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Hypothesis-driven weight of evidence analysis to determine potential endocrine activity of MTBE

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ABSTRACT

Endocrine-related endpoints in animals have been reported to respond to high doses of methyl tertiary-butyl ether (MTBE), however, a systematic and transparent evaluation of endocrine potential has not been published. Resolving whether MTBE exhibits endocrine activity is important given regulatory and public interest in endocrine disrupting substances and their potential for causing adverse effects in humans or wildlife. A weight-of-evidence (WoE) analysis was conducted, focusing on hypotheses related to the potential for MTBE to interact with estrogen, androgen, and thyroid pathways, and steroidogenesis. To reach scientifically justified conclusions based on the totality of evidence, this WoE procedure involved a semi-quantitative relevance weighting of each endpoint for each hypothesis and systematic consideration of each endpoint in various study designs. This procedure maximized use of an extensive body of relevant and reliable literature on MTBE with evidence supporting or opposing a given mode of action hypothesis. Evaluating the strength and consistency of observations from many MTBE studies also provided a way to assess whether high doses used in experiments with MTBE confound identification of direct endocrine system responses. Based on results of studies using mammalian and fish models and *in vitro* screening assays, this WoE assessment reveals that MTBE lacks direct endocrine activity.

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1. Introduction and background

Methyl tertiary-butyl ether (MTBE, CAS RN 1634-04-4) is an additive in gasoline used to increase the efficiency of combustion and bring associated environmental benefits. MTBE also has minor uses as a solvent in closed systems. Endocrine-related responses in rodents have been observed at high doses of MTBE, and endocrine activity has been theorized as a mode of action (MoA) responsible for some of these effects (Bird et al., 1997; McGregor, 2006; Cruzan et al., 2007). However, to date, there has been no comprehensive, detailed, and systematic review of the database on MTBE that weighs the evidence with attention focused on specific endocrine pathways. Given regulatory and public interest in endocrine active substances and their potential for causing adverse effects in humans or wildlife, resolving whether MTBE exhibits endocrine activity is important. The goal of this paper is to determine if MTBE acts through an endocrine MoA. To achieve this we used a robust and transparent WoE process. The WoE analyses performed were focused on specific hypotheses addressing the estrogen, androgen, and thyroid hormonal pathways and steroidogenesis, including an analysis of studies related to aromatase, the steroidogenic P450 enzyme that converts androgens to estrogens. Studies pertaining to both mammalian and non-mammalian organisms were considered and used in the WoE assessment if relevant to any of the hypotheses.

A WoE approach has been in the health risk assessment lexicon for many years but may have different meanings to different people (Weed, 2005). The US Environmental Protection Agency Endocrine Disruptor Screening Program (US EPA EDSP) has developed guidance for using a WoE approach to evaluate and integrate all relevant scientific and technical information from Tier 1 screening tests to draw conclusions about the need for further testing (US EPA, 2011). Others further recommend objective, systematic and structured hypothesis-driven approaches for WoE evaluations (Rhomberg et al., 2010; Bars et al., 2011, 2012; Borgert et al., 2011a,b, 2014). The WoE approach chosen for this analysis is semi-quantitative, based on ranking of the relevance of responses following a pre-defined framework that should be blind to the study outcomes, with an assessment of the reliability of data applied to each endpoint and study (Borgert et al., 2011a,b, 2014).

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Briefly, this involved systematic consideration of each endpoint observed in one or more study designs, and a semi-quantitative weighting of the relevance of each type of response to a given hypothesis, to reach scientifically justified conclusions based on the totality of the evidence. It is important to emphasize that, unlike some approaches that weigh relevance of an effect observed strictly on the basis of type of study design (e.g., *in vivo* vs. *in vitro*), this analysis is aimed at discerning which endpoints are the best predictors of mode of action. Study purpose, study design and the ability of the design to meet that intended purpose are also carefully considered in terms of reliability. In the end, conclusions were based on a simple classification system of pro- or anti-hormone effects, and then evidence for (or against) direct interaction with the endocrine system versus secondary effects of toxicity is discussed in more detail. Indirect effects of systemic toxicity, unrelated to a primary endocrine mode of action, are an important consideration for regulatory classification of any chemical, including MTBE.

This evaluation is timely since the US EPA has included MTBE on a list of chemicals to be screened under the EDSP. Listing is based on potential exposure and other statutory considerations, but does not imply that the US EPA suspects those chemicals of interacting with endocrine systems of humans or other species. Tier 1 of the EDSP uses a battery of assays to determine if a chemical has the potential to interact with the endocrine system. Identifying adverse effects is not the focus of the Tier 1 battery, and no single assay alone is sufficient to determine if there is the potential for the chemical to interact with the endocrine system. Further testing of those chemicals that show potential to interact would be needed to identify adverse effects and, with an understanding of relevant environmental exposure, quantify the risk.

The main focus on endocrine activity is not intended to imply that this should be viewed as the most important toxicological concern for MTBE. Acknowledging that many study endpoints can be affected by multiple modes of action is key to conducting an objective and scientifically sound WoE analysis (Rhomberg et al., 2010). The appearance of increased (or decreased) hormone-dependent cell growth, changes in hormone-dependent organ weights and histology, *in vitro* hormone receptor-ligand changes, altered hormone concentrations, include some of the observations that may collectively imply the potential for endocrine activity when there is sufficient strength and consistency of the responses. However, none alone is sufficient evidence of an endocrine mode of action, and these may also be affected by non-endocrine mechanisms, for example induced or diminished catalytic activity of liver enzymes that metabolize steroid hormones.

An objective evaluation of potential endocrine activity must also consider the fact that many toxicology studies administer doses of chemicals that far exceed possible occupational, consumer, or environmental exposures. This conservative approach to testing chemicals has some justification, but one must also always keep in mind that excessive doses of any chemical increase the probability of systemic toxicity and effects on endocrine endpoints mediated indirectly by other effects. Typical concentrations of MTBE in air and water are many orders of magnitude below the exposures tested in MTBE toxicology studies using experimental animals. Likewise, tissue concentrations of MTBE measured in human populations when MTBE was in widespread use are also many orders of magnitude below the concentrations tested in *in vitro* toxicology studies.

2. Methods

2.1. Literature search

National Library of Medicine search engines, PubMed and Toxnet, were used with the following keywords: methyl t(ertiary)-butyl ether, MTBE, tert-butyl methyl ether, 2-methoxy-2-methyl-propane and toxicity. Open peer-reviewed literature was supplemented with additional unpublished and other 'grey' literature resources in comprehensive MTBE reviews (ATSDR, 1996; McGregor, 2006), as well as studies listed in the European Union risk assessment report (EU, 2002) and updated in the REACH submission (ECHA, 2012).

Maximal use of all existing relevant and sufficiently reliable information was a goal. Consistent observations in multiple studies and multiple species with different experimental designs can reduce unknown bias or confounding and increase support for or against a given mode of action hypothesis (Boobis et al., 2008). Risk assessors and others increasingly acknowledge that all of these types of studies can have merits, whether or not they were conducted with strict adherence to standard guidelines and good laboratory practices (GLP) (McCarty et al., 2012; Batke et al., 2013). All studies were first considered for relevance and then for reliability, if endpoints measured were deemed sufficiently relevant.

An MTBE study did not have to have an endocrine focus in order to be considered relevant. Results of reproduction/fertility and prenatal developmental toxicity studies that encompass different life stages, and also studies that provide carcinogenicity information on endocrine organs were all considered relevant to a consideration of endocrine activity (OECD, 2012). All endpoints associated with the endocrine pathways addressed by the hypotheses were considered relevant, including, for example, subchronic, or chronic general toxicology studies reporting gonadal, thyroid or hormone-dependent weights of organs or histopathology. If an MTBE study had an endocrine organ endpoint related to any of the hypotheses then it was included in the initial literature review.

A few studies were eliminated from consideration after the initial review of literature. For example, a study with exposure to gasoline vapor condensate with and without MTBE (Benson et al., 2011) was not included due to the confounding effects of exposure to various gasoline components. Three *in vitro* studies (Li and Han, 2006; Li et al., 2007, 2009) using cells from a rodent endocrine organ, specifically mouse or rat testis, had no measured endpoints that could be related to the hypotheses so were also excluded before reliability scoring.

2.2. Data quality assessment

Considerable toxicity testing of MTBE was conducted during the 1980s (Duffy et al., 1992) before adoption of study protocols and guidance for evaluating the potential for endocrine activity. Some study designs were generally similar to a standard test protocol, but relatively few were similar enough to qualify as guideline studies in the context of current methods for evaluating endocrine activity. While it is appropriate to have greater confidence in studies that have employed standardized and validated test methods and were conducted according to GLPs (Becker et al., 2009; Borgert et al., 2011b; McCarty et al., 2012), a non-guideline study could still be considered reliable if the methods were sufficiently well-documented and the results transparently and thoroughly reported. A prevailing objective was to retain as many sufficiently reliable studies as possible for the WoE analysis.

The data quality discussion about primary, secondary and tertiary validity in Borgert et al. (2011a and supplement) was consulted, as were other consensus opinions on principles and processes for judging reliability and quality of study designs and data (Klimisch et al., 1997; Schneider et al., 2009; Tluczkiewicz et al., 2013). Sufficient transparency in the documentation (secondary validity) and classification by Klimisch score became one of the most important criteria, insofar as adequate documentation of study details is necessary for making further judgments about study and specific endpoint reliability for the WoE analysis.

Primary validity judgments were also an important focus of the data quality assessment; for example, if results were likely to have been confounded by other factors or the result was not repeated either within the study or between studies then that was noted during the literature review. Whether or not the study design was capable of addressing the hypothesis in question and establishing causality (tertiary validity) was considered, although the lack of specific and probative positive controls in most studies reduced the ability to confirm biological plausibility. Evaluating validity of specific studies took place first during the detailed review of each study to extract potentially relevant endpoints, and continued throughout the analysis of the WoE information included in the tables.

In the current assessment, a Klimisch reliability score of 1 was possible only for studies performed according to a regulatory or near-regulatory guideline following GLP, so those that were not so identified initially ranked no higher than a 2. Recommendations from the "ToxRTool" (Schneider et al., 2009) were taken into consideration when evaluating the level of detail in study reports. Some publications describing non-guideline studies contained multiple experiments. A few included both *in vitro* and *in vivo* experiments that were evaluated independently, so it was possible for data from different experiments in a series described in a single publication to receive different scores. Scoring and interpretation of scores in the final WoE evaluation was as follows: 1 – Reliable without restriction (useful, check relevance for intended purpose); 2 – Reliable with restrictions (potentially useful, check relevance for intended purpose); 3 – Not reliable (significant methodological or documentation deficiencies, might be useful as supportive information); and 4 – Not assignable (documentation insufficient, but may still be useful as supportive information).

One abstract was never published as a full peer-reviewed paper and was eliminated at this stage with a Klimisch score of 4 for lack of detail (Almeida et al., 2004). Reliability of a published male rat study (Zavgorodnij et al., 2013) became questionable and was given a score of 3 after a close review of content revealed that method descriptions lacked sufficient detail for assessing reliability of the results. A published paper could be relevant and even sufficiently well documented, but one or more endpoints considered unreliable because of some shortcoming in methods or details missing from the description of that method that caused concern about reliability. For example, an *in vitro* experiment involving MTBE exposure of testosterone-producing isolated rat Leydig cells (de Peyster et al., 2003) lacked a nonspecific cytotoxicity measurement for comparison. That experiment was ultimately assigned a score of 3, whereas the *in vivo* companion studies in that report were considered sufficiently reliable to be scored as 2. Study information with reliability scores of 1 or 2 qualified for use in the final WoE analysis.

2.3. Weight of evidence analysis methods

The WoE approach used to evaluate the MTBE data was based on a relatively new method (Borgert et al., 2011a, 2014). This approach is transparent and systematic, and is also referenced in OECD and US EPA guidance for evaluating chemicals for endocrine disruption (OECD, 2012; US EPA, 2011). The method is a semi-quantitative hypothesis-testing approach for ranking or weighting study endpoints based on their relevance for a particular hypothesis. The hypotheses considered were whether or not MTBE exhibits the potential to interact with components of the estrogen, androgen, or thyroid pathways as an agonist or antagonist, or if MTBE induces or inhibits the steroidogenic pathway. While the Borgert et al. (2011a, 2014) procedure for WoE was primarily developed to evaluate the data coming from the US EPA EDSP, the framework and methodological approach is broadly applicable.

Endocrine relevant endpoints were identified from the large MTBE database. Study endpoints were assigned one of three ranks using the criteria designated in Borgert et al. (2014). Ranking the endpoints *a priori* allows for a transparent and objective assessment of the data and their importance in informing each hypothesis.

> Rank 1 endpoints are specific and sensitive for the hypothesis being evaluated. These can be interpreted without clarification from other endpoints and are rarely confounded by non-specific activity. Rank 1 endpoints are *in vivo* measurements only as *in vitro* responses are typically not able to identify a relevant biological effect.
> Rank 2 endpoints are also specific and sensitive for the hypothesis being evaluated but are less informative than Rank 1 as these are often subject to confounding influences or other modes of action. Rank 2 endpoints include both *in vitro* and *in vivo* data.
> Rank 3 endpoints are relevant for the hypothesis being evaluated but only when corroborative of Rank 1 and 2 endpoints. Rank 3 endpoints are not specific for a particular hypothesis and include some *in vitro* and many apical *in vivo* endpoints.

Not all study endpoints are equally meaningful for each hypothesis. In assigning ranks or "weighting" the endpoints, consideration was given to how well the data informed the specific hypothesis being tested. Thus, study endpoints do not always fall into the same rank for each of the hypotheses. For example, vitellogenin (VTG), the egg yolk protein in fish, is sensitive and specific for the estrogen agonist hypothesis. However, it is not relevant for the thyroid agonist or antagonist hypotheses. In addition, non-specific endpoints such as decreases in concentrations of hormone or VTG or altered gonado-somatic index (GSI) may be related to effects on the liver and are not necessarily a primary endocrine-related response. The endpoints identified from the review of the MTBE literature database relevant for each of the eight hypotheses were ranked and listed in tables. There are a number of endpoints in the MTBE literature that appear relevant for inclusion in an assessment of endocrine activity but are not in the US EDSP screens, and thus assignment of relevance ranks has not previously been proposed. Endpoints not evaluated by Borgert et al. (2014), including endpoint responses in adult animals versus pubertal animals, were included in the MTBE ranking tables only if literature was found that established the ability of that endpoint to address one of the hypotheses. The authors acknowledge that these proposed rankings could change as better understanding is achieved in evaluating and interpreting potential endocrine activity. However, ranking the endpoints *a priori* allows for a transparent and objective assessment of the data and their importance in informing each hypothesis.

After the endpoints were ranked in tables the applicable studies and responses were entered. Each hypothesis was evaluated independently. According to Borgert et al. (2011a, 2014), positive responses in rank 1 endpoints are a preliminary indication that the hypothesis is supported. Positive responses in both ranks 1 and 2 endpoints provide additional evidence that the hypothesis is supported. Rank 3 endpoints were then reviewed to determine consistency in response across the tested hypothesis. Conversely, negative responses in rank 1 endpoints are a preliminary indication that the hypothesis is not supported. Consistent negative responses in ranks 1 and 2 provide sufficient evidence that the hypothesis is not supported. In those cases, rank 3 endpoints are not meaningful since these are not as sensitive and specific for the hypothesis, being easily impacted by other mechanisms or modes of action. In situations where rank 1 endpoints are negative or there are none for that hypothesis, but there are positive rank 2

endpoints, rank 3 endpoints were consulted for consistency and relevance and the strength of the response was considered.

