or kine-tically derived maximum dose in some reports, Saghir et al. 2012). Data from the EOGRT TK range finder study (Saghir et al. 2013) showed that the high dose for male rats (800 ppm; 41 mg/kg/day) was close to, but slightly above the TSRC. Following 28 days of dietary treatment prior to mating, the 2,4-D plasma area under the curve (AUC) in the 800 ppm dose was a dose-disproportionate 11-fold higher relative to the AUC at the 8-fold lower 100 ppm dose (5 mg/kg/day). The high dose of 600 ppm selected for females in the EOGRT study, however, substantially exceeded the TSRC during the 28-day pre-mating treatment. During the 28-day pre-mating dosing period, the female plasma 2,4-D AUCs at 200, 400 and 600 ppm doses (14, 25–27 and 41 mg/kg/day, respectively) were 3–8, 11- and 31-fold higher relative to the AUC at 100 ppm (6–7 mg/kg/day). An even larger 33-fold difference in plasma AUC was observed between the 100 and 600 ppm doses on gestation day 17 rat dams (Marty et al. 2013), likely due to increased food consumption in the latter stages of pregnancy. Based on the EOGRT range finder TK data, the TSRC in adult male rats is 30–40 mg/kg/day and 15–20 mg/kg/day in adult non-pregnant females. Males are more efficient at excreting 2,4-D than females because androgens increase the expression of the saturable OAT-1
transporter (Ljubojevic et al. 2004). Thus, the sex-dependent difference in expression of the organic ion transporter likely accounts for the differential thresholds for saturation of 2,4-D between male and female rats.

Differences in species sensitivity to 2,4-D also appear related to the presence or absence of the OAT-1 transporter in the renal tubules. The implications of species-specific differences in 2,4-D TK to selecting the appropriate species for deriving the point of departure (POD) for human risk assessment have been summarized in a review by the Industry Task Force II on 2,4-D Research Data (Bus & Hammond 2007):

"Knowledge of the dose and species-dependent pharmacokinetic behavior of 2,4-D significantly enhances the understanding of the relevance of toxicity findings of 2,4-D in rodents, and particularly in dogs, to predicting potential human health risks. Once absorbed, 2,4-D is rapidly and completely excreted in urine by both rats and humans, but not dogs (Van Ravenswaay et al. 2003; Timchalk, 2004). In rodents and humans, renal excretion of 2,4-D is facilitated by a saturable organic anion active transporter located in the renal tubules (Timchalk, 2004). The transporter does not effectively function in dogs. Studies in rats indicate the renal clearance of 2,4-D is clearly saturated at oral (gavage) dose levels of 50 mg/kg, resulting in nonlinear increases in 2,4-D blood concentrations at this dose and above (Gorzinski et al. 1987; Van Ravenswaay et al. 2003). Given this non-linear behavior, saturation of 2,4-D renal clearance at 50 mg/kg suggests that animal toxicity findings observed at this dose level and higher overestimate potential human risks. In the case of dogs, both subchronic and chronic studies indicate this species, with an overall NOAEL of 1 mg/kg/day (Charles et al. 1996b), is more sensitive to 2,4-D-induced toxicity than rodents, with an overall NOAEL of 5 mg/kg/day (Charles et al. 1996c). Since the dog is lacking an effective renal organic anion clearance mechanism, this differential species response has been attributed to an inability of the dog to effectively clear 2,4-D from the body, resulting in significantly higher 2,4-D blood concentrations in dog relative to rats and humans at an equivalent oral dose of 5 mg/kg (Van Ravenswaay et al. 2003; Timchalk, 2004). In this case the rat represents a more relevant species for deriving data for [human] risk assessment."

Because of the substantial differences in TK of 2,4-D in dogs relative to other species including humans, EPA, Canadian PMRA and European Food Safety Authority (EFSA) regulatory assessments of 2,4-D have concluded that the dog is an inappropriate species for human risk assessment (US EPA 2005; PMRA 2007; EFSA 2014). As a consequence, the animal no-observed-adverse-effect level (NOAEL) used as the primary reference point to establish acceptable chronic human 2,4-D exposures is 21 mg/kg/day based on toxicity in chronic dietary studies in rats (Marty et al. 2013). This NOAEL is based on renal toxicity, not on endocrine or reproductive effects. Alexander et al. (2007) reported that children living on farms on which 2,4-D was being actively applied had systemic doses (geometric mean) of 0.32 (children 4–11) to 0.12 (children >12 years old) μg/kg based on five days of comprehensive urinary biomonitoring. These dose levels were 65 625–175 000-fold below the overall NOAEL of 21 mg/kg/day (21 000 μg/kg/day) used to set the EPA chronic reference dose for 2,4-D. Large margins of exposure (MOEs) were similarly noted for both applicators and spouses (geometric mean systemic doses of 2.46 and 0.8 μg/kg/day, respectively). Biomonitoring equivalent determinations in this and other populations similarly demonstrate conservatively large MOEs (Aylward et al. 2010; Hays et al. 2012). Since the inflection points for onset of non-linear TK in male and female rats are in the range of 15–40 mg/kg/day, toxicity studies such as the EOGRT fulfilled recent dose selection guidance recommending use of a KMD dose selection strategy, i.e. for 2,4-D using doses at or below the TSRC, when the non-linear TK inflection point is well separated from human exposures. Thus, TK data are a key contextual consideration facilitating interpretation of the potential human relevance of 2,4-D toxicity findings limited to doses above the TSRC, including potentially endocrine-related endpoints.

Organization of the WoE evaluation

The WoE is organized to summarize the 2,4-D in vitro studies, followed by studies from the ecotoxicological and mammalian toxicological databases for 2,4-D with endpoints relevant to evaluating EAT and steroidogenesis endpoints. EDSP Tier 1 studies, and the quail one-generation reproductive toxicity study, the EOGRT EDSP Tier 2-equivalent and multi-generation rat studies are summarized briefly in the appropriate sections because these studies provide the most relevant information for characterizing EAT or steroidogenesis interactions. In each case, the available studies from the regulatory databases and the published literature are tabulated with a brief description of method, results, Klimisch score and rationale. The published in vitro studies are presented alphabetically by first author, because many of these publications cover multiple types of in vitro assays. The ecotoxicological and mammalian toxicological studies are organized by study type. Following the review of mammalian studies is a brief overview of epidemiological studies that assessed relevant endpoints.

The article continues with the WoE assessments for potential interactions with the estrogen, androgen or thyroid pathways or for interaction with steroidogenesis or HPG axis integrating the data from all studies considered high quality (Klimisch 1 or 2).

There are several supplementary appendices. The first, Appendix I, provides the search strategy used in to identify potentially relevant published literature. Six appendices follow that include more comprehensive summaries of the regulatory toxicological studies and published studies:

- Appendix II: in vitro studies (Klimisch criteria 1 or 2), including
  - EDSP in vitro studies
  - In vitro studies in the published literature
  - Further detail on ToxCast™ assays
- Appendix III: in vivo ecotoxicological studies (Klimisch criteria 1 or 2), including
  - EDSP in vivo ecotoxicological studies
    - Amphibian metamorphosis assay (AMA)
    - Fish short term reproduction (FSTR) assay
    - Quail one-generation reproductive toxicity study
  - In vivo ecotoxicological studies in the published literature
Appendix IV: in vivo mammalian toxicological studies (Klimisch criteria 1 or 2), including:

- Reproductive toxicity
  - EDSP Tier 2 equivalent EOGRT study
  - Guideline two-generation rat reproductive toxicity study
- Developmental toxicity studies in rat and rabbit
- Subchronic and chronic toxicity studies in rats, mice, and dogs
- In vivo mammalian toxicological studies in the published literature

Appendix III includes summaries of the regulatory ecotoxicological summaries on 2,4-D acid, followed by published ecotoxicological studies. Appendix IV includes summaries of the regulatory mammalian toxicological summaries on 2,4-D acid, followed by published mammalian studies organized by study type, with priority given to the types of studies with the most relevant endpoints for assessing potential endocrine pathway interactions, e.g., reproductive toxicity evaluations.

Studies considered to be of less than optimal quality for inclusion in the WoE (Klimisch 3 or 4), or found to not contain relevant data are summarized in Appendices V (in vitro studies); VI (in vivo-ecotoxicological studies) and VII (in vivo-mammalian studies).

In vitro studies of 2,4-D relevant to assessment of potential endocrine pathway interactions

In general, in vitro studies may assist in defining adverse outcome pathways and clarifying in vivo findings, but are not indicative by themselves of an adverse endocrine-disrupting effect. Further, results of these studies may be influenced by incompletely or unassessed cytotoxicity, artifacts from transient cell transfection, lack of metabolic co-factors or irrelevant compound concentrations tested.

EDSP tier I in vitro studies

The in vitro EDSP screening assays of 2,4-D are described in a recent publication (Coady et al. 2014). These assays followed the published US EPA guidelines for ER binding (rat uterine cytosol ER binding assay), ER-mediated transcriptional activation (HeLa-9903-ERα transactivation assay), AR binding (rat prostate cytosol AR binding assay), aromatase enzymatic activity inhibition (recombinant human CYP19 aromatase inhibition assay) and interference with steroidogenesis (H295R steroidogenesis assay).

The single exception to the guidelines for these assays was that it was considered appropriate to limit the high concentration in the first four EDSP in vitro assays to 100 μM, rather than the guideline-recommended 1 mM, because the 100 μM concentration was equivalent to serum concentrations at or slightly above the TSRC (slightly above the inflection point for non-linear TK) in rats dosed with 2,4-D in the diet in the EOGRT study (Marty et al. 2010; Marty et al. 2013; Saghir et al. 2013). As noted previously, responses seen only in the non-linear TK range are not regarded as relevant to human risk assessment. Thus, endocrine receptor binding or activation observed only at in vitro concentrations equal to or exceeding serum concentrations at the TSRC are not regarded as relevant to human risk and therefore not informative of potential human endocrine risk; testing high-exaggerated concentrations was considered not appropriate or useful. The maximum concentration recommended in the steroidogenesis assay is 100 μM, and was used in that assay.

These EDSP studies are considered to meet Klimisch criteria 1 because they were conducted according to US EPA guideline recommendations, methodology was validated extensively, deviations from the method were minor and performance and reporting complied with GLP. Table 3 below summarizes the assay type, concentration range tested, results and study quality evaluation from the EDSP in vitro studies; detailed summaries of methods and results are provided in Supplementary Appendix IIA1. The ER binding, ER transactivation, AR binding and aromatase assays showed no effects of 2,4-D, predicting no interactions with the estrogen or androgen pathways either as an antagonist or agonist. There was no effect on testosterone level in the steroidogenesis assay. There was a statistically significant increase in estradiol at the highest concentration tested in all three replicates of the steroidogenesis assay; however, the magnitude of the change was very low (1.2 fold) and did not meet the 1.5-fold cutoff criterion for an exposure-related increase established in the steroidogenesis assay validation studies (Hecker et al. 2008). Therefore, it was concluded that there was no robust evidence for an exposure-related effect.

Published in vitro studies

In addition to the in vitro Tier 1 EDSP screening assays described by Coady et al. (2014) and summarized above, sixteen additional published in vitro studies investigating the potential endocrine activity of 2,4-D were identified. Many of these publications contain multiple assays. These are listed in Table 4 and include: studies of ER and AR agonist and antagonist activity as measured in transactivation assays, assays of ER, AR and progesterone receptor (PR) binding, tissue steroid hormone production and the proliferation of estrogen-responsive cells.

Studies with Klimisch scores of 1 or 2 are summarized in Supplementary Appendix II B; other studies are summarized in Supplementary Appendix V. Supplementary Appendix V also provides a general explanation for the exclusion of yeast-based assays, although these assays were reviewed.

ToxCast™ assays of 2,4-D

EPA developed the ToxCast™ program as a high throughput in vitro screen (HTS) using primarily proprietary assays to screen for potential biological activity and to be used, in conjunction with exposure information, to prioritize chemicals for future testing. EPA has recognized that ToxCast assays offer quantitative data informing the potential reactivity of substances with endocrine pathways and thus can serve as alternatives to current Tier 1 receptor binding, transactivation and uterotrophic assays (US EPA 2016). Regardless, the,
proprietary methods used in ToxCast™ eliminate the opportunity to score study quality. However, ToxCast™ evaluates possible endocrine-receptor-related interactions in multiple assays, including examination of potential binding at both whole receptors and ligand-binding domains only, as well as examination of both agonist and antagonist activity in reporter-based systems (Judson et al. 2010). An evaluation of endocrine related ToxCast™ assays (Rotroff et al. 2013) demonstrated that:

“ToxCast™ estrogen receptor and androgen receptor assays predicted the results of relevant EDSP Tier 1 assays with balanced accuracies of 0.91 (p < 0.001) and 0.92 (p < 0.001), respectively. Uterotrophic and Hershberger assay results were predicted with balanced accuracies of 0.89 (p < 0.001) and 1 (p < 0.001), respectively.”

A more recent analysis (Cox et al. 2014) using a case study of HTS-derived models for predicting in vivo androgen, estrogen and thyroid endpoints showed that the more robust cross validation models (based on a set of endocrine ToxCast™ assays and guideline in vivo endocrine screening studies) have balanced accuracies from 79 to 85% for androgen or estrogen pathway interactions, but predicted substantially less accuracy for thyroid endpoints.

2,4-D purity was greater than 90% for all ToxCast™ assays. ToxCast™ assays for 2,4-D included:

**Cell-free HTS assays.** 2,4-D was tested at eight concentrations in the range of 0.00229–50 μM at receptor proteins of relevance to potential estrogen or androgen endocrine modulation. At the seven receptor proteins tested, inhibition of radio-ligand binding was less than 50% at all 2,4-D concentrations tested. This includes at the bovine and human ERs; bovine and human PRs; rat and human ARs; and human thyroid hormone receptor-α. Additionally, 2,4-D tested at eight concentrations in the range of 0.00914–20 μM exhibited less than 50% inhibition of human aromatase enzyme activity.

**Cell-based HTS assays.** In the cell-based HTS assays, 2,4-D was tested at 15 concentrations in the range of 0.0010–76.6 μM. Under these conditions, 2,4-D was considered inactive for agonist activity at the human AR, human ER-α and the human thyroid hormone receptor-β. Furthermore, it did not block (or antagonize) the activity of established ligands at these receptor sites.

**Multiplex transcription reporter assay.** 2,4-D did not activate chimeric transcriptional proteins containing ligand-binding domains for the human AR, human ER-α, human estrogen-related receptor-α, human estrogen-related receptor-γ or human thyroid hormone receptor-α. The chemical also did not activate transcription at a human estrogen response element.

**Aromatase.** 2,4-D did not inhibit aromatase activity.

**Thyroid.** Although the thyroid pathway-related ToxCast™ assays for 2,4-D were negative, it should be noted that EPA has recently concluded that the ToxCast™ in vitro thyroid assays are not predictive of all relevant thyroid modes of

<table>
<thead>
<tr>
<th>Study</th>
<th>Assay and test system</th>
<th>Purity 2,4-D</th>
<th>Concentration range tested</th>
<th>Result</th>
<th>Klimisch score</th>
<th>Rationale for Klimisch score</th>
</tr>
</thead>
<tbody>
<tr>
<td>LeBaron et al. 2011a</td>
<td>Estrogen receptor (ER) binding assay (US EPA 2009a) with uterine cytosol from Sprague-Dawley rats</td>
<td>98.5%</td>
<td>10⁻¹¹–10⁻⁴ M</td>
<td>Negative, non-binder to ER</td>
<td>1</td>
<td>Guideline compliant study; dose range based on mammalian TK data to be below the TSRC</td>
</tr>
<tr>
<td>LeBaron &amp; Kan 2011</td>
<td>Estrogen transcriptional activation assay (US EPA 2009b) with human ERa-HeLa-9903 cells</td>
<td>98.5%</td>
<td>10⁻¹¹–10⁻⁴ M</td>
<td>Negative; no ER transactivation</td>
<td>1</td>
<td>Guideline compliant study; dose range based on mammalian TK data to be below the TSRC</td>
</tr>
<tr>
<td>LeBaron et al. 2011b</td>
<td>Androgen receptor (AR) binding assay (US EPA 2009c) with ventral prostate cytosol from Sprague-Dawley rats</td>
<td>98.5%</td>
<td>10⁻¹¹–10⁻⁴ M</td>
<td>Negative, non-binder to AR</td>
<td>1</td>
<td>Guideline compliant study; dose range based on mammalian TK data to be below the TSRC</td>
</tr>
<tr>
<td>Coady &amp; Sosinski 2011</td>
<td>Aromatase assay (US EPA 2009d) with human recombinant aromatase and titrated androstenedione</td>
<td>98.5%</td>
<td>10⁻¹⁰–10⁻⁴ M</td>
<td>Negative, non-inhibitor of aromatase activity</td>
<td>1</td>
<td>Guideline compliant study; dose range based on mammalian TK data to be below the TSRC</td>
</tr>
<tr>
<td>LeBaron et al. 2011c</td>
<td>Steroidogenesis assay (US EPA 2009e) with H295R cells</td>
<td>98.5%</td>
<td>10⁻¹⁰–10⁻⁴ M</td>
<td>Small, significant ↑ estradiol at 10⁻⁴ M; magnitude of change less than criterion used to define a positive response in validation studies (Hecker et al. 2008); considered negative</td>
<td>1</td>
<td>Guideline compliant study</td>
</tr>
</tbody>
</table>

Table 3. Results from EDSP in vitro studies of 2,4-D (data from individual study reports; published in Coady et al. 2014).
<table>
<thead>
<tr>
<th>Study</th>
<th>Assay and Test system</th>
<th>Purity 2,4-D</th>
<th>Concentration range tested</th>
<th>Result</th>
<th>Klimisch score</th>
<th>Rationale for Klimisch score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blair et al. 2000</td>
<td>Competitive rat ER binding assay in uterine tissue homogenates from ovariec-tomized Sprague-Dawley rats</td>
<td>99%</td>
<td>&quot;2 high concentrations spanning 3 log concentrations&quot;</td>
<td>Negative</td>
<td>3</td>
<td>Strengths: Well documented; method close to validated Guideline design; Weaknesses: 2,4-D specific data not provided; Only tested at 2 concentrations which were not reported</td>
</tr>
<tr>
<td>Fang et al. 2003</td>
<td>Binding to recombinant rat AR</td>
<td>Purity not specified; Supplier (Supelco) produces analytical standards but also mixtures</td>
<td>$4.28 \times 10^{-9}$–$4.28 \times 10^{-10}$ M</td>
<td>Negative</td>
<td>3</td>
<td>Adequate study but lack of information on purity of 2,4-D</td>
</tr>
<tr>
<td>Jung et al. 2004</td>
<td>ER antagonist activity in yeast-based reporter system</td>
<td>&quot;Highest grade commercially available&quot;</td>
<td>Not specified</td>
<td>Negative</td>
<td>3</td>
<td>Weaknesses: concentrations tested not reported; possibly commercial formulation tested and purity unspecified. Not relevant/reliable: Yeast two-hybrid detection system</td>
</tr>
<tr>
<td>Jungbauer &amp; Beck 2002</td>
<td>ER antagonist activity in yeast two-hybrid system</td>
<td>Not specified</td>
<td>Not specified</td>
<td>Negative</td>
<td>3</td>
<td>Weaknesses: concentrations tested not reported; purity unspecified. Not relevant/reliable: Yeast two-hybrid detection system</td>
</tr>
<tr>
<td>Kim et al. 2005</td>
<td>AR+ 22Rv1 cell proliferation with 2,4-D and its metabolite DCP</td>
<td>&gt;98%</td>
<td>$10^{-13}$–$10^{-5}$ M</td>
<td>Negative with 2,4-D or DCP alone, but positive with 2,4-D or DCP with dihydrotestosterone (DHT) added</td>
<td>3</td>
<td>Weakness: Lack of solvent only control; data for DHT alone appears to have been generated for a single subset of tests only (set A in graphs); no rationale for 10 nM concentration of DHT added (possibly supra physiological); measured cell proliferation, which may be stimulated by ER-independent factors, including epidermal growth factor (based on information in the ATCC website (<a href="http://www.atcc.org">www.atcc.org</a>) for this particular strain of cells. See also Sramkoski et al. 1999)</td>
</tr>
<tr>
<td>Transactivation reporter assays with AR+ 22Rv1 and AR-PC3 cells</td>
<td>&gt;98%</td>
<td>$10^{-12}$–$10^{-6}$ M</td>
<td>Negative with 2,4-D or DCP alone, but positive with 2,4-D or DCP with DHT</td>
<td>3</td>
<td>Weakness: Lack of solvent only control; no rationale for concentration of DHT added (possibly supra physiological); only one concentration of 2,4-D tested in AR-PC3 cells.</td>
<td></td>
</tr>
<tr>
<td>AR expression (mRNA and total protein levels) in 22Rv1 cells</td>
<td>&gt;98%</td>
<td>$10^{-7}$ M 2,4-D; $10^{-10}$ M DCP</td>
<td>Negative</td>
<td>3</td>
<td>Weakness: Single concentration of 2,4-D tested; Lack of solvent only control</td>
<td></td>
</tr>
<tr>
<td>AR binding assay in monkey kidney COS cells</td>
<td>&gt;98%</td>
<td>$5 \times 10^{-9}$ M to $5 \times 10^{-7}$ M</td>
<td>Positive (50% inhibition with both 2,4-D and DCP)</td>
<td>3</td>
<td>Weakness: Lack of solvent only control; did not use reagent to separate unbound from bound ligand; evaluated limited number of test compound concentrations; typical dose-response</td>
<td></td>
</tr>
<tr>
<td>Study</td>
<td>Assay and Test system</td>
<td>Purity 2,4-D</td>
<td>Concentration range tested</td>
<td>Result</td>
<td>Klimisch score</td>
<td>Rationale for Klimisch score</td>
</tr>
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</tr>
<tr>
<td>Kojima et al. 2004</td>
<td>Estrogenic and anti-estrogenic activity in CHO cells transiently transfected with human ERβ</td>
<td>≥95%</td>
<td>10⁻⁸–10⁻⁶ M</td>
<td>Negative</td>
<td>2</td>
<td>Strengths: Method very close to validated guideline; well documented; positive control used; weakness: specific response data for 2,4-D not provided. Well documented but method not formally validated. Weakness: specific response data for 2,4-D not provided.</td>
</tr>
<tr>
<td>Androgenic and anti-androgenic activity in CHO cells transiently transfected with human AR</td>
<td>≥95%</td>
<td>10⁻⁸–10⁻⁶ M</td>
<td>Negative</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dual activity as ER agonists and AR antagonists</td>
<td>≥95%</td>
<td>10⁻⁸–10⁻⁶ M</td>
<td>Negative</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lee et al. 2006</td>
<td>Estrogenic activity in yeast one-hybrid and two-hybrid systems</td>
<td>NR</td>
<td>10⁻⁷–10⁻⁴ M</td>
<td>Negative in one-hybrid system; Positive in two hybrid system</td>
<td>3</td>
<td>Weakness: test material uncharacterized. Not relevant: Yeast one-hybrid and two-hybrid detection system</td>
</tr>
<tr>
<td>Lemaire et al. 2006</td>
<td>Estrogenic and anti-estrogenic activity in HeLa cells stably transfected with human ERα or human ER-β</td>
<td>≥95%</td>
<td>10⁻⁶ M</td>
<td>Negative</td>
<td>3</td>
<td>Weakness: Only one concentration tested; otherwise adequate quality</td>
</tr>
<tr>
<td>Lin &amp; Garry 2000</td>
<td>MCF-7 proliferation</td>
<td>2,4-D (reagent grade) 2,4-D isooctyl ester (reagent grade)</td>
<td>0.1–10 μg/mL</td>
<td>Negative</td>
<td>2</td>
<td>Weakness: MCF-7 cell line may provide variable responses and result may not be specific for estrogenicity (Odum et al. 1998)</td>
</tr>
<tr>
<td>MCF-7 proliferation</td>
<td>2,4-D LV4 (commercial grade 66.24% 2,4-D isooctyl ester); 2,4-D amine (commercial grade) 46.5% 2,4-D dimethylamine salt</td>
<td>0.1–10 μg/mL</td>
<td>Positive</td>
<td>3</td>
<td>Weaknesses: Results in commercial grade materials appear confounded due to formulation excipients as reagent grade materials did not show effects; MCF-7 proliferation may be a non-estrogen specific response and cell line may provide markedly variable responses (Odum et al. 1998)</td>
<td></td>
</tr>
<tr>
<td>Nishihara et al. 2000</td>
<td>Estrogenic activity using the yeast two-hybrid system</td>
<td>“Highest grade commercially available”</td>
<td>NR</td>
<td>Negative</td>
<td>3</td>
<td>Weaknesses: formulation and test material purity not characterized concentrations tested not reported; Not relevant: Yeast two-hybrid detection system</td>
</tr>
<tr>
<td>Orton et al. 2009</td>
<td>Xenopus laevis ovulation and ovarian steroidogenesis (production of progesterone)</td>
<td>≥97%</td>
<td>6.25 × 10⁻⁶ and 62.5 × 10⁻⁶ M</td>
<td>Negative</td>
<td>3</td>
<td>Weaknesses: Methods not specific regarding stage of oocytes used (ranges given) or how</td>
</tr>
<tr>
<td>Study</td>
<td>Assay and Test system</td>
<td>Purity 2,4-D</td>
<td>Concentration range tested</td>
<td>Result</td>
<td>Klimisch score</td>
<td>Rationale for Klimisch score</td>
</tr>
<tr>
<td>-------------------------------------------</td>
<td>----------------------------------------------------------------------------------------</td>
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<td>---------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>testosterone and estradiol</td>
<td></td>
<td>≥97%</td>
<td>4.9 × 10⁻²–1 × 10⁻³ M</td>
<td>Negative</td>
<td>3</td>
<td>these were distributed to the test wells; methods not specific regarding number of replicates or criteria for accepting or rejecting replicate findings; non-validated assay; only two concentrations tested</td>
</tr>
<tr>
<td>Petit et al. 1997</td>
<td>ER and AR agonist and antagonist activity in yeast</td>
<td>NR</td>
<td>10⁻⁴ M</td>
<td>Equivocal</td>
<td>3</td>
<td>Weaknesses: Not relevant; Yeast assay; otherwise well reported and conducted</td>
</tr>
<tr>
<td></td>
<td>VTG in mRNA expression in primary hepatocyte cultures derived from male rainbow trout</td>
<td>NR</td>
<td>10⁻⁴–10⁻⁸ M</td>
<td>Negative</td>
<td>3</td>
<td>Weaknesses: Only tested at a single concentration; test material uncharacterized. VTG mRNA expression only 8% increase compared to control (cannot determine if ineffective or weakly responsive).</td>
</tr>
<tr>
<td></td>
<td>β-galactosidase activity in yeast cells stably transfected with rainbow trout ER</td>
<td>NR</td>
<td>10⁻⁴–10⁻⁸ M</td>
<td>Negative</td>
<td>3</td>
<td>Weakness: test material uncharacterized; Not relevant Yeast-based system</td>
</tr>
<tr>
<td>Soto et al. 1995</td>
<td>ER competitive binding assay in yeast cells stably transfected with rainbow trout ER</td>
<td>NR</td>
<td>&gt;10⁻⁴ M</td>
<td>Negative</td>
<td>3</td>
<td>Weaknesses: only tested at a single concentration; test material uncharacterized: Not relevant: Yeast-based system</td>
</tr>
<tr>
<td></td>
<td>Estrogenic activity by measuring MCF-7 proliferation</td>
<td>NR</td>
<td>NR</td>
<td>Negative</td>
<td>3</td>
<td>Weaknesses: test material uncharacterized and concentrations tested not reported; MCF-7 cell line may provide markedly variable responses (Odum et al. 1998)</td>
</tr>
<tr>
<td>Sun et al. 2012</td>
<td>Estrogenic and anti-estrogenic activity in Vero cells</td>
<td>≥99%</td>
<td>0.003–3.0 mg/L</td>
<td>Negative</td>
<td>2</td>
<td>Well-conducted and reported assay. Rationale for dose selection questionable; high dose exceeds potential human exposure although it falls within the linear TK range</td>
</tr>
<tr>
<td></td>
<td>Androgenic and anti-androgenic activity in Vero cells</td>
<td>≥99%</td>
<td>0.003–3.0 mg/L</td>
<td>Negative for androgenicity or anti-androgenicity; at 3.0 mg/L increased the effects of testosterone (in anti-androgenic assay); other concentrations negative</td>
<td>2</td>
<td>Well-conducted and reported assay. Rationale for dose selection questionable; effect seen only at 3.0 mg/L which exceeds predicted concentrations for potential human exposure although it falls within the linear TK range; biological relevance of the finding questionable as the test was designed to measure anti-androgenic activity rather than potentiation or androgenic activity</td>
</tr>
<tr>
<td></td>
<td>Agonist and antagonist activity to TR in Vero cells</td>
<td>≥99%</td>
<td>0.003–3.0 mg/L</td>
<td>Negative</td>
<td>3</td>
<td>Well-conducted and reported assay; however In vitro thyroid assay model not validated</td>
</tr>
<tr>
<td>Vonier et al. 1996</td>
<td>Competitive binding to ER and PR extracted from the oviparous tissues of adult female alligators</td>
<td>≥99%</td>
<td>NR</td>
<td>Negative</td>
<td>3</td>
<td>Well-conducted study with positive control; Weaknesses: concentrations tested not reported.</td>
</tr>
</tbody>
</table>
action in *in vivo* studies (Rotroff et al. 2013), concordant with findings in Cox et al. 2014.