It is important to consider consistency of response in evaluating the data. Not unexpected for substances with a large database of studies conducted by a variety of methodologies and researchers, there are a number of examples in the MTBE database of conflicting responses (i.e., positive and negative for the same endpoint), as well as patterns of responses across endpoints that are not what would be expected by a particular mode or mechanism of action. It is likely that the extremely high doses used in many studies result in toxicity or physiological changes that do not have a primary basis in an endocrine pathway interaction. For example, Marty et al. (2011) has noted that systemic toxicity can impact many of the endocrine endpoints in the pubertal rat studies. Others have alerted investigators to the confounding effects of stress and systemic toxicity on measures of endocrine and other effects (Everds et al., 2013; Brown et al., 2000; Pellegrini et al., 1998). Reduced body weight gain, as well as ataxia and lethargy after dosing, have been noted in a number of MTBE studies (see Discussion Section 5). US EPA and OECD test guidelines specify that the highest dose should be at or just below the maximum tolerated dose (MTD), up to a limit of 1000 mg/kg/day in the mammalian studies (US EPA, 2009a,b,c,d; OECD, 2007a, 2009). This is also the case with many of the guideline chronic studies evaluating reproductive and developmental endpoints (e.g., OECD, 2007b). Most of the studies performed with MTBE, however, used top doses well above this limit dose guidance, making it difficult to differentiate between primary endocrine activity and responses that are secondary to overt toxicity or significantly altered toxicokinetics.

2.4. Examples of ranking the relevance of responses related to the different hypotheses

Scientific justifications for most of the endpoint ranking undertaken in this analysis are explained in Borgert et al. (2014) so will not be reiterated in detail. Some examples are discussed here for readers unfamiliar with the approach.

When considering estrogen or androgen pathway-related hypotheses, few *in vivo* assays are recognized as being specific and sensitive enough to be rank 1 for detecting "estrogen-like" and "androgen-like" compounds. A study based on a rodent uterotrophic assay design can detect estrogen agonists and antagonists using uterine weight as an endpoint, but only the estrogen agonist response of an increase in uterine weight has been formally validated for the uterotrophic assay.

The guideline Hershberger assay requires the measurement of five androgen-dependent tissues (US EPA, 2009a). A Hershberger-like assay (de Peyster et al., 2003) measured weights of prostates and seminal vesicles in castrated near-peripubertal rats. Although it differed in several respects from a guideline study (see Supplemental material), weights of prostates and seminal vesicles from this study were still considered rank 1 endpoints.

The fish short-term reproduction test (FSTRA) (US EPA, 2009e) is a multi-modal assay with endpoints that address estrogen, androgen, and steroidogenic pathways. VTG, in particular, is a sensitive and specific indicator of estrogen agonist potential when increased in male fish. Most of the other endpoints measured in this study, however, are ranks 2 or 3 as these are predominately apical in response. Higher level endpoints that reflect an integration of all underlying biological processes contribute to an assessment of a chemical's overall effect, but are not typically helpful for elucidating a mode of action.

Several *in vitro* assays have been sufficiently studied and validated for use in endocrine activity screening programs. As the *in vitro* screens lack metabolic capability and can be confounded by cytotoxicity, these are considered ranks 2 or 3 in the WoE ranking framework. Estrogen (ERB) and androgen receptor binding (ARB) assays were not designed to distinguish agonistic from antagonistic effects. This limitation of dependence on responses in other endpoints to further distinguish agonism from antagonism resulted in receptor binding responses in no higher than rank 2. The estrogen receptor transcriptional activation (ERTA) assay can distinguish estrogen agonists from antagonists so was assigned a rank of 2 for both estrogen hypotheses. The two hormone endpoints (testosterone and estradiol) measured in the *in vitro* steroidogenesis H295R cell screening assay, which was developed specifically to determine if a chemical alters the steroidogenic pathway by induction or inhibition, were both ranked 2 in the steroidogenesis hypotheses. The *in vitro* aromatase activity assay using recombinant human microsomes ranked 3 when assessing steroidogenesis inhibition. Although specific to measuring the aromatase effect of interest, the assay can detect an inhibitory effect on only one steroidogenic enzyme and generalized cytotoxicity, if not controlled for, could produce an apparent decrease in aromatase activity.

Reproductive, pre-/post-natal developmental effects studies, and also carcinogenicity bioassays of MTBE that characterize histopathology or weight of some hormone-producing or hormone-dependent organs, provided additional information for a few ranked endpoints. Other types of apical endpoints (e.g., pregnancy and fertility indices, embryofetal mortality, skeletal and soft tissue malformations and variations) are accepted as contributing to an overall assessment of a chemical's ability to cause some endocrine system perturbation; however, these are not usually helpful for elucidating a mode of action. Positive findings in these types of reproductive or developmental endpoints do not usually help to distinguish whether the effect observed is a result of direct or indirect involvement of the endocrine system or whether it should be classified as pro-hormonal or anti-hormonal.

Numerous factors must be considered when interpreting concentrations of serum hormones, organ weights, and histopathology. Altered circulating hormone concentrations could be secondary to many other primary changes involving non-endocrine mechanisms (e.g., increased metabolism of steroids by the liver, excessive weight loss, fluid retention in the body causing dilution of blood components, to name but a few). Homeostatic feedback control of hormone concentrations also means that single time-point measurements in blood can sometimes miss an effect or otherwise be unrepresentative of the trend and/or typical status of hormone concentrations the animal experienced throughout the study. Unless the response is well understood to be pro- or anti-hormonal, the change is hard to interpret and apply the result to an analysis of a specific hypothesis without additional information from more direct measures of endocrine function. For these reasons, hormone endpoints from these studies were not ranked 1 in this WoE analysis. That said, however, absence of any significant adverse findings in these types of studies, especially if consistent across studies, would be considered useful information that lends support for the position that the chemical has neither agonist nor antagonistic interactions with a given hormonal pathway.

3. Literature summary

3.1. Health effects studies of MTBE with endpoints relevant to endocrine activity

A brief overview of the types of studies relevant for this WoE evaluation is included here specifying route of exposure and range of doses used for each study. These basic elements of study designs complement other information about species and key endpoints examined shown in the WoE tables discussed in Section 4. Readers

wanting more detail about these studies are encouraged to consult the online Supplemental material containing much more about experimental designs, strains of the species tested, other treatment information (e.g., detailing timing and frequency if not daily, full range of MTBE doses/concentrations tested, vehicles or other substances co-administered for comparison), and other findings in these studies that helped to provide context for effects observed (or not observed), but ultimately were not considered specific or predictive enough to be included in the WoE tables. The Supplemental material also contains additional discussion of the reliability of some studies and offers specific concerns about methods and results that call into question the validity of their findings or conclusions. Other results, such as reduced amount of body-weight gain or other significant changes in non-endocrine organs, were noted in the Supplemental material to help place any endocrine findings into perspective.

Relevant positive (statistically significant) and negative data specifically related to ranked endocrine hypothesis endpoints were extracted from the studies for the WoE tables. Additional key information was also added to the tables to further facilitate analysis. For example, the test species was noted with the study reference in the tables in order to understand any species-specific response. In addition, differences in endpoints evaluated and experiment timing were noted in the Tables if results differed across multiple experiments described in a report.

3.1.1. In vitro studies

In vitro studies of estrogen receptor binding (ERB) and estrogen receptor transcriptional activation (ERTA) (Moser et al., 1998) were part of a series of experiments investigating the suggestion that MTBE had increased the frequency of liver tumors in female mice in a cancer bioassay (Bird et al., 1997) by a MoA involving estrogen insufficiency. These would not be considered as US EDSP screening guideline studies but the test methods employed were similar. No effect was observed when concentrations of MTBE were tested in the ERB assay ranging from 10^{-11} M to 10^{-4} M. Concentrations of MTBE tested in the ERTA assay, also revealing no activity, ranged from 10^{-8} M to 10^{-4} M. Formaldehyde and tert-butanol (TBA) were also tested in the ERB assay and found to be without effect.

In addition, three GLP-compliant endocrine screening assays following accepted guidelines were conducted using MTBE and TBA, the predominant metabolite of MTBE (de Peyster et al., 2014): (1) androgen receptor (AR) competitive binding in rat prostate cytosol following the US EPA OPPTS 890.1150 guideline; (2) aromatase inhibition activity in human recombinant microsomes following the US EPA guideline OPPTS 890.1200; and (3) steroidogenesis using the adrenal corticocarcinoma cell line H295R that expresses all enzymes needed to synthesize testosterone and estradiol, following the US EPA guideline OPPTS 890.1550 which is equivalent to the OECD test guideline 456. Concentrations of MTBE and TBA evaluated were 10^{-10} M–10^{-3} M in the AR binding and steroidogenesis assays, and 10^{-10} M–10^{-4} M in the aromatase inhibition assay. All of these *in vitro* endocrine assays showed absence of an effect.

3.1.2. Endocrine-focused studies in female and male rodent models

Studies included in this section were focused on determining whether MTBE altered one or more endocrine parameters in male or female rodents. In general, these studies were designed to address specific questions about effects of MTBE observed at high doses in previous studies. In most instances, these studies were targeted at answering specific questions, and included similarly high doses to try to replicate conditions in the previous MTBE studies.

Female mice were used in several MTBE gavage and inhalation studies designed to investigate MTBE mode of action using exposures of 3 days to 8 months duration (Moser et al., 1996, 1998). A central question investigated in these studies was whether the increased hepatic adenomas and decreased uterine cystic hyperplasias observed in female mice in an earlier cancer bioassay (Bird et al., 1997) could be linked to hormonal modulation, and, if so, whether the underlying cause could be increased hepatic metabolism of estrogen. To be consistent with the highest MTBE concentration used in that earlier cancer bioassay, 8000 ppm was chosen for the inhalation concentration in these follow up experiments examining mode of action. One of these studies (Moser et al., 1996) focused on the involvement of the liver in effects seen with MTBE, also comparing rates of metabolism of estradiol by hepatocytes from female mice in treated and vehicle-control groups. In that study, mice were gavaged with an 1800 mg/kg dose of MTBE for 3 days. Specific P450 enzymes involved in steroid metabolism were also examined in that study. In Moser et al. (1998), the focus was more on effects on female mouse endocrine organ weights, histopathology and tissue proliferation, estrous cycle length, and evidence of involvement of estrogen receptors. These female mice were exposed for 3 or 21 days or 4 or 8 months to the MTBE vapor dose observed to be hepatocarcinogenic (8000 ppm). This report also included the companion *in vitro* ERB and ER transcriptional activation studies mentioned in the previous section. Main conclusions drawn from these studies were that although effects observed in female mice administered MTBE involve estrogen-dependent organs, these effects do not appear to be mediated through estrogen receptors.

Another MTBE study in immature female mice with the same general objective included an uterotrophic assay-type design augmented by other measurements not required by the standard test guideline (Okahara, 1999). In this study, 1500 mg/kg doses of MTBE administered by gavage according to the standard test protocol caused no effect on uterine weight whether or not animals were also dosed with estradiol, further supporting the notion that MTBE does not directly act upon estrogen receptors. Collectively, this experiment and the studies by Moser et al. provided information about effects of MTBE on the female reproductive organs and endocrine axis (uterine, ovarian, vaginal/cervical, pituitary), histopathology and weight changes, estrous cycle length, circulating estrogen concentrations, and in some cases other endpoints giving insights at a more molecular level, specifically changes in ER immunoreactivity, cell proliferation, and uterine peroxidase in estrogen-sensitive tissues.

Male rodent models were used in other peer-reviewed experimental studies, most consisting of multiple 14- or 28-day experiments examining potential endocrine effects of high gavage doses of MTBE in male rats (Williams et al., 2000; Williams and Borghoff, 2000; de Peyster et al., 2003; Li et al., 2008). Responses of male mice have been examined in a few additional experiments discussed later (Billitti et al., 2005; de Peyster et al., 2008). The highest doses used in the studies with rats ranged from 800 to 1600 mg/kg BW/day. The majority of these studies administered MTBE by gavage to intact adult animals, although one experiment used castrated rats gavaged with 800 mg/kg daily for 5 days in a Hershberger-like study design (de Peyster et al., 2003, experiment 3). Because the main research question of that study was slightly different from that of a routine endocrine screening study, the castrated rats were slightly older than pubertal, and weights of only two of the five androgen-dependent tissues recommended in standard guidelines were measured in animals with/without testosterone propionate supplementation. Li et al. (2008) reported having purchased rats 28–30 days of age followed by a 10-day acclimation, which would have made these animals only 38–40 days old at study start and suggested that they might not have reached full sexual maturity. If this is indeed the case, then results of those 2-week and 4-week experiments may not be entirely consistent with results seen in otherwise comparable studies involving older rats.

Another study exposed juvenile male mice to MTBE added to drinking water at concentrations ranging from 80 to 8000 ppm from 25 to 26 days of age through post-natal day (PND) 77 (de Peyster et al., 2008). Adult male mice were exposed to MTBE daily by gavage for 1 week to as much as 2000 mg/kg BW/day (Billitti et al., 2005). Neither of these studies in mice observed any effect on the endocrine-related endpoints evaluated.

High gavage doses (1000–1600 mg/kg) were included in the majority of these male rodent studies. It was explicitly noted in several of the studies that an objective was to understand the basis of a reported increase in Leydig cell tumors seen in rats exposed to high doses of MTBE (Belpoggi et al., 1995; Bird et al., 1997). Those results are now considered highly suspect, but the findings were taken seriously at that time and warranted follow up. Concerns raised about the reliability of these Leydig cell carcinogenicity study results included use of a non-standard cancer bioassay protocol in one of the studies (Belpoggi et al., 1995), and an unusually low tumor incidence observed in a control group used for comparison with MTBE-treated groups in the other study (Bird et al., 1997).

Collectively, those types of studies provided information on many different endocrine-related endpoints. Weights of testis and male accessory sex organs were most often reported. Histopathology of testis was also evaluated in a few of these studies. When hormone concentrations were measured, they often included testosterone in blood and sometimes also in testicular interstitial fluid (TIF). Some experiments also measured one or more other hormone concentrations in blood; for example, dihydrotestosterone (DHT), estradiol, follicle stimulating hormone (FSH), luteinizing hormone (LH), and prolactin. Information on effects of MTBE on sperm quality parameters (count, motility, morphology) was found in a few studies. Additional studies furnished information on effects of MTBE on biochemical and molecular endpoints like aromatase and other P450 activities, and androgen binding protein mRNA in male rat tissues. One of these studies found increased activity of liver P450 isozymes playing a major role in testosterone metabolism (Williams and Borghoff, 2000). One of the male rat studies mentioned above (Williams et al., 2000, experiments designated A and B) provides some additional information about thyroid-stimulating hormone (TSH), triiodothyronine (T3), and thyroxine (T4), while another measured thyroid weights in one of several experiments (de Peyster et al., 2003, experiment 2). Literature discussing the effect of MTBE on the thyroid hormone pathways is not as extensive as it is for estrogens and androgens, although some of the subchronic and chronic repeat-dose mammalian general toxicology studies discussed next contain one or more relevant endpoints.