Reif et al. (2010) provides a “Tox-pi” diagram for 2,4-D which confirms that the full range of endocrine-related ToxCast™ assays for 2,4-D were negative.

Based on the multiple assays and consistency of results with *in vivo* and *in vitro* studies of 2,4-D, the ToxCast™ program endocrine-relevant results are considered supportive for concluding 2,4-D is not likely to show potential interactions with either the estrogen or androgen pathways. These data are consistent with the regulatory (EDSP Tier I) *in vitro* data and with the majority of *in vitro* studies of 2,4-D in the published literature.

**Ecotoxicological studies of 2,4-D relevant to assessment of potential endocrine pathway interactions**

The AMA and FSTRA conducted to meet EDSP Tier 1 screening requirements, and a one-generation reproductive toxicity study in quail (Mitchell et al. 2000) conducted to meet prior regulatory testing requirements, provide the most relevant and substantive ecotoxicological data for studying the possible endocrine activity of 2,4-D. These studies are briefly summarized below for ready reference. Specific data are provided for the quail study because this study was unpublished and it is the only relevant bird study identified. Further details on these studies may be found in Supplementary Appendix III and, for the frog and fish assays, in Coady et al. 2013. Regulatory and published studies with Klimisch scores of 2 or higher that are relevant to evaluation of potential endocrine interactions are summarized in Supplementary Appendix III. Studies with lower Klimisch scores, or those adequate studies that were found not to include relevant endpoints, are summarized in Supplementary Appendix VI.

**Coady et al. 2010 (published in Coady et al. 2013)**

The study design of the AMA (Coady et al. 2010) corresponded with guidelines: OPPTS 890.1100 (US EPA 2009) and OECD 231. In brief, African clawed frog (*Xenopus laevis*) tadpoles were exposed to 2,4-D (98.6% purity) under continuous flow-through conditions for 21 days. Nominal test concentrations of 0, 0.4, 4, 40 and 100 mg/L were tested, with the high concentration selected based on prior acute toxicity studies, and equivalent to a limit concentration in the guideline. Concentrations were monitored over the course of the study. Although decreases from the nominal concentration (probably due to biodegradation) were noted, particularly at the lowest concentration tested, the concentrations tested were well documented and the study is considered valid with a Klimisch score of 1 for this guideline compliant study. The mean measured concentrations of 2,4-D in this assay were 0.273, 3.24, 38.0 and 113 mg/L for the 0.4, 4, 40 and 100 mg/L nominal concentrations, respectively.

There was no indication of systemic toxicity in this study, with no effects on survival, clinical signs or body weights (evaluated days 7 and 21). There were no effects on days 7 or 21 on snout-vent length, Nieuwkoop and Faber (1994) developmental stage, hind limb length (normalized to snout-vent length or asynchronous development). There were no exposure-related findings on histopathological evaluation of the thyroid gland or the morphological endpoints of this assay under thyroid control (hind limb length and developmental stage), and there is no evidence of a potential interaction with the HPT axis in this Tier 1 EDSP AMA tested to the assay limit concentration of 100 mg 2,4-D/L.

**Marino et al. 2010 (published in Coady et al. 2013)**

Marino et al. 2010 tested 2,4-D in a FSTRA (US EPA 2009 g). The study was conducted in compliance with OPPTS 890.1350 and OECD 229. Sexually mature fathead minnows (*Pimephales promelas*) were exposed to 2,4-D (98.6% purity) under continuous flow-through conditions for 21 days at nominal concentrations of 0, 0.4, 4, 40 and 100 mg/L. The high concentration was selected based on acute toxicity tests and an early life stage test with fathead minnows (Alexander et al. 1983; Mayes et al. 1990); and also represents a limit concentration for the assay. The negative control was untreated laboratory dilution water. Although decreases from the nominal concentration (probably due to biodegradation) were noted, particularly at the two lowest concentrations tested, the concentrations tested were well documented and the study is considered valid with a Klimisch score of 1 for this guideline-compliant study.

Results are summarized in Table 5. The only statistically significant finding compared to the controls was a decrease in fecundity (considered a non-specific finding) among fish exposed to the highest concentration of 2,4-D. In the absence of effects upon other more specific endocrine-mediated endpoints, the isolated effect on fecundity at 100 mg a.i./L is considered most likely to reflect systemic toxicity and a generalized stress response. This concentration is relatively high (approximately 1/3 of the acute LC50 value in fish), is the limit concentration for the FSTRA, and is a concentration which exceeds the maximum acceptable toxicant concentration (MATC) for larval fish survival in an early life stage toxicity test with fathead minnows (Mayes et al. 1990).

In conclusion, 2,4-D does not appear to interact with the estrogen, androgen or steroidogenic pathways, or with the HPG axis in fathead minnows tested up to the limit concentration in this EDSP Tier 1 FSTRA.

**Mitchell et al. 2000**

An avian single generation reproductive toxicity study (Mitchell et al. 2000) of 2,4-D showed no systemic toxicity to quail and a lack of potential endocrine-related effects. This study complied with Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) Guideline 71–4 and OECD Guideline 206 and was conducted under GLP, and is therefore scored a Klimisch 1.
2,4-D acid (96.9% pure) was administered to adult Northern Bobwhite male and female quail (Colinus virginianus) for 21 weeks via the diet at 0, 160, 400 and 1000 ppm. The high dose level complies with the limit dose recommended in OECD Guideline 206. The no-observed effect concentration for northern bobwhite quail exposed to 2,4-D acid in the diet during the study was 1000 ppm, the highest concentration tested. There were no effects on mortality, clinical signs, body weight or feed consumption of adult birds and no exposure-related findings at necropsy. Two high-dose deaths were attributable to injury. Other results are summarized in Table 6 below. Slight but statistically significant decreases in the percent of hatchlings/eggs set and 14/day survivors/eggs set and a non-statistically significant decrease in the mean percent of viable embryos as a percent of eggs set were observed at the low dose. These findings were attributable primarily to one pen, in which no eggs were fertile, and the male showed quiescent testes at necropsy. This fact and the lack of dose response led to the conclusion that this finding was not exposure-related. Eggshell thickness was statistically significantly increased at 400 ppm; primarily attributable to results from one pen with an elevated eggshell thickness. This finding was not considered exposure related based on the slight nature of the finding, the attribution to one pen and the lack of dose response. This study is considered valid; it predicts a very low hazard of reproductive toxicity of 2,4-D to birds and a low likelihood of endocrine-related effects on birds.

### Review of studies for study quality

The EDSP Tier 1 ecotoxicological studies, one-generation quail and published ecotoxicological studies identified as possibly relevant to assessment of potential endocrine pathway interactions are tabulated in Table 7. There were no findings in the regulatory toxicological studies considered likely to reflect endocrine pathway interactions. A series of studies by Crain et al. (1997; 1999) was considered valid; these studies using 2,4-D applied to alligator eggs was validated with a positive control and showed no effects of 2,4-D (summarized in Supplementary Appendix III). Other studies are summarized in Supplementary Appendix VI.
Mammalian toxicological studies of 2,4-D relevant to assessment of potential endocrine pathway interactions

Selection of regulatory mammalian toxicology studies for inclusion in review

Regulatory (unpublished) mammalian toxicity studies of 2,4-D acid conducted for pesticide registration purposes were reviewed. Fourteen studies with the most relevant endpoints for evaluation of potential endocrine toxicity and most comprehensive reporting were selected for this WoE evaluation. Data from several of these studies have also been published; citations to both the reports and publications are provided.

These studies included, most critically, an EOGRT study of 2,4-D (Marty et al. 2010 published in Marty et al. 2013) and the two-generation reproductive study findings (Rodwell & Brown 1985), which, as noted, used TK data to inform dose selection, and which included multiple endpoints specifically added in consultation with the US EPA and the Canadian PMRA to provide additional information on potential endocrine interactions of 2,4-D with the estrogen, androgen or thyroid pathways. At the time this study was conducted, the guideline for an EOGRT study was still under development; however, based on the extensive vetting of the study design, similarity to the adopted test guideline, and involvement of two regulatory authorities in both the study design and critical decision points, it met all the objectives of the current OECD (2012a) study guideline (443).

Other selected regulatory studies include:

- US EPA Office of Pesticide Program (OPP) 83-4 guideline two-generation reproductive toxicity study (Rodwell & Brown 1985);
- OPP 83-3 guideline developmental toxicity study in rats (Rodwell 1983; Charles et al. 2001);
- OPP 83-3 guideline developmental toxicity study in rabbits (Hoberman 1990; Charles et al. 2001);
- OPP 82-1 guideline 13-week rat subchronic toxicity study (Schulze 1991a; Charles et al. 1996a);
- non-guideline 13-week rat subchronic toxicity studies (Gorzinski et al. 1981a, 1981b);
- OPP 83-5 guideline two-year rat chronic toxicity/oncogenicity study (Jeffries et al. 1995; Charles et al. 1996c);
- OPP 82-1 guideline 13-week mouse subchronic toxicity (Schulze 1991b);
- OPP 83-2 guideline-equivalent mouse oncogenicity evaluations (Stott 1999a, 1999b; Charles et al. 1996c);
- OPP 82-1 non-guideline 13-week dog subchronic toxicity study (Schulze 1990);
- OPP 82-1 guideline 13-week dog subchronic toxicity (Dalgard 1993a; Charles et al. 1996b); and
- OPP 83-1 guideline dog chronic toxicity study (Dalgard 1993b; Charles et al. 1996b).

The regulatory mammalian toxicological database provides an opportunity to evaluate consistency of responses across species and strains, and also across exposure durations. The Tier 2 EDSP-equivalent EOGRT study (Marty et al. 2010; published in Marty et al. 2013) and the two-generation reproductive study findings (Rodwell & Brown 1985) are briefly summarized in this section because these two studies provide by far the most comprehensive and relevant endpoints for evaluating the potential EAT and steroidogenesis interactions of 2,4-D.

Subsequently, other subchronic and chronic mammalian regulatory studies and studies identified in the published literature are tabulated and scored for study quality. Further details on these studies may be found in Appendix IV for studies considered to meet Klimisch criteria 1 or 2, and in Appendix VII for studies considered to meet Klimisch criteria 3 or 4.

The primary caveat regarding regulatory studies other than the EOGRT, and the majority of the published mammalian toxicological studies is that the high dose level was based on (or in some cases exceeded) a classic maximum tolerated dose (MTD) and far exceeds the TSRC. High dose level findings above the TSRC are presented but, as discussed in the Introduction, are not considered relevant for human hazard characterization or risk assessment.

Additionally, as discussed previously, information from the dog studies is not considered relevant for human risk assessment because the dog lacks an effective organic acid renal transport mechanism (Timchalk 2004); however, data from the dog studies are included because they may be useful in predicting potential effects on other species lacking an
### Table 7. 2,4-D ecotoxicological studies possibly relevant to assessment of potential endocrine interactions.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Assay and test system</th>
<th>Purity 2,4-D</th>
<th>Concentration range tested</th>
<th>Result</th>
<th>Klimisch score</th>
<th>Klimisch score rationale</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Amphibians</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coady et al. 2010; Coady et al. 2013</td>
<td>Amphibian metamorphosis assay (US EPA 2009f, OECD 231). African clawed frog tadpoles exposed in continuous flow-through system for 21 days.</td>
<td>98.6%</td>
<td>0.4–100 mg a.e./L</td>
<td>No exposure-related effects</td>
<td>1</td>
<td>Guideline and GLP compliant study; tested to limit concentration</td>
</tr>
<tr>
<td>Aronzon et al. 2011</td>
<td>South American toad exposed to 2,4-D DBE or formulated product either through embryogenesis or in pulsed exposures</td>
<td>99%</td>
<td>2,4-D di-butyl ether 1–15 mg/L 2,4-D DBE for continuous exposure</td>
<td>“Teratogenic” to toads</td>
<td>3</td>
<td>No controls were included in this study; results cannot be interpreted</td>
</tr>
<tr>
<td>Heggstrom 2009</td>
<td>Wood frog tadpoles in microcosms exposed to 2,4-D dimethylamine</td>
<td>99%</td>
<td>0.1–100 μg/L</td>
<td>Negative for survival, deformities, effects on time to metamorphic climax; total length decreased in mid dose group only; no effect on plasma corticosterone levels</td>
<td>3</td>
<td>High non-exposure related mortality</td>
</tr>
<tr>
<td>Heggstrom 2009</td>
<td>Wood frog tadpoles in field ponds (agricultural and forested) exposed to 2,4-D dimethylamine</td>
<td>99%</td>
<td>10 μg/L</td>
<td>Tadpoles from the agricultural pond in the absence of 2,4-D dimethylamine and tadpoles from the agricultural pond applied with 10 μg/L 2,4-D dimethylamine gave similar results in the measured endpoints</td>
<td>3</td>
<td>Control tadpoles from the two ponds showed some marked differences; only one concentration level evaluated</td>
</tr>
<tr>
<td>Stebbins-Boaz et al. 2004</td>
<td>Xenopus oocytes exposed to 2,4-D sodium salt</td>
<td>NR</td>
<td>0.6–2.43 g/L (2.5–10 mM)</td>
<td>Germinal vesicle breakdown inhibited</td>
<td>3</td>
<td>Mechanistic study; extremely high doses; purity not reported</td>
</tr>
<tr>
<td>LaChapelle et al. 2007</td>
<td>Xenopus oocytes exposed to 2,4-D</td>
<td>NR</td>
<td>2.43 g/L (10 mM)</td>
<td>Irreversible dysfunction of meiotic signaling</td>
<td>3</td>
<td>Mechanistic study; only one extremely high dose evaluated; purity not reported</td>
</tr>
<tr>
<td>Morgan et al. 1996</td>
<td>Frog embryo teratogenic assay in Xenopus</td>
<td>Commercial formulation (99%)</td>
<td>180–270 mg/L</td>
<td>Teratogenic only at high concentrations</td>
<td>2</td>
<td>Adequate study but irrelevant to evaluation of potential endocrine effects; formulation tested</td>
</tr>
<tr>
<td>Vardia et al. 1984</td>
<td>Indian tadpoles exposed to 2,4-D in a static renewal exposure system</td>
<td>NR</td>
<td>7.5–11 mg/L</td>
<td>96-h LC50: 8.05 mg/L</td>
<td>4</td>
<td>Experiment not described in detail; lacks information on potential endocrine effects</td>
</tr>
<tr>
<td><strong>Fish</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Marino et al. 2010; Coady et al. 2013</td>
<td>Fish short term reproduction assay (OPPTS 890.1350, OECD 229, US EPA 2009g). Adult fat head minnows were exposed via continuous flow-through for 21</td>
<td>98.6%</td>
<td>0.4–100 mg a.e./L</td>
<td>No interaction with endocrine pathways; decreased fecundity at highest concentration tested attributed to systemic toxicity</td>
<td>1</td>
<td>Guideline and GLP compliant study; tested to limit concentration</td>
</tr>
<tr>
<td>Reference</td>
<td>Assay and test system</td>
<td>Purity 2,4-D</td>
<td>Concentration range tested</td>
<td>Result</td>
<td>Klimisch score</td>
<td>Klimisch score rationale</td>
</tr>
<tr>
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<td>------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Holcombe et al. 1995</td>
<td>Acute larval survival and growth tests in Japanese Medaka exposed to 2,4-D acid</td>
<td>99% 2,4-D acid</td>
<td>567–8970 mg/L</td>
<td>96-h LC₅₀: 2780 mg/L 2,4-D Acid</td>
<td>2</td>
<td>Adequate study but no specific endocrine endpoints evaluated</td>
</tr>
<tr>
<td>Holcombe et al. 1995</td>
<td>Chronic larval survival and growth tests in Japanese Medaka exposed to 2,4-D acid</td>
<td>99% 2,4-D acid</td>
<td>27.2–425 mg/L</td>
<td>Survival and growth reduced at 56.5 mg/L</td>
<td>2</td>
<td>Adequate study but no specific endocrine endpoints evaluated</td>
</tr>
<tr>
<td>Holcombe et al. 1995</td>
<td>Chronic larval survival and growth tests in Japanese Medaka exposed to 2,4-D acid</td>
<td>99% 2,4-D acid</td>
<td>2.37–60.2 mg/L</td>
<td>Survival and growth reduced at 60.2 mg/L</td>
<td>2</td>
<td>Adequate study but no specific endocrine endpoints evaluated</td>
</tr>
<tr>
<td>Koç &amp; Akbulut 2012</td>
<td>Acute toxicity to ovary of zebrafish</td>
<td>NR</td>
<td>0.1–1 mg/L</td>
<td>Ovarian histopathological changes and atretic follicles following 5 days exposure</td>
<td>3</td>
<td>Source and purity not defined; report says 2,4-D “available as a formulation” so possible a formulation was tested; methods not well defined; potential sectioning artifacts in slides</td>
</tr>
<tr>
<td>Padilla et al. 2012</td>
<td>Zebrafish developmental screening assay in zebrafish embryos exposed to 2,4-D</td>
<td>&gt;90%</td>
<td>0.001–80 μM</td>
<td>Negative in concentration range study; weak positive in single high concentration study</td>
<td>2</td>
<td>Reported findings too non-specific to be useful for WoE</td>
</tr>
<tr>
<td>Rehwold et al. 1977</td>
<td>Acute toxicity tests with striped bass, the banded killifish, pumpkinseed, white perch, American eel, carp, and guppy exposed to 2,4-D</td>
<td>NR</td>
<td>NR</td>
<td>96-h LC₅₀ ranged from 26.7 mg/L (banded killifish) to 300.6 mg/L (American eel)</td>
<td>4</td>
<td>Experimental detail not provided; fish field collected</td>
</tr>
<tr>
<td>Rehwold et al. 1977</td>
<td>Chronic toxicity tests with striped bass, the banded killifish, pumpkinseed, white perch, American eel, carp, and guppy exposed to 2,4-D</td>
<td>NR</td>
<td>0.1 mg/L</td>
<td>No observable physiological symptoms</td>
<td>4</td>
<td>Experimental detail not provided; fish field collected</td>
</tr>
<tr>
<td>Rehwold et al. 1977</td>
<td>Breeding effects on guppy chronically exposed to 2,4-D</td>
<td>NR</td>
<td>0.1 mg/L</td>
<td>No observable physiological symptoms</td>
<td>4</td>
<td>Experimental detail not provided</td>
</tr>
<tr>
<td>Xie et al. 2005</td>
<td>Juvenile rainbow trout exposed to 2,4-D dimethylamine in static test system</td>
<td>NR</td>
<td>0.00164–1.64 mg/L</td>
<td>VTG levels significantly greater at 0.164 and 1.64 mg/L</td>
<td>3</td>
<td>High variability; small sample size, sex of fish not determined, purity of test material not reported</td>
</tr>
<tr>
<td>Mitchell et al. 2000</td>
<td>Avian single generation reproductive study in Northern Bobwhite quail. US EPA OPP 71-4;</td>
<td>96.9%</td>
<td>160, 400, 1000 ppm (No mg/kg/d dose calculated)</td>
<td>No treatment-related effects including on potentially endocrine related parameters</td>
<td>1</td>
<td>Guideline compliant</td>
</tr>
<tr>
<td>Reference</td>
<td>OECD Guideline 206; Subjects exposed via diet for 21 weeks.</td>
<td>Purity 2,4-D</td>
<td>Concentration range tested</td>
<td>Result</td>
<td>Klimisch score</td>
<td>Klimisch score rationale</td>
</tr>
<tr>
<td>-------------------</td>
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<td>---------------------------------------------------</td>
</tr>
<tr>
<td>Somers et al. 1974</td>
<td>Fertilized hen's eggs exposed to 2,4-D by spraying</td>
<td>NR</td>
<td>2.8–3.4 kg/ha and 44.8 kg/ha</td>
<td>No mortalities in ovo, at pip, or at hatch; no weight gain effects</td>
<td>3</td>
<td>Non-conventional method of application; purity not reported; no positive control; evidence of poor absorption into the egg content</td>
</tr>
<tr>
<td>Somers et al. 1978a</td>
<td>Hens and cockerels exposed to 2,4-D by spraying</td>
<td>Formulation purity NR</td>
<td>111.2 kg/ha</td>
<td>No effect on chicken reproduction in various endpoints measured</td>
<td>3</td>
<td>Non-conventional method of application; formulation; single concentration and purity not reported; no positive control; evidence of poor absorption into the egg content</td>
</tr>
<tr>
<td>Somers et al. 1978b</td>
<td>Hens eggs exposed to 2,4-D by spraying</td>
<td>Formulation purity NR</td>
<td>111.2 kg/ha</td>
<td>No effect on hatching success, weight gain, or mortality;</td>
<td>3</td>
<td>Non-conventional method of application; formulation; single concentration; purity not reported; no positive control; evidence of poor absorption into the egg content</td>
</tr>
</tbody>
</table>

**Reptiles**

- **Crain et al. 1997**
  - American alligator eggs exposed topically to 2,4-D prior to sexual differentiation
  - 97.6% 0.14–14 ppm
  - No effects on sex reversal, plasma steroid concentrations, gonadal aromatase activity
  - 2
  - Non-conventional study design; validated with estradiol positive control

- **Crain et al. 1999**
  - American alligator eggs exposed topically to 2,4-D prior to sexual differentiation
  - 97.6% 0.14–14 ppm
  - No effects on hepatic aromatase activity or testicular histopathology
  - 2
  - Non-conventional study design; validated with estradiol positive control

- **Spiteri et al. 1999**
  - American alligator eggs exposed topically to 2,4-D prior to sexual differentiation, at two temperatures
  - 97.6% 0.14–14 ppm
  - No effects on sex reversal, hepatic aromatase activity or gonadal histopathology
  - 2
  - Non-conventional study design; validated with estradiol positive control

**Mixed species**

- **Relyea 2005**
  - Algal microcosms and other microcosms containing 25 species exposed to 2,4-D
  - Formulation (44.5%) 0.117 mL/m²
  - No effect on community diversity, survival, or biomass
  - 3
  - Formulation tested and single concentration; No specific endocrine endpoints evaluated

NR: not reported.
effective OAT-1 transporter (if any) in the environment and because they provide information suggesting that the thyroid findings for 2,4-D may be rodent-specific.