3.1.3. Mammalian general toxicology

Acute/single dose studies were not considered helpful except to provide some lethal dose information for test species used in studies that were considered relevant to the WoE analysis of endocrine responses. There are a number of relevant subchronic and chronic MTBE studies that report histology of ovarian, uterine, vaginal/cervical, mammary, testis, prostate, seminal vesicle, epididymal, thyroid, and pituitary tissues. Where weights of organs were measured in these studies, they were also considered relevant to either the estrogen, androgen or thyroid hypotheses. A few of these studies also reported concentrations of TSH, T4, T3, estradiol and/or testosterone measured in blood samples. Observations were extracted from the following subchronic studies, all but one (Zhou and Ye, 1999) including both female and male animals. A 28-day study was conducted in mice by Chun and Kintigh (1993, inhalation, 400–8000 ppm). Studies in rats were conducted by Robinson et al. (1990, gavage, 357–1428 mg/kg/day for 14 days); IIT Research Institute (1992, gavage, 90–750 mg/kg/day for 28 days); Chun and Kintigh (1993, inhalation, 400–8000 ppm for 28 days); and Dong-mei et al. (2009, gavage, 400–1600 mg/kg/day for 2 or 4 weeks). Longer-term studies, all in rats, consisted of the following: Greenough et al. (1980, inhalation, 250–1000 ppm for 90 days); Robinson et al. (1990, gavage, 100–1200 mg/kg/day for 90 days); Lington et al. (1997, inhalation, 800–8000 ppm for 13 weeks); Zhou and Ye (1999, gavage, 200–1000 mg/kg/day for 13 weeks, males only); and Bermudez et al. (2012, 13-week and 1-year studies using drinking water exposures with male rats receiving 37–972 mg/kg BW/day and female rats receiving 50–1153 mg/kg BW/day as calculated from water consumption data for each sex and target MTBE concentrations of 0.5–15 mg/ml in their drinking water). Most of these studies are consistent in showing no significant effect on weights of organs and histopathology. Potential confounding effects (e.g., significant loss of body weight) were noted that could explain most inconsistencies. Cell proliferation in testis was examined using bromodeoxyuridine (BrdU) uptake in one of the 13-week studies (Bermudez et al., 2012), and no effect was seen. An unpublished report (Dodd et al., 2010) and other unpublished results from the study furnished some additional information about a few endpoints in the 13-week and 1-year studies. For example, there was no apparent effect of MTBE on TSH, T3, and T4 in either sex, serum estradiol in the females, serum testosterone in males, or intratesticular testosterone when analyzed in 28-day samples taken during the 13-week drinking water study.

The rats in one of these 13-week studies (Lington et al., 1997) were also given a thorough neurotoxicology evaluation (Daughtrey et al., 1997). That specialized testing was also of interest in this WoE evaluation since the central nervous system is a well-recognized primary target of high MTBE exposures. It has also been recognized that alterations in the brain can be induced by endocrine active compounds, not only during *in utero* development but also possibly in mature neurons (Masuo and Ishido, 2011). Hypothalamic hormones play essential roles in regulating the hypothalamic–pituitary–gonadal and hypothalamic–pituitary–thyroid axes, and other peptides elsewhere in the brain influence hypothalamic hormones. Although ultimately this neurotoxicology evaluation contained no ranked endpoints, the overall results showing no significant effects were considered useful because of the apical nature of the parameters studied.

Most of these subchronic and chronic general toxicology studies followed protocols at least similar to standard testing guidelines current at that time, exceptions being Dong-mei et al. (2009) and Zhou and Ye (1999). Many additional details about consistency of individual studies discussed in this section with standard test protocol expectations can be found in the Supplemental material.

3.1.4. Reproduction, developmental, and carcinogenicity studies

Several reproductive and developmental toxicology studies have been conducted with MTBE using mice or rats, all using the inhalation route of exposure and MTBE concentrations as high as 3400 ppm in the mouse studies and 8000 ppm in the rat studies. Mouse studies were reported in Conaway et al. (1985, teratology, 250–2500 ppm) and Bevan et al. (1997b, development, 300–3400 ppm). Rat studies were conducted by Conaway et al. (1985, teratology, 250–2500 ppm); Biles et al. (1987, single generation reproduction, 300–3400 ppm); Bevan et al. (1997b, development, 1000–8000 ppm); and Bevan et al. (1997a, two-generation reproduction, 400–8000 ppm). One teratology study using rabbits was conducted by Bevan et al. (1997b, 1000–8000 ppm by inhalation). Although this is an important study insofar as no adverse effects were observed in offspring of pregnant rabbits exposed to extremely high concentrations of MTBE, responses in rabbits have not been evaluated yet to establish any appropriate rankings of rabbit endpoints in the WoE method used here.

These studies provide many high level apical endpoints that could reveal some endocrine system dysregulation, or conversely, if no significant effects were seen, indirectly support a conclusion of no endocrine effect significant enough to cause an adverse response on reproduction or embryo-fetal development. Reproductive endpoints reported in those studies typically included pregnancy index (number of females with confirmed pregnancies/ total number of females used for mating × 100), maternal weight gain, consumption of water and food, and male fertility index (number of males siring a litter/total number of males used for mating × 100). Adverse developmental responses include: decreased numbers of corpora lutea or uterine implantations; increased early or late resorptions, or decreased numbers of live fetuses observed at C-section; reduced fetal body weight or crown rump length; altered gender ratio or ano-genital distance; and, increased percentages of fetuses and litters with external and soft tissue malformations, or abnormal ossification and other variations. In the absence of other endpoints, however, none of these endpoints specifically indicate an endocrine mode of action or distinguish hormone agonistic from antagonistic behavior. In the end, very few effects were observed in these studies, and then only at doses that were maternally toxic. No effect on fragility of oocytes of female rats was observed after they were dosed with MTBE added to their drinking water at a concentration of 0.3% (Berger and Horner, 2003).

These studies sometimes provided more specific information about highly ranked endpoints like reproductive organ weights and/or histology. Most notably, because the two-generation study in rats reported in Bevan et al. (1997a) exposed the F1 animals throughout maturation to mating, information relating to pre-/ peripubertal exposures and effects on maturing rodent testis, uterus, and ovaries could be extracted from this study. Pituitary, testes, epididymis, prostate and seminal vesicles, and vagina, uterus, ovaries, and respiratory tract were examined microscopically from all parent animals (e.g., F0 and F1) of the control and high dose groups. Livers from F1-animals were also weighed and those of the control and high dose group were studied microscopically. No histopathological changes in the organs examined were observed. The F1/first generation was exposed *in utero*, through maternal milk throughout weaning on PND 28, and then by direct inhalation starting on PND28. The F1s experienced exposures through sexual maturation and were then mated to produce an F2 generation.

Three cancer bioassays with MTBE have been conducted in rats (Belpoggi et al., 1995; Bird et al., 1997; Dodd et al., 2013) and mice (Bird et al., 1997). Although these high level apical cancer studies were not expected to distinguish between the hypotheses being examined in any definitive way, an important objective of this WoE evaluation was to include as many reliable studies as possible as supporting evidence, if not with specifically ranked endpoints. Other than a questionable increase (see Supplemental material for details) in rat Leydig cell tumors seen in two of these long-term studies using gavage with 250–1000 mg/kg doses of MTBE (Belpoggi et al., 1995) or inhalation exposures of 400–8000 ppm (Bird et al., 1997), no MTBE-related cancers have been seen in endocrine organs. No increase in rat Leydig cell tumors was found in a more recent MTBE cancer bioassay using a drinking water exposure protocol (Dodd et al., 2013). The actual overall calculated intake of MTBE in the highest dose group in that later bioassay of MTBE when consumed in drinking water (972 mg/kg BW/day) was similar to the highest individual bolus gavage doses (1000 mg kg BW/d, 4×/week) used in the study by Belpoggi et al. (1995). Dodd et al. (2013) also reported some relevant organ weight measurements made at the end of that 2-year drinking water exposure, so those elements of the study were included in the WoE evaluation.

3.1.5. Fish and other non-mammalian studies

Most ecotoxicological studies with MTBE are focused on acute survival effects in short-term tests (ECHA, 2012). Chronic invertebrate studies were not considered useful in this assessment for addressing potential endocrine activity due to the lack of relevant diagnostic endpoints for estrogen, androgen, and thyroid pathways and steroidogenesis. Two guideline fish short-term reproduction studies (FSTRA), one with fathead minnow (*Pimephales promelas*) and one with zebrafish (*Danio rerio*) are available for the WoE assessment. The studies followed the US EPA OCSPP 890.1350 and OECD 229 guidelines with the objective to determine if MTBE might interact with the estrogenic or androgenic hormone axes of fish.

In the zebrafish study, exposure of fish to 0.122 and 3.04 mg MTBE/L had no effect on any of the endpoints measured except for a statistically significant elevation in plasma VTG concentrations in male fish exposed to 3.04 mg/L. This elevation was marginal (3.4-fold) with individual values within the range normally observed in adult male zebrafish. Exposure of fish to 147 mg/L MBTE resulted in a statistically significant reduction in the total number of eggs produced and the number of eggs produced per female per reproductive day. This reduction in fecundity was accompanied by a statistically significant increase in the incidence of oocyte atresia along with a statistically significant increase in the accumulation of oocyte debris in the oviduct. Oocyte atresia is a common finding, even in control fish, and its occurrence in this study did not impact the fertility of the fish.

In the fathead minnow study, there were no apparent effects on survival, growth, reproduction, secondary sex characteristics, GSI, VTG or gonad histopathology in male or female fish exposed to MTBE for 21 days to measured concentrations up to 64 mg/L. Based on the endpoints evaluated, MTBE does not appear to interact with the estrogenic or androgenic hormone axes of fathead minnows.

These two fish studies were performed to address findings of a non-dose responsive increase in male VTG and the suppression of sperm mobility in an earlier study by Moreels et al. (2006). Deficiencies in the Moreels et al. study resulted in a Klimisch score of 3 so it is not included in the relevance ranking tables.

A study with the European common brown frog (*Rana temporaria*) contributed information on metamorphosis and growth to the WoE assessment (Paulov, 1987). Tadpoles were exposed to 100–10,000 mg MTBE/L. Weights of tadpoles and frogs and timing of metamorphosis observed in this study were considered rank 3 endpoints because of the lack of coincident information on thyroid histopathology and specific measurements on other growth parameters such as hind limb length.

4. Weight of evidence results

Tables 1–8 summarize evidence used in the WoE analysis, identifying statistically significant findings in ranked endpoints. Some study reports describe multiple experiments. If a report described more than one experiment in which a given endpoint was measured, and the type of result was consistent (i.e., always no effect, decrease, or increase), then no specific experiment designation was shown. However, if results differed between experiments in the same report then a specific experiment is indicated. When sufficient information was available as to the expected direction of change for an endpoint then that was noted in the tables.

4.1. Estrogen agonist hypothesis

VTG induction in male fish and an increase in uterine weight in a uterotrophic study are two endpoints that are ranked 1 for the

Table 1
Ranked endpoints for relevant MTBE data for the estrogen agonist hypothesis. Endpoint responses compared to controls for each study are noted as having no effect (**NE**), an increase (**IN**), or a decrease (**DE**). Expected responses of the endpoint to known positives for the hypothesis are noted in the heading with an arrow when available.

Rank 1 endpoints	Rank 2 endpoints	Rank 3 endpoints
FSTRA ***VTG ↑ males*** **NE** (Mihaich et al., in preparation)	**Female rodent studies** ***Ovarian weight ↓*** **NE** (Dodd et al., 2013 (rat); Bermudez et al., 2012 (rat); IIT, 1992 (rat); Robinson et al., 1990 (rat); Biles et al., 1987 (rat); Greenough et al., 1980 (rat)) **DE** (Moser et al., 1998 (mouse); Moser et al., 1996 (mouse))	**Female rodent studies** ***Estrous cyclicity*** **IN** (Moser et al., 1998 (mouse))
Uterotrophic-like assay ***Uterus weight ↑*** **NE** (Okahara, 1999 (mouse))	***Ovarian histopathology*** **NE** (Bermudez et al., 2012 (rat); Okahara, 1999 (mouse); Moser et al., 1998 (mouse); Lington et al., 1997 (rat); Bevan et al., 1997a (rat); Moser et al., 1996 (mouse);Chun and Kintigh, 1993 (mouse, rat); IIT, 1992 (rat); Robinson et al., 1990 (rat); Biles et al., 1987 (rat); Greenough et al., 1980 (rat))	***Blood estradiol*** **NE** (Bermudez et al., 2012 (rat); Moser et al., 1998 (mouse); Chun and Kintigh, 1993 (mouse)) ***Mammary tissue histopathology*** **NE** (Lington et al., 1997 (rat); IIT, 1992 (rat); Greenough et al., 1980 (rat)) ***Uterus weight ↑*** **NE** (Bermudez et al., 2012 (rat); Bevan et al., 1997a (rat); Chun and Kintigh, 1993 (mouse, rat); Greenough et al., 1980 (rat)) **DE** (Moser et al., 1998 (mouse); Moser et al., 1996 (mouse))
	Male rodent studies ***Testis weight ↓*** **NE** (de Peyster et al., 2014 (rat); Dodd et al., 2013 (rat); Bermudez et al., 2012 (13-wk exposure, rat);Dong-mei et al., 2009 (Expt B/4 wk, rat); de Peyster et al., 2008 (mouse); Billitti et al., 2005 (mouse); de Peyster et al., 2003 (rat); Williams et al., 2000 (Expts B and C, rat); Lington et al., 1997 (rat); Bird et al., 1997 (mouse, rat); IIT, 1992 (rat); Robinson et al., 1990 (rat), Biles et al., 1987 (rat); Greenough et al., 1980 (rat)) **IN** (Bermudez et al., 2012 (1 yr exposure, rat); Williams et al., 2000 (Expt A, (rat)) **DE** (Dong-mei et al., 2009 (Expt A/2 wk, rat)) ***Testis histopathology*** **NE** (Bermudez et al., 2012 (rat); de Peyster et al., 2008 (mouse); Billitti et al., 2005 (mouse); Williams et al., 2000 (rat); Zhou and Ye, 1999 (rat); Lington et al., 1997 (rat); Bevan et al., 1997a (rat)); Chun and Kintigh, 1993 (mouse, rat); IIT, 1992 (rat); Robinson et al., 1990 (rat); Biles et al., 1987 (rat); Greenough et al., 1980 (rat)) **IN** (Li et al., 2008 (rat)) **FSTRA** ***Male tubercle score ↓*** **NE** (Mihaich et al., in preparation) ***Male gonad histopathology*** **NE** (Mihaich et al., in preparation) ***Male behavior*** **NE** (Mihaich et al., in preparation) **ERTA assay** ***ER agonism*** **NE** (Moser et al., 1998)	***Uterus histopathology*** **NE** (Bermudez et al., 2012 (rat); Okahara, 1999 (mouse); Lington et al., 1997 (rat); Bevan et al., 1997a (rat); Moser et al., 1996 (mouse); Chun and Kintigh, 1993 (mouse, rat); IIT, 1992 (rat); Greenough et al., 1980 (rat)) **IN** (Moser et al., 1998 (mouse)) ***Vaginal/cervical histopathology*** **DE** (Bermudez et al., 2012 (rat)); **IN** (Moser et al., 1998 (mouse)) ***ER immunoreactivity in estrogen sensitive tissue*** **NE** (Moser et al., 1998 (mouse)) ***Oocyte fragility*** **NE** (Berger and Horner, 2003 (rat)) **Hepatic estradiol metabolism** **IN** (Moser et al., 1996 (mouse))
		Male rodent studies ***Prostate weight*** **NE** (de Peyster et al., 2014 (rat); Bermudez et al., 2012 (rat); Dong-mei et al., 2009 (Expt A/2 wk, rat); de Peyster et al., 2003 (rat); Williams et al., 2000 (rat); Biles et al., 1987 (rat); Greenough et al., 1980 (rat)) **DE** (Dong-mei et al., 2009, Expt B/4 wk, rat)) ***Epididymis histopathology*** **NE** (de Peyster et al., 2008 (mouse); Bevan et al., 1997a (rat); Lington et al., 1997 (rat); Chun and Kintigh, 1993 (mouse, rat); IIT, 1992 (rat); Greenough et al., 1980 (rat)) **FSTRA** **Fecundity** **NE** (Mihaich et al., in preparation (fathead minnow)) **DE** (Mihaich et al., in preparation (zebrafish)) **Female behavior** **NE** (Mihaich et al., in preparation) **GSI: ↓ males, ↑ females** **NE** (Mihaich et al., in preparation) **Follicular atresia** **NE** (Mihaich et al., in preparation (fathead minnow)) **IN** (Mihaich et al., in preparation (zebrafish)) **Fertilization success** **NE** (Mihaich et al., in preparation) **Steroidogenesis assay** **Estradiol concentrations** **NE** (de Peyster et al., 2014) **ER binding assay** **Competitive binding** **NE** (Moser et al., 1998)

Table 2
Ranked endpoints for relevant MTBE data for the estrogen antagonist hypothesis. Endpoint responses compared to controls for each study are noted as having no effect (**NE**), an increase (**IN**), or a decrease (**DE**). Expected responses of the endpoint to known positives for the hypothesis are noted in the heading with an arrow when available.

Rank 1 endpoints	Rank 2 endpoints	Rank 3 endpoints
Uterotrophic assay	**FSTRA**	**Female rodent studies**
Uterus weight ↑	**VTG ↓ females**	**Estrous cyclicity**
NE (Okahara, 1999 (mouse))	**NE** (Mihaich et al., in preparation)	**IN** (Moser et al., 1998 (mouse))
	Female gonad histopathology	**Blood estradiol**
	NE (Mihaich et al., in preparation (fathead minnow)) **IN** (Mihaich et al., in preparation (zebrafish))	**NE** (Bermudez et al., 2012 (rat); Moser et al., 1998 (mice); Chun and Kintigh, 1993 (mice))
	ERTA	**Ovarian weight ↓** **NE** (Dodd et al., 2013 (rat); Bermudez et al., 2012 (rat); IIT, 1992 (rat); Robinson et al., 1990 (rat); Biles et al., 1987 (rat); Greenough et al., 1980 (rat)) **DE** (Moser et al., 1998 (mouse); Moser et al., 1996 (mouse))
	ER antagonism **NE** (Moser et al., 1998)	**Ovarian histopathology** **NE** (Bermudez et al., 2012 (rat); Okahara, 1999 (mouse); Moser et al., 1998 (mouse); Lington et al., 1997 (rat); Bevan et al., 1997a (rat); Moser et al., 1996 (mouse); Chun and Kintigh, 1993 (rat); IIT, 1992 (rat); Robinson et al., 1990 (rat); Biles et al., 1987 (rat); Greenough et al., 1980 (rat))
	ER binding assay	**Mammary tissue histopathology** **NE** (Lington et al., 1997 (rat); IIT, 1992 (rat); Greenough et al., 1980 (rat))
	Competitive binding **NE** (Moser et al., 1998)	**Uterus weight ↓** **NE** (Bermudez et al., 2012 (rat); Bevan et al., 1997a (rat); Chun and Kintigh, 1993 (mouse, rat); Greenough et al., 1980 (rat)) **DE** (Moser et al., 1998 (mouse); Moser et al., 1996 (mouse))
		Uterus histopathology (Bermudez et al., 2012 (rat); Okahara, 1999 (mouse); Lington et al., 1997 (rat); Bevan et al., 1997a (rat); Moser et al., 1996 (mouse); Chun and Kintigh, 1993 (mouse, rat); IIT, 1992 (rat); Greenough et al., 1980 (rat)) **IN** (Moser et al., 1998 (mouse))
		Vaginal/cervical histopathology **NE** (Bermudez et al., 2012 (rat)) **IN** (Moser et al., 1998 (mouse))
		ER immunoreactivity in estrogen sensitive tissue **NE** (Moser et al., 1998 (mouse))
		Oocyte fragility **NE** (Berger and Horner, 2003 (rat))
		Hepatic estradiol metabolism **IN** (Moser et al., 1996 (mice))
		FSTRA
		Fecundity **NE** (Mihaich et al., in preparation (fathead minnow)) **DE** (Mihaich et al., in preparation (zebrafish))
		Male/female behavior **NE** (Mihaich et al., in preparation)
		GSI **NE** (Mihaich et al., in preparation)
		Fertilization success **NE** (Mihaich et al., in preparation)
		Steroidogenesis assay
		Estradiol concentrations **NE** (de Peyster et al., 2014)
		Aromatase assay
		Inhibition **NE** (de Peyster et al., 2014)

estrogen agonist hypothesis (Table 1). Two FSTRA were performed to assess the potential for MTBE to interact with the endocrine system in fathead minnow (*P. promelas*) and zebrafish (*D. rerio*) (Mihaich et al., in preparation). As this assay screens for disturbances in the hypothalamic–pituitary gonadal axis (HPG), it is relevant to six of the eight hypotheses being evaluated. Only involvement of the thyroid pathway is not addressed in this assay. While there was a statistically significant increase in male VTG at the mid dose of 3.04 mg/L in the zebrafish study, it was only 3.4-fold higher than the controls and within the normal historical control range for the laboratory, so the increase in VTG in the mid-dose only in the zebrafish study was not considered to be test-substance related. In the study with fathead minnow, there was no difference in VTG concentration between control or MTBE-exposed fish.

No guideline uterotrophic assays are available, although a one dose, uterotrophic-like study performed in immature mice at or near the maximum tolerated dose and above the limit dose suggested in the EPA 890.1600 guideline resulted in no change in uterine weight (Okahara, 1999). Uterine weight in cycling adult female rodents is highly variable (Stoker and Zorilla, 2010) and is therefore a rank 3 corroborative endpoint. Uterine weight measurements were reported in a number of studies with MTBE that can help clarify other ranked endpoints in the WoE, especially when the ranked study was not performed according to a regulatory guideline. Studies by Bermudez et al. (2012), Bevan et al. (1997a), Chun and Kintigh (1993), and Greenough et al. (1980), using adult rats or mice with a functionally intact HPG axis, reported no changes in uterine weight compared to control at any doses used in any of these studies (see Literature Review for doses used). In contrast, Moser et al. (1996, 1998) both reported a decrease in uterine weight in mice. Given that for the estrogen agonist hypothesis a uterine weight increase, not a decrease, would

Table 3
Ranked endpoints for relevant MTBE data for the androgen agonist hypothesis. Endpoint responses compared to controls for each study are noted as having no effect (**NE**), an increase (**IN**), or a decrease (**DE**). Expected responses of the endpoint to known positives for the hypothesis are noted in the heading with an arrow when available.

Rank 1 endpoints	Rank 2 endpoints	Rank 3 endpoints
Hershberger-like study	**Male rodent studies**	**Male rodent studies**
Prostate weight **NE** (de Peyster et al., 2003 (Expt 3, rat))	**Testis weight** **NE** (de Peyster et al., 2014 (rat); Dodd et al., 2013 (rat); Bermudez et al., 2012 (13-wk exposure, rat); Dong-mei et al., 2009 (Expt B/4 wk, rat); de Peyster et al., 2008 (mouse); Billitti et al., 2005 (mouse); de Peyster et al., 2003 (rat); Williams et al., 2000 (Expts B and C, rat); Lington et al., 1997 (rat); Bird et al., 1997 (mouse, rat); IIT, 1992 (rat); Robinson et al., 1990 (rat), Biles et al., 1987 (rat); Greenough et al., 1980 (rat)) **IN** (Bermudez et al., 2012 (1 yr exposure, rat); Williams et al., 2000 (Expt A, (rat)) **DE** (Dong-mei et al., 2009 (Expt A/2 wk, rat))	**Blood testosterone** **NE** (de Peyster et al., 2014 (Expt 1, rat); Bermudez et al., 2012 (rat); de Peyster et al., 2008 (mouse); Billitti et al., 2005 (mouse); de Peyster et al., 2003 (Expt 1[a], Expt 2[b], rat); Williams et al., 2000 (Expts A and C, rat)) **DE** (de Peyster et al., 2014 (Expt 2, rat); de Peyster et al., 2003 (Expt 1[a]-, Expt 2[b], Expt 5 (rat)); Williams et al., 2000 (Expt B, rat)) Li et al., 2008 (Expt A/2 wk, rat)) **IN** (Li et al., 2008 (Expt B/4 wk, rat))
Seminal vesicle weight **NE** (de Peyster et al., 2003 (Expt 3, rat)	**Testis histopathology** **NE** (Bermudez et al., 2012 (rat); de Peyster et al., 2008 (mouse); Billitti et al., 2005 (mouse); Williams et al., 2000 (rat); Zhou and Ye, 1999 (rat); Lington et al., 1997 (rat); Bevan et al., 1997a (rat); Chun and Kintigh, 1993 (mouse, rat); IIT, 1992 (rat); Robinson et al., 1990 (rat); Biles et al., 1987 (rat); Greenough et al., 1980 (rat)) **IN** (Li et al., 2008 (rat))	**Blood LH** **NE** (Bermudez et al., 2012 (rat); Li et al., 2008 (B/4 wk, rat); Williams et al., 2000 (Expts B and C, rat); de Peyster et al., 2003 (Expts 2 and 3, rat)) **DE** (Williams et al., 2000 (Expt A, rat)); de Peyster et al., 2003 (Expt 5, rat)) IN (Li et al., 2008 (A/2 wk, rat))
FSTRA	**Epididymides weight** **NE** (de Peyster et al., 2014 (rat); Dodd et al., 2013 (rat); Bermudez et al., 2012); de Peyster et al., 2008 (mouse); Li et al., 2008 (rat); de Peyster et al., 2003 (Expt 1, 2, rat); Williams et al., 2000 (rat); Biles et al., 1987 (rat))	**Blood DHT** **NE** (Williams et al., 2000, (Expt B, rat)) **DE** (Williams et al., 2000, (Expt A, rat))
Tubercles in females **NE** (Mihaich et al., in preparation)	**Epididymides histopathology** **NE** (de Peyster et al., 2008 (mouse); Bevan et al., 1997a (rat)); Lington et al., 1997 (rat); Chun and Kintigh, 1993 (mouse, rat); IIT, 1992 (rat); Greenough et al., 1980 (rat))	**Blood FSH** **NE** (Bermudez et al., 2012 (rat); Li et al., 2008 (B/4 wk, rat); Williams et al., 2000 (Expts A and B, rat)) **IN** (Li et al., 2008 (A/2 wk, rat))
	Prostate weight **NE** (de Peyster et al., 2014 (rat); Bermudez et al., 2012 (rat); Dong-mei et al., 2009 (Expt A/2 wk (rat); de Peyster et al., 2003 (rat); Williams et al., 2000 (rat); Biles et al., 1987 (rat); Greenough et al., 1980 (rat)) **DE** (Dong-mei et al., 2009 (Expt B/4 wk (rat))	**Blood prolactin** **NE** (Bermudez et al., 2012 (rat); de Peyster et al., 2003 (rat); Williams et al., 2000, rat))
	Seminal vesicle weight **NE** (de Peyster et al., 2014 (rat); Bermudez et al., 2012 (rat); de Peyster et al., 2008 (mouse); de Peyster et al., 2003 (rat); Williams et al., 2000 (rat); Biles et al., 1987 (rat))	**Testosterone biotransformation enzymes activity** **IN** (Williams and Borghoff, 2000, (rat))
	FSTRA	**Liver and testis microsomal aromatase activity** **NE** (de Peyster et al., 2014 (rat)) **DE** (de Peyster et al., 2003 (rat))
	VTG ↓ females **NE** (Mihaich et al., in preparation)	**Aromatase mRNA** **NE** (de Peyster et al., 2014 (rat liver)) **IN** (de Peyster et al., 2014 (rat testis))
	Gonad histopathology **NE** (Mihaich et al., in preparation (fathead minnow)) **IN** (Mihaich et al., in preparation (zebrafish))	**Sperm count** **NE** (de Peyster et al., 2008 (mouse); (Li et al., 2008 (rat))
		Sperm motility **NE** (de Peyster et al., 2008 (mouse)) **Androgen binding protein mRNA in testis** **DE** (Li et al., 2008 (rat)) **Testosterone measured in testis** **NE** (de Peyster et al., 2014 (rat);Bermudez et al., 2012 (rat);Williams et al., 2000 (rat)) **Pituitary weight** **NE** (Bermudez et al., 2012 (rat); de Peyster et al., 2003 (rat); Williams et al., 2000 (Expt A and C, rat); Greenough et al., 1980 (rat)) **IN** (Williams et al., 2000 (Expt B, rat)) **DE** (Moser et al., 1998 (mouse)) **Pituitary histopathology** **NE** (Bermudez et al., 2012 (rat); Lington et al., 1997 (rat); Chun and Kintigh, 1993 (mouse, rat); IIT, 1992 (rat); Greenough et al., 1980 (rat)) **Female rodent studies** **Uterus weight** **NE** (Bermudez et al., 2012 (rat); Bevan et al., 1997a (rat); Chun and Kintigh, 1993 (mouse, rat); Greenough et al., 1980 (rat)) **DE** (Moser et al., 1998, (mouse); Moser et al., 1996 (mouse)) **Ovarian weight** **NE** (Dodd et al., 2013 (rat); Bermudez et al., 2012 (rat); IIT, 1992 (rat); Robinson et al., 1990; Biles et al., 1987; Greenough et al., 1980 (rat))

(*continued on next page*)

Table 3 (*continued*)

Rank 1 endpoints	Rank 2 endpoints	Rank 3 endpoints
		DE (Moser et al., 1998 (mouse); Moser et al., 1996 (mouse)) **Uterine histopathology** **NE** (Bermudez et al., 2012 (rat); Okahara, 1999 (mouse); Lington et al., 1997 (rat); Bevan et al., 1997a (rat); Moser et al., 1996 (mouse); Chun and Kintigh, 1993 (mouse, rat); IIT, 1992 (rat); Greenough et al., 1980 (rat)) **IN** (Moser et al., 1998 (mouse)) **Ovarian histopathology** **NE** (Bermudez et al., 2012 (rat); Okahara, 1999 (mouse); Moser et al., 1998 (mouse); Lington et al., 1997 (rat); Bevan et al., 1997a (rat); Moser et al., 1996 (mouse); Chun and Kintigh, 1993 (mouse, rat); IIT, 1992 (rat); Robinson et al., 1990 (rat); Biles et al., 1987 (rat); Greenough et al., 1980 (rat)) **FSTRA** **Fecundity** **NE** (Mihaich et al., in preparation (fathead minnow)) **DE** (Mihaich et al., in preparation (zebrafish)) **Behavior** **NE** (Mihaich et al., in preparation) **GSI** **NE** (Mihaich et al., in preparation) **Fertilization success** **NE** (Mihaich et al., in preparation) **Steroidogenesis assay** **Testosterone concentrations** **NE** (de Peyster et al., 2014) **Aromatase assay** **Inhibition** **NE** (de Peyster et al., 2014) **AR binding assay** **Competitive binding** **NE** (de Peyster et al., 2014)

[a] NE in Expt 1 when sampled on Day 14, by tail vein, or on Day 28 at necropsy, both sampling times 12–20 h after the final MTBE dose, but DE in the same Expt 1 when blood was sampled by tail vein 4–5 h after the first MTBE dose.
[b] NE in Expt 2 when sampled on Day 14 by tail vein just prior to dosing, but DE on Day 28 at necropsy 16–20 h after final MTBE dose.

be expected, the results from many studies provide corroborative evidence for the preliminary finding that the estrogen agonist hypothesis is not supported. Similarly, there was no change in ovarian weight, a rank 2 endpoint, compared to controls in a number of studies with rats (Dodd et al., 2013; Bermudez et al., 2012; IIT Research Institute, 1992; Robinson et al., 1990; Biles et al., 1987; Greenough et al., 1980) although there was a decrease in ovarian weight in mice in the studies by Moser et al. (1996, 1998). Ovarian weight would be expected to decrease with exposure to estrogenic compounds (Stoker and Zorrilla, 2010; Biegel et al., 1998). However, the decrease in uterine weight in the same species (Moser et al., 1996, 1998) when an increase would be expected with an estrogen agonist, suggests that MTBE is not consistently exhibiting estrogenic potential in mice. There were no ovarian histopathological changes noted when compared to controls with either female rats or mice (Table 1). The only histopathological finding reported in female estrogen-dependent reproductive tissue was a description of mouse uterus and vagina being similar in appearance to what would be expected in ovariectomized mice (Moser et al., 1998). This finding was not mentioned in an earlier study of comparable duration and high inhalation dose levels (up to 8000 ppm) using the same CD-1 strain of mouse (Chun and Kintigh, 1993).