**Marty et al. 2010 (published in Marty et al. 2013)**
The EOGRT study of 2,4-D (Marty et al. 2010, published in Marty et al. 2013) was specifically designed to provide sufficient information to assess whether endocrine targets are, in fact, altered with in vivo exposure, and to provide the basis for robust risk assessment of 2,4-D, including risk assessment protective for any potential endocrine effects. This study design provides a reliable basis for establishing the potential of 2,4-D to interact with the estrogen, androgen or thyroid pathways and is considered a Tier 2-equivalent EDSP assessment. The OECD (2012b) considers the EOGRT study a preferable method for evaluation of in vivo endocrine disruption in that it evaluates endocrine-sensitive endpoints not found in conventional 2-generation bioassays. This study is assigned a Klimisch score of 1.

As discussed in the Introduction, this study used extensive TK information on 2,4-D to set doses. Based on blood levels obtained during the EOGRT study, the high dose in males (800 ppm) adequately approximated or slightly exceeded the TSRC, but the high dose in females (600 ppm) clearly exceeded the TSRC.

A summary of the study design and discussion of results and endocrine-related parameters is included in Appendix IV. EOGRT study key parameters and findings are summarized in Table 8.

In conclusion, there was no evidence of adversely altered endocrine function in a comprehensive EOGRT study of 2,4-D. Slight adaptive effects were seen on thyroid hormone homeostasis at the high dose in a single life-stage, at a dose exceeding the TSRC and not relevant for human risk assessment.

**Rodwell and Brown, 1985**
This study was a two-generation OPP 83–4 Guideline reproductive toxicity study in Fischer 344 rats. 2,4-D (97.5% purity) was administered in the diet at nominal dose levels of 0, 5, 20 and 80 mg/kg/day (30/sex/dose) for one full generation and at 0, 5 and 20 mg/kg/day for the second generation. The 80 mg/kg/day group was dropped after the first generation because it exceeded a MTD, based on excessive mortality among the F1b pups following a mis-dosing during gestation and lactation. The mis-dosing resulted in all groups of F1b dams and pups being exposed to greater than nominal doses; high-dose dam exposure was ≥100 mg/kg/day. There was no dose concentration adjustment in this study and the high dose exceeded the TSRC. Because of the mis-dosing and several study deficiencies, this study is scored a Klimisch score of 2. Details on the study are provided in Supplementary Appendix IV; a summary of key parameters evaluated and results for the Rodwell and Brown (1985) study are presented in Table 9.

In summary, there were no robust indications of interaction with the estrogen or androgen pathways in this study; thyroid function was not evaluated.

A summary of regulatory developmental, subchronic and chronic toxicity studies follows in Table 10, and published mammalian toxicological studies in Table 11.

The key points from the mammalian regulatory developmental, subchronic and chronic toxicity studies (Table 10) and published toxicological studies (Table 11) are:

- 2,4-D has not been shown to have exposure-related changes in endpoints potentially related to EAT pathway or steroidogenesis endpoints at doses below the TSRC in high quality studies, including in a comprehensive EOGRT. Findings at higher (in most cases much higher) doses are not considered relevant to human risk assessment.
- No data provide robust indications of interactions with the estrogen or androgen pathways or with steroidogenesis. Adaptive effects on thyroid parameters are seen at doses exceeding the TSRC in rodents, but not in dogs.
- Developmental and subchronic toxicity studies of 2,4-D esters and amines show no unique endocrine-related toxicity and results are generally consistent and predictable based on the acid studies. Note that these compounds break down rapidly to the acid form.

**Occupational and epidemiological investigations**

**Male reproductive health**

**Lerda and Rizzi, 1991**
Thirty-two farmers occupationally exposed to 2,4-D and 25 non-exposed controls were studied for the following reproduction-related effects: ejaculatory volume, sperm count, sperm motility and sperm morphology. Exposure level was estimated by measuring the concentration of 2,4-D in the urine. Mean 2,4-D concentrations were 9.02 milligrams per liter (mg/L) in the exposed group, while 2,4-D was not detectable in the control group.

The investigators reported that the incidence of asthenospermia, necrospermia and teratospermia were greater in the exposed group, and that sperm motility was decreased. However, many study-specific details were not reported, including background of controls, number of participants excluded due to spermatogenesis-affecting health conditions, method used in “consideration” of external factors, detection limit for 2,4-D in urine, time period of urine collection and ranges of sperm parameters and 2,4-D urine levels evaluated (only means provided in the paper). The selection of controls appeared inappropriate, as comparison was to workers in the field exposed to 2,4-D, but the controls were not agricultural workers or doing similar field work. Field work involves exposure to other factors (e.g. increased temperature, dusts and allergens) that could potentially alter sperm parameters. In addition, no attempt was made to correlate 2,4-D urine levels with any specific sperm parameter changes or anomalies. Therefore, this study is considered too limited in scope and relevant details, and is not considered to provide reliable
Table 8. F1-extended one generation dietary toxicity study summary table (Marty et al. 2010).

<table>
<thead>
<tr>
<th>Dose (ppm)</th>
<th>100</th>
<th>300</th>
<th>600F/800</th>
<th>30F/40M</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Targeted mg/kg/day doses</td>
<td>5</td>
<td>15</td>
<td>30F/40M</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Systemic toxicity**
- **Mortality**: N N N No exposure-related mortality in any group
- **Live birth index**: N N N No effect
- **Mean number of pups**: N N N No effect
- **Clinical signs**: N N N No effect
- **Body weight**: N N ↓ Decreased P1 F bw during lactation; decreased F1 pup weight
- **Feed and water consumption**: N N ↓ P1 F Decreased P1 F feed consumption during lactation
- **Liver weight**: N N N No effect
- **Kidney weight**: N ↓ P1 F M and F; F1 F Exposure related; very slight and considered non-adverse at 300 ppm
- **Kidney histopathology**: N F1 M P M; F1 M and F Exposure related; very slight and considered non-adverse at 300 ppm

**Estrogen pathway-potentially mediated endpoints**
- **Sexual maturation (vaginal opening)**: N N N No effect F1
- **Nipple retention (male)**: N N N No effect F1
- **Estrous cycling**: N N N No effect P or F1
- **Female mating**: N N N No effect P
- **Mean duration of gestation**: N N N No effect P
- **Gestation index**: N N N No effect P
- **Pup sex ratio**: N N N No effect P or F1
- **Ovaries (paired) weights**: N N N No effect P or F1
- **Differential ovarian follicle count**: N N N No effect F1
- **Uterus weight with oviducts and cervix**: N N ↓(N) Non-statistically significant; increase within HCD; stage of estrus uncontrolled at necropsy; estrous cyclicity not changed; conclusion no effect P or F1
- **Uterine histopathology**: N N N No effect P or F1
- **Ovarian histopathology**: N N N No effect P or F1
- **Vaginal histopathology**: N N N No effect P or F1

**Androgen pathway-potentially mediated endpoints**
- **Sexual maturation (prepubial separation)**: N N ↓(N) Slight delay F1 M attributed to decreased body weight before and post weaning (artifact from group assignments)
- **Sperm parameters**: N N N No effect P or F1
- **Male fertility**: N N N No effect P
- **Epididymis weight**: N N N No effect P or F1
- **Testicular weight**: N N ↓ P F1 pups; N P F1 adults Decreased testis weights in F1 PND 21 pups at high dose strongly correlated with decreased body weight; did not persist in adults.
- **Prostate weight**: N N ↓(N) P1; N F1 P1 prostate weights not statistically different from control; decreases not considered exposure-related because the absolute and relative prostate weights in the control group were atypical, exceeding the laboratory HCD ranges; not seen in F1
- **Seminal vesicles with coagulating glands weight**: N N ↓(N) P1; N F1 P1 males decreased seminal vesicle weight not considered exposure-related because the absolute and relative seminal vesicle weight in the control group were atypical, exceeding the laboratory HCD ranges; not seen in F1
- **Testicular histopathology**: N N N No effect P or F1
- **Prostate histopathology**: N N N No effect P or F1
- **Epididymides histopathology**: N N N No effect P or F1
- **Seminal vesicle histopathology**: N N N No effect P or F1
- **Coagulation gland histopathology**: N N N No effect P or F1

**Thyroid pathway-potentially mediated endpoints**
- **T3**: N N ↓(GD17 satellite F N Other life stages) No adverse findings; response in dams considered adaptive
- **T4**: N N ↓(GD17 satellite F N Other life stages) No adverse findings; response in dams considered adaptive
- **TSH**: N N ↓(GD17 satellite F N Other life stages) No adverse findings; response in dams considered adaptive
- **Thyroid weight**: N N N No adverse findings

(continued)
evidence of male reproductive toxicity or endocrine disruption resulting from occupational exposure to 2,4-D.

**Garry et al. 2001**

Twenty-four applicators and 15 minimally exposed foresters (control subjects) were studied for biomarker outcomes compared to urinary levels of 2,4-D. Categorized by applicator method, men who used hand-held, backpack sprayer applicators showed the highest average level (453.6 ppb) of 2,4-D in urine. No significant differences in follicle-stimulating hormone (FSH), total testosterone or free testosterone levels between application methods were reported. Significantly increased luteinizing hormones (LH) levels were reported in either backpack applicators or boom-sprayer applicators combined; however, no significant effect on LH levels was observed in either backpack applicators or boom-sprayer applicators alone.

No correlation was shown between FSH, free testosterone or total testosterone concentrations with 2,4-D urinary levels at the time of maximum 2,4-D usage. In contrast, LH levels were reported to correlate with 2,4-D urinary levels at the time of maximum 2,4-D usage (using 21 of 24 applicators). LH levels are subject to considerable inherent variation and single samples from individuals are unlikely to provide a reliable profile (Partsch et al. 1994). Total testosterone levels after the application season were reported to correlate with 2,4-D urinary levels at the time of peak 2,4-D use. The study authors acknowledged that the limited sample size warrants cautious interpretation of the data. This study is considered too limited in scope to provide substantive evidence of endocrine modulation caused by exposure to 2,4-D.

**Swan et al. 2003**

Swan et al. (2003) in a case-control study evaluated semen quality, sperm concentration, morphology and motility in general population participants in two states, evaluating levels of pesticide metabolites taken close to the time of sample collection as surrogates for exposure. They found no statistically significant effects on sperm concentration or quality, or increased abnormal sperm at urinary 2,4-D levels above the limit of detection (LOD). (It should be noted that very few samples were above the LOD for 2,4-D.) The authors commented that the results for 2,4-D should be "considered borderline, with small and somewhat inconsistent associations." The abstract to the paper indicates that 2,4-D was "associated with poor semen quality in some analyses." Based on the results presented in the paper this statement in the abstract appears speculative, and unsupported by the data, unless the "analyses" in the statement refers to one for all pesticides combined, and not 2,4-D specifically. This study does not provide any robust evidence that 2,4-D exposure is associated with poor semen quality in humans. It is considered too limited, due to the low numbers of control and case subjects with urinary 2,4-D levels above the LOD, to be considered in the WoE as evidence for presence or absence of an association.

**Thyroid**

**Knopp, 1994**

The urinary excretion of 2,4-D was measured during eight biological monitoring studies over a five-year period (1985–1989) of 27 men and 18 women employees exposed during the production and formulation of 2,4-D and related sodium and dimethylamine salts (DMAs). In addition, venous blood samples were collected in three legs of the studies, and thyroid hormone concentrations in blood were measured.

Results showed that 2,4-D was detectable in serum and urine of all persons, but in varying amounts. The highest urinary concentration was 19.5 ppm, and the 2,4-D urinary concentration profile for a weekly interval showed an increase in exposure during the work week.

No notable abnormalities of thyroid hormone concentrations in blood were found. It should be noted, however, that...
the thyroid hormone content in blood was measured during a routine biennial health monitoring of the staff (population size not provided), and no attempt was made to correlate thyroid hormone levels with the urine and blood 2,4-D levels. Therefore, although this study does not provide any evidence of thyroid-modulating potential of 2,4-D, it is considered of limited reliability.

Goldner et al. 2010 and Goldner et al. 2013

There are two publications on thyroid disease using data from the Agricultural Health Study (AHS). The first, (Goldner et al. 2010) evaluated exclusively women in the AHS, and the second, (Goldner et al. 2013), evaluated men. The 2010 publication was based on about 16,500 women. This represented about 70% of the overall AHS female cohort. The authors found no association with hypothyroidism and working/living on a farm or using pesticides. They found no statistically significant associations with thyroid disease and 2,4-D.

The 2013 publication included 22,246 men. This group represented only 62% of the overall AHS male cohort because of requirements for complete data on thyroid disease. Contrary to the 2010 Goldner et al. study results, a number of statistically significant odds ratios were reported for various pesticides. These included six herbicides (including 2,4-D), most organochlorines, two insecticides and one carbamate. According to the authors, this is the first epidemiology study to report these associations. With respect to 2,4-D, the authors cite a 2007 paper by Stoker et al. (see Table 11) as supportive evidence of biological plausibility. The latter assertion will be discussed in the thyroid WoE discussion.

The authors list the limitations as “potential for recall bias affecting exposure estimates, reliance on self-reported disease, and possible selection bias due to high dropout rates.”

Table 9. 2,4-D: two-generation reproductive toxicity study summary table (Rodwell & Brown 1985).

<table>
<thead>
<tr>
<th>Dose (mg/kg/day)</th>
<th>Vehicle control</th>
<th>5</th>
<th>20</th>
<th>80*</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Systemic toxicity</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mortality</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>Y</td>
<td>Pup mortality increased in the F1b litters (overdosed during mating, gestation and lactation).</td>
</tr>
<tr>
<td>Mean number of pups</td>
<td>NA</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>No effect</td>
</tr>
<tr>
<td>Live birth index</td>
<td>NA</td>
<td>N</td>
<td>N</td>
<td>Y</td>
<td>Increased stillbirths in F1b litters (overdosed during mating, gestation and lactation).</td>
</tr>
<tr>
<td>Clinical signs</td>
<td>NA</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>No effect</td>
</tr>
<tr>
<td>Body weight</td>
<td>NA</td>
<td>N</td>
<td>N</td>
<td></td>
<td>Decreased body weights in F1a and F1b litters at ≥80 mg/kg/day</td>
</tr>
<tr>
<td>Feed consumption</td>
<td>NA</td>
<td>N</td>
<td>N</td>
<td></td>
<td>Decreased in dams producing F1b litters (overdosed during mating, gestation and lactation).</td>
</tr>
<tr>
<td>Liver weight</td>
<td>NA</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>No dose related effect</td>
</tr>
<tr>
<td>Kidney weight</td>
<td>NA</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>No dose-related effect</td>
</tr>
<tr>
<td><strong>Estrogen pathway —potentially indicative endpoints</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Estrous cycling</td>
<td>(extrapolated)</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>No effect (extrapolated from time to mating)</td>
</tr>
<tr>
<td>Mating index</td>
<td>NA</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>No effect</td>
</tr>
<tr>
<td>Fertility index</td>
<td>NA</td>
<td>N</td>
<td>N</td>
<td></td>
<td>Decreased (not statistically significantly) in mating to produce F1b litters at ≥80 mg/kg/day (overdosed during mating, gestation and lactation).</td>
</tr>
<tr>
<td>Mean duration of gestation</td>
<td>NA</td>
<td>N</td>
<td>N</td>
<td></td>
<td>Increased 1 day in F1b litters at ≥80 mg/kg/day (may be hormone mediated but not due to estrogen or androgen interactions; may be secondary to systemic toxicity at exaggerated high dose due to mis-dosing)</td>
</tr>
<tr>
<td>Gestation index</td>
<td>NA</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>No effect</td>
</tr>
<tr>
<td>Pup sex ratio</td>
<td>NA</td>
<td>N</td>
<td>N</td>
<td>(N)</td>
<td>Increased number of male pups at 80 mg/kg/day in F1a litters; no effect on F1b litters dosed at a higher level; therefore, considered incidental</td>
</tr>
<tr>
<td>Uterine histopathology</td>
<td>NA</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>No effect (weanlings)</td>
</tr>
<tr>
<td>Ovarian histopathology</td>
<td>NA</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>No effect</td>
</tr>
<tr>
<td><strong>Androgen pathway —potentially indicative endpoints</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fertility index</td>
<td>NA</td>
<td>N</td>
<td>N</td>
<td></td>
<td>Decreased (not statistically significantly) in mating to produce F1b litters at ≥80 mg/kg/day</td>
</tr>
<tr>
<td>Pup sex ratio</td>
<td>NA</td>
<td>N</td>
<td>N</td>
<td>(N)</td>
<td>Increased number of male pups at 80 mg/kg/day in F1a litters; no effect on F1b litters dosed at a higher level; therefore, considered incidental</td>
</tr>
<tr>
<td>Testes weight</td>
<td>NA</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>No effect</td>
</tr>
<tr>
<td>Testes histopathology</td>
<td>NA</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>No effect</td>
</tr>
<tr>
<td>Prostate histopathology</td>
<td>NA</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>No effect (weanlings)</td>
</tr>
<tr>
<td>Epididymides</td>
<td>NA</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>No effect</td>
</tr>
<tr>
<td>histopathology</td>
<td>NA</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>No effect</td>
</tr>
<tr>
<td>Seminal vesicle</td>
<td>NA</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>No effect</td>
</tr>
<tr>
<td>histopathology</td>
<td>NA</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>No effect</td>
</tr>
</tbody>
</table>

*Mis-dosing during F1b gestation probably to a dose approximating 100 mg/kg/day.
NA: not applicable; N: no effect; (N): finding but not likely exposure related.
↑Increase
↓Decrease

CRITICAL REVIEWS IN TOXICOLOGY 23
Table 10. Regulatory 2,4-D developmental, subchronic and chronic mammalian studies and evaluation of study quality.

<table>
<thead>
<tr>
<th>Study</th>
<th>Assay and test system</th>
<th>Purity 2,4-D</th>
<th>Concentration range or doses tested (mg/kg/day)</th>
<th>Result</th>
<th>Klimisch score</th>
<th>Weaknesses</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Developmental studies</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rodwell 1983</td>
<td>OPP 83-3 Guideline developmental toxicity evaluation in F344 rats. Dosing by oral gavage from GD 6-15.</td>
<td>97.5%</td>
<td>8, 25, 75</td>
<td>Maternal: 75 mg/kg/day produced slight toxicity Developmental: No adverse effects observed</td>
<td>1</td>
<td>None noted; however note current Guideline requires a longer exposure period; high dose exceeds TSRC</td>
</tr>
<tr>
<td>Hoberman 1990</td>
<td>OPP 83-3 Guideline developmental toxicity evaluation in New Zealand white rabbits. Dosing by oral gavage from GD 6-18.</td>
<td>97.5%</td>
<td>10, 30, 90</td>
<td>Maternal: High dose produced two abortions Developmental: No toxicity observed.</td>
<td>1</td>
<td>None noted; however note current Guideline requires a longer exposure period and more animals per dose level (20 vs 12); high dose exceeds TSRC in rabbits</td>
</tr>
<tr>
<td><strong>Subchronic and chronic toxicity studies</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Schulze 1991a</td>
<td>OPP 82-1 guideline subchronic study with F344 rats. Dosing via the diet for 13 weeks.</td>
<td>96.1%</td>
<td>1, 15, 100, 300</td>
<td>Systemic toxicity: 300 mg/kg/day: marked (excessive) systemic toxicity, ▼ body weight (28%), renal toxicity and effects on eyes, hearts, and lungs. 100 mg/kg/day: renal toxicity Endocrine endpoints: 300 mg/kg/day (F): ▼ T3, T4, ▼ thyroid/parathyroid weights; thyroid follicular cell hypertrophy ▼ pituitary weights. 100 mg/kg/day (F): ▼ T3 and T4 300 mg/kg/day (M): ▼ T4, ▼ thyroid/parathyroid weights, ▼ testes weight and atrophy; ▼ pituitary weights. 100 mg/kg/day (M): ▼ T4; ▼ thyroid/parathyroid weights No histopathological changes: pituitary, epididymides, ovary, uterus, and vagina.</td>
<td>1</td>
<td>High dose excessive; exceeded MTD; doses ≥100 mg/kg/day exceed TSRC.</td>
</tr>
<tr>
<td>Gorzinski et al. 1981a</td>
<td>OPP 82-1 non-Guideline study with F344 rats. Dosing via the diet for 13 weeks.</td>
<td>97.3%</td>
<td>15, 60, 100, 150</td>
<td>150 mg/kg/day: marked Systemic toxicity in both genders. ≥100 mg/kg/day (F): ▼ T4. ≥100 mg/kg/day (M): ▼ testes weight</td>
<td>2</td>
<td>Not all guideline endpoints evaluated. Doses ≥60 mg/kg/day exceed TSRC.</td>
</tr>
<tr>
<td>Gorzinski et al. 1981b</td>
<td>Non-guideline subchronic study with F344 rats. Dosing via the diet for 13 weeks.</td>
<td>100%</td>
<td>15, 60, 100, 150</td>
<td>Systemic toxicity in both genders at ≥100 mg/kg/day. ≥ 60 mg/kg/day (F): ▼ T4. (M): No effects on endocrine relevant endpoints including testes weights</td>
<td>2</td>
<td>Doses ≥60 mg/kg/day exceed TSRC. Very limited endpoints evaluated. Study done primarily to evaluate testes weight change in prior study</td>
</tr>
</tbody>
</table>