Testis weights and histopathology endpoints are also rank 2. For the estrogen agonist hypothesis, a decrease in testis weight would be expected if the chemical is acting like an estrogen (Cook et al., 1998). Many measurements of weight of the testes have been made in MTBE experiments, some with gavage doses as high as 1000–1600 mg/kg BW/day in subchronic studies and 1000 mg/kg BW/day orally or 8000 ppm by inhalation in studies lasting up to 1–2 years. Results from 14 studies reveal no effect (Table 1). Only three studies indicated weight changes compared to controls in testis and two of them reported an increase in relative weight, rather than a decrease. Bermudez et al. (2012) reported an increase in left testis weight:BW ratio in the two higher dose groups with no change in the same ratio on the right side, no change in absolute weights of either testis, and no testis histopathology findings after 1 year of exposure to MTBE in drinking water where the highest target concentration used was 15 mg MTBE/ml (calculated later to be 972 mg/kg BW/day). Significantly reduced body weights were observed in all MTBE-treated groups in that experiment, so this finding could simply be due to the comparatively lower body weights observed in these dose groups. In the study by Williams et al. (2000) in which testis:body weight (BW) ratio was also increased this was seen only at the highest gavage dose (1500 mg/kg/day). This finding could also simply be due to the comparatively lower body weights observed in that dose group, an interpretation supported by the absolute testis weights in that experiment that were no different from controls. The interpretation in both experiments of a possible BW-driven difference in ratios is a reasonable alternative to an effect on the testis itself. In a third study (Dong-mei et al., 2009), investigators gavaging with doses up to 1600 mg/kg BW/day reported a decrease in testis:BW ratio seen in a 2-week experiment (experiment A) but not in a companion 4-week experiment (experiment B) and, at 2 weeks, the decrease was of the same magnitude (11–12%) in all MTBE dose groups. The significance of effect is questionable when they are not consistent across similar experiments and the magnitude does not increase or decrease as doses increase. The lack of consistency and the equivocal nature of the interpretations is evidence that MTBE does not alter testis weights in rodents, even when administered at high doses.

Table 4
Ranked endpoints for relevant MTBE data for the androgen antagonist hypothesis. Endpoint responses compared to controls for each study are noted as having no effect (**NE**), an increase (**IN**), or a decrease (**DE**). Expected responses of the endpoint to known positives for the hypothesis are noted in the heading with an arrow when available.

Rank 1 endpoints	Rank 2 endpoints	Rank 3 endpoints
Hershberger-like study	**Male rodent studies**	**Male rodent studies**
Prostate weight **NE** (de Peyster et al., 2003 (Expt 3, rat))	**Testis weight** **NE** (de Peyster et al., 2014 (rat); (Dodd et al., 2013 (rat); Bermudez et al., 2012 (13 wk, rat); Dong-mei et al., 2009 (Expt B/4 wk, rat); de Peyster et al., 2008 (mouse); Billitti et al., 2005 (mouse); de Peyster et al., 2003 (rat); Williams et al., 2000 (Expts B and C, rat); Lington et al., 1997(rat); Bird et al., 1997 (mouse, rat); IIT, 1992 (rat); Robinson et al., 1990 (rat), Biles et al., 1987 (rat); Greenough et al., 1980 (rat)) **IN** (Bermudez et al., 2012 (1 yr exposure, rat); Williams et al., 2000 (Expt A, (rat)) **DE** (Dong-mei et al., 2009 (Expt A/2 wk, rat))	**Blood testosterone** **NE** (de Peyster et al., 2014 (Expt 1, rat); Bermudez et al., 2012 (rat); de Peyster et al., 2008 (mouse); Billitti et al., 2005 (mouse); de Peyster et al., 2003 (Expt 1[a], Expt 2[b], rat); Williams et al., 2000 (Expts A and C, rat)) **DE** (de Peyster et al., 2014 (Expt 2, rat); de Peyster et al., 2003 (Expt 1[a]-, Expt 2[b], Expt 5 (rat)); Williams et al., 2000 (Expt B, rat); Li et al., 2008 (Expt A/2 week, rat)) **IN** (Li et al., 2008 (Expt B/4 week, rat))
Seminal vesicle weight **NE** (de Peyster et al., 2003 (Expt 3, rat))	**Testes histopathology** **NE** (Bermudez et al., 2012 (rat); de Peyster et al., 2008 (mouse); Billitti et al., 2005 (mouse); Williams et al., 2000 (rat); Zhou and Ye, 1999 (rat); Lington et al., 1997 (rat); Bevan et al., 1997a (rat)); Chun and Kintigh, 1993 (mouse, rat); IIT, 1992 (rat); Robinson et al., 1990 (rat); Biles et al., 1987 (rat); Greenough et al., 1980 (rat)) **IN** (Li et al., 2008 (rat))	**Blood LH** **NE** (Bermudez et al., 2012 (rat); Li et al., 2008 (Expt B/4 wk, rat); Williams et al., 2000 (Expts B and C, rat); de Peyster et al., 2003 (Expts 2 and 3, rat)) **DE** (Williams et al., 2000 (Expt A, rat); de Peyster et al., 2003 (Expt 5, rat)) **IN** (Li et al., 2008 (Expt A/2 wk, rat))
	Epididymides weight **NE** (Bermudez et al., 2012); de Peyster et al., 2008 (mice); Li et al., 2008 (rat); de Peyster et al., 2003 (rat); Williams et al., 2000 (rat); Biles et al., 1987 (rat))	**Blood DHT** **NE** (Williams et al., 2000, (Expt B, rat)) **DE** (Williams et al., 2000, (Expt A, rat))
	Epididymides histopathology **NE** (de Peyster et al., 2008 (mouse); Bevan et al., 1997a (rat); Lington et al., 1997 (rat); Chun and Kintigh 1993 (mouse, rat); IIT, 1992 (rat); Greenough et al., 1980 (rat))	**Blood FSH** **NE** (Bermudez et al., 2012 (rat); Li et al., 2008 (Expt B/4 wk, rat); Williams et al., 2000 (Expts A and B, rat)) **IN** (Li et al., 2008 (Expt A/2 wk, rat))
	Prostate weight **NE** (de Peyster et al., 2014 (rat); Bermudez et al., 2012 (rat); Dong-mei et al., 2009 (Expt A/2 wk (rat); de Peyster et al., 2003 (rat); Williams et al., 2000 (rat); Biles et al., 1987 (rat); Greenough et al., 1980 (rat)) **DE** (Dong-mei et al., 2009 (Expt B/4 wk (rat))	**Blood prolactin** **NE** (Bermudez et al., 2012 (rat); de Peyster et al., 2003 (rat); Williams et al., 2000, (rat))
	Seminal vesicle weight **NE** (de Peyster et al., 2014 (rat); Bermudez et al., 2012 (rat); de Peyster et al., 2008 (mouse); de Peyster et al., 2003 (rat); Williams et al., 2000 (rat); Biles et al., 1987 (rat))	**Testosterone biotransformation enzymes activity** **IN** (Williams and Borghoff, 2000 (rat))
	FSTRA	**Liver and testis microsomal aromatase activity** **NE** (de Peyster et al., 2014 (rat)) **DE** (de Peyster et al., 2003 (rat))
	VTG ↑ females **NE** (Mihaich et al., in preparation)	**Aromatase mRNA** **NE** (de Peyster et al., 2014 (rat liver)) **IN** (de Peyster et al., 2014 (Exp 2 – rat testis))
	Male secondary sex characteristics ↓ **NE** (Mihaich et al., in preparation)	**Sperm count** **NE** (de Peyster et al., 2008 (mouse); Li et al., 2008 (rat)) **Sperm motility** **NE** (de Peyster et al., 2008 (mouse))
	Gonad histopathology **NE** (Mihaich et al., in preparation (fathead minnow)) **IN** (Mihaich et al., in preparation (zebrafish))	
		Androgen binding protein mRNA in testis **DE** (Li et al., 2008 (rat)) **Testosterone measured in testis** **NE** (de Peyster et al., 2014 (rat); Bermudez et al., 2012 (rat); Williams et al., 2000 (rat)) **Pituitary weight** **NE** (Bermudez et al., 2012 (rat); Williams et al., 2000 (Expts A and C, rat)) **IN** (Williams et al., 2000 (Expt B, rat)) **DE** (de Peyster et al., 2003 (rat); Moser et al., 1998

(continued on next page)

Table 4 (*continued*)

Rank 1 endpoints	Rank 2 endpoints	Rank 3 endpoints
		(mouse)) **Pituitary histopathology** **NE** (Bermudez et al., 2012 (rat); Lington et al., 1997 (rat); Chun and Kintigh, 1993 (mouse, rat); IIT, 1992 (rat); Greenough et al., 1980 (rat)) **FSTRA** **Fecundity** **NE** (Mihaich et al., in preparation (fathead minnow)) **DE** (Mihaich et al., in preparation (zebrafish)) **Behavior** **NE** (Mihaich et al., in preparation) GSI **NE** (Mihaich et al., in preparation) **Fertilization success** **NE** (Mihaich et al., in preparation) **Steroidogenesis assay** **Testosterone concentrations** **NE** (de Peyster et al., 2014) **AR binding assay** **Competitive binding** **NE** (de Peyster et al., 2014)

[a] NE in Expt 1 when sampled on Day 14, by tail vein, or on Day 28 at necropsy, both sampling times 12–20 h after the final MTBE dose, but DE in the same Expt 1 when blood was sampled by tail vein 4–5 h after the first MTBE dose.
[b] NE in Expt 2 when sampled on Day 14 by tail vein just prior to dosing, but DE on Day 28 at necropsy 16–20 h after final MTBE dose.

Table 5
Ranked endpoints for relevant MTBE data for the thyroid agonist hypothesis. Endpoint responses compared to controls for each study are noted as having no effect (**NE**), an increase (**IN**), or a decrease (**DE**). Expected responses of the endpoint to known positives for the hypothesis are noted in the heading with an arrow when available.

Rank 1 endpoints	Rank 2 endpoints	Rank 3 endpoints
No relevant rank 1 endpoints available	**Male rodent studies** **Thyroid weight** **NE** (Dodd et al., 2013 (rat); Bermudez et al., 2012 (13 wk, rat); Chun and Kintigh, 1993 (mouse, rat); Greenough et al., 1980 (rat)) **IN** (de Peyster et al., 2003 (rat)) **DE** (Bermudez et al., 2012 (1 yr, rat)) **Blood T4** **NE** (Bermudez et al., 2012 (27 wk and 1-yr, rat); Dodd et al., 2010 (rat); Williams et al., 2000 (rat)) **IN** (Chun and Kintigh, 1993 (mouse)) **DE** (Bermudez et al., 2012[a] (rat)) **Blood T3** **NE** (Bermudez et al., 2012, rat); Dodd et al., 2010 (rat); Williams et al., 2000 (Expt B, rat); Chun and Kintigh, 1993 (mouse)) **DE** (Williams et al., 2000 (Expt A, rat)) **Blood TSH** **NE** (Bermudez et al., 2012 (rat); Dodd et al., 2010 (rat); Williams et al., 2000 (rat)) **IN** (Chun and Kintigh, 1993 (mouse))	**Male rodent studies** **Pituitary weight** **NE** (Bermudez et al., 2012 (rat); de Peyster et al., 2003 (rat); Williams et al., 2000 (Expts A and C, rat); Greenough et al., 1980 (rat)) **IN** (Williams et al., 2000 (Expt B, rat)) **DE** (Moser et al., 1998 (mouse)) **Pituitary histopathology** **NE** (Bermudez et al., 2012 (rat); Lington et al., 1997 (rat); Chun and Kintigh, 1993 (mouse, rat); IIT, 1992 (rat); Greenough et al., 1980 (rat)) **Thyroid histopathology** **NE** (Bermudez et al., 2012 (1 yr); Lington et al., 1997 (rat); Chun and Kintigh, 1993 (mouse); IIT, 1992 (rat); Greenough et al., 1980 (rat))
	Female rodent studies **Thyroid weight** **NE** (Dodd et al., 2013 (rat); Bermudez et al., 2012 (rat); Chun and Kintigh, 1993 (mouse, rat); Greenough et al., 1980 (rat)) **Blood T4** **NE** (Bermudez et al., 2012 (rat); Dodd et al., 2010 (rat); Chun and Kintigh, 1993 (mouse)) **Blood T3** **NE** (Bermudez et al., 2012 (rat); Dodd et al., 2010 (rat); Chun and Kintigh, 1993 (mouse)) **Blood TSH** **NE** (Bermudez et al., 2012 (rat); Dodd et al., 2010 (rat); Chun and Kintigh, 1993 (mouse))	**Female rodent studies** **Pituitary weight** **NE** (Bermudez et al., 2012 (rat); Greenough et al., 1980 (rat)) **DE** (Moser et al., 1998 (mouse)) **Pituitary histopathology** **NE** (Bermudez et al., 2012 (rat); Lington et al., 1997 (rat); Chun and Kintigh, 1993 (mouse, rat); IIT, 1992 (rat); Greenough et al., 1980 (rat)) **Thyroid histopathology** **NE** (Bermudez et al., 2012 (1 yr); Lington et al., 1997 (rat); Chun and Kintigh, 1993 (mouse); IIT, 1992 (rat); Greenough et al., 1980 (rat)) **Amphibian study** **Frog metamorphosis rate and weight** **IN** (Paulov, 1987 (*Rana temporaria*))

[a] High dose group statistically lower than controls in cardiac blood samples from five males per concentration at day 28 of the 13-week exposure experiment. TSH and T3 were no different from controls. (No organ weights or histopathology measurements were taken at that time.)