(continued)
<table>
<thead>
<tr>
<th>Study</th>
<th>Assay and test system</th>
<th>Purity 2,4-D</th>
<th>Concentration range or doses tested (mg/kg/day)</th>
<th>Result</th>
<th>Klimisch score</th>
<th>Weaknesses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jeffries et al. 1995</td>
<td>Guideline chronic toxicity/oncogenicity study with F344 rats. Dosing via the diet for 12 or 24 months.</td>
<td>96.4%</td>
<td>5, 75, 150</td>
<td>150 mg/kg/day (F): ↓ body weight; renal and other systemic toxicity; ↓ secretory material in thyroidal epithelial cells; ↓ ovary weight; ↓ incidence of benign adenomas in pituitary, and ↓ mammary gland hyperplasia. ≥ 75 mg/kg/day: renal tox, ↓ T4 levels; ↑ thyroid weights; ↑ focal cystic dilatation. 150 mg/kg/day (M): ↑ thyroid weights; ↓ testes weights. 75 mg/kg/day (M); ↓ T4 levels at 100 and 300 mg/kg/day, no effect on testes or ovary weights. No histopath. findings: pituitary, adrenal, thyroid, parathyroid, testes, epididymides, ovary, uterus.</td>
<td>1</td>
<td>Doses ≥75 mg/kg/day exceed TSRC; guideline study; thyroid tissue accountability issue in females at terminal sacrifice.</td>
</tr>
<tr>
<td>Schulze 1991b</td>
<td>Guideline B6C3F1 mice subchronic toxicity study. Dosing via the diet for 13 weeks.</td>
<td>96.1%</td>
<td>1, 15, 100, 300</td>
<td>100 mg/kg/day: no effect on testes or ovary weights. No histopath. findings: pituitary, adrenal, thyroid, parathyroid, testes, epididymides, ovary, uterus, vagina, mammary.</td>
<td>1</td>
<td>Doses ≥100 mg/kg/day likely exceed TSRC</td>
</tr>
<tr>
<td>Stott 1995a</td>
<td>Oncogenicity study B6C3F1 mice (females only). Dosing via diet for 24 months; 12-month interim sacrifice. Guideline when considered in conjunction with Stott 1995b study.</td>
<td>96.4%</td>
<td>5, 150, 300</td>
<td>≥150 mg/kg/day: systemic toxicity (renal). No histopath. findings: pituitary, adrenal, thyroid, ovary, uterus, vagina, mammary.</td>
<td>1</td>
<td>Doses ≥150 mg/kg/day likely exceed TSRC</td>
</tr>
<tr>
<td>Stott 1995b</td>
<td>Oncogenicity study with B6C3F1 mice (males only). Dosing via the diet for 12 or 24 months. Guideline when considered in conjunction with Stott 1995a study.</td>
<td>96.4%</td>
<td>5, 62.5, 125</td>
<td>No significant exposure-related effects. No histopath. findings: testes, epididymides, prostate, seminal vesicles, adrenal, thyroid, pituitary</td>
<td>1</td>
<td>125 mg/kg/day likely exceeds TSRC</td>
</tr>
<tr>
<td>Schulze 1990</td>
<td>Guideline subchronic study with dogs (82-1). Dosing via capsule for 13 weeks.</td>
<td>96.1%</td>
<td>0.3, 1, 3, 10</td>
<td>10 mg/kg/day: Severe systemic toxicity including lethality; ↓ testes weight; testicular atrophy. No effects thyroid: T3, T4, weight and histopath; no effect ovary weight; no histopath findings: ovary, uterus, epididymides, pituitary.</td>
<td>1</td>
<td>Guideline study: 10 mg/kg/day dose exceeds MTD; ≥ 3 mg/kg/day exceeds dog TSRC; immaturity of dogs at study initiation limits ability to detect any exposure-related testes lesions due to very high background incidence in published HCD</td>
</tr>
</tbody>
</table>

(continued)
With respect to bias of exposure, we know that the AHS participants have adequate recall of what was applied but are less reliable with respect to how often and for how many years. Further, application (i.e. use) is a poor proxy for exposure, because the range of exposures from a single application is highly variable as demonstrated from biomonitoring studies (such as the Farm Family Exposure Study (Alexander et al. 2007) and the AHS biomonitoring study (Thomas et al. 2010)). As a result, the efforts to evaluate exposure-response in the AHS are very limited at best. Reliance on self-reporting is less of a concern with the thyroid outcome, as the outcome is physician-diagnosed, but it may be reflected in the declining participation in the AHS over time. It is also possible that persons with health concerns have selectively participated in Phase II and III, which may bias toward an increase in observed disease rates.

There is a lack of correlation between the genders. The authors reported no association of 2,4-D use and hypothyroidism in women (OR = 0.96; 95% CI 0.8–1.1) and a statistically significant but relatively weak association in men (OR = 1.35; 95% CI 1.04–1.76) if the association were causal for thyroid disease, one would expect to see an association in both men and women. According to the Mayo Clinic, women over 50 are at risk for an underactive thyroid, hypothyroidism (http://www.mayoclinic.com/health/hypothyroidism/R8067). It is unclear why the authors observed associations with several pesticides in men but not in women. It may be due to unintentional bias, such as related to exposure misclassification or to participation.

### WoE for potential endocrine pathway interactions

As discussed in the Introduction, the following WoE reflects an assessment of whether results might signal a potential endocrine pathway interaction, the relative weight or rank placed on that parameter for specifically and sensitively flagging a potential interaction, and whether a finding (if any) was made only at a systematically toxic or excessive dose. The WoE tables developed for each pathway hypothesis provide primarily a visual representation that assists in identifying patterns of findings that may indicate a potential endocrine pathway interaction. The following format is used for the WoE table contents addressing each potential endocrine pathway interaction.

#### Parameters relevant to and negative for specific potential pathway interactions

- Parameters with findings potentially supporting an endocrine-pathway-related finding seen only at high doses exceeding the TSRC and/or excessive doses are indicated in light gray and marked as "N" for negative.
- Parameters relevant to and negative for specific potential pathway interactions are indicated in dark gray and marked as "N" for negative.
- Parameters with findings potentially supporting an endocrine-pathway-related finding seen only at high doses exceeding the TSRC and/or excessive doses are indicated in dark gray and marked as "N" for negative.

#### Parameters with findings at the limit dose (in the FSTRA, in which TK data were not available to advise dose selection) in which TK data were not available to advise dose selection but showing a test article-related response are indicated in light gray and marked as "L" for limit dose.

#### Parameters with findings potentially supporting an endocrine-pathway-related finding seen only at high doses exceeding the TSRC and/or excessive doses are indicated in light gray and marked as "O" for over the TSRC.

#### Parameters with findings at the limit dose (in the FSTRA, in which TK data were not available to advise dose selection) in which TK data were not available to advise dose selection but showing a test article-related response are indicated in light gray and marked as "L" for limit dose.

#### Table 10. Continued

<table>
<thead>
<tr>
<th>Study</th>
<th>Assay and test system</th>
<th>Purity 2,4-D</th>
<th>Concentration range or doses tested (mg/kg/day)</th>
<th>Result</th>
<th>Klimisch score</th>
<th>Weaknesses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dalgard 1993a</td>
<td>Guideline subchronic study with dogs. Dosing via diet for &gt;13 weeks.</td>
<td>96.7%</td>
<td>0.5, 1, 3.75, 10 (lowered after 8 weeks to 7.5)</td>
<td>Body weight at mid and high dose. Thyroid weight not considered exposure-related. No clearly dose-related effects on testes or prostate histopath; no effects thyroid histopath.</td>
<td>1</td>
<td>Guideline study; doses ≥3.75 mg/kg/day exceed dog TSRC.</td>
</tr>
<tr>
<td>Dalgard 1993b</td>
<td>Guideline chronic study with dogs. Dosing via diet for 1 year.</td>
<td>96.7%</td>
<td>1, 5, 10 (lowered after 8 weeks to 7.5)</td>
<td>Body weight</td>
<td>1</td>
<td>Guideline study; doses ≥5 mg/kg/day exceed dog TSRC.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Study</th>
<th>Assay and test system</th>
<th>Purity 2,4-D</th>
<th>Concentration range or doses tested (mg/kg/day)</th>
<th>Result</th>
<th>Klimisch score</th>
<th>Weaknesses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dalgard 1993a</td>
<td>Guideline subchronic study with dogs. Dosing via diet for &gt;13 weeks.</td>
<td>96.7%</td>
<td>0.5, 1, 3.75, 10 (lowered after 8 weeks to 7.5)</td>
<td>Body weight at mid and high dose. Thyroid weight not considered exposure-related. No clearly dose-related effects on testes or prostate histopath; no effects thyroid histopath.</td>
<td>1</td>
<td>Guideline study; doses ≥3.75 mg/kg/day exceed dog TSRC.</td>
</tr>
<tr>
<td>Dalgard 1993b</td>
<td>Guideline chronic study with dogs. Dosing via diet for 1 year.</td>
<td>96.7%</td>
<td>1, 5, 10 (lowered after 8 weeks to 7.5)</td>
<td>Body weight</td>
<td>1</td>
<td>Guideline study; doses ≥5 mg/kg/day exceed dog TSRC.</td>
</tr>
</tbody>
</table>
Table 11. Published literature references for mammalian studies and evaluation of study quality.

<table>
<thead>
<tr>
<th>Study</th>
<th>Assay and test system</th>
<th>Purity 2,4-D</th>
<th>Concentration range or doses tested (mg/kg/day)</th>
<th>Result</th>
<th>Klimisch score</th>
<th>Weaknesses</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Developmental studies</strong></td>
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<tr>
<td>Bage et al. 1973</td>
<td>Pregnant NMRI mice dosed subcutaneously from GD 6–14</td>
<td>Mixture of formulated products, purity of 2,4-D not defined</td>
<td>50 and 110 (2:1 2,4-D and 2,4,5-T)</td>
<td>Increases in fetal resorptions, cleft palate. Decreased fetal body weights; no malformations characteristic of endocrine modulators</td>
<td>3</td>
<td>Weaknesses: 2,4-D not tested separately (although 2,4,5-T was); purity of 2,4-D not defined; maternal toxicity not evaluated; subcutaneous dosing not relevant for risk assessment; Strength: formulation excipients were added to the control group</td>
</tr>
<tr>
<td>Cavieres et al. 2002</td>
<td>ND4 mice dosed orally by gavage from GD 6–15</td>
<td>Formulation (Purity unspecified)</td>
<td>0.01–100</td>
<td>Decreases in litter sizes and purported decreases in implantations</td>
<td>3</td>
<td>Weaknesses: Formulation; purity of 2,4-D unspecified; data discrepancies; lack of appropriate concurrent controls; lack of biological plausibility for reported findings</td>
</tr>
<tr>
<td>Charles et al. 2001</td>
<td>OPP 83-3 guideline study. Sprague-Dawley rats dosed orally by gavage with 2,4-D 2-butoxyethyl ester (BEE) from GD 6–15</td>
<td>2,4-D BEE 95.6%</td>
<td>25, 75, 185 ai (17, 51, 125 ae)</td>
<td>Maternal toxicity at 125 mg/kg/day ae;</td>
<td>2</td>
<td>Overall high quality study; minor limitations in amount of detail reported due to multiple studies and compounds; e.g. nature of visceral malformations not specified; ae doses ≥51 mg/kg/day exceed TSRC.</td>
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<tr>
<td></td>
<td>OPP 83-3 guideline study. Sprague-Dawley rats dosed orally by gavage with 2,4-D 2-ethylhexyl ester (EHE) from GD 6–15</td>
<td>2,4-D EHE 95.0%</td>
<td>14.1, 45.2, 135.7 ai (10, 30, 90 ae)</td>
<td>Maternal toxicity (slight) at 30 mg/kg/day ae;</td>
<td>2</td>
<td>Overall high quality study; minor limitations in amount of detail reported due to multiple studies and compounds; e.g. skeletal variations at 30 mg/kg/day ae not shown in Table; Table identifies wavy ribs as malformations; typically classified as deviations (Kimmel et al. 2014); doses ≥30 mg/kg/day ae exceed TSRC.</td>
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<tr>
<td>Charles et al. 2001</td>
<td>OPP 83-3 guideline study. Sprague-Dawley rats dosed orally by gavage with 2,4-D 2-ethylhexyl ester (EHE) from GD 6–15</td>
<td>2,4-D IPE 95.1%</td>
<td>12.3, 36.9, 123 ai (10, 30, 100 ae)</td>
<td>Maternal toxicity ≥30 mg/kg/day ae;</td>
<td>2</td>
<td>Overall high quality study; minor limitations in amount of detail reported due to multiple studies and compounds;</td>
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<td>(for publication)</td>
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<tr>
<td>Charles et al. 2001</td>
<td>OPP 83-3 guideline study. Sprague-Dawley rats dosed orally by gavage with 2,4-D diethanolamine salt (DEA) from GD 6–15</td>
<td>2,4-D DEA Aqueous based manufacturing concentrate (73.1%)</td>
<td>15, 75, 150 ai (10.2, 50.8, 101.6 ae)</td>
<td>Maternal toxicity ≥50.8 mg/kg/day ae;</td>
<td>2</td>
<td>Overall high quality study; minor limitations in amount of detail reported due to multiple studies and compounds;</td>
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<td></td>
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<td>(for publication)</td>
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</tr>
<tr>
<td>Charles et al. 2001</td>
<td>OPP 83-3 guideline study. Sprague-Dawley rats dosed orally by gavage with 2,4-D dimethylamine (DMA) from GD 6–15</td>
<td>2,4-D DMA Aqueous based manufacturing concentrate (66.2%)</td>
<td>15, 60.2, 120.4 ai (12.5, 50, 100 ae)</td>
<td>Maternal toxicity ≥50 mg/kg/day ae;</td>
<td>2</td>
<td>Overall high quality study; minor limitations in amount of detail reported due to multiple studies and compounds;</td>
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<td></td>
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<td>(for publication)</td>
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<td>(continued)</td>
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<tr>
<td>Study</td>
<td>Assay and test system</td>
<td>Purity 2,4-D</td>
<td>Concentration range or doses tested (mg/kg/day)</td>
<td>Result</td>
<td>Klimisch score</td>
<td>Weaknesses</td>
</tr>
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<tr>
<td>OPP 83-3 guideline study. Sprague-Dawley rats dosed orally by gavage with 2,4-D isopropylamine (IPA) from GD 6–15</td>
<td>2,4-D IPA</td>
<td>Aqueous based manufacturing concentrate (50.2%)</td>
<td>22, 65, 190 ai (17, 51, 150 ae)</td>
<td>Slight maternal toxicity (150 mg/kg/day ae); no exposure related visceral malformations</td>
<td>2</td>
<td>(for publication)</td>
</tr>
<tr>
<td>OPP 83-3 guideline study. Sprague-Dawley rats dosed orally by gavage with 2,4-D triisopropylamine (TIPA) from GD 6–15</td>
<td>2,4-D TIPA</td>
<td>Aqueous based manufacturing concentrate (72.2%)</td>
<td>32.5, 100, 325 ai (17, 51, 175 ae)</td>
<td>Maternal toxicity (severe) 175 mg/kg/day ae; fetal body weights, skeletal changes and malformations 175 mg/kg/day ae. No exposure related visceral malformations</td>
<td>2</td>
<td>(for publication)</td>
</tr>
<tr>
<td>OPP 83-3 guideline study. New Zealand white rabbits dosed orally by gavage with 2,4-D BEE from GD 7–19</td>
<td>2,4-D BEE</td>
<td>95.6%</td>
<td>15, 45, 110 ai (10, 30, 75 ae)</td>
<td>Severe maternal toxicity at ≥30 mg/kg/day ae; resorptions at 30 mg/kg/day ae; no exposure related visceral malformations</td>
<td>2</td>
<td>(for publication)</td>
</tr>
<tr>
<td>OPP 83-3 guideline study. New Zealand white rabbits dosed orally by gavage with 2,4-D DEA from GD 6–18</td>
<td>2,4-D DEA</td>
<td>Aqueous based manufacturing concentrate (73.1%)</td>
<td>15, 30, 60 ai (10.2, 30.3, 40.6 ae)</td>
<td>Maternal toxicity and resorptions and skeletal variations at 40.6 mg/kg/day ae; no exposure related visceral malformations</td>
<td>2</td>
<td>(for publication)</td>
</tr>
<tr>
<td>OPP 83-3 guideline study. New Zealand white rabbits dosed orally by gavage with 2,4-D DMA from GD 6–18</td>
<td>2,4-D DMA</td>
<td>Aqueous based manufacturing concentrate (66.2%)</td>
<td>12, 36.1, 108.4 ai (10, 30, 90 ae)</td>
<td>Equivocal maternal toxicity; deaths at mid dose; litter size low and high dose; no evidence exposure related; no exposure related visceral malformations</td>
<td>2</td>
<td>(for publication)</td>
</tr>
<tr>
<td>OPP 83-3 guideline study. New Zealand white rabbits dosed orally by gavage with 2,4-D IPA from GD 7–19</td>
<td>2,4-D IPA</td>
<td>Aqueous based manufacturing concentrate (50.2%)</td>
<td>13, 38, 95 ai (10, 30, 75 ae)</td>
<td>Severe maternal toxicity at 75 mg/kg/day ae; no exposure related visceral malformations</td>
<td>2</td>
<td>(for publication)</td>
</tr>
<tr>
<td>OPP 83-3 guideline study. New Zealand white rabbits dosed orally by gavage with 2,4-D TIPA from GD 7–19</td>
<td>2,4-D TIPA</td>
<td>Aqueous based manufacturing concentrate (72.2%)</td>
<td>19, 56, 140 ai (10, 30, 75 ae)</td>
<td>Severe maternal toxicity at 75 mg/kg/day ae; no exposure related visceral malformations</td>
<td>2</td>
<td>(for publication)</td>
</tr>
<tr>
<td>Study</td>
<td>Assay and test system</td>
<td>Purity 2,4-D</td>
<td>Concentration range or doses tested (mg/kg/day)</td>
<td>Result</td>
<td>Klimisch score</td>
<td>Weaknesses</td>
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<tr>
<td>Collins &amp; Williams 1971</td>
<td>Syrian golden hamsters dosed orally by gavage from GD 6–10</td>
<td>Three different lots of technical 2,4-D</td>
<td>20–100</td>
<td>Increased incidence of fetal abnormalities at high doses; decreased fetal viability</td>
<td>3</td>
<td>Poor methodology (use of different lots of test material), purity undefined; and reporting deficiencies; additionally hamster is a poor model for developmental toxicity evaluations due to multiple spontaneous malformations</td>
</tr>
<tr>
<td>Dinamarca et al. 2007</td>
<td>Pregnant mice dosed with technical and formulated 2,4-D in drinking water from GD 0–9</td>
<td>Purity unspecified</td>
<td>0.01–100</td>
<td>No changes in maternal toxicity, body weight gain, implantation sites, resorptions; no endocrine related effects</td>
<td>2</td>
<td>Purity unspecified for technical and formulated 2,4-D; no analytical confirmation of doses; otherwise study quality seemed adequate; refuted findings of Cavieres et al. (2002)</td>
</tr>
<tr>
<td>Duffard et al. 1996</td>
<td>Wistar rats orally exposed to 2,4-D DBE</td>
<td>Formulation; concentration and purity of 2,4-D unspecified</td>
<td>70</td>
<td>Increased serotonin levels</td>
<td>3</td>
<td>Unclear methodologies; insufficient data provided; results not specific to endocrine activity</td>
</tr>
<tr>
<td>Pochettino et al. 2013</td>
<td>Prenatal and postnatal 2,4-D exposure in pregnant Wistar rats evaluated at PND 45, 60, and 90</td>
<td>Purity unspecified</td>
<td>70</td>
<td>Evidence of potential oxidative stress in various tissues</td>
<td>3</td>
<td>Source of 2,4-D not identified; purity not stated; single dose; no evidence dose analysis conducted; no evidence potential litter effects were accounted for</td>
</tr>
<tr>
<td>Maternal nursing behavior and pup body weight effects</td>
<td></td>
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</tr>
<tr>
<td>Stürtz et al. 2000</td>
<td>Wistar rats exposed to 2,4-D via intraperitoneal injection</td>
<td>Purity unspecified</td>
<td>50–100</td>
<td>Decreased pup body weights; detectable 2,4-D residues in stomach, blood, brain, and kidney of breast-fed neonates</td>
<td>3</td>
<td>Irrelevant route of exposure (intraperitoneal); purity not specified; no evidence litter effects accounted for</td>
</tr>
<tr>
<td>Stürtz et al. 2006</td>
<td>Wistar rats dosed orally with 2,4-D</td>
<td>98%</td>
<td>15–70</td>
<td>Decreased pup body weight gains; decreased lipid content of milk; altered fatty acid content</td>
<td>3</td>
<td>Unclear methodology; no evidence litter effects accounted for; dose formulation procedures questionable; neither effects nor internal dosimetry consistent with other 2,4-D studies</td>
</tr>
<tr>
<td>Stürtz et al. 2008</td>
<td>Wistar rats dosed orally with 2,4-D</td>
<td>98%</td>
<td>15–50</td>
<td>Altered dam-pup interactions; increased catecholamine levels; decreased indolamine and prolactin levels</td>
<td>3</td>
<td>Unclear methodology; no evidence litter effects accounted for; dose formulation procedures questionable; limited assessment of maternal nursing behavior, pup observations inconsistent with typical findings in pups suffering from maternal neglect</td>
</tr>
<tr>
<td>Study</td>
<td>Assay and test system</td>
<td>Purity 2,4-D</td>
<td>Concentration range or doses tested (mg/kg/day)</td>
<td>Result</td>
<td>Klimisch score</td>
<td>Weaknesses</td>
</tr>
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</tr>
<tr>
<td>Stürz et al. 2010</td>
<td>Wistar rats dosed orally or IP with 2,4-D</td>
<td>98%</td>
<td>2.5–70</td>
<td>Decreased pup weight gain; decreases in amount of milk ejected, plasma prolactin and oxytocin altered</td>
<td>3</td>
<td>Unclear methodology; no evidence litter effects accounted for; dose formulation procedures questionable; no bedding material mentioned — absence could stress dams; procedure for evaluation of milk production flawed; incorrect statistical procedures</td>
</tr>
<tr>
<td>Male reproductive toxicity</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Lamb et al. 1981a</td>
<td>Male C57BL/6N mice dosed orally via diet mixture of 2,4-D and 2,4,5-T; mated with untreated females</td>
<td>98.5% (2,4-D Purity specified in Lamb et al. 1981c toxicity study)</td>
<td>20 and 40 mg/kg/day 2,4-D</td>
<td>No changes in fertility, sperm number, motility, or morphology</td>
<td>2</td>
<td>2,4-D not tested in isolation, but purity and doses of 2,4-D defined; a negative response reported; provides useful information re potential male repro. toxicity</td>
</tr>
<tr>
<td>Lamb et al. 1981b</td>
<td>Male C57BL/6N mice dosed orally via diet mixture of 2,4-D and 2,4,5-T; mated with untreated females</td>
<td>98.5% (2,4-D Purity specified in Lamb et al. 1981c toxicity study)</td>
<td>20 and 40 mg/kg/day 2,4-D</td>
<td>No impact of 2,4-D on reproductive performance of males; no changes in development and survival of fetuses and pups</td>
<td>2</td>
<td>2,4-D not tested in isolation, but a negative response reported and purity and doses of 2,4-D defined; provides useful information regarding potential male reproductive toxicity</td>
</tr>
<tr>
<td>Blakley et al. 1989</td>
<td>CD-1 male mice exposed to a mixture of 2,4-D and picloram via drinking water mated to untreated females</td>
<td>Mixture of chemicals; purity of 2,4-D not specified</td>
<td>84–336</td>
<td>High mortality in males at high concentration; no changes in resorptions, implantations; fetal weight at highest dose; maternal toxic effects all doses</td>
<td>3</td>
<td>2,4-D not tested in isolation; types of malformations not reported; small group size</td>
</tr>
<tr>
<td>Hassanein 2012</td>
<td>Male Sprague-Dawley rats exposed via gavage</td>
<td>Purity unspecified</td>
<td>30</td>
<td>Congestion of blood vessels in testicles and epididymis after two months; necrosis and sloughing seminiferous tubules, necrobiosis changes in epithelial lining of epididymis</td>
<td>3</td>
<td>Unknown purity; single dose exposure</td>
</tr>
<tr>
<td>Kim et al. 2002</td>
<td>Hershberger assay in CD rats</td>
<td>Purity unspecified</td>
<td>50</td>
<td>Increased ventral prostate, Cowper's gland and glans penis weights; in testosterone (T) supplemented phase of assay; authors hypothesize: size 2,4-D synergizes with T in increasing accessory sex tissue weights or inhibited T metabolizing enzymes activity</td>
<td>3</td>
<td>T dose reporting discrepant (different doses reported in different sections of paper); only one 2,4-D dose tested; inconsistencies in reported methods; T-supplemented Hershberger phase tests anti-androgenicity; not validated to assess androgenicity; organ weights not similar to weights for the same organs reported by other investigators (note article is in Korean; no translation)</td>
</tr>
<tr>
<td>Study</td>
<td>Assay and test system</td>
<td>Purity 2,4-D</td>
<td>Concentration range or doses tested (mg/kg/day)</td>
<td>Result</td>
<td>Klimisch score</td>
<td>Weaknesses</td>
</tr>
<tr>
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</tr>
<tr>
<td>Oakes et al. 2002a</td>
<td>Male Sprague-Dawley rats dosed orally via gavage</td>
<td>Formulation; 2,4-D concentration and purity unspecified</td>
<td>37.5–150</td>
<td>No effects on plasma testosterone; decreased weight gain and decreased testicular weight at high dose</td>
<td>3</td>
<td>Formulation; concentration and purity unspecified poor study design; small sample size; insufficient reporting of data</td>
</tr>
<tr>
<td>Stoker et al. 2007</td>
<td>Male Wistar rats exposed to 2,4-D orally by gavage</td>
<td>Purity unspecified</td>
<td>100 and 200 reported in abstract; per Stoker and Zorilla (2010) book chapter 3 and 30 were also evaluated</td>
<td>Delayed PPS at high dose; decreased ventral prostate weight; T and androstenedione decreased at high dose; no change in LH and prolactin; T3 decreased at both high doses; no effects at 30mg/kg/day based on Stoker and Zorilla 2010 book chapter</td>
<td>4</td>
<td>Insufficient information provided (only reported as abstract and as limited information in book chapter); doses reported in abstract exceed threshold for renal clearance</td>
</tr>
<tr>
<td>Charles et al. 1996a</td>
<td>OPP 82-1 Guideline study; Fischer 344 rats exposed to 2,4-D in the diet for 13 weeks</td>
<td>96.1%</td>
<td>1, 15, 100, 300</td>
<td>Reviewed above with regulatory toxicology studies on 2,4-D based on study report (Schulze 1991a)</td>
<td>2</td>
<td>Limited detail due to multiple studies in one publication</td>
</tr>
<tr>
<td>Charles et al. 1996a</td>
<td>OPP 82-1 Guideline study; Fischer 344 rats exposed to 2,4-D dimethyl amine (DMA), 2,4-D ethylhexyl amine (EHE) in the diet for 13 weeks</td>
<td>66.2% a i (95.3% dry wt) 2,4-D DMA</td>
<td>1–300 mg/kg/day a.e. (acid equivalent)</td>
<td></td>
<td>2</td>
<td>Limited detail due to multiple studies in one publication</td>
</tr>
<tr>
<td>Charles et al. 1996a</td>
<td>OPP 82-1 Guideline study; Fischer 344 rats exposed to 2,4-D ethylhexyl amine (EHE) in the diet for 13 weeks</td>
<td>95.1% a i 2,4-D EHE</td>
<td>1–300 mg/kg/day a.e. (acid equivalent)</td>
<td></td>
<td>2</td>
<td>Limited detail due to multiple studies in one publication</td>
</tr>
<tr>
<td>Oakes et al. 2002b</td>
<td>Male Sprague-Dawley rats dosed orally with gavage with a formulation of 2,4-D and picloram</td>
<td>Formulation with multiple active ingredients; 24-D concentration and purity not specified</td>
<td>37.5–150 (2,4-D)</td>
<td>No effects on plasma T; weight gain and testicular weight at 150mg/kg/day</td>
<td>3</td>
<td>Formulation; 2,4-D concentration and purity not specified; 2,4-D not tested in isolation; poor study design; small sample size; insufficient reporting of data</td>
</tr>
<tr>
<td>Kobal et al. 2000</td>
<td>Wistar rats dosed by gavage for ten days</td>
<td>Formulation (2,4-D in formulation 98% pure, but inert in formulation unspecified)</td>
<td>11 and 110</td>
<td>↓ serum T4 at 110mg/kg/day; ↓T3 males day 6 at 110mg/kg/day. Statistically significant differences in the low-dose group ↓ in females and ↓ in males; examination of the figures suggests that these changes were not biologically significant and may reflect inherent variability of the assays. No other thyroid-related parameters were assessed in this study; the biological significance of the findings cannot be assessed</td>
<td>3</td>
<td>T4 pre-test values showed considerable variance; no data are presented for thyroid hormone measurements, only figures, which show mean values only without confidence limits or standard errors. Formulated commercial material with unknown formulation excipients. High dose exceeds TSRC; other toxicity to animals not monitored. TSH, thyroid weight, and thyroid histopathology not evaluated.</td>
</tr>
<tr>
<td>Study</td>
<td>Assay and test system</td>
<td>Purity 2,4-D</td>
<td>Concentration range or doses tested (mg/kg/day)</td>
<td>Result</td>
<td>Klimisch score</td>
<td>Weaknesses</td>
</tr>
<tr>
<td>-------</td>
<td>----------------------</td>
<td>-------------</td>
<td>-----------------------------------------------</td>
<td>--------</td>
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<td>------------</td>
</tr>
<tr>
<td>Charles et al. 1996b</td>
<td>OPP 82-1 subchronic and 83-1 chronic guideline studies in purebred beagle dogs exposed to 2,4-D in diet for 90 days or one year</td>
<td>96.7% 2,4-D</td>
<td>0, 0.5, 1.0, 3.75 and 7.5 in subchronic study; 0, 1, 5 and 10/7.5 in chronic study</td>
<td>Studies (Dalgaard 1993a, 1993b) reviewed above with regulatory toxicology studies based on study reports</td>
<td>2 (for publication)</td>
<td>Studies reviewed above based on actual study reports</td>
</tr>
<tr>
<td></td>
<td>OPP 82-1 guideline subchronic study in purebred beagle dogs exposed to 2,4-D dimethylamine salt (DMA) for 90 days</td>
<td>66.7% aqueous solution (93.5% dry weight basis)</td>
<td>0, 1, 3.75 and 7.5 (a.e. basis)</td>
<td>[ body weight gains at high dose; no effects on thyroids; slightly [ textes weight at 7.5 mg/kg/day ae; non-exposure related testicular histopathology; slightly [ incidence of “juvenile/ inactive” prostate at 7.5 mg/kg/day ae ] ]</td>
<td>2 (for publication)</td>
<td>Limited details included due to multiple studies presented</td>
</tr>
<tr>
<td></td>
<td>OPP 82-1 guideline subchronic study in purebred beagle dogs exposed to 2,4-D 2-ethylhexyl ester (2-EHE) for 90 days</td>
<td>95.1%</td>
<td>0, 1, 3.75 and 7.5 (a.e. basis)</td>
<td>[ body weight gains at mid and high dose; thyroid weights [; not statistically significant or dose related; no thyroid histopath; no effects testes weights; no testicular path. ] ]</td>
<td>2 (for publication)</td>
<td>Limited details included due to multiple studies presented</td>
</tr>
<tr>
<td>Obidike et al. 2012</td>
<td>West African Dwarf goats dosed via diet</td>
<td>Formulation; purity of 2,4-D not specified</td>
<td>75–125 mg/kg of 720 g/L formulation of 2,4-D</td>
<td>Decreases in testicular sperm reserves and in epididymal sperm reserves; hyperemia and edema of the stroma; decrease in Sertoli cells</td>
<td>3</td>
<td>Non-conventional test system; formulation tested; small number of animals of varying ages obtained from different sources; no assurance control animals representative</td>
</tr>
<tr>
<td>Rawlings et al. 1998</td>
<td>Female ewes dosed directly to the rumen via gelatin capsules</td>
<td>Formulation; concentration and purity of 2,4-D not defined</td>
<td>10 mg/kg; 3 times per week</td>
<td>Decreased serum T4; no effect on LH, FSH, P, E2, cortisol; insulin; no changes in histopathology of any organs tested</td>
<td>3</td>
<td>Small sample size; formulation tested; concentration and purity of test material not defined; only one dose tested; direct administration into rumen of questionable relevance</td>
</tr>
<tr>
<td>Thyroid mechanism related</td>
<td>Florsheim &amp; Velcoff 1962</td>
<td>Sprague-Dawley rats on low iodine diet subcutaneously injected with 2,4-D</td>
<td>Formulation</td>
<td>Decrease in serum protein-bound iodine; decrease in thyroid to serum radioiodide ratio; no changes in pituitary TSH concentrations, thyroidal cell height, or thyroid histopathology</td>
<td>“5”</td>
<td>Subcutaneous injection, formulation, unspecified purity of test material; dose likely exceeds TSRC; mechanistic study</td>
</tr>
<tr>
<td>Florsheim et al. 1963</td>
<td>Sprague-Dawley rats</td>
<td>Formulation</td>
<td>80</td>
<td>Decreased serum thyroxine concentration; increased brain and liver thyroxine concentration; no change in thyroxine half-life, thyroxine distribution or in thyroid histopathology</td>
<td>“5”</td>
<td>Route of administration not specified (probably subcutaneous injection); small sample size formulation, unspecified purity of test material; mechanistic study; dose likely exceeds TSRC</td>
</tr>
</tbody>
</table>

(continued)
Endpoints either not relevant to a specific study type, or not assessed in a particular assay are noted with a “–” and are white.

Note: Many of the findings marked “O” have explanations that rule out attribution to an endocrine mechanism or make this much less likely. These findings are indicated in the table footnotes and discussed in more detail in the text following the tables.

Overall, there were no findings for 2,4-D that are considered clearly positive for a direct endocrine pathway potential interaction in vitro, and no in vivo findings in mammals in robust studies (Klimisch criteria 1 or 2) relevant to potential endocrine pathway interactions at doses below the TSRC.

**WoE evaluation for the estrogen hormonal pathway**

As outlined in EPA’s WoE guidance (US EPA 2011), generally five Tier 1 EDSP screening studies provide data relevant to assessing whether a compound potentially interacts with the estrogen hormonal pathway. In the case of the 2,4-D EDSP requirements, EPA waived the requirement for the EDSP Tier 1 uterotrophic and female pubertal assays, based on a recent EDSP Tier 2 equivalent EOGRT study (Marty et al. 2010). This study provides similar information to the female pubertal study (OPPTS 890.1450; US EPA 2009h), and is considerably more comprehensive because it includes assessment of reproductive parameters and offspring development. Endpoints highly sensitive to estrogenic effects are included in the EOGRT study; however, this study does not provide the more specific (Rank 1) information on potential estrogenicity provided by the uterotrophic assay.

The Rodwell and Brown two-generation reproductive toxicity study provides additional information, including an assessment of uterine histopathology in immature animals, which, as they are unlikely to be cycling, provide a more sensitive model for assessing estrogenic activity than do adults. The subchronic and chronic regulatory toxicity studies in rats, mice and dogs also provide information on relevant endpoints and provide a resource for evaluating potential species sensitivity.

Additionally, the EDSP Tier 1 in vitro ER binding or activation studies provide information relevant to assessing the potential receptor-mediated estrogenic activity of 2,4-D. The Tier 1 EDSP FSTRA provides information from an aquatic species. Endpoints and results relevant to the estrogen pathway from key regulatory studies, including the EDSP Tier 1 FSTRA and the EOGRT study are summarized in Tables 12–14. The one-generation quail reproductive toxicity study also provides relevant information and is discussed in the text following the tables. High quality in vitro and in vivo data from the published literature and ToxCast™ screening results are also considered important in developing the WoE and are discussed in the text.

The only type of in vivo assay considered to provide a clear rank 1 result for estrogenic pathway activity are: 1) the uterotrophic assay (none available for 2,4-D), in which statistically significant and marked uterine weight increases are

<table>
<thead>
<tr>
<th>Study</th>
<th>Assay and test system</th>
<th>Purity 2,4-D</th>
<th>Concentration of 2,4-D tested (mg/kg)</th>
<th>Result</th>
<th>Klimisch score</th>
<th>Weaknesses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Van den Berg et al. 1991</td>
<td>Male WAG/MBL rats exposed to 2,4-DB in diet; 2,4-D tested in vitro</td>
<td>“Highest purity available”</td>
<td>0.06 mmol/kg 2,4-DB</td>
<td>Lower plasma T4 levels in vivo following 2,4-D exposure; decreased T4 binding to serum protein in vitro after 2,4-D exposure.</td>
<td>5</td>
<td>2,4-D not tested in vivo; intra-peritoneal route irrelevant; only one concentration tested. In vitro mechanistic study: 2,4-D demonstrated a high (70-100%) competition for binding of T4 to trans-thyretin at a concentration of 100 µg/ml—a concentration well above TSRC.</td>
</tr>
</tbody>
</table>

CRITICAL REVIEWS IN TOXICOLOGY 33
Potential evidence of pathway interaction at limit dose only
No evidence of pathway interaction
All studies:
† Relevant, but useful only if corroborating Rank (1) or (2) endpoints
(2) Potentially sensitive for the hypothesis; stronger if correlated with Rank 1 data
(1) Specific and sensitive to the hypothesis

There are no in vitro studies.

In vitro studies
There are no in vitro data supporting a direct interaction of 2,4-D with the ER. The EDSP in vitro ER binding (LeBaron et al. 2011a) and ER transactivation assays (LeBaron & Kan 2011) were negative.

Kojima et al. (2004) examined the ability of 2,4-D to act as either an agonist or an antagonist of the human ERs or human ERß in transiently transfected Chinese hamster ovary cells (CHO K1) that also expressed a luciferase reporter plasmid containing an estrogen-responsive element (ERE). There were no 2,4-D related effects in these assays. Kojima et al. is considered to provide reliable corroborating data regarding the lack of ability of 2,4-D to act in vitro as an agonist or antagonist at the ER.

One high quality in vitro study examined the ability of reagent-grade 2,4-D to induce the proliferation of an estrogen-responsive cell line, MCF-7 (Lin & Garry 2000). Reagent-grade 2,4-D had no effect on MCF-7 cell proliferation. Commercial grade 2,4-D LV4 and 2,4-D amine caused a proliferative response, but, because the reagent-grade 2,4-D was negative, this response in the commercial grade ingredients was thought to be due to other components present in the commercial formulations. The proliferation of MCF-7 cells can occur via either an estrogen-related pathway or a non-estrogen-related mechanism; additionally the reliability of the test method may be influenced by the derivation of the cell line and culture conditions (Odum et al. 1998; Payne et al. 2000). Therefore, a positive response in this assay alone is not necessarily a reliable indicator of a compound’s estrogenic potential.

The ToxCast™ assays lack the details in methods and results required to fully establish validity, but serve to generally confirm the absence of in vitro effects on ER-binding or transactivation. As noted previously, a recent analysis by Cox et al. (2014) shows good concordance between the ToxCast™ results and EDSP endpoints indicative for potential ER interaction. ToxCast™ assays showed no ER binding or transactivation potential for 2,4-D.

Ecotoxicological studies
In the EDSP Tier 1 FSTRA (Marino et al. 2010, published in Coady et al. 2013), 2,4-D exposed female fish showed decreased fecundity at the high exposure concentration only (nominal limit dose of 100 mg/L). Secondary sex characteristics were not affected in males or females. There were no effects on male or female VTG, fertility or gonadal histopathology in the FSTRA, and it is considered likely that the high dose effects on fecundity reflect stress or uncharacterized systemic toxicity rather than an effect relating to interaction with the estrogen pathway. Notably, there were no effects signifying increased male VTG, which as noted is considered a rank 1, i.e. relatively specific and sensitive endpoint for assessing potential estrogenicity (Borgert et al. 2014).

Data from an acceptable reproductive toxicity study in quail (Mitchell et al. 2000) show no effects potentially related to an estrogen pathway interaction at any dietary concentration. Ottinger et al. (2002) indicates that female quail show declines in productivity following exposure to estrogenic chemicals; no changes in egg production or hatching success were seen in the 2,4-D quail reproduction study.

There are limited data from high quality ecotoxicological studies from the published literature. Crain et al. 1997 and Spiteri et al. 1999 reported results after dosing alligator’s eggs with 2,4-D. Estradiol was used as a positive control. In the first study, on pipping, chorio-allantoic fluid was analyzed and blood from hatchlings (10-days post hatch) was analyzed for estradiol. The sex of hatchlings was also determined. In the second study, which followed a similar exposure regimen
Table 13. Results for 2,4-D from regulatory reproductive/developmental toxicity studies relevant to potential interaction with the estrogen pathway.

<table>
<thead>
<tr>
<th>Study</th>
<th>Time to mating</th>
<th>Gestation duration</th>
<th>Gestation index</th>
<th>Fertility index</th>
<th>Implantations</th>
<th>Pub sex ratio</th>
<th>Anogenital distance (AGD)</th>
<th>Vaginal opening</th>
<th>Estrous cyclicity</th>
<th>Urogenital malformations</th>
<th>Uterus weight</th>
<th>Uterus histopathology</th>
<th>Ovaries weight</th>
<th>Ovaries histopathology</th>
<th>Vaginal histopathology</th>
</tr>
</thead>
<tbody>
<tr>
<td>EOGRT</td>
<td></td>
<td></td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Two Gen</td>
<td>N (2)</td>
<td>N (3)</td>
<td>N (2)</td>
<td>N (3)</td>
<td>N (3)</td>
<td>N (3)</td>
<td>N (3)</td>
<td>N (2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dev Tox Rat</td>
<td></td>
<td></td>
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<tr>
<td>Dev Tox Rabbit</td>
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</tbody>
</table>

*EOGRT: F1-extended one-generation reproductive toxicity study (Marty et al. 2010); Two Gen: OPP 83-4 Two-generation reproductive toxicity study (Rodwell & Brown 1985); Dev Tox Rat: OPP 83-3 Rabbit Developmental toxicity study (Rodwell 1983); Dev Tox Rabbit: OPP 83-3 Rabbit Developmental toxicity study (Hoberman 1990).

†Marty et al. 2010 Uterine weight differences were not statistically different from control and attributable to non-exposure related difference from control in stage of estrous cycle at termination; uterine weights were within HCD.
‡Rodwell and Brown 1985 The length of gestation was statistically significantly prolonged (by 1 day) in the production of the F1b pups at ≥80 mg/kg/day, compared with controls; may be attributable to the very excessive dose of 2,4-D to the dams during production of the F1b litters; high pup mortality was seen at this excessive dose.
§80 mg/kg/day F1a pups showed a statistically significant change in sex ratio (increased M pups), compared with the controls. This finding was not repeated in the F1b pups (at a higher dose) and is considered unlikely to be exposure-related because of the lack of consistency.
¶The gross and microscopic evaluation of uteri in the F1b PND 28 offspring showed no evidence of imbibed uterine or uterine lining proliferation.
||Evaluated but not sensitive; as implantations complete and offspring sex determined prior to initiation of dosing.
#Increase in female sex ratio present; not attributed to sexing sex determined prior to initiation of dosing; implantations complete prior to dosing; no evidence selective loss of females
All studies
††No evidence of potential interaction
Finding over the TSBC
†Increased relative to control
–Endpoint not evaluated
Endpoint scores in parentheses based on Borger et al. 2014 (modified in some cases based on context or strength of response)
(1) specific and sensitive to the hypothesis
(2) potentially sensitive for the hypothesis; stronger if correlated with Rank 1 data
(3) relevant, but useful only if corroborating Rank (1) or (2) endpoints

Mammalian studies

There were no effects on females or offspring in the EOGRT study (Marty et al. 2010, published in Marty et al. 2013) supporting either estrogenicity or anti-estrogenicity. Additionally, gonadal histopathology was not found in contrast to estradiol. Mammalian treatments showed no effects of 2,4-D on the reproductive organs, including AGD or age at vaginal opening.

There were no exposure-related effects on developmental landmarks, including AGD or age at vaginal opening.

There were no effects on estrous cycle length or estrous cycle pattern, including a lack of either persistent estrus or interrupted cycling at any dose level.

There were no exposure-related effects on gonadal histopathology, including ovarian follicle counts.

Thus, there was no pattern suggesting estrogenicity or anti-estrogenicity in a developmental context. There were no exposure-related changes in reproductive organ histopathology, including ovarian follicles.

Litter size and pup survival were not affected by 2,4-D.

There were no biologically significant effects on reproductive organ weights in adults or offspring at any dose of 2,4-D and there was no exposure-related change in reproductive organ histopathology, including ovarian follicles.

There were no exposure-related effects on developmental landmarks, including AGD or age at vaginal opening.

There were no effects on estrous cycle length or estrous cycle pattern, including a lack of either persistent estrus or interrupted cycling at any dose level.

There were no exposure-related effects on gonadal histopathology, including ovarian follicle counts.

There were no signs of oocytes in 24-D-exposed F1 dams.

There were no effects on estrous cycle length or estrous cycle pattern, including a lack of either persistent estrus or interrupted cycling at any dose level.

There were no exposure-related effects on gonadal histopathology, including ovarian follicle counts.

There were no signs of oocytes in 24-D-exposed F1 dams.

There were no effects on estrous cycle length or estrous cycle pattern, including a lack of either persistent estrus or interrupted cycling at any dose level.

There were no exposure-related effects on gonadal histopathology, including ovarian follicle counts.

There were no signs of oocytes in 24-D-exposed F1 dams.

There were no effects on estrous cycle length or estrous cycle pattern, including a lack of either persistent estrus or interrupted cycling at any dose level.
Table 14. Results for 2,4-D from regulatory toxicity subchronic and chronic toxicity studies relevant to potential interaction with the estrogen pathway.

<table>
<thead>
<tr>
<th>Study*</th>
<th>Uterus histopathology</th>
<th>Ovaries weight</th>
<th>Ovaries histopathology</th>
<th>Mammary histopathology</th>
<th>Vaginal histopathology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat SC (1)</td>
<td>N(2)</td>
<td>–</td>
<td>N(2)</td>
<td>–</td>
<td>N(2)</td>
</tr>
<tr>
<td>Rat SC (2)</td>
<td>N(2)</td>
<td>–</td>
<td>N(2)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Rat C</td>
<td>N(3)</td>
<td>O(3)†</td>
<td>N(2)</td>
<td>O(3)†</td>
<td>N(2)</td>
</tr>
<tr>
<td>Mouse SC</td>
<td>N(2)</td>
<td>–</td>
<td>N(2)</td>
<td>–</td>
<td>N(2)</td>
</tr>
<tr>
<td>Mouse SC (F)</td>
<td>N(3)</td>
<td>–</td>
<td>N(2)</td>
<td>–</td>
<td>N(3)</td>
</tr>
<tr>
<td>Dog SC (1)</td>
<td>N(2)</td>
<td>–</td>
<td>N(2)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Dog SC (2)</td>
<td>N(2)</td>
<td>–</td>
<td>N(2)</td>
<td>–</td>
<td>N(2)</td>
</tr>
<tr>
<td>Dog C</td>
<td>N(2)</td>
<td>–</td>
<td>N(2)</td>
<td>–</td>
<td>N(2)</td>
</tr>
</tbody>
</table>

* Rat SC (1): OPP 82-1 13-week rat subchronic toxicity study (Schulze 1991a); Rat SC (2): OPP 82-1 13-week rat subchronic toxicity study (Gorzinski et al. 1981a); Rat C: OPP 83-5 two year rat chronic/oncogenicity study (Jeffries et al. 1995); Mouse SC: OPP 82-1 13 week mouse subchronic toxicity study (Schulze 1991b); Mouse C(F): OPP 83-2 mouse oncogenicity study females (Stott 1995a); Dog SC (1): OPP 82-1: 13-week dog subchronic toxicity study (Schulze 1990); Dog SC (2): OPP 82-1: 13-week dog subchronic toxicity study (Dalgard 1993a) and Dog C (1): OPP 83-1: dog chronic toxicity study (Dalgard 1993b).
† Schulze 1991a Ovary weight increase at 300 mg/kg/day high dose; no correlating histopathological findings and stage of estrous cycle not controlled at necropsy.
‡ Jeffries et al. 1995 Ovary weight decrease at 150 mg/kg/day high dose terminal sacrifice attributable to body weight loss, no correlating histopathological findings.
¶ Decreased incidence of mammary gland hyperplasia at 150 mg/kg/day terminal sacrifice; likely attributable to body weight loss. All studies
N No evidence of potential interaction
O Finding over the threshold for renal saturation
† Increased relative to control
‡ Decreased relative to control
– Endpoint not evaluated

It should be noted that the uterus was evaluated both grossly and histopathologically in F1b PND 28 F344 female rats (Rodwell & Brown 1985). This evaluation provided information on uterine growth and/or stimulation in weanling animals that, while approaching puberty, were unlikely to be cycling based on the time of puberty onset in F344 rats. The absence of cycling makes these young animals less variable and more sensitive to potential estrogenic effects. There were no effects on the uterine histopathology in these animals even at a dose well above the TSRC.