Thirteen studies have evaluated testicular histopathology, with 12 studies reporting no effect. Only one, Li et al. (2008), reported an increase in less compact cells with some shedding of cellular material in seminiferous epithelium in rats. Unfortunately, testis weight was not assessed in this study and there is no indication of either severity scores or number of rats with this finding, which rate this aspect of the study a Klimisch 4. Other than these few findings, there were no changes compared to controls in testis weight or histopathology in many studies with both rats and mice (Table 1).

Table 6
Ranked endpoints for relevant MTBE data for the thyroid antagonist hypothesis. Endpoint responses compared to controls for each study are noted as having no effect (**NE**), an increase (**IN**), or a decrease (**DE**). Expected responses of the endpoint to known positives for the hypothesis are noted in the heading with an arrow when available.

Rank 1 endpoints	Rank 2 endpoints	Rank 3 endpoints
Male rodent studies	**Male rodent studies**	**Male rodent studies**
Thyroid weight **NE** (Dodd et al., 2013 (rat); Bermudez et al., 2012 (13 wk, rat); Chun and Kintigh, 1993 (mouse, rat) Greenough et al., 1980 (rat)) **IN** (de Peyster et al., 2003 (rat)) **DE** (Bermudez et al., 2012 (1 yr, rat))	**Blood T4** **NE** (Bermudez et al., 2012 (27 wk and 1-yr, rat); Dodd et al., 2010 (rat); Williams et al., 2000 (rat)) **IN** (Chun and Kintigh, 1993 (mouse)) **DE** (Bermudez et al., 2012[a] (rat))	**Pituitary weight** **NE** (Bermudez et al., 2012 (rat); de Peyster et al., 2003 (rat); Williams et al., 2000 (Expts A and C, rat); Greenough et al., 1980 (rat)) **IN** (Williams et al., 2000 (Expt B, rat)) **DE** (Moser et al., 1998 (mouse))
Thyroid histopathology **NE** (Bermudez et al., 2012 (1 yr, rat); Lington et al., 1997 (rat); Chun and Kintigh, 1993 (mouse); IIT, 1992 (rat); Greenough et al., 1980 (rat))	**Blood T3** **NE** (Bermudez et al., 2012 (rat); Dodd et al., 2010 (rat); Williams et al., 2000 (Expt B, rat); Chun and Kintigh, 1993 (mouse)) **DE** (Williams et al., 2000 (Expt A, rat))	**Pituitary histopathology** **NE** (Bermudez et al., 2012 (rat); Lington et al., 1997 (rat); Chun and Kintigh, 1993 (mouse, rat); IIT, 1992 (rat); Greenough et al., 1980 (rat))
Female rodent studies	**Blood TSH** **NE** (Bermudez et al., 2012 (rat); Dodd et al., 2010 (rat); Williams et al., 2000 (rat)) **IN** (Chun and Kintigh, 1993 (mouse))	**Female rodent studies**
Thyroid weight **NE** (Dodd et al., 2013 (rat); Chun and Kintigh, 1993 (mouse, rat); Greenough et al., 1980 (rat))	**Female rodent studies**	**Pituitary weight** **NE** (Bermudez et al., 2012 (rat); Greenough et al., 1980 (rat)) **DE** (Moser et al., 1998 (mouse))
Thyroid histopathology **NE** (Chun and Kintigh, 1993 (mouse); IIT, 1992 (rat); Greenough et al., 1980 (rat))	**Ovarian weight ↓** **NE** (Dodd et al., 2013 (rat); Bermudez et al., 2012 (rat); IIT, 1992 (rat); Robinson et al., 1990 (rat); Biles et al., 1987 (rat); Greenough et al., 1980 (rat)) **DE** (Moser et al., 1998 (mouse); Moser et al., 1996 (mouse)) **Blood TSH** **NE** (Bermudez et al., 2012 (rat); Dodd et al., 2010 (rat); Chun and Kintigh, 1993 (mouse)) **Blood T4** **NE** (Bermudez et al., 2012 (rat); Dodd et al., 2010 (rat); Chun and Kintigh, 1993 (mouse)) **Blood T3** **NE** (Bermudez et al., 2012 (rat); Dodd et al., 2010 (rat); Chun and Kintigh, 1993 (mouse))	**Pituitary histopathology** **NE** (Bermudez et al., 2012 (rat); Lington et al., 1997 (rat); Chun and Kintigh, 1993 (mouse, rat); IIT, 1992 (rat); Greenough et al., 1980 (rat)) **Amphibian study** **Frog metamorphosis rate and weight** **IN** (Paulov, 1987 (*Rana temporaria*))

[a] High dose group statistically lower than controls in cardiac blood samples from five males per concentration at day 28 of the 13-week exposure experiment. TSH and T3 were no different from controls. No organ weights or histopathology measurements were taken at that time.

Some responses in males in the FSTRA are also rank 2. In both the zebrafish and fathead minnow studies there were no changes in male gonad histopathology or male behavior (Mihaich et al., in preparation). Tubercles are only assessed in male fathead minnow and there were no changes in the tubercle score in fish exposed to up to 62 mg MTBE/L compared to controls.

The final rank 2 study available is the estrogen receptor transcriptional activation study (ERTA) which measures the ability of a chemical to bind to the ER and activate transcription (Table 1). The ERTA is considered a rank 2 study while the estrogen receptor binding (ERB) screen is a rank 3 because the ERTA can distinguish between agonist and antagonist responses while the ERB currently is not validated to differentiate the responses (Borgert et al., 2014). MTBE did not exhibit ER agonism in the ERTA study (Moser et al., 1998).

For the most part, all rank 3 endpoints including estradiol concentrations in serum of both female mice and rats and in the *in vitro* steroidogenesis assay, prostate weights, and the ERB exhibited no differences compared to controls. Estrous and non-estrous stages were both lengthened in one study but this occurred at a dose where there was a significantly decreased body weight gain (Moser et al., 1998). The absence of histopathology findings in rat mammary tissue (Lington et al., 1997; IIT Research Institute, 1992; Greenough et al., 1980) lends further support that MTBE is not acting as an estrogen agonist.

In the fish model, while the increase in VTG in male zebrafish is a rank 1 endpoint suggestive of an estrogenic response, it conflicts with the decrease in fecundity, a potentially androgenic response if controlled by a hormonal MoA. With only minor and non-consistent ranks 2 and 3 responses in high dose rodent and fish (rank 3 only) studies there is no clear supportive evidence of a direct agonist effect on the estrogen pathway.

4.2. Estrogen antagonist hypothesis

As described in Borgert et al. (2014), none of the endpoints in the current US EDSP Tier 1 screens are considered sufficiently sensitive and specific to assign them rank 1 status, although it was noted that the antagonist mode of the uterotrophic study would be so ranked when validated. Okahara (1999) did evaluate both agonist and antagonist responses in an uterotrophic-like bioassay (see Supplemental material for additional information). Immature CD-1 mice were exposed to MTBE with/without 17β-estradiol. To test estrogen antagonist behavior, the MTBE+17β-estradiol responses were compared to a 17β-estradiol-only control. The MTBE treatment in these experiments produced no statistically significant effects on immature mouse uterus or ovary. A positive antagonist control, clomiphene citrate, was also employed in the Okahara study. While uterine weight was not impacted by the positive control, clomiphene citrate administered to mice also treated with 17β-estradiol did cause a statistically significant decrease relative to the 17β-estradiol-only control group in uterine peroxidase activity also measured in that study. Uterine peroxidase is an enzyme that responds to estrogenic/antiestrogenic effects of chemicals (Lyttle and DeSombre, 1977). MTBE administered to mice also treated with 17β-estradiol did not cause a statistically

Table 7
Ranked endpoints for relevant MTBE data for the steroidogenesis induction hypothesis. Endpoint responses compared to controls for each study are noted as having no effect (**NE**), an increase (**IN**), or a decrease (**DE**). Expected responses of the endpoint to known positives for the hypothesis are noted in the heading with an arrow when available.

Rank 1 endpoints	Rank 2 endpoints	Rank 3 endpoints
	Steroidogenesis assay	**Male rodent studies**
No relevant rank 1 endpoints identified	**Estradiol concentrations** **NE** (de Peyster et al., 2014 (rat))	**Blood testosterone** **NE** (de Peyster et al., 2014 (Expt 1, rat); Bermudez et al., 2012 (rat); de Peyster et al., 2008 (mouse); de Peyster et al., 2003 (Expt 1[a], Expt 2[b], rat); Williams et al., 2000 (Expts A and C, rat)) **DE** (de Peyster et al., 2014 (Expt 2, rat); de Peyster et al., 2003 (Expt 1[a]-, Expt 2[b], Expt 5, rat); Williams et al., 2000 (Expt B, rat); Li et al., 2008 (Expt A/2 week, rat)) **IN** (Li et al., 2008 (Expt B/4 week, rat))
	Testosterone concentrations **NE** (de Peyster et al., 2014 (rat))	**Blood estradiol** **NE**: (de Peyster et al., 2014 (rat); de Peyster et al., 2008 (mouse)) **IN** (de Peyster et al., 2003 (rat))
		Liver and testis microsomal aromatase activity **NE** (de Peyster et al., 2014 (rat)) **DE** (de Peyster et al., 2003 (rat)) **Aromatase mRNA** **NE** (de Peyster et al., 2014 (rat liver)) **IN** (de Peyster et al., 2014 (rat testis)) **Testosterone measured in testis** **NE** (de Peyster et al., 2014 (rat);Bermudez et al., 2012 (rat);Williams et al., 2000 (rat)) **Female rodent studies** **Blood estradiol** **NE** (Moser et al., 1998 (mouse); Chun and Kintigh, 1993 (mice))

[a] NE in Expt 1 when sampled on Day 14, by tail vein, or on Day 28 at necropsy, both sampling times 12–20 h after the final MTBE dose, but DE in the same Expt 1 when blood sampled by tail vein 4–5 h after the first MTBE dose.
[b] NE in Expt 2 when sampled on Day 14 by tail vein just prior to dosing, but DE on Day 28 at necropsy 16–20 h after final MTBE dose.

significant decrease in uterine peroxidase activity relative to the 17β-estradiol-only control group, indicating lack of estrogen antagonist behavior of MTBE.

MTBE did not decrease female VTG in either fathead minnow or zebrafish compared to controls in the two FSTRA studies (Table 2). There was an increase in oocyte atresia, along with a reduction in fecundity, in the zebrafish study however, there were no impacts on fertilization success of the fish in this study. Oocyte atresia is a common finding in control fish (McCormick et al., 1989; Hunter and Macewicz, 1985; Henderson, 1963) and is often a result of general toxicity or stress unrelated to a sex hormone pathway MoA (Clearwater and Pankhurst, 1997; Milla et al., 2009). No female gonad histopathology differences from controls were identified in the fathead minnow study that used slightly lower doses than in the zebrafish study.

While the ERB was considered a rank 3 endpoint for the estrogen agonist hypothesis, it is a rank 2 for the antagonist hypothesis because intrinsic activity is not required to evaluate an estrogen antagonist (Borgert et al., 2014), as binding alone is sufficient. MTBE did not exhibit any difference compared to controls in the ERB or the ERTA experiments (Moser et al., 1998) (Table 2).

Estrogen antagonists would be expected to decrease uterine weights (Ashby et al., 2002). However, uterine weights are extremely variable in cycling rodents (Stoker and Zorrilla, 2010) so it is considered a rank 3 endpoint that would need corroboration of rank 1 or 2 endpoints to be meaningful. Estrogen antagonists would also be expected to decrease ovary weights and cause ovary atrophy (Borgert et al., 2014). While there were no differences compared to controls in uterus or ovary weights in rats (Dodd et al., 2013; Bermudez et al., 2012; Bevan et al., 1997a; Chun and Kintigh, 1993; IIT Research Institute, 1992; Robinson et al., 1990; Biles et al., 1987; Greenough et al., 1980), and in one general toxicology study that reported on mouse uterus weights (Chun and Kintigh, 1993), there were decreases in uterine and ovary weights seen in two studies with mice (Moser et al., 1996, 1998) (Table 2).

Moser et al. (1996) found a twofold increase in the rate of 17β-estradiol metabolism in hepatocytes isolated from mice treated with MTBE at 1800 mg/kg BW/day for 3 days, and Moser et al. (1998) reported that the uteri of mice exposed by inhalation to MTBE at 8000 ppm resembled what would be expected in overiectomized mice, which suggests low estrogen concentrations. A reduction in circulating estradiol could also be responsible for the increase in estrous cycle length that was noted in the Moser et al. (1998) study, as could MTBE-induced decreases in body weight gain and stress (Moser et al., 1998; Everds et al., 2013). However, serum estradiol concentrations, a rank 3 endpoint, were unchanged compared to controls. Otherwise, there were no uterine or ovarian histopthological changes noted when compared to controls with either female rats or mice (Table 2).

Other rank 3 endpoints from two *in vitro* studies targeting the steroidogenic pathway assess endpoints that could be relevant to estrogen antagonists. Aromatase, the enzyme responsible for the conversion of androgens to estrogens, was not inhibited in the aromatase assay and estradiol concentrations were not different from control in the steroidogenesis assay (de Peyster et al., 2014) (Table 2).

In summary, rank 1 uterus weight was not decreased compared to controls and the rank 2 endpoints in the FSTRA, ERTA, and ER binding were also not impacted by exposure to MTBE, except as noted for the zebrafish. While the results of the ranks 1 and 2 endpoint assessment are sufficient to conclude a lack of potential for the estrogen antagonist hypothesis, the majority of the rank 3 endpoints are also negative, thus providing additional corroboration for no direct antagonist effect on the estrogen pathway.

4.3. Androgen agonist hypothesis

Accessory sex organ weight in a Hershberger study and secondary sex characteristics (e.g., tubercles) in fish are rank 1 endpoints for the androgen agonist hypothesis. In the US EDSP, the Hershberger study is used to assess effects on accessory sex organs in the castrated male peripubertal rat (US EPA, 2009a) by measuring the weight of five androgen-dependent tissues. An increase in weight compared to controls in two of the five is

Table 8
Ranked endpoints for relevant MTBE data for the steroidogenesis inhibition hypothesis. Endpoint responses compared to controls for each study are noted as having no effect (**NE**), an increase (**IN**), or a decrease (**DE**). Expected responses of the endpoint to known positives for the hypothesis are noted in the heading with an arrow when available.