In other regulatory guideline toxicity studies, including a two-generation rat reproductive toxicity study, rat and rabbit developmental toxicity studies in rats, and subchronic and chronic toxicity studies in mice, rats and dogs, few endpoints were observed suggesting either estrogenic or anti-estrogenic activity, even at dose levels causing significant systemic toxicity.

One finding in the two-generation reproductive toxicity (Rodwell & Brown 1985) suggesting possible endocrine toxicity (but not necessarily an interaction with the estrogen pathway) was that the length of gestation was statistically significantly prolonged (by 1 day) in the production of the F1b pups at ≥80 mg/kg/day (HDT), compared with controls. (Due to an inadvertent dosing error to the P animals during production of the F1b treatment group, the actual 2,4-D dose for that generation/littering was ≥100 mg/kg/day). Length of gestation is considered a Rank 3 endpoint. Gestation may be prolonged because of difficulties in parturition, hormonal imbalance, delays in implantation or decreased intrauterine growth; other uncharacterized factors may also result in prolonged gestation. The first alternative is unlikely because no evidence of dystocia was reported. Because of the mis-dosing, the high dose was well above the TSRC and significant toxicity was observed, and this delay may simply be due to excessive toxicity. There were no similar findings in the F1a littering (high dose confirmed as 80 mg/kg/day) in the Rodwell and Brown (1985) study or in the Saghir et al. (2008a) range-finding study, in which a similar high and toxic dose did not result in prolonged gestation. At most, the finding of prolonged gestation in the F1b litters provides equivocal evidence of a potentially treatment-related hormonal imbalance resulting from 2,4-D exposure at a dose significantly exceeding the TSRC (and exceeding a classically defined MTD).

With a single exception, higher significance (Rank 2) endpoints in the mammalian regulatory studies showed no effects of 2,4-D suggesting an interaction with the estrogen pathway. The only exception was a change in ovary weight in a subchronic rat study (Schulze 1991a) at a high dose exceeding both the MTD and the TSRC; however, there were no correlating histopathological changes. The stage of the estrous cycle was not controlled in this study and the ovarian weight findings could have been due to chance. No other possibly estrogen pathway-related effects were seen in females in the subchronic rat toxicity study. More robust ovarian assessment, such as in the EOGRt study in which ovarian follicular counts were performed, showed no exposure-related effects.

Two Rank 3 endpoints were affected in the chronic rat study (Jeffries et al. 1995): ovary weight was decreased (with no histopathological correlate) and mammary hyperplasia was decreased, both at the terminal sacrifice at the high excessively toxic dose of 150 mg/kg/day. There was also a decreased incidence of benign adenomas of the pars distalis in the pituitary (which is an estrogen-sensitive tumor; Dinse et al. 2010), in females at 150 mg/kg/day. These findings might point toward anti-estrogenicity, but are confounded by the systemic toxicity which included marked body weight...
loss. Note that mammary histopathology in the chronic rat study is ranked a 3 for sensitivity (as opposed to 2 for this endpoint in subchronic studies) because the high background incidence of mammary tumors in the F344 rat strain is a potential confounder.

The mouse subchronic (Schulze 1991b) and chronic (Stott 1995a) studies showed no effects on uterine or ovarian histopathology. The dog subchronic (Schulze 1990 and Dalgard 1993a) and chronic studies (Dalgard 1993b) similarly showed no effects on ovary weights, or on uterine, vaginal or mammary gland histopathology.

As noted previously, estrogens could alter male reproductive system endpoints including testes weight, sperm development or histopathology. The Marty et al. (2010) EOGRT study showed no testicular weight or histopathology findings attributable to a potential endocrine pathway interaction. Sperm parameters including testicular and epididymal sperm counts, motility and morphology were not altered in the Marty et al. (2010) study. Testicular weights and histopathology in the Rodwell and Brown (1985) two-generation reproductive toxicity study showed no exposure-related findings. Findings in these studies in males and on male reproductive tissues in the subchronic and chronic toxicity studies will be discussed in detail in the assessment of potential interactions with the androgen pathway.

Other mammalian studies relevant to potential interactions with the estrogen pathway

In a study by Dinamarca et al. (2007), ICR/Jcl mice were mated, and subsequently administered 2,4-D as a "pure compound" (purity unspecified) or as a commercially available formulation available in Chile (unspecified) in drinking water at concentrations providing mg/kg/day doses of 0, 0.01, 0.10 or 100 mg/kg/day from gestational day (GD) 0–9. The dose spacing in this study was designed to address the low dose hypothesis proposed by Cavieres et al. (2002). (The Cavieres et al. research is summarized in Supplementary Appendix VII.) Maternal toxicity was evaluated. Mice were bled at GD 9 for biochemical evaluations and cesarean-sectioned. Ovaries were evaluated for numbers of corpora lutea and uterine horns were evaluated for number of implantation sites, resorptions and live embryos. There were no signs of maternal toxicity nor differences in body weight gain between the dosed groups and the control. Numbers of corpora lutea, implantation sites, resorptions and live embryos were similar between the dose groups and control. The Dinamarca et al. study demonstrated that the finding of decreased implantations reported in mice exposed to 2,4-D by Cavieres et al. (2002) could not be replicated, even with an exposure period correctly designed to explore this possibility. The only limitations we identified in the Dinamarca et al. study was the failure to identify the specific purity of the "pure" test substance, and the lack of confirmatory dose analyses.

Regulatory developmental toxicity studies in rats and rabbits on various esters, amines and salts of 2,4-D summarized by Charles et al. (2001) do not predict any estrogenic activity. This publication is considered Klimisch criteria 2 based on relative absence of detail in reporting because of the large number of studies covered. The individual studies were guideline compliant, with the exception of the rabbit developmental toxicity study of 2,4-D DMA, which had a reduced number of litters available for evaluation. However, developmental toxicity was considered to be adequately characterized in this study. None of the rat or rabbit developmental toxicity studies showed any effects on maintenance of pregnancy, or urogenital malformations of the type that may signify endocrine modulating activity.

Another article by Charles et al. (1996a) presents data from several rat subchronic toxicity studies conducted with 2,4-D DMA, or 2,4-D 2-ethylhexyl ester (2-EHE), and with the 2,4-D acid study by Schulze (1991a) discussed above. This publication is considered Klimisch criteria 2 based on relative absence of detail in reporting because of the large number of studies covered. The studies were GLP guideline studies conducted to satisfy US EPA regulatory testing requirements. Fischer 344 rats (10/sex/dose group) were dosed in the diet with target doses of 0, 1, 15, 100 and 300 mg/kg/day (expressed as acid equivalent doses) for 90 days. Endocrine endpoints relevant to potential interactions with the estrogen pathway included: ovary organ weight, and mammary gland, ovary, and uterine histopathological evaluations. There was no evidence of potential interaction with the estrogen pathway in these studies.

A third article by Charles et al. (1996b) presents data from dog subchronic toxicity studies conducted with 2,4-D DMA, or 2,4-D 2-EHE. It also includes results from subchronic and chronic dog studies on 2,4-D acid (Dalgard 1993a, 1993b), which were reviewed based on the study reports. This publication is considered Klimisch criteria 2 based on relative absence of detail in reporting because of the number of studies covered. These studies were GLP guideline studies conducted to satisfy US EPA testing requirements. Beagle dogs (4/sex/dose group) were dosed in the diet with target doses of 0, 1.0, 3.75 and 7.5 mg/kg/day (expressed as acid equivalent doses).

Endocrine endpoints evaluated in these subchronic and chronic studies most relevant to the estrogen pathway included ovary weights, and histopathological evaluations of mammary gland, ovary and uterus. Clinical signs, body weight, feed consumption, clinical pathology and evaluation of standard target organ histopathology were also performed in these studies. There were no dose-related findings in possible estrogen pathway-related female endpoints, even though the high dose caused marked systemic toxicity.

In conclusion, the EDSP Tier 1 assays of 2,4-D considered relevant to the estrogen hormonal pathway, and the Marty et al. (2010) Tier 2 EDSP EOGRT study were judged to be quality studies. Further, the results were considered reliable for assessing the potential interaction with the estrogen pathway for 2,4-D, supplemented by information in other regulatory studies of 2,4-D as well as the high-quality studies available in the published literature. Based on a WoE evaluation of the available data, including the absence of potentially estrogenic or anti-estrogenic exposure-related findings in the Marty et al. (2010) EOGRT study, the lack of evidence for potential estrogen pathway interactions predicted by the other regulatory mammalian toxicity studies at doses below
the TSRC, the weak and non-specific response in the FSTRA, the absence of any adverse effects in the quail dietary reproductive toxicity study, the absence of adverse effects in high quality studies in the published literature and the negative Tier 1 EDSP in vitro ER binding and ER transactivation assays as well as negative ToxCast™ and other high quality in vitro screening data relevant to the estrogen pathway, it is concluded that 2,4-D does not show evidence for direct interaction with the estrogen pathway.

**WoE evaluation for the androgen hormonal pathway**

Two EDSP Tier 1 screening assays, including the AR binding assay (LeBaron et al. 2011b, published in Coady et al. 2014) and the FSTRA (Marino et al. 2010, published in Coady et al. 2013), provide data relevant to assessing whether 2,4-D potentially interacts with the androgen hormonal pathway. No Hershberger or male pubertal assays were required for 2,4-D because of the recently completed EOGRT study Marty et al. (2010) (published in Marty et al. 2013). The EOGRT study provides information on all endpoints included in the male pubertal assay, with the exception of serum testosterone levels, and provides additional endpoints sensitive to androgen deficiency not assessed in the pubertal study including AGD and nipple retention in males. Endpoints considered most relevant for assessing potential interactions with the androgen pathway are findings from the Marino et al. (2010) Tier 1 EDSP FSTRA, the Marty et al. (2010) Tier 2 EOGRT study, the Rodwell and Brown (1985) two-generation reproductive toxicity study, the Rodwell (1983) developmental toxicity study, the Hoberman (1990) developmental toxicity study. These studies, along with the subchronic and chronic toxicity studies are summarized below in Tables 15–17.

There were no findings for 2,4-D that suggest a potential androgen pathway interaction at doses less than the TSRC in any mammalian species, and only very limited in vitro data suggesting a direct interaction with this pathway is possible. The ranking used in the following tables reflects an assessment of the validity of the endpoints for assessing anti-androgenicity.

As noted previously, the WoE for each pathway depends on multiple other factors besides the rankings, and the rankings themselves may be modified based on the strength of a particular response. The WoE may also be influenced by context, e.g. decreased secondary sex characteristics in male fish is probably a stronger signal for potential anti-androgenicity than for estrogenicity, but may be due to other toxicity or to effects on steroidogenesis or interaction with the HPG axis rather than due specifically to potential anti-androgenicity. In contrast, an increase in nuptial tubercles in female fish appears closely associated with androgenicity and is considered a rank 1 endpoint for assessing the potential interaction with the AR (Borgert et al. 2014). Additionally, typical variance in a parameter in an assay should be considered in ranking that parameter.

More confidence in a potential anti-androgenic pathway interaction would come from corroborative findings in the in vitro AR binding and transactivation assays. Findings such as markedly delayed (2–3 day) preputial separation in a reproductive toxicity assay, urogenital anomalies in fetuses or pups exposed in utero in developmental or reproductive toxicity studies and alterations in male gonad histopathology in repeat dose studies and diminished male secondary sex characteristics in the FSTRA would strongly signal anti-androgenicity, particularly if the in vivo findings occurred at not otherwise systemically toxic concentrations. Importantly, no such corroborative evidence of anti-androgenicity was observed across the 2,4-D studies.

**In vitro studies**

The in vitro EDSP Tier 1 AR binding assay (LeBaron et al. 2011b, published in Coady et al. 2013) for 2,4-D was negative.

In a high quality published in vitro study, Kojima et al. (2004) examined the ability of 2,4-D to act as either an agonist or an antagonist of the human AR in transiently transfected CHO K1 that also expressed a luciferase reporter plasmid containing an androgen responsive element (ARE) over a range of test compound concentrations (10⁻⁸–10⁻⁵ M). Kojima et al. found no effects associated with 2,4-D exposure and is considered to provide reliable data regarding the lack of ability of 2,4-D to act as an agonist or antagonist at the AR in vitro.

Sun et al. (2012) reported the results of luciferase reporter gene assays to measure the effects of 2,4-D on AR in Vero cells, derived from African green monkey kidney epithelium, which do not express the AR endogenously. Transfected cells were exposed to a range of concentrations of 2,4-D based on the maximum contaminant level (MCL) in Chinese drinking water, i.e. cells were treated with 0.003, 0.03, 0.3 and 3.0 mg/L 2,4-D. There was no detectable androgenic or anti-androgenic activity at any of the 2,4-D concentrations tested; however, at the high concentration defined as 100x the MCL, 2,4-D was reported to enhance the effects of testosterone in the AR antagonist assay. The rationale for dose selection was questionable in this study; the effect was seen only at a high concentration far exceeding potential human exposure (Aylward & Hays 2008) although it falls within the linear TK range in rats. Further, the biological basis of this assay and relevance of this finding is questionable as the test was specifically designed to measure anti-androgenic activity rather than potentiation or androgenic activity.

The ToxCast™ assay battery developed and run under the auspices of the US EPA showed no evidence of interaction with the AR in either a cell-based or cell-free system. ToxCast™ assays showed no AR binding or transactivation potential for 2,4-D.

Similar to findings for estrogen pathway-related activity, an analysis by Rotroff et al. (2013) and a separate analysis by Cox et al. (2014) showed relatively good concordance between the ToxCast™ results and EDSP Tier 1 endpoints indicative for potential AR interaction, including Hershberger assay results.

**Ecotoxicological studies**

In the FSTRA (Marino et al. 2010, published in Coady et al. 2013), there were no specific effects on male fish suggesting
an androgen pathway interaction. There were no effects on secondary sex characteristics in female fish (occurrence/increase nuptial tubercles in female fathead minnows is a Rank 1 endpoint for androgenicity), and no effects on VTG or gonadal histopathology in either males or females.

A reproductive toxicity study in quail (Mitchell et al. 2000) did not show any findings potentially associated with interaction with the androgen pathway.

There are limited data from high quality ecotoxicological studies from the published literature. Crain et al. (1997, 1999) and Spiteri et al. (1999) reported results after dosing alligator’s eggs with 2,4-D. Estradiol was used as a positive control for estrogen pathway mediated effects; there was no positive control for androgen-mediated effects. In the first study, at pipping, chorio-allantoic fluid was analyzed and blood from hatchlings (10 days post hatch) was analyzed for testosterone. The sex of hatchlings was also determined. The 1999 study included evaluation of testicular histopathology. Gonadal histopathology was evaluated in a study by Spiteri et al. (1999) which followed a similar exposure regimen, but incubated eggs at two different temperatures. (Incubation temperature normally determines sex of alligator eggs.) There were no effects of 2,4-D on testosterone concentrations or on testicular histopathology following 2,4-D exposure. The studies were considered valid (Klimisch 2). One reservation was that the amount of 2,4-D penetrating the egg was not determined. Consequently, predictions based on these studies are limited to the conditions of exposure, i.e. application directly to the egg. These studies provide support that 2,4-D applied to alligator eggs at concentrations up to 14 ppm do not alter testosterone concentrations or affect male reproductive system histopathology in the exposed offspring.

**Mammalian studies**

There were no effects in the Marty et al. (2010) EOGRT study (published in Marty et al. 2013) considered to reflect an androgenic or anti-androgenic mode of action. Although there were several study findings that could support potential anti-androgenicity, these were found either not exposure-related, not replicated across generations, or were attributable to other factors such as randomization artifacts.

Preputial separation was slightly delayed (1.6 days) in the F1 males at 800 ppm, which was attributed to decreased growth during lactation and post-weaning. Although preputial separation delay is a Rank 2 finding for anti-androgenicity, the magnitude of the effect was very slight. Body weight at the time of puberty onset was similar in 800 ppm males and controls, despite the delay in onset for the high dose group. These data indicate that 800 ppm 2,4-D had an effect on the rate of growth in peri-pubescent male rats. The magnitude of the delay in preputial separation was consistent with reductions in body weight as demonstrated by a feed restriction study (Marty et al. 2003), and supports that the decreased growth, as opposed to anti-androgenicity, was responsible for the delay in preputial separation in the 2,4-D EOGRT study.

In P1 adult males, decreased seminal vesicle and prostate weights (without corresponding histopathological changes) were seen at ≥300 ppm; prostate weights were not statistically different from control. The control weights for the seminal vesicles and prostate were atypically high compared to HCD, and the high dose findings were within HCD. Two high dose males showed testicular atrophy (within HCD). None of these Rank 2 findings were reproduced in the F1 generation, which had higher and longer duration exposures. As noted previously, based on the absence of dose-related increased implantation loss or fetal deaths, there was no evidence that a sensitive sub-population was removed.

Decreased testis weights in 600-ppm PND 22 F1 weanlings were attributed to decreased body weights. A previous feed restriction study with untreated rats showed weanling testes weights alter with decreased body weight (Carney et al. 2004). In contrast, testes weights are conserved in the presence of similar/modest body weight decrements in adult male rats (Chapin et al. 1993). In the EOGRT study, there were no histopathological findings in the testes, and no effects on testis weights or histopathology were seen in adult F1 males.

Other endpoints sensitive to anti-androgenicity, including AGD and nipple retention in F1 offspring, were not altered. As noted in Marty et al. 2013, “These endpoints are considered highly sensitive to altered androgen status (Clark 1999; McIntyre et al. 2001; Wolf et al. 2002; Hotchkiss et al. 2004).”

Other androgen-sensitive endpoints examined in F1 generation adults, including sperm parameters, male reproductive
organ weights, and testicular and accessory sex gland weights or histopathology, were not altered.

Further, although this has not been tested formally, the lower sensitivity to males to systemic toxicity from 2,4-D exposure compared to females in the EOGRT study correlates with the higher predicted androgen-mediated expression of OAT-1 in males (Ljubojevic et al. 2004). This sex-specific difference would not be expected to be prominent if 2,4-D had potent anti-androgenic activity.

There were relatively few relevant parameters evaluated in the Rodwell and Brown (1985) two-generation study. Mating and fertility were not affected, nor were testicular weights and histopathology, or histopathology of the epididymides, seminal vesicles and prostate at doses up to 80 mg/kg/day.

A single finding in the Rodwell and Brown (1985) two-generation study potentially showing an androgen pathway interaction was that the 80 mg/kg/day F1a pups showed a statistically significant change in sex ratio (109 males and 71 females), compared with the controls. This finding was not repeated in the F1b pups (at a higher dose, due to mis-dosing during gestation) and is considered unlikely to be exposure related because of the lack of consistency. Additionally, there were no parallel exposure related effects on the sex ratio in the EOGRT study for 2,4-D (Marty et al. 2010), or in the range-finding for the latter study (Saghir et al. 2008a) at equivalent or higher concentrations to those tested by Rodwell and Brown (1985). Therefore, the finding is considered not likely to be exposure related.

There were no changes in fetal sex ratio noted in the developmental study in rats (Rodwell 1983). This is not considered a relevant endpoint in this study design because the genotypic sex of offspring is determined prior to the time during gestation that dosing was initiated. The developmental toxicity study in rabbits (Hoberman 1990) showed an altered sex ratio (more males than females) at the high dose. This finding is not considered exposure-related because dosing in this study was initiated shortly after implantation, at a time when genotypic sex of offspring has already been determined. There was no exposure-related selective loss of female offspring in utero based on a lack of effects on post-implantation loss or on litter size. Neither developmental study showed exposure-related urogenital visceral malformations which could reflect anti-androgenicity.

The Schulze (1991a) subchronic rat study showed flaccid testes and testicular atrophy at the very high and systemically toxic dose of 300 mg/kg/day, far exceeding the TSRC and MTD. Decreased testes weights were seen in a subchronic rat toxicity study at doses ≥100 mg/kg/day, also exceeding the TSRC (Gorzinski et al. 1981a); however, a follow up study by the same authors (Gorzinski et al. 1981b) failed to replicate this finding. Neither of the two Gorzinski et al. studies showed exposure-related histopathological lesions in the testes. Decreased testes weight was also seen at the excessively systemically toxic high dose (150 mg/kg/day) in the chronic rat study (Jeffries et al. 1995). Testicular atrophy was noted in 2/10 animals at the interim sacrifice in this study; however, no exposure-related testicular lesions were evident at the terminal sacrifice. The high-dose testes findings in the rat
**Table 17.** Results for 2,4-D from regulatory subchronic and chronic toxicity studies in rat, mouse and dog relevant to potential interaction with the androgen pathway.

<table>
<thead>
<tr>
<th>Study</th>
<th>Testes histopathology</th>
<th>Epididymides weight</th>
<th>Epididymides histopathology</th>
<th>Seminal vesicles histopathology</th>
<th>Prostate histopathology</th>
<th>Coagulating glands histopathology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat SC (1)</td>
<td>O (2)</td>
<td>N (2)</td>
<td>N (2)</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Rat SC (2)</td>
<td>O (2)</td>
<td>N (2)</td>
<td>N (2)</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Rat SC (3)</td>
<td>N(2)</td>
<td>N(2)</td>
<td>N(2)</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Rat C</td>
<td>O (2)</td>
<td>N(2)</td>
<td>N(2)</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Mouse SC</td>
<td>N(2)</td>
<td>N(2)</td>
<td>N(2)</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Mouse C (M)</td>
<td>N(2)</td>
<td>N(2)</td>
<td>N(2)</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Dog SC (1)</td>
<td>O (3)</td>
<td>N(2)</td>
<td>N(2)</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Dog SC (2)</td>
<td>O (3)</td>
<td>N(2)</td>
<td>N(2)</td>
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<tr>
<td>Dog C</td>
<td>N(2)</td>
<td>N(2)</td>
<td>N(2)</td>
<td>–</td>
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<td>–</td>
</tr>
</tbody>
</table>

* Rat SC (1): 13 week rat subchronic toxicity study (Schulze 1991a); SC (2): 13 week rat subchronic toxicity study; SC (3): 13 week rat subchronic toxicity study (Gorzinski et al. 1981a); SC (1): OPP 82-1 13 week rat subchronic toxicity study (Jeffries et al. 1995); Mouse SC: OPP 82-1 13 week mouse subchronic toxicity study (Schulze 1991b); Mouse C (M): OPP 83-2 mouse oncogenicity study (males) (Stott 1995b); Dog SC (1): OPP 82-1: 13-week dog subchronic toxicity study (Schulze 1990); Dog SC (2): OPP 82-1: 13-week dog subchronic toxicity study (Dalgard 1993a) and Dog C: OPP 83-1: dog chronic toxicity study (Dalgard 1993b).

† Schulze 1991a and Gorzinski et al. 1981a: Testes weight: decrease at ≥100 mg/kg/day; atrophy at 300 mg/kg/day (doses far exceeded TSRC and systemically toxic).

‡ Jeffries et al. 1995: Atrophy in 2/10 animals at 150 mg/kg/day in interim sac; not evident at termination (dose far exceeded TSRC and systemically toxic).

¶ Hypospermia in 2 of the 10 mg/kg/day dose group surviving dogs; equivocal relationship to exposure (dose exceeded MTD) and juvenile animals tested.

* Epididymal weight taken with testes weight; see comments on testes; no histopathological correlates in epididymides.

** Dalgard 1993b: Testes weight decreased at 7.5 mg/kg/day dose (dose systemically toxic and exceeded TSRC); juvenile animals tested.

†† Juvenile prostate noted in all groups; not exposure-related

All studies:

- N: no evidence of potential interaction
- O: finding over the threshold for renal saturation
- |: increased relative to control
- |: decreased relative to control
- –: Endpoint not evaluated

End point scores in parenthesis based on Borgert et al. 2014 (modified in some cases based on context or strength of response)

1) specific and sensitive to the hypothesis
2) potentially sensitive for the hypothesis; stronger if correlated with Rank 1 data
3) relevant, but useful only if corroborating Rank (1) or (2) endpoints

Studies could hypothetically be associated with anti-estrogenicity or androgenicity; they could as readily hypothetically reflect changes in steroidogenesis caused by the severe systemic toxicity at the excessive dose levels which far exceed the TSRC, or simply reflect direct non-endocrine mediated target organ toxicity, such as that induced by oxidative stress. Given that there is no other potential evidence of androgenicity or anti-androgenicity in these studies, alternative mechanisms present a more likely hypothesis.