Rank 1 endpoints	Rank 2 endpoints	Rank 3 endpoints
No rank 1 endpoints	**Steroidogenesis assay** **Estradiol concentrations** **NE** (de Peyster et al., 2014 (rat))	**Aromatase assay** **Inhibition** **NE** (de Peyster et al., 2014 (rat))
	Testosterone concentrations **NE** (de Peyster et al., 2014 (rat))	**FSTRA**
	FSTRA	**Fecundity** **NE** (Mihaich et al., in preparation (fathead minnow)) **DE** (Mihaich et al., in preparation (zebrafish))
	VTG ↓ females **NE** (Mihaich et al., in preparation)	**Behavior** **NE** (Mihaich et al., in preparation)
	Male gonad histopathology **NE** (Mihaich et al., in preparation)	**GSI** **NE** (Mihaich et al., in preparation)
		Fertilization success **NE** (Mihaich et al., in preparation)
		Male rodent studies
		Blood testosterone **NE** (de Peyster et al., 2014 (Expt 1, rat); Bermudez et al., 2012 (rat); de Peyster et al., 2008 (mouse); de Peyster et al., 2003 (Expt 1[a], Expt 2[b], rat); Williams et al., 2000 (Expts A and C, rat)) **DE** (de Peyster et al., 2014 (Expt 2, rat); de Peyster et al., 2003 (Expt 1[a]-, Expt 2[b], Expt 5, rat); Williams et al., 2000 (Expt B, rat); Li et al., 2008 (Expt A/2 week, rat)) **IN** (Li et al., 2009 (Expt B/4 week, rat))
		Blood estradiol **NE**: (de Peyster et al., 2014 (rat); de Peyster et al., 2008 (mouse)) **IN** (de Peyster et al., 2003 (rat))
		Testis weight **NE** (de Peyster et al., 2014 (rat); Dodd et al., 2013 (rat); Bermudez et al., 2012 (13-wk exposure, rat); Dong-mei et al., 2009 (Expt B/4 wk, rat); de Peyster et al., 2008 (mouse); Billitti et al., 2005 (mouse); de Peyster et al., 2003 (rat); Williams et al., 2000 (Expts B and C, rat); Lington et al., 1997(rat); Bird et al., 1997 (mouse, rat); IIT, 1992 (rat); Robinson et al., 1990 (rat), Biles et al., 1987 (rat); Greenough et al., 1980 (rat)) **IN** (Bermudez et al., 2012 (1 yr exposure, rat); Williams et al., 2000 (Expt A, rat)) **DE** (Dong-mei et al., 2009 (Expt A/2 wk, rat))
		Prostate weight **NE** (de Peyster et al., 2014 (rat); Bermudez et al., 2012 (rat); Dong-mei et al., 2009 (Expt A/2 wk, rat); de Peyster et al., 2003 (rat); Williams et al., 2000 (rat); Biles et al., 1987 (rat); Greenough et al., 1980 (rat)) **DE** (Dong-mei et al., 2009 (Expt B/4 wk (rat))
		Seminal vesicle weight **NE** (de Peyster et al., 2014 (rat); Bermudez et al., 2012 (rat); de Peyster et al., 2008 (mouse); de Peyster et al., 2003 (rat); Williams et al., 2000 (rat); Biles et al., 1987 (rat))
		Liver and testis microsomal aromatase activity **NE** (de Peyster et al., 2014 (rat)) **DE** (de Peyster et al., 2003 (rat))
		Aromatase mRNA **NE** (de Peyster et al., 2014 (rat liver)) **IN** (de Peyster et al., 2014 (rat testis))
		Testosterone measured in testis **NE** (de Peyster et al., 2014 (rat); Bermudez et al., 2012 (rat); Williams et al., 2000 (rat))

[a] NE in Expt 1 when sampled on Day 14, by tail vein, or on Day 28 at necropsy, both sampling times 12–20 h after the final MTBE dose, but DE in the same Expt 1 when blood was sampled by tail vein 4–5 h after the first MTBE dose.
[b] NE in Expt 2 when sampled on Day 14 by tail vein just prior to dosing, but DE on Day 28 at necropsy 16–20 h after final MTBE dose.

necessary to conclude a positive response according to the guideline. The study by de Peyster et al. (2003) was performed in a similar manner to a Hershberger method, although weights of only two androgen-sensitive tissues, prostate and seminal vesicles, were evaluated. In that study, MTBE did not increase the weights of prostate or seminal vesiscles in rats not supplemented with testosterone propionate. Further corroboration of no impacts on prostate and seminal vesicles in both rats and mice comes from the results of a number of studies with endpoints considered as rank 2 (de Peyster et al., 2014, 2008, 2003; Bermudez et al., 2012; Williams et al., 2000; Biles et al., 1987; Greenough et al., 1980) (Table 3). An androgen agonist would be expected to increase prostate weight – not decrease it – a finding reported by one rat study using a very high dose of MTBE (1600 mg/kg BW/day) (Dong-mei et al., 2009).

Increases in the presence of tubercles in female fathead minnow is specifically linked to positive activity in the androgen pathway (Ankley et al., 2003; Ankley and Gray, 2013). In the fathead minnow FSTRA, no tubercles were present on female fish suggesting that MTBE is not androgenic in that test system.

In addition to prostate and seminal vesicle weights already discussed earlier, other rank 2 endpoints include testis and epididymal weights and histopathology, female VTG and gonadal histopathology in fish, and AR competitive binding. Testis weight and histopathology were largely unaffected in both rats and mice after exposure to MTBE (Table 3). There were two studies that

showed an increase in testis weight, although, in both, only relative not absolute weight was increased coinciding with a significant reduction in body weight gain indicating an exceedance of the maximum tolerated dose (Bermudez et al., 2012; Williams et al., 2000). Conversely, there was a decrease in relative testis weight in a 2-wk study by Dong-mei et al. (2009). However, the weight difference was not seen in a companion 4-wk experiment and the magnitude of the change in the 2-wk experiment was the same at all doses of MTBE making the finding suspect. As noted in the discussion of the estrogen agonist hypothesis, testis histopathology differences were only noted in one rat study (Li et al., 2008), however, there were no details in the report concerning severity and incidence of the finding. No difference from control in epididymides weight or histopathology was observed in any study (Table 3).

No change in prostate and seminal vesicle weight was already noted in the rank 1 endpoint evaluation of a Hershberger-like study (de Peyster et al., 2003). In rank 2, additional studies that measured the weights of these two organs in intact rodents were added to the table (Table 3). The only change in weight was a slight but statistical decrease at the highest dose (1600 mg/kg BW/d) in one 4-wk experiment in one study assessing prostate weight (Dong-mei et al., 2009). This was not observed at an earlier time point in a companion experiment, and neither experiment resulted in a clear dose response so the decrease in weight could have been an incidental finding.

In the rank 2 endpoints in the fish study, no differences compared to controls in concentrations of VTG were observed in female fathead minnow and zebrafish. There were no gonadal histopathological findings of note with fathead minnows, however, there was an increase in oocyte atresia in the zebrafish study (Mihaich et al., in preparation). As previously noted, changes in gonadal histopathology that are in relative isolation to related corroborative responses can be the result of other toxic stressors apart from primary perturbation of endocrine relevant pathways and some oocyte atresia is relatively common even in control fish (McCormick et al., 1989; Hunter and Macewicz, 1985; Henderson, 1963). In rank 3 endpoints, fecundity was decreased in zebrafish but not fathead minnow, while there were no effects in either species with regard to fertilization success, GSI, and behavior (Table 3).

The *in vitro* AR binding was considered a rank 2 endpoint for androgen agonists in the Borgert et al. (2014) paper even though the assay does not indicate what kind of activity might result from binding to the receptor. The caveat was that if binding occurred, the collective WoE would be necessary to identify the potential MoA for the compound. In the current assessment, AR binding is considered a rank 3 endpoint to be consistent with the placement of the ER binding study. MTBE was a non-binder with the androgen receptor (de Peyster et al., 2014) (Table 3). Two guideline *in vitro* screens, considered rank 3 endpoints, were performed to identify impacts on the steroidogenic pathway. One is the steroidogenesis screen that detects both inducers and inhibitors of enzymes responsible for the production of male and female steroid sex hormones (US EPA, 2009f). The endpoints in this cell-based screen are testosterone and estradiol concentrations (Table 3). MTBE does not interfere with steroidogenesis as measured by steroid hormone concentrations in H295R cells (de Peyster et al., 2014). Aromatase is an enzyme complex responsible for estrogen biosynthesis, converting androgens to estrogens in the steroidogenic pathway. The screen detects chemicals that inhibit aromatase activity. MTBE did not inhibit aromatase activity (de Peyster et al., 2014) (Table 3).

There are many endpoints that are listed as rank 3 for the androgen agonist hypothesis. Given that the ranks 1 and 2 endpoints are predominately negative, suggesting that MTBE does not have the potential to act as an androgen agonist, a few of the rank 3 endpoints will be discussed to add additional support to the higher ranked results. Although androgen agonists can decrease testosterone concentrations (O'Connor et al., 2000), hormonal measures are a rank 3 endpoint because there are a number of confounding factors that can impact circulating concentrations including overt toxicity, food intake, and metabolic enzyme induction (Marty, 2013; Laws et al., 2007). Testosterone concentrations were measured in a number of studies with both rats and mice (Table 3). There were statistically significant reductions in circulating concentrations reported in a few experiments at the highest dose levels although there was not a consistent response even within other experiments reported by the same authors (Li et al., 2008; de Peyster et al., 2003; Williams et al., 2000). MTBE exposure *in vivo* increased activity of testosterone biotransformation enzymes in rat liver microsomes (Williams and Borghoff, 2000), the implication being that catabolism and elimination could be faster after exposure to MTBE so circulating concentrations would be reduced. However, comparing studies where there were reported differences from controls in testosterone concentrations with rank 2 responses in testes and epididymides weights and histopathology, prostate weight, and seminal vesicle weight highlights the lack of concordance in hormonal and organ responses.

The androgen agonist, testosterone, has been shown to increase uterine weight and uterine stromal cell proliferation, increase serum testosterone and prolactin, and decrease serum FSH and LH in the pubertal female study (O'Connor et al., 2000). However, in a similar study by Kim et al. (2002), exposure to testosterone at 0.05, 0.2, and 1.0 mg/kg/day by subcutaneous injection resulted in a decrease in uterine (top two doses) and ovarian (all three doses) weights albeit at lower doses than employed in the O'Connor et al. (2000) study. Divergent responses in similar studies reduces the weight that can be attributed to the endpoint for the hypothesis which is a reason that hormone concentrations have been considered a rank 3 endpoint. In studies with MTBE, uterine weight was either not changed or decreased compared to controls (Table 3).

No effect in the rank 1 endpoints from the Hershberger-like assay and the FSTRA, no binding in the rank 2 AR binding assay and inconsistent responses in other *in vivo* rank 2 endpoints such as testis weight and histopathology and rank 3 endpoints (e.g. uterus and ovary weight, histopathology, testosterone concentrations) are not supportive of MTBE having the potential to act as an androgen agonist.

4.4. Androgen antagonist hypothesis

Ranks 1 and 2 endpoints for the mammalian studies in the androgen antagonist hypothesis are the same as for the androgen agonist hypothesis (Table 4). In the study performed in a similar manner to a Hershberger method (de Peyster et al., 2003), androgen-dependent organs weighed from rats treated with MTBE and testosterone propionate were not different from those receiving testosterone propionate only. The two studies showing an increase in relative (but not absolute) testis weight (Bermudez et al., 2012; Williams et al., 2000), one reporting a decrease (Dong-mei et al., 2009) in relative testis weight (absolute weight not provided for comparison), and one reporting unusual testis appearance at the microscopic level (Li et al., 2008) but without sufficient detail, make these findings equivocal, as discussed in the previous section. No differences from control in epididymides weight and histopathology, and seminal vesicle weight, were observed in any study. Prostate weight, a rank 2 endpoint, was decreased in one rat study (Dong-mei et al., 2009) although using a very high dose of MTBE (1600 mg/kg BW/day), while many others reported no change (Table 4). Other than the few inconsistent changes, rank 1 and 2 mammalian endpoints are not affected by MTBE exposure in a way that suggests androgen antagonism.

The FSTRA endpoints and rankings are slightly different than in the agonist hypothesis. There are no endpoints considered appropriately sensitive and specific to be rank 1 in the FSTRA. Rank 2 endpoints are an increase in female VTG, a decrease in male secondary sex characteristics, and changes in gonad histopathology indicative of an androgen antagonist. No changes in female VTG or male secondary sex characteristics were observed in the FSTRA studies with fathead minnow and zebrafish (Mihaich et al., in preparation). Reduced oocyte maturation is a hallmark of antiandrogen exposure in fish (Martinović et al., 2008; US EPA, 2007). In the zebrafish study, the increase in oocyte atresia along with a statistically significant increase in the accumulation of oocyte debris in the oviduct could indicate exposure to an androgen antagonist. Given the previous discussion of oocyte atresia being a common occurrence in fish and often related to general toxicity or stress unrelated to a sex hormone pathway and the lack of any other androgen sensitive response in the fish studies, it is unlikely that MTBE is exhibiting a potential to act as an androgen antagonist. Rank 3 endpoints were discussed with respect to the androgen agonist hypothesis and a similar discussion could be made here. However, no rank 1 endpoint was impacted by exposure to MTBE and the lack of consistent androgen antagonist effects in the rank 2 rodent and fish endpoints suggest that MTBE is not acting like an antiandrogen.

4.5. Thyroid agonist hypothesis

Borgert et al. (2014) list asynchronous development and thyroid histopathology in *Xenopus* tadpoles from the amphibian metamorphosis assay (AMA) (US EPA, 2009g) as rank 1 endpoints for the thyroid agonist hypothesis. No relevant amphibian studies with rank 1 endpoints exist for MTBE.

Rank 2 endpoints include thyroid weight and blood hormone concentrations in both female and male rodents (Table 5). No differences compared to controls were identified with respect to thyroid weight in either female rats or mice. Of the six MTBE studies that reported thyroid weight in males, three reported no effect (Dodd et al., 2013; Chun and Kintigh, 1993; Bermudez et al., 2012 (13-wk study)). One detected an increase in relative thyroid:body weight and thyroid:brain weight ratios, although there was also a significant reduction in body weight at the same dose (800 mg/kg by gavage) which could contribute to the greater organ:body weight ratio. Another study detected a decrease in absolute weights of thyroid/parathyroid in males but weight was not significantly different from controls when normalized to body weight (Bermudez et al., 2012 (1 year study)). The thyroid hormones T3, T4 and TSH were measured in one study with mice (Chun and Kintigh, 1993) and two studies in rats (Dodd et al., 2010; Williams et al., 2000 (males only)). No differences compared to controls were identified in females with respect to T3, T4, and TSH measurements. In males, thyroid hormone measurements were primarily unchanged although there were some increases and/or decreases in a few studies. The effects of thyroid hormone are complex making it difficult to firmly establish an expected pattern of response. However, of the studies that reported changes compared to controls in T4, T3 or TSH concentrations, T4 and TSH increased while T3 was not impacted in one study and only in males (Chun and Kintigh, 1993), the decrease in T4 in Bermudez et al. (2012) was only observed in the high dose on day 28 of a 13-week study, and T3 was decreased in only one experiment of a multiple experiment study (Williams et al., 2000) with no supporting changes in T4 and TSH and no measurement of thyroid weight or histopathology. In other words, any thyroid hormone or weight changes reported were neither consistent nor accompanied by supporting evidence of thyroid histopathology. Tentative conclusions from rank 2 endpoints are suggestive that MTBE is not exhibiting a potential to act as a thyroid agonist, however, consulting rank 3 endpoints will help to add confidence in the assessment.

Pituitary weight is a rank 3 endpoint. Like thyroid weight, pituitary weight was primarily unchanged, although there are two studies with conflicting results. Williams et al. (2000) reported an increase in pituitary weight after doses of 1500 mg/kg BW/day MTBE for 2 weeks. This increase was seen in only one of six studies with male rats that reported weights of pituitary, including two companion experiments, one 15-day and one 28-day, conducted by the same investigators where no weight change was seen. A decrease was seen in the other study, this one using female mice (Moser et al., 1998). Thyroid histopathology was considered a rank 3 endpoint, primarily because not enough information has been documented yet about expected patterns of response to distinguish agonists from antagonists. However, given there are no observed effects on thyroid histopathology in conjunction with no consistent effects in the rank 2 thyroid weight and hormone measurements, there is good support for MTBE not interacting with the thyroid pathway in rodents.