Decreased testicular weights were noted in two subchronic dog studies (Schulze 1990; Dalgard 1993a) at high (lethal or close to lethal) dose levels clearly exceeding the TSRC (and classically defined MTD) in that species. The Schulze (1990) subchronic dog study also showed an increased incidence of testicular lesions (giant cells and hypoplastic/emasculatory) at a lethal dose. Evaluation of the two 2,4-D dog subchronic studies together, which were conducted at the same laboratory, however, shows a high combined incidence of these lesions. A high historical control incidence has also been reported for these findings in the histopathological literature, particularly for juvenile dogs. Immature dogs (less than 9 months of age) have been reported to have control incidences as high as 75% of both decreased testes weight and hypospermia (Goedken et al. 2008). In the Goedken et al. analyses of data from a large population of control dogs, atrophic/hypoplastic tubules in the testes were seen in 26.3% of all dogs, with 25–40% of dogs under 12 months old showing this finding. Evaluation of body weights, and control testicular and prostate histopathology (detailed in Supplementary Appendix IV) demonstrates the dogs in these subchronic studies of 2,4-D were on the lower end of the stated age range of 4–6 months old at study initiation and clearly juvenile (less than 9 months old) at terminal sacrifice. Additionally, the high incidence of finding of “juvenile prostate” in the Dalgard 1993a study (prostate was not evaluated in the Schulze 1990 study) supports that the dogs were immature. It seems likely that the decreased testes weights in both subchronic studies and histopathological findings in the testes of the dogs in the Schulze (1990) study are an artifact related to the young age of these animals, or at most, represent delayed development caused by the extremely toxic high dose. Supporting this possibility, a chronic study in dogs (Dalgard 1993b) showed no exposure-related effects on testes weights or histopathology following a one-year exposure to 2,4-D at a high dose level (10 reduced to 7.5 mg/kg/day), generally equivalent to the high dose in the prior subchronic studies. Reversibility of an exposure-related testicular finding such as atrophy in a continuous exposure situation is very unlikely; so the absence of effects in the dog chronic study supports that the findings in the subchronic dog studies were not exposure-related.

**Published mammalian studies relevant to the androgen pathway**

Two studies by Lamb et al. (1981a, 1981b) evaluated potential male reproductive effects. In both studies, male C57BL/6N...
mice were dosed for 8 weeks with combinations of organochlorine chemicals including 2,4-D. Approximate exposures to 2,4-D were 40 mg/kg/day and 20 mg/kg/day (in combination with varied concentrations of the other compounds). The high dose of 2,4-D is considered likely to approximate the TSRC (rats and mice have similar expression of the OAT-1 transporter (Buist & Klaassen 2004)), so the 2,4-D dose was appropriate and not limited by the toxicity of the other mixture components. Controls received untreated diet. Lamb et al. (1981a, 1981b) included evaluation of male fertility (mating the dosed males with untreated females), and sperm number, motility and morphology. Females were either cesarean sectioned at gestation day 18 for fetal evaluation or allowed to deliver and rear their pups until PND 21 for evaluation of offspring birth weight and viability. There was no effect on male fertility, sperm parameters or reproductive performance of the dosed males; development and survival of fetuses and pups in the dosed groups were similar to that of the control mice. The study provided no evidence of male-mediated reproductive toxicity or of endocrine disrupting activity of 2,4-D in these mixtures of chemicals and provide data supporting that there is no evidence of potential androgen pathway interactions for 2,4-D. The studies are high quality; the primary weakness in these studies, as it relates to 2,4-D specifically, is that they test only mixtures.

Regulatory developmental toxicity studies in rats and rabbits on various esters, amines and salts of 2,4-D summarized by Charles et al. (2001) do not predict any androgenic activity. None of the rat or rabbit developmental toxicity studies showed any urogenital malformations of the type that may signify endocrine modulating activity.

An article by Charles et al. (1996a) presents data from rat subchronic toxicity studies conducted with 2,4-D DMA, or 2,4-D 2-EHE as well as with the acid (discussed above (Schulze 1991a)). These studies were GLP guideline studies conducted to satisfy US EPA testing requirements. Fischer 344 rats (10/sex/dose group) were dosed in the diet with target doses of 0, 1, 15, 100 and 300 mg/kg/day (expressed as acid equivalent doses) for 90 days. Endocrine endpoints relevant to potential interactions with the androgen pathway included: testes weight; and epididymides, prostate and testes, histopathological evaluations. Clinical signs, body weight, feed consumption, clinical pathology and evaluation of standard target organ histopathology were also performed in these studies. Findings were similar to those reported for 2,4-D acid, and all occurred at a very high and severely systemically toxic dose level. Relative testes weights were decreased at 300 mg/kg/day (acid equivalent), and testicular atrophy was noted at the same dose level. This dose far exceeds the TSRC; and the marked systemic toxicity could have contributed to the testes findings.

A second article by Charles et al. (1996b) presents data from dog subchronic toxicity studies conducted with 2,4-D DMA or 2,4-D 2-EHE (and also included one of the dog subchronic studies (Dalgard 1993a) and the dog chronic toxicity study (Dalgard 1993b) on 2,4-D acid discussed above). These studies were GLP guideline studies conducted to satisfy US EPA testing requirements. Beagle dogs (4/sex/dose group) were dosed in the diet with target doses of 0, 1, 3, 7.5 mg/kg/day (expressed as acid equivalent doses). Endocrine endpoints evaluated in these studies possibly relevant to the androgen pathway included testes weight; and epididymides, prostate and testes histopathological evaluations. Clinical signs, body weight, feed consumption, clinical pathology and evaluation of standard target organ histopathology were also performed in these studies.

There were two findings in these studies that potentially could be related to androgen pathway modulation, although, similar to the hypothesis for the subchronic dog findings with 2,4-D acid, they more likely reflect the immature age of the dogs. Relative testes weights were decreased in the mid dose but not high dose of both the 2,4-D DMA and 2,4-D EHE subchronic studies. (In the subchronic study of 2,4-D acid (Dalgard 1993a) reviewed above, testes weight decreases were seen at the high dose). The absence of dose response in the 2,4-D DMA and 2,4-D EHE studies supports that the finding in the 2,4-D acid study was related to the immature age of the test animals, as discussed previously, and occurred by chance. Additionally, there were no exposure-related histopathological lesions in the testes in the 2,4-D DMA and EHE subchronic studies.

The second finding in the dog studies reported by Charles et al. (1996b) is that inactive/juvenile prostates were noted in high-dose males in the subchronic studies of 2,4-D DMA and EHE; the authors concluded this finding is likely to be related to delayed development from poor nutrition. We consider it likely that this finding also (or possibly primarily) reflects the immature age of the test animals. First, this finding was not made in the chronic 2,4-D acid dog toxicity study which tested a similar high dose on an acid equivalent basis. Dogs at study initiation in the subchronic 2,4-D DMA and EHE studies were relatively young (4–6 months old at study initiation according to Charles et al. (1996b)); review of body weight data suggests many of the dogs were on the low end of this age range. In the subchronic 2,4-D acid dog study (Dalgard 1993a), the incidence of the juvenile/inactive prostate finding shows a clearly non-dose related pattern, including the presence of this finding in control dogs. As discussed above in the context of the 2,4-D acid dog studies, a very high incidence of decreased testes weights and the same testicular lesions observed in these studies has been reported in young control dogs; an increased incidence of juvenile/inactive prostate is also a typical finding in juvenile dogs (as indicated by the terminology for this finding).

The evaluations by Charles et al. (1996a, 1996b) also support that findings from the rat and dog subchronic studies on 2,4-D acid are generally consistent with findings with the salts and esters of 2,4-D (when doses are expressed as acid equivalents), and that there are no unique toxicities, endocrine-mediated or otherwise, associated with these forms.

**Epidemiological studies**

Three limited epidemiological studies evaluating potential associations of 2,4-D and changes in human sperm parameters (Lerda & Rizzi 1991; Swan et al. 2003) or hormonal biomarkers (Garry et al. 2001) were identified. The Lerda and
Rizzi (1991) study is considered too limited in scope and relevant details, and is not considered to provide reliable evidence of male reproductive toxicity or endocrine disruption resulting from occupational exposure to 2,4-D. Swan et al. (2003) is considered too limited, due to the low numbers of control and case subjects with urinary 2,4-D levels above the LOD, to be considered in the WoE as evidence for presence or absence of an association. Garry et al. (2001) found no correlation between FSH, free testosterone, or total testosterone concentrations with 2,4-D urinary levels at the time of maximum 2,4-D usage. LH levels were reported to show a correlation, but the authors indicated the limited sample size warrants caution in drawing any conclusions from this study. It should also be noted that the animal studies showed no findings congruent with altered LH levels, such as increased Leydig cell tumors.

Based on a weight of evidence evaluation of the available data, including the absence of evidence for potential androgen pathway interactions in the Marty et al. (2010) Tier 2 EDSP equivalent EOGRT study, the lack of evidence for potential androgen pathway interactions predicted by the other key mammalian toxicity studies at doses below the TSRC, the absence of androgen-pathway-related responses in the FSTRA, the absence of adverse effects in the quail dietary reproductive toxicity study, and the negative Tier 1 EDSP in vitro AR binding, the negative ToxCast™ AR binding and AR transactivation and other high-quality published in vitro screening data and in vivo studies relevant to the androgen pathway, it is concluded that 2,4-D does not show evidence for direct interaction with the androgen pathway (either androgenic activity or anti-androgenic activity) at exposure levels relevant for human or ecological risk assessment.

**WoE evaluation for the steroidogenic pathway or HPG axis interactions**

Three EDSP Tier 1 screening assays relevant to steroidogenesis are available for 2,4-D, including the steroidogenesis (LeBaron et al. 2011c) and aromatase (Coady & Sosinski 2011) in vitro assays (published in Coady et al. 2014) and the FSTRA (Marino et al. 2010, published in Coady et al. 2013). The Marty et al. (2010) EOGRT study is an EDSP Tier 2 equivalent mammalian assay that also provides data possibly relevant to assessing whether 2,4-D interacts with the steroidogenic pathway, as do the key regulatory toxicity studies. The in vivo Tier 1 assay and key toxicity study results that may indicate potential interaction with the steroidogenesis pathway overlap substantially with those relevant to assessing potential interactions with the estrogen and androgen hormonal pathways, because the steroidogenic pathway is critical for the production of both estrogens and androgens. Positive effects in the in vitro steroidogenesis or aromatase assays provide supporting evidence that in vivo findings are influenced by interactions with these pathways, but negative findings do not rule out potential interactions. Endpoints and findings potentially relevant for addressing interactions of 2,4-D with the steroidogenic pathway are found in Tables 18–20.

Also relevant are selected parameters from the avian reproductive toxicity study, and results from published in vitro and in vivo studies. It should be noted that limited high quality published data are available investigating this potential mechanism. Supplementary information from the ToxCast™ aromatase assay is noted; no relevant aromatase or steroidogenesis assays were found in the peer reviewed literature.

Evaluation of potential interactions with the HPG axis is based on studies in intact mammals; relevant endpoints are not evaluated in other studies. Data are not separately tabulated to evaluate this interaction, as the gonadal findings are adequately captured by the evaluation for steroidogenesis interactions. Pituitary findings are discussed in the text.

As mentioned previously, the WoE for each pathway depends on multiple other factors. Changes in steroidogenic activity may be an underlying mechanism for changes in parameters that are also affected by androgen and/or estrogen pathways. The reader should be mindful that interaction with steroidogenesis pathway is also difficult to distinguish from findings secondary to systemic toxicity, because steroidogenesis depends on a complex and interrelated system of hormonal synthesis and feedback which may be influenced markedly by factors such as a decrease in the cholesterol starting material (from poor nutrition or disruption of synthesis or metabolism in the liver) or changes in membrane transport of steroid precursors, changes in mitochondrial function or other effects mediated by oxidative stress or through other membrane toxicity. Non-specific stress due to excessive toxicity or non-compound-related factors such as immobilization may also affect the steroidogenic process (Orr et al. 1994; Orr & Mann 1990). Adrenal weight and specific histopathological changes may point toward a steroidogenic or stress-related mechanism, but it should be noted there are multiple sites for steroidogenesis in the intact organism.

Note there are no exposure-related or equivocal findings that suggest altered steroidogenesis for 2,4-D in mammalian studies below the TSRC, and no specific evidence of modulation of steroidogenesis in the FSTRA.

**In vitro studies**

In the Tier 1 EDSP steroidogenesis assay (LeBaron et al. 2011c, published in Coady et al. 2014), there was a slight increase in estradiol in all runs at the high concentration only (100 μM). The 1.2-fold increase was below the 1.5-fold response threshold established in the EDSP steroidogenesis validation assays as a positive response (Hecker et al. 2008), and therefore this finding is not considered biologically meaningful.

The Tier 1 EDSP aromatase assay (Coady & Sosinski 2011, published in Coady et al. 2014) was negative. The ToxCast™ aromatase assay developed under the auspices of the US EPA also showed no evidence of aromatase inhibition.

The published in vitro literature lacked high quality studies evaluating potential effects on steroidogenesis or aromatase.

Based on the above data, it is unlikely that 2,4-D affects steroidogenesis in vitro.
The FSTRA (Marino et al. 2010; Coady et al. 2013) showed no findings likely to be associated with increased or decreased testosterone or estradiol. Secondary sex characteristics, fertility, gonadal somatic index (GSI), gonadal histopathology, and VTG levels were not affected in this study in males or females. There was only the single finding of decreased fecundity, at the high concentration only, which could potentially be associated with altered steroidogenesis. As previously discussed, this is a non-specific finding and is considered likely confounded by stress or uncharacterized systemic toxicity. As the steroidogenesis pathway is generally considered well conserved among vertebrate species, the absence of effects on this pathway in the EOGRT study also supports the concept that the non-specific decreased fecundity in the high concentration group of the FSTRA is likely due to stress or uncharacterized systemic toxicity and not to a potential interaction with steroidogenesis. While there are differences in exposure route (oral compared to via the gills), 2,4-D is completely absorbed via the oral route in rats and would be expected to be similarly readily absorbed through the gills. Further, both fish and rats would be exposed to parent 2,4-D as 2,4-D would avoid first-pass liver metabolism in fish due to the route of entry, and 2,4-D is not highly metabolized in rats.

A high dose reproductive toxicity study in quail (Mitchell et al. 2000) does not provide evidence of any effects that could be associated with increased or decreased testosterone or estradiol.

In published studies, a steroidogenesis assay using alligator eggs directly exposed to 2,4-D has been performed, which showed no effect of 2,4-D on steroidogenesis (Crain et al. 1997). This study used unconventional methodology but is of interest because it expands the range of species tested. Estradiol was used as a positive control, resulting in development of ovaries in embryos incubated at male-producing temperatures that was also associated with increased gonadal–adrenal mesonephros complex aromatase activity.
<table>
<thead>
<tr>
<th>Study</th>
<th>Anogenital distance (AGD)</th>
<th>Nipple retention</th>
<th>Time to mating</th>
<th>Mating indices</th>
<th>Fertility indices</th>
<th>Pup sex ratio</th>
<th>Developmental abnormalities</th>
<th>Ovary weight</th>
<th>Ovary histopathology</th>
<th>Uterus weight</th>
<th>Uterine histopathology</th>
<th>Vaginal opening</th>
<th>Estrous cyclicity</th>
<th>Mammary histopathology</th>
<th>Testes weight</th>
<th>Testes histopathology</th>
<th>Epididymides weight</th>
<th>Epididymides histopathology</th>
<th>Prostate weight</th>
<th>Prostate histopathology</th>
<th>Seminal vesicles weight</th>
<th>Seminal vesicles histopathology</th>
<th>Sperm parameters</th>
<th>Preputial separation</th>
<th>Adrenal weight</th>
<th>Adrenal histopathology</th>
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</table>

EOGRT: EOGRT study (Marty et al. 2010); Two Gen: OPP 83-4 Two-generation reproductive toxicity study (Rodwell & Brown 1985); Dev Tox Rat: OPP 83-3 developmental toxicity study in rats (Rodwell 1983); Dev Tox Rabbit: OPP 83-3 developmental toxicity study in rabbit (Hoberman 1990).

†With coagulating gland.

§Marty et al. 2010 High dose uterine weight increases not considered exposure related (non-statistically significant change in cycling females; no correlating histopathology except normal estrous cycle related changes; within HCD).

¶Decreased testes weight F1 pups at weaning, attributable to decreased body weight, not apparent in F1 adults.

††Testicular atrophy in 2 high-dose F1 males, considered spontaneous, not present in F1 dosed animals.

‡‡Rodwell and Brown 1985 The length of gestation was statistically significantly prolonged (by 1 day) in the production of the F1b pups at ≥80 mg/kg/day, compared with controls; likely attributable to the very excessive dose of 2,4-D to the dams during production of the F1b litters; high pup mortality was seen at this excessive dose (≥100 mg/kg/day).

††80 mg/kg/day F1a pups showed a statistically significant change in sex ratio (increased M pups), compared with the controls. This finding was not repeated in the F1b pups (at a higher dose) and is considered unlikely to be exposure related because of the lack of consistency across generations or with other 2,4-D studies.

#Evaluated but not sensitive; as offspring sex was determined prior to initiation of dosing.

### Change in fetal sex ratio present; not attributed to test article as offspring sex determined prior to initiation of dosing; no selective loss of females

#### All studies:

- **No evidence of potential interaction**
- **Observation over the threshold for renal saturation**
- **Increased relative to control**
- **Decreased relative to control**

Endpoint scores in parentheses based on Borgert et al. 2014 (modified in some cases based on context or strength of response)

- **(1) specific and sensitive to the hypothesis**
- **(2) potentially sensitive for the hypothesis; stronger if correlated with Rank 1 data**
- **(3) relevant, but useful only if corroborating Rank (1) or (2) endpoints**
### Table 20. Results for 2,4-D from regulatory subchronic (SC) and chronic (C) toxicity studies relevant to potential interaction with steroidogenesis.

<table>
<thead>
<tr>
<th>Study*</th>
<th>Ovary weight</th>
<th>Ovary histopathology</th>
<th>Uterine histopathology</th>
<th>Vaginal histopathology</th>
<th>Mammary histopathology</th>
<th>Testes weight</th>
<th>Testes histopathology</th>
<th>Epididymides weight</th>
<th>Epididymides histopathology</th>
<th>Prostate histopathology</th>
<th>Seminal vesicles histopathology</th>
<th>Adrenal weight</th>
<th>Adrenal histopathology</th>
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</thead>
<tbody>
<tr>
<td>Rat SC (1)</td>
<td>O(2)†</td>
<td>N(2)</td>
<td>N(2)</td>
<td>N(3)</td>
<td>–</td>
<td>O(2)†</td>
<td>O(2)†</td>
<td>N(3)</td>
<td>N(2)</td>
<td>–</td>
<td>–</td>
<td>O(2)† M F</td>
<td>O(2)† MF</td>
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<td>N(2)</td>
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<td>O(2)†</td>
<td>N(2)†</td>
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<td>N(2)</td>
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<td>Rat C</td>
<td>O(1) (3)†</td>
<td>N(2)</td>
<td>N(2)</td>
<td>N(3)</td>
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<tr>
<td>Mouse SC</td>
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<tr>
<td>Mouse C (1 F)</td>
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<td>N(3)</td>
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<td>N(3)</td>
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</table>

*Rat SC (1): OPP 82-1 13-week rat subchronic toxicity study (Schulze 1991a); Rat SC (2): 13 week rat subchronic toxicity study 82-1 (Gorzinski et al. 1981a); Rat SC (3): 13 week rat subchronic toxicity study (Gorzinski et al. 1981b); Rat C: OPP 83-5 Two year rat chronic/oncogenicity study (Jeffries et al. 1995); Mouse SC: OPP 82-1 13 week mouse subchronic toxicity study (Schulze 1991b); Mouse C (1 F): OPP 83-2 mouse oncogenicity study (females) (Stott 1995a); Mouse C (2 M): OPP 83-2 mouse oncogenicity study (males) (Stott 1995b); Dog SC (1): OPP 82-1: 13-week dog subchronic toxicity study (Schulze 1990); Dog SC (2): OPP 82-1: 13-week dog subchronic toxicity study (Dalgaard 1993a); Dog C: OPP 83-1: dog chronic toxicity study (Dalgaard 1993b).

†Schulze 1991a Increased ovary weight at 300 mg/kg/day (far exceeded TSRC and systemically toxic), no histopathological correlate.

‡Schulze 1991a and Gorzinski et al. 1981a Testes weight: decrease at <100 mg/kg/day; atrophy at 300 mg/kg/day (far exceeded TSRC and systemically toxic).

¶Schulze 1991a Adrenal: weight changes and histopathology (hypertrophy zona glomerulosa) at 300 mg/kg/day (far exceeded TSRC and systemically toxic); zona glomerulosa does not respond to HPA axis.

§Jeffries et al. 1995 Ovary: weight decrease at high dose attributable to body weight decrease; no correlating histopathological changes.

‖Tests: absolute and relative testes weights decreased at 150 mg/kg/day at both interim and terminal sacrifices; no corresponding histopathological findings at terminal sacrifice.

#Adrenal: weights in females decreased at 75 and 150mg/kg/day at 2 yr sac.; may be attributable to body weight decreases.

††Schulze 1991b Adrenal weights increased; no dose response; considered unlikely to be exposure related.

†Schulze 1991b Adrenal: weight changes and histopathology (hypertrophy zona glomerulosa) at 300 mg/kg/day (far exceeded TSRC and systemically toxic); zona glomerulosa does not respond to HPA axis.

‡‡Stott 1995b Relative testes weight increased but not absolute weight at 24 month only; attributed to body weight decrease.

§§Dalgaard 1993a Testes weight decreased at 10 mg/kg/day (dose exceeded MTD).

#### Endpoint scores in parentheses based on Borgert et al. 2014 (modified in some cases based on context or strength of response)

1. (1) specific and sensitive to the hypothesis
2. (2) potentially sensitive for the hypothesis; stronger if correlated with Rank 1 data
3. (3) relevant, but useful only if corroborating Rank (1) or (2) endpoints
studies, few endpoints were observed suggesting increased or decreased estradiol, even at dose levels causing significant systemic toxicity.

Ovary weights were increased at 300 mg/kg/day in the Schulze (1991a) subchronic rat study; there were no histopathological findings in the ovaries correlating with this change. No other changes suggesting increased estradiol were seen in this study, nor were any findings in females suggesting decreased estradiol. In contrast, the Jeffries et al. (1995) chronic rat study showed decreased ovarian weights at the high dose (which caused marked systemic toxicity and weight loss), again without histopathological correlates. The Jeffries et al. study also showed a decreased incidence of pituitary tumors of the pars distalis, which is an estrogen-sensitive tumor (Dinse et al. 2010), and a decreased incidence of mammary gland hyperplasia in 2,4-D exposed rats, also at the severely toxic high dose. The extent of weight loss at this dose confounds any attribution of these findings to decreased estradiol. The mouse subchronic (Schulze 1991b) and chronic studies (Stott 1995a) showed no effects on uterine or ovarian histopathology. The dog subchronic (Schulze 1990; Dalgard 1993a) and chronic (Dalgard 1993b) studies similarly showed no effects on uterine, vaginal or mammary gland histopathology.

There were no effects in the Marty et al. (2010) EOGRTS study considered indicative of either increased or decreased testosterone:

- Preputial separation was slightly delayed in the F1 males at 800 ppm, which was attributed to decreased growth during lactation and post-weaning, as discussed previously;
- There were no exposure-related effects on developmental landmarks, including AGD (measured in all F1 pups),
nipple retention (measured in non-culled F1 pups in all
dose groups);
- There were no effects on sperm counts, motility or
morphology;
- There were no exposure-related effects on reproductive
indices, including mating, fertility and time to mating;
- There were no exposure-related effects on reproductive
organ or accessory sex tissue weights at any dose of 2,4-
D; and
- 2,4-D did not alter reproductive organ or accessory sex
tissue histopathology.

There were comparatively a few relevant parameters eval-
uated in the Rodwell and Brown (1985) two-generation study.
Mating and fertility were not affected, nor were testicular
weights and histopathology, or histopathology of the epidi-
ymides, seminal vesicles (weanlings only) and prostate
(weanlings only).

There were no exposure-related effects on testicular weight
or histopathology in the Marty et al. (2010) EOGRT study attrib-
utable to interactions with steroidogenesis, or in the Rodwell
and Brown (1985) two-generation reproductive toxicity study.
A single finding in the Rodwell and Brown (1985) two-gener-
ation study potentially showing a steroidogenesis interaction
was that the 80 mg/kg/day F1a pups showed a statistically sig-
nificant change in sex ratio (109 males and 71 females), com-
pared with the controls. As discussed previously, this finding
was not repeated in the F1b pups at a higher dose and is con-
sidered unlikely to be exposure-related.

The Schulze (1991a) subchronic rat study showed flaccid
testes and testicular atrophy at the very high and systemically
toxic dose of 300 mg/kg/day (that exceeded both the TSRC
and a classic MTD). The high-dose testes findings could hypo-
thetically be associated with changes in steroidogenesis
(excess estradiol and/or decreased testosterone) secondary to
systemic toxicity; they could as readily reflect changes to the
HPG axis caused by the severe systemic toxicity at these
excessive doses, or reflect direct target organ toxicity not
mediated by an endocrine interaction, such as that related to
stress. Given that there is no substantive evidence of
increased estradiol or decreased testosterone in the 2,4-D
mammalian studies as a whole, the stress or systemic toxicity
seem the most likely hypotheses. Decreased testes weight
was also seen at the systemically toxic high dose in the
chronic rat study (Jeffries et al. 1995). Testicular atrophy
was noted in 2/10 animals at the interim sacrifice in this study;
however, no exposure-related testicular lesions were evident
at the terminal (two-year) sacrifice.

Mice in a subchronic study (Schulze 1991b) showed no
effects on male reproductive tissues. Increased testicular
weight was seen at the high dose in a chronic mouse study
(Stott 1995b); there was no histopathological correlate to the
testicular weight finding.

Dogs in 2,4-D subchronic toxicity studies (Schulze 1990;
Dalgard 1993a) showed decreased testes weights at systemic-
ally toxic doses; as discussed previously, this finding is not
considered likely to be endocrine-mediated, but rather to
reflect a combination of the immature age of the test animals
and, possibly, delayed development due to systemic toxicity.

These findings were at doses exceeding the TSRC in dogs.
There were no testicular or prostate effects in the chronic
dog study (Dalgard 1993b). As discussed previously, dogs are
not relevant for human health risk assessment; however, as a
susceptible species, the dog may predict potential effects on
other species deficient in the OAT-1 transporter. Therefore,
data from the dog studies are included in the WoE evalu-
ation. It should be noted particularly that all potentially endo-
crine-related effects in the dogs were seen at dose levels that
also caused other marked systemic toxicity. An EPA Science
Advisory Panel has agreed with the EPA position that
responses observed in endocrine disruption assays in the
presence of overt toxicity are “not useful for interpretation of
whether a compound has an endocrine effect” (US EPA 2013).
Thus, there does not appear to be any particular susceptibil-
ity to potentially endocrine-related effects.

There were no effects on adrenal weight or histopathology
in the EOGRT study (Marty et al. 2010). The Schulze (1991a) rat
subchronic study showed adrenal weight changes and histo-
pathology (hypertrophy of the zona glomerulosa) at 300 mg/
kg/day (far above the TSRC and systemically excessively toxic).
The adrenal zona glomerulosa is responsible for production of
mineralocorticoids such as aldosterone and does not respond
to changes in the hypothalamic–pituitary–adrenal (HPA) axis.
Consequently, this finding is not considered evidence of an
interaction with the steroidogenesis pathway. Adrenal weight
changes (in opposite directions) were also seen in the mouse
subchronic study (Schulze 1991b) and in the chronic rat study
(Jeffries et al. 1995). No exposure-related histopathology was
found in the adrenals in these studies.

In conclusion, 2,4-D does not show robust evidence of
interaction with the steroidogenesis pathway(s) at environ-
mentally relevant exposure levels. Mammalian studies, includ-
ing a comprehensive EDSP Tier 2 equivalent EOGRT study, fail
to show coherent evidence of alterations in estradiol synthe-
sis or testosterone synthesis at doses below the TSRC. Even
at high doses, findings are limited and may reflect direct tar-
get organ toxicity without an endocrine-mediated mechan-
ism, e.g. effects associated with excessive systemic toxicity.
There are no robust effects in the FSTRA indicating altered
steroidogenesis, and a quail reproductive toxicity study
showed no findings suggesting altered steroidogenesis. The
in vitro EDSP steroidogenesis and aromatase assays were
negative, as was the ToxCast™ aromatase assay.

**Evaluation of potential interaction with the HPG axis**

The majority of parameters potentially under control of the
HPG axis were unaffected in the Marty et al. (2010) EOGRT
study as discussed above. Absolute and relative (fixed) pituit-
ary gland weights were significantly decreased by 9 and 8%,
respectively, in the high dose males in one set of F1 adult
animals. The magnitude of the differences from pituitary
weights in control animals, however, was extremely slight
and the absolute and relative pituitary weights in the
800 ppm males were within the historical control range. No
exposure-related pathological changes were seen in these
tissues. Additionally, toxicologically significant alterations in
pituitary function would be expected to alter numerous other
study endpoints, including reproductive and accessory sex gland weights and sperm parameters. These endpoints were not affected by 2,4-D exposure in this study. It is concluded that this study shows no robust evidence of an HPG axis interaction.

There is limited and inconsistent evidence of an HPG axis interaction in the other key toxicity studies of 2,4-D. As previously discussed, in the Rodwell and Brown (1985) reproductive toxicity study, there was an altered pup sex ratio in the F1a litters; this finding as noted above is considered unlikely to be exposure related. Pituitary weights were decreased in females in the Schulze (1991a) rat subchronic toxicity study at the high and systemically toxic dose of 300 mg/kg/day (exceeding an MTD and far exceeding the TSRC) but were increased in males; there were no histopathological correlates in either sex and the exposure relationship is considered equivocal. A decrease in tumors in the pars distalis of the pituitary was seen in the Jeffries et al. (1995) rat chronic toxicity study; the decreased incidence of this estrogen-responsive tumor is attributed to the marked weight loss at the excessively toxic high dose.

WoE evaluation for potential effects of 2,4-D on the HPT axis

The HPT axis is an integrated system involving various positive and negative feedback systems to control production of thyroid hormones. To characterize these feedback systems, the use of an intact, in vivo model is required. For evaluation of potential HPT axis interaction, it is useful to characterize whether (if there are effects) the compound is acting as a thyroid agonist or antagonist. Agonists show increases in circulating thyroid hormone levels (e.g. T4) but may or may not show increased TSH and slight evidence of thyroid histopathology (reduced colloid) at the 600-ppm dose level suggesting an adaptive exposure-related effect. There is a mechanistic basis for this finding, in that high dose 2,4-D has been shown to displace thyroxine from serum binding protein in the rat (Van den Berg et al. 1991). This displacement could lead to easier excretion or hepatic sequestration of the free hormone. Further, thyroids in dams are stressed during gestation by the need to supply thyroid hormone to the developing fetuses, making the dams relatively hypothyroid and vulnerable to such an effect. In addition, because of increased doses, feed consumption during gestation and the lack of dietary concentration adjustment during this critical stage, dams were receiving 2,4-D at a significantly higher internal dose than animals did at most other time points in the study. The lack of adversity is demonstrated by: the lack of replication in dams at lactation day 22, showing reversibility; the slight severity of the findings; and the lack of adverse findings that might be associated with decreased thyroid function in the F1 pups. For example, there were no findings in the developmental neurotoxicity (DNT) component of the EOGRT study (Marty et al. 2010) consistent with thyroid hormone modulation. No exposure related effects on auditory startle, brain morphometry or myelin deposition changes were seen, demonstrating the lack of an adverse thyroid hormone deficiency during fetal and pup development.

The Schulze (1991a) subchronic rat study showed stronger effects on thyroid hormone economy at doses...
≥100 mg/kg/day. Gorzinski et al. (1981a, 1981b) showed decreased T4 at 100 mg/kg/day. Schulze (1991a) also identified exposure-related effects on thyroid histopathology in female rats at a dose exceeding an MTD (300 mg/kg/day); even then, the histopathological effect was limited to follicular cell hypertrophy, which may be regarded as adaptive. Mice in the subchronic study (Schulze 1991b) showed decreased T4 at the high dose but no changes in thyroid weight or histopathology; mice in the chronic studies (Stott 1995a, 1995b) showed no effect on thyroid histopathology in either females or males.

In contrast to rodent study results, the subchronic dog study by Schulze (1990) tested dogs to doses above the MTD and saw no consistent effects on the thyroid. (Relative thyroid weight was increased at the high dose, but absolute weight was not affected; this finding is attributed to body weight loss at the high dose.) There were no effects in this study on thyroid hormone measurements (T3 and T4), nor on thyroid histopathology. The other subchronic dog study (Dalgard 1993a) and the chronic dog study (Dalgard 1993b) showed no effects on thyroid weights or histopathology, despite testing systemically toxic doses exceeding the dog TSRC. The dog studies provide data supporting that rodent species (especially rats) are particularly vulnerable to changes in thyroid hormone economy.

Charles et al. (1996a) presented data from rat subchronic toxicity studies conducted with 2,4-D DMA, or 2,4-D 2-EHE. Endocrine endpoints relevant to the thyroid pathway included: thyroid hormones (T3 and T4); thyroid (and parathyroid) organ weights and histopathological evaluations. Decreased T4 and/or T3 was observed at dose levels of ≥100 mg/kg/day, with T4 appearing somewhat more sensitive than T3 and females more sensitive than males. Correlating with these findings were increases in relative thyroid weights (primarily at 300 mg/kg/day); however, no correlating histopathological evidence of thyroid follicular cell hypertrophy or hyperplasia was evident at any dose. Therefore, these changes are considered slight in severity and non-adverse.

Charles et al. (1996b) also described data from dog subchronic toxicity studies conducted with 2,4-D DMA, or 2,4-D 2-EHE. Endocrine endpoints evaluated in these studies relevant to the HPT axis included: pituitary, thyroid (and parathyroid) organ weights and histopathological evaluations. Thyroid hormone analyzes were not performed in these studies, which is a weakness for evaluating potential thyroid effects. In contrast to the rat, there were no findings in dogs supporting an interaction with the HPT axis, even though the high doses in these studies were markedly systemically toxic.

Discussion of mechanism of high dose-specific effect on HPT axis in rodents

Plasma protein binding appears to protect circulating thyroid hormone from metabolism and clearance by the liver. Thus, if 2,4-D is a relatively weak competitor with thyroid hormone for binding sites or transport/carryer protein, high exposures to 2,4-D would be anticipated to result in increased free thyroid hormone which would be subject to enhanced sequestration and/or excretion by the liver. This would not be an outcome anticipated at low exposure levels, however, which is consistent with the thyroid-related findings being limited to high-dose 2,4-D exposure in the rodent subchronic and chronic toxicity studies summarized above.

The Florsheim et al. (1963) study supports the idea that high doses of 2,4-D in the rat may modulate thyroid hormone levels. These data, in conjunction with the Van den Berg et al. (1991) study, support that the likely primary mechanism is 2,4-D competition for the thyroxine serum binding sites, particularly transthyretin. It should be noted that even in the chronic rat toxicity study of 2,4-D (Jeffries et al. 1995) there was no frank progression to thyroid follicular cell hyperplasia or neoplasia, suggesting changes in circulating thyroid hormone levels were sufficiently mild to not elicit biologically adaptive and sustained elevations of TSH. Therefore, these findings provide a possible mechanistic explanation for decreases in circulating thyroid concentrations in rats selective to high dosages of 2,4-D, but do not provide evidence of a biologically significant adverse effect.

The rat is more likely to be susceptible to this mechanism than the human because the predominant thyroid hormone binding protein binds thyroxine less tightly than that of the human. In humans and other primates, thyroxine-binding globulin (TBG) is the principal protein that binds T4 (Dohler et al. 1979). It has a very high affinity for T4: only about 0.03% of the T4 in serum is in the free unbound form (Hill et al. 1989). Binding sharply reduces clearance of T4 from serum. Rats do not have TBG, and most T4 in rat serum is bound to albumin and transthyretin. The binding affinity of T4 for TBG is more than a 100 times greater than that of albumin or transthyretin (Hill et al. 1989), and the difference contributes to the higher rate of T4 clearance in rats. Further evidence that the rat is an overly sensitive species is the lack of thyroid findings in the subchronic and chronic 2,4-D dog studies, conducted at doses clearly exceeding the TSRC in that species.

Overall, there are no findings in the 2,4-D studies suggesting an adverse effect on the thyroid or clear evidence for an HPT axis interrelationship at doses below the TSRC. Adaptive changes were seen in pregnant dams during a susceptible life stage in the Marty et al. 2010 EOGRT study (also at a dose exceeding the TSRC); the mechanism for these high-dose specific findings has been characterized. No adverse effects were observed on offspring, either for thyroid parameters or in assessment of potential developmental neurotoxicity. The thyroid does not appear to provide a POD or driving effect for 2,4-D risk assessment because more sensitive indicators of toxicity are present, which occur within the linear TK range. It should be further noted that the serum protein binding to thyroxine in humans is considerably stronger than in rats (Jahnke et al. 2004), providing an extra margin safety for humans to any potential thyroid toxicity.

Interestingly, despite reports of good interspecies concordance for the HPT axis, the AMA (Coady et al. 2010) was negative up to the limit dose tested. This lack of concordance may relate to 2,4-D’s postulated mechanism for thyroid hormone effect, which is displacing bound thyroxine from the binding proteins used for systemic transport, making the thyroxine more available for excretion or hepatic storage. It is
reasonable to hypothesize that the affinity of the binding protein to circulating thyroid hormone in frogs is different from the affinity of the binding protein in rat and that the difference in interspecies response might be attributable to that difference; no research directly addressing potential differences has been identified.

The WoE shows that, though there is weak evidence of 2,4-D potentially interacting with the HPT axis, this is very unlikely to result in any adverse effects at exposure below the relatively high doses characterizing the onset of the TSRC, even in rodents. No adverse effects on the thyroid, or adverse sequelae to the offspring, including effects on myelination or brain morphometric parameters, were identified in the Tier 2 equivalent EOGRT study up to the highest dose tested. The interaction has been studied across life stages and there is a high degree of confidence in this conclusion. Exposure to 2,4-D did not result in any thyroid-related effects in frogs tested up to the limit dose. The interaction has been studied across life stages and there is a high degree of confidence in this conclusion. Exposure to 2,4-D did not result in any thyroid-related effects in frogs tested up to the limit dose.

The Goldner et al. (2013) assertion of biological plausibility for a specific association of 2,4-D with hypothyroidism or thyroid disease in humans is very tenuous. Although the hypothyroid associations reported in Goldner et al. (2013) included positive associations with multiple herbicides and insecticides, the Stoker “paper” used by Goldner et al. to justify biological plausibility of their reported epidemiological findings specifically for 2,4-D is an abstract of an extremely high-dose study conducted in rats (100 and 200 mg/kg/day by oral gavage) which reported reductions in circulating thyroid hormone at both of the very high doses. This is consistent with findings in regulatory rodent toxicity studies of high dose decreases in T4 and adaptive changes in thyroid histopathology (limited to colloid depletion and in some cases hypertrophy, without evidence of hyperplasia or follicular cell tumors) at doses substantially exceeding the TSRC. Importantly, however, Stoker later published an abbreviated summary of these findings in a book chapter (Stoker & Zorrilla 2010) in which it was noted that the 2,4-D thyroid effects were not detected at the next lower dose of 30 mg/kg/day: “... and the herbicide 2,4-diphenoxycetic acid (2,4-D) (sic), which induced renal toxicity at both 3 and 30 mg/kg and did not alter thyroid hormone (T4) or any of the other male pubertal endpoints until 100 mg/kg …”.

The Stoker and other related toxicity and biomonitoring data are thus not causally supportive of human thyroid disease, and in fact demonstrate an extremely low biological plausibility for any such outcome for the following reasons. First, it is well established that oral gavage doses of 100 mg/kg are well above the TSRC of 2,4-D in rats, and regulatory guidance addressing dose selection for animal bioassays, including the EOGRT, has cautioned that toxicity observed above saturating doses is not relevant for human risk assessment if there is a large disparity between doses reflecting onset of the TSRC compared to real-world human exposures (OECD 2012a, 2012b, 2012c). Second, weakly active non-adverse thyroid effects were observed in the high dose only in pregnant dams in the robust EOGRT study, but importantly, that high dose also was demonstrated to be well above the TSRC in females, and particularly in pregnant females. Third, dog studies of 2,4-D showed no evidence of thyroid toxicity even at lethal doses. In addition, 2,4-D blood concentrations are substantially higher in dogs than rats administered equivalent external doses (van Ravenswaay et al. 2003), primarily because dogs do not clear 2,4-D as efficiently as rats and humans do (Timchalk 2004). Fourth, the recently completed EDSP Tier 1 assays failed to detect any signal of adverse thyroid activity in frogs, in which certain developmental changes are specific for thyroid toxicity. The lack of findings in the dogs and the frog supports the conclusion that the rat is uniquely susceptible to hypothyroidism due to the poor binding of T4 to the carrier proteins in rat blood (Jahnke et al. 2004) making the rat T4 uniquely susceptible to competitive displacement by 2,4-D (van den Berg et al. 1991). Finally, a lack of biological plausibility is further affirmed by the extremely large margin of exposure between biomonitored 2,4-D doses reported for male farm-worker applicators in the Ag Health Study itself and the NOEL dose for thyroid effects in rats reported by Stoker. The Alexander et al. (2007) study of farm families identified a geometric mean exposure dose for male applicators of 2.46 μg/kg/day, which is approximately 10 000X below the NOEL of 30 mg/kg/day (30 000 μg/kg/day) for thyroid effects identified by Stoker and Zorrilla (2010). Importantly, the geometric mean dose for female spouses living in close proximity to active 2,4-D application operations was 0.08 μg/kg/day, and was substantially disparate (> 300 000) from the approximately 25 mg/kg/day dietary dose identified as the inflection point for onset of TSRC in female rats (a non-thyroid toxic dose in rats). These large margins of exposures have been confirmed in other high quality biomonitoring studies of farmer-applicators in which a geometric mean dose of 1.6 μg/kg/day was reported (Thomas et al. 2010).

Thus, a WoE evaluation of potential effects of 2,4-D on the HPT axis indicates no concern for a hypothyroid disease or thyroid tumor outcome in humans.

Conclusions

The Tier 1 EDSP studies and the mammalian Tier 2 EDSP equivalent EOGRT dietary toxicity study of 2,4-D are reliable studies and provide a robust basis for assessing interactions of 2,4-D with the estrogen, androgen and steroidogenesis pathways, and the HPT axis. Key conclusions from the WoE evaluation of the EDSP studies and key toxicological studies include:

- 2,4-D clearly does not demonstrate the potential to interact directly with the estrogen pathway in toxicological studies, including an EDSP Tier 2 equivalent mammalian EOGRT dietary toxicity study in which the top dose exceeded the TSRC, a FSTRA tested to the limit concentration, and a quail reproductive toxicity study, or in high quality studies from the published literature. In addition, EDSP Tier 1 in vitro assays, high quality published in vitro assays, and ToxCastTM in vitro screening studies were negative for estrogen pathway interactions.
- 2,4-D does not demonstrate the potential to interact directly with the androgen pathway in toxicological studies, including an EDSP Tier 2 equivalent mammalian EOGRT dietary toxicity study in which the top dose exceeded the
TSRC, a FSTRA tested to the limit concentration and a quail reproductive toxicity study, or in high quality studies from the published literature. In addition, EDSP Tier 1 in vitro, high quality published in vitro assays and ToxCast in vitro studies were negative for androgen pathway interactions.

- 2,4-D showed no robust evidence of interaction with the steroidogenesis pathway in an EDSP Tier 2 equivalent mammalian EOGRT study in which the top dose exceeded the TSRC. 2,4-D effects on steroidogenesis parameters in other studies are likely related to high-dose specific systemic toxicity at doses exceeding the TSRC and are not likely to be endocrine mediated.

- 2,4-D showed no adverse interactions with the HPT axis in an EDSP Tier 2 equivalent mammalian EOGRT study in which the top dose exceeded the TSRC. It interacts with the HPT axis in rats (which is clearly a species susceptible to thyroid interactions and not predictive of thyroid effects in other species for compounds acting on the thyroid by the mechanism demonstrated for 2,4-D—displacement of thyroxine from plasma-binding sites) at high doses exceeding the TSRC in mammals and substantially exceeding human systemic doses identified in high quality biomonitoring studies. The thyroid-sensitive AMA tested to the assay limit concentration was negative.

- The EOGRT dietary toxicity study is an acceptable EDSP Tier 2-equivalent mammalian EOGRT study in which the top dose exceeded the TSRC, and predicts no adverse endocrine-related toxicity to mammals. This study provides a robust basis for concluding that the NOAEL for any endocrine effects is higher than the NOAELs currently used as points of departure for acute, subchronic or chronic human health risk assessment.

- No studies, including high quality studies in the published literature, predict significant endocrine-related toxicity or functional decrements in any species at environmentally relevant concentrations, or, in mammals, at doses below the TSRC that are relevant for human hazard and risk assessment.

Overall, there is no basis for concern regarding a potential for interaction of 2,4-D with endocrine pathways or axes (estrogen, androgen, steroidogenesis, or thyroid), and thus 2,4-D is unlikely to pose a threat from endocrine disruption to wildlife or humans under conditions of real-world exposures. This conclusion is consistent with a similar but less comprehensive WoE review of the 2,4-D endocrine disruption data conducted the US EPA (US EPA 2015), which stated that there was “no convincing evidence of potential interaction of [2,4-D] with the estrogen, androgen or thyroid pathways.” In addition, EPA concluded there was no need for additional EDSP Tier 2 testing given the availability of the EOGRT study that was regarded as equivalent to the EDSP Tier 2 study.

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In memoriam, Barbara Neal, DABT (prepared by James Lamb):

Barbara Neal and I worked together for over 20 years. The response by her colleagues to her passing remind me how valued she was in the field of toxicology. Barbara and I evaluated dozens of various issues in reproductive, developmental and endocrine responses. She approached work and life with a sense of humor and passion that will be missed by me and others in toxicology. Very few will ever match her keen observations and care at interpretation.

Barbara would dig into data more deeply than most. She would find interesting and useful information often missed by others. The paper by Barbara in this issue of Critical Reviews in Toxicology is a perfect example of Barbara’s passion for paying attention to detail. She has analyzed and understood the vast dataset on 2,4-D in a way that no one else could match.

Barbara was a creative and open-minded toxicologist who always looked for more clear ways to describe data, and stronger methods to test a hypothesis. She had no patience with sloppy or convenient interpretations. She would become annoyed with scientists who worked to prove their own pre-existing views, which she felt had become far too common and too adversarial. Barbara always sought an honest answer to an honest question without malice or some hidden agenda. She had no patience for hiding or overlooking results to prove a point.

Barbara worked with her own unique sense of humor. She enjoyed puns and surprising twists in a story. You could often hear her chuckling in a crowd, often at her own joke. She laughed often, even at herself, but never at the expense of others.

Barbara Neal was a special scientist and person who will be deeply missed by many of us in toxicology, which she called home.

Barbara passed away on October 19, 2015, during the final stages of this manuscript preparation. She was a member of the Society of Toxicology and a Diplomate of the American Board of Toxicology, and held the position of Senior Managing Scientist at Exponent, Inc. since 2010. Her career in toxicology extended over 30 years, and included earlier positions at The Weinberg Group, Inc., BBL Sciences, and Battelle Columbus Laboratories.

Declaration of interest

The employment affiliation of the authors is as shown on the cover page. This review was funded by the Industry Task Force II on 2,4-D Research Data [Authors Neal (deceased), Bus, Williams, Staveley and Lamb work for Exponent, which is a consulting company that has performed work for the Industry Task Force II on 2,4-D Research Data, as well as for individual member companies of the Task Force who manufacture 2,4-D. Authors Coady and Marty work for The Dow Chemical Company, which manufactures 2,4-D. On behalf of a previous employer and manufacturer of 2,4-D (The Dow Chemical Company), author Bus has engaged in a single litigation case (defendant deposition).] The review is the exclusive work product of the authors. The professional opinions expressed and the conclusions drawn are those of the authors and not necessarily those of their employers or the sponsors. This review was funded by the Industry Task Force II on 2,4-D Research Data.

Supplemental material

Supplemental material for this article is available online here.

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