In a one-dose study at 100 mg/L, weights of tadpoles and frogs were increased and time to metamorphosis was reduced by 2 days compared to controls after exposure to MTBE (Paulov, 1987). The lack of coincident information on thyroid histopathology and specific measurements on other growth parameters make these apical endpoints difficult to put into context in the WoE and can only be viewed as corroborative in the entire WoE analysis.

In summary, the consistent rank 2 endpoints along with little or no change in pituitary weights and no impacts on pituitary and thyroid histopathology are suggestive that MTBE is not exhibiting a potential to act as a thyroid agonist.

4.6. Thyroid antagonist hypothesis

Metamorphosis and thyroid histopathology in amphibians and thyroid weight and histopathology in rodents are rank 1 endpoints for the thyroid antagonist hypothesis. As was stated for the thyroid agonist hypothesis, no relevant amphibian studies are available. No differences from controls for the female rodent endpoints of thyroid weight and histopathology were noted in studies with either rats or mice. In male rodent studies, no histopathology differences between control and MTBE treated rats and mice were observed. Thyroid weight was primarily unchanged although there are two studies with conflicting results (e.g., an increase in thyroid weight in one and a decrease in the other).

Similar conflicting results were seen in rank 2 endpoints where thyroid hormone measurements were primarily unchanged as noted in the previous discussion under the thyroid agonist hypothesis. In females, there was no change in ovary weights in a number of studies in rats but there was a decrease in ovary weight in two studies with mice (Moser et al., 1996, 1998) which would be expected with a thyroid antagonist (Marty et al., 1999). In males, thyroid hormone measurements were primarily unchanged although there were some increases and/or decreases in a few studies that did not fit a consistent pattern (Table 6).

No consistent patterns were evident when considering the rodent experiments that measured ranked thyroid endpoints and reported any statistically significant findings, all at very high doses (Table 6). The inconsistencies could simply be indicative of incidental findings; limitations of single-time point thyroid hormone measurements; different levels of stress; weight gain suppression in the test animals; and/or other systemic toxicities co-occurring in animals in different experiments. Closer scrutiny was warranted to avoid overlooking any more subtle signs of known modes of action involving the thyroid; for example, liver enzyme induction with secondary effects on thyroid (Marty, 2013). If this were the

case, then expectations would include liver enlargement with centrilobular hepatic hypertrophy, indicating increased liver P450 and suggesting faster catabolism of T4/T3, increased TSH as a consequence of that, and, if that occurs, an expectation of some sign of effect in the thyroid itself like increased weight or hyperplasia of follicular cells. Both liver and thyroid endpoints had to be measured in the same test animals for this comparison to be meaningful. After reviewing the studies that reported on one or more key endpoints for both thyroid and liver that are necessary to demonstrate applicability of that MoA, this thyroid–liver connection that has been identified as relevant to some potent P450 inducers apparently does not apply to MTBE. This is perhaps not too surprising insofar as MTBE is recognized as only a weak liver enzyme inducer.

Similar to the thyroid agonist hypothesis, the lack of any consistent change in the rank 1 thyroid weight and histopathology results, no difference in the rank 2 female thyroid hormone measurements in both rats and mice and the conflicting changes in thyroid hormones in the male studies suggest that MTBE does not have thyroid antagonist properties.

4.7. Steroidogenesis induction hypothesis

This hypothesis refers specifically to the synthesis of steroids in the estrogen and androgen pathways by steroid-producing endocrine organs. Although corticosterone is a steroid produced by adrenal glands, blood concentrations of corticosterone, adrenal weights and histopathology are not currently considered diagnostic of endocrine modes of action (OECD, 2012) Steroidogenesis also does not include how steroids are handled by the body after they are produced (e.g., concentrations and binding to sex hormone binding globulin produced primarily in liver, or catabolism and excretion of steroids, all of which are largely dependent on non-endocrine organs). Information on expected responses to a chemical that induces steroidogenesis is lacking, thus no rank 1 endpoints were identified (Table 7). To address the possibility that MTBE would interact with the steroidogenic pathway, estradiol and testosterone concentrations from the guideline *in vitro* steroidogenesis study were identified as rank 2 endpoints. MTBE was classified negative for effects on testosterone and estradiol in the steroidogenesis assay. In the Borgert et al. (2014) relevance rankings, testosterone concentrations in the male pubertal assay are considered a rank 3 endpoint as it could be assumed that steroid hormone concentrations would increase with inducers of steroidogenesis. Only testosterone and thyroid hormones are evaluated in the *in vivo* EDSP assays, otherwise estradiol would also be relevant as a rank 3 corroborative endpoint so we included it as additional evidence. Plasma testosterone and estradiol concentrations from male and female rodent studies with MTBE could thus, potentially be supportive of the *in vitro* responses. Only one experiment of two reported in the same study has reported an increase in testosterone concentration (Li et al., 2008). The rest of the supporting studies either reported no changes or a decrease in testosterone concentrations compared to controls. No change has been seen in estradiol concentrations when measured in female mice. A significant increase in circulating estradiol was seen in only one of three male rodent experiments in which this has been measured. Ongoing adjustments occurring at the level of the hypothalamus and pituitary to maintain normal circulating concentrations make it especially important to see consistency in blood hormone results in multiple studies. The activity and mRNA of aromatase, a steroidogenic enzyme, have been measured in tissues from male rats treated with high doses of MTBE. Results have been mixed, with activity decreased in a study with only one high (1200 mg/kg) MTBE dose group (de Peyster et al., 2003) but not statistically different in a more extensive follow up experiment (de Peyster et al., 2014), and mRNA showing an increasing tendency in testis related to dose that was statistically significant but with a weak correlation, and no effect in liver – none of which consistently supports significant aromatase induction. The lack of effect in the *in vitro* steroidogenesis assay along with the lack of consistent hormonal response in the *in vivo* rodent studies suggests that MTBE is not inducing steroidogenesis.

4.8. Steroidogenesis inhibition hypothesis

Similar to the steroidogenesis induction hypothesis, no rank 1 endpoints were identified for the steroidogenesis inhibition hypothesis (Table 8). Hormone concentrations in the *in vitro* steroidogenesis assay as well as female VTG and male gonad histopathology in the FSTRA are considered sensitive and specific enough endpoints to be rank 2. There were no differences from controls in estradiol or testosterone concentrations in the steroidogenesis study. Similarly, in the fish study there were no effects on female VTG or male gonadal histopathology.

There are many rank 3 endpoints for the steroidogenesis inhibition hypothesis. Aromatase inhibition in the aromatase *in vitro* assay is a strong corroborative endpoint in the hypothesis as it directly relates to the conversion of testosterone to estradiol, however it is only one specific step in the pathway thus was considered a rank 3 endpoint. While Borgert et al. (2014) did not include male androgen-dependent weights, Stoker and Zorrilla (2010) and O'Connor et al. (2002b) both noted that the steroidogenesis inhibitor ketoconazole reduced the weight of androgen-dependent tissues in both the pubertal male study and the 15-day intact adult male study. For completeness, testis, prostate, and seminal vesicle weights, as well as hormone concentrations, aromatase activity and mRNA from *in vivo* rodent studies have been listed as rank 3 endpoints from the MTBE studies. Using the Borgert et al. (2014) approach we would not need to consider their response because they are much less sensitive and specific for the hypothesis. In summary, the lack of consistent supporting statistically significant findings in steroid hormone measurements from rank 3 studies, the preponderance of no effects reported in steroid hormone-dependent organ weights or histology, as well as the absence of impacts on aromatase and steroidogenesis enzyme activity *in vitro* and the fish study *in vivo*, suggest that it is unlikely that MTBE is inhibiting steroidogenesis.

5. Discussion

The definition of an endocrine disruptor is a substance that alters endocrine system function and consequently causes adverse health effects in an intact organism, or its progeny or in (sub) populations (WHO, 2002). We are now in a position to characterize the many responses seen as a consequence of exposure to MTBE in experiments using rodent and fish models. Using a transparent and systematic WoE approach, the eight endocrine pathway hypotheses have been tested with the large database available for MTBE. Of the rank 1 and 2 endpoints for the eight hypotheses, no consistent pattern emerged that supported agonistic or antagonistic effects of MTBE on estrogen, androgen, thyroid or steroidogenesis pathways. The majority of the endpoints were not different in MTBE-treated animals relative to the controls in the clear majority of the studies. Many of the studies are long-term – 13-weeks to 1–2 years. Most tests used adults but some testing used young animals, providing an opportunity to observe differences if they were occurring. In addition, in a series of studies comparing sensitivity of adults to pubertal animals, O'Connor et al. (2002a,b, 2000) used positive control compounds in a 15-day intact male assay to compare to the response of the male

pubertal assay and the Hershberger assay for their ability to identify endocrine active chemicals. A primary endpoint in the pubertal study not assessed in the adult study is onset of puberty. Using patterns of hormonal response and histopathology, the authors of that comparison study concluded that the ability of the intact adult male study to identify a potential MoA was comparable to that of the pubertal studies for the positive control compounds used. So, while puberty onset measurements are not available for MTBE, the wealth of existing hormonal and histopathological data provides sufficient information to assess potential MoA.

A few of the MTBE reproductive and pre-/post-natal developmental effects studies (Bevan et al., 1997a; Biles et al., 1987), and also a more recent publication describing a 2-year carcinogenicity bioassay of MTBE (Dodd et al., 2013) provided some additional endocrine organ-specific weight and histopathology information that could be ranked. As was noted earlier, these types of studies, and also a comprehensive neurotoxicological guideline GLP evaluation of MTBE (Daughtrey et al., 1997), provide many other apical effects endpoints, such as fertility and reproductive indices, fetal examination results, neuropathology evaluation with behavioral correlates, and tumor incidences that were not ranked. Despite not being ranked, they contribute substantially to a more general assessment of a chemical's ability to cause an adverse effect possibly by some endocrine system perturbation even if they do not identify an MoA or distinguish between endocrine agonistic and antagonistic properties. The absence of any significant adverse findings in these types of studies, especially if consistent across studies as is the case with MTBE, lends support for the position that this chemical has neither agonist nor antagonistic interactions with the hormonal pathways examined in detail in this analysis. In addition, no endocrine organ has been unequivocally recognized as a target of MTBE in carcinogenicity bioassays.

Because MTBE appears to have had some sporadic effects on endocrine endpoints in some of the studies we reviewed, the question of what could cause this other than a direct effect on the endocrine system should be addressed. Several MTBE study endpoints do not appear in the hypothesis ranking tables because they were not considered sufficiently related to endocrine activity or, even if related, could not help to distinguish between agonist and antagonist behavior. However, they are worth mentioning because these additional responses seen concurrently with more specifically endocrine organ-related effects are consistent with the idea that at least the high dose MTBE-treated animals experience significantly more stress than vehicle-treated controls. Most of the studies reviewed report one or more effects associated with non-endocrine organ toxicities. Those that could impact the endocrine system as well include central nervous system disturbances (manifested as transient lethargy, hypoactivity, ataxia, and lack of startle response), significant reductions in body weight gain, dehydration, increased adrenal gland weights, elevated serum corticosterone, and microscopic changes in adrenal glands (specifically loss of zona reticularis) (Dodd and Kintigh, 1989; Robinson et al., 1990; IIT Research Institute, 1992; Chun and Kintigh, 1993; Daughtrey et al., 1997; Lington et al., 1997; Bird et al., 1997; Bevan et al., 1997a,b; Moser et al., 1998; Zhou and Ye, 1999; Williams and Borghoff, 2000; Williams et al., 2000; de Peyster et al., 2003, 2008, 2014; Dong-mei et al., 2009). Exposures at which one or more of these occur are in the range of 3000–8000 ppm by inhalation, and 800–1600 mg/kg BW/day when MTBE is given by gavage. It should probably be clarified here that changes in corticosterone are not considered to be evidence of endocrine modulation by the tested substance when general stress is known or suspected as the likely underlying cause.

Stress responses in both female and male rodents include decreased concentrations of gonadotropin-releasing hormone (GnRH), gonadotropin hormones (FSH and LH), gonadal sex steroids (testosterone, progesterone, and estradiol) (Everds et al., 2013). Using immobilization of male rats to create repeated stress, although of temporary duration, researchers have found higher plasma concentrations of corticosterone and progesterone, reduced body weight gain, lipid depletion of the zona fascicularis of the adrenal glands, and lower plasma concentrations of the androgens testosterone and androstenedione (Pellegrini et al., 1998). Microscopic examination of the testes of those stressed animals revealed disorganized seminiferous tubular epithelium and enlargement of the interstitial spaces with considerable individual variability in extent and severity of the damage. "Disordered arrangement of seminiferous epithelium" is also how the investigators described their findings in the only MTBE study that reported abnormal testis in male rats, in that case after daily gavage with 800 or 1600 mg/kg BW/d MTBE for 28-days (Dong-mei et al., 2009). In reviewing that study, we also noted a surprising number of mortalities (3 of 10) in the lowest 400 mg/kg/day MTBE dose group and even one vehicle control animal, suggesting the possibility of improper or inconsistent gavaging. Caution is advised when interpreting effects that could be related to a general stress response (Everds et al., 2013), and this study is but one example of an MTBE study where stress may be the underlying cause.

Daily gavaging of animals is a common and accepted method in toxicology studies, but it still causes stress even if done properly (Brown et al., 2000). Unexpected test animal mortality is not uncommon especially in studies of MTBE administered by gavage, and gavage errors are not always distinguished from chemically-caused deaths in all study reports. Vehicle-treated animals are also gavaged to control for stress, but MTBE has a very pungent taste and odor and a low taste and odor threshold. Therefore, one can imagine an experimental animal gavaged with MTBE, regardless of the dose, might find that experience more stressful than being gavaged only with vehicles used in the MTBE studies.

Decrements in body weight can also confound interpretation of potential compound-related effects on the endocrine system (de Peyster et al., 2014; O'Connor et al., 2000). Significantly reduced final body weights relative to controls are often reported along with endocrine endpoints in MTBE experiments using rodents. Reduced concentrations of testosterone can be a function of suppressed body weight gain (Bergendahl et al., 1989; Chen et al., 2005). Decreased production of LH, FSH, estrogen and thyroid hormones has also been shown in studies on food restriction in rodents (Ahmed et al., 2012; Boelen et al., 2008). Even when a study does not report a statistically significant difference between control and treated animal body weights, further comparison of mean body weight gains across the test groups – which not all studies report but can often be ascertained from data provided – sometimes reveals markedly reduced weight gain in MTBE groups relative to controls.

In an attempt to fully understand underlying causes of the relatively few effects seen in endocrine organs after exposure to high doses of MTBE, an additional alternative explanation proposed involves the liver as a primary target with secondary effects on the endocrine system. This idea has been introduced in several WoE hypothesis discussions. Reduced concentrations of hormones, including estrogens, androgens, and TSH in serum, can occur when the enzymes involved in their catabolism are induced. Experiments focusing on steroid metabolic enzymes have demonstrated an ability of MTBE to increase total P450 and more specifically activity of enzymes involved in estradiol and testosterone metabolism (Moser et al., 1996; Williams and Borghoff, 2000). MTBE is not considered a strong inducer. Furthermore because it appears to accelerate its own metabolism (Williams and Borghoff, 2000), over time the degree of induction and its consequences would diminish. Varying

degrees of liver enzyme induction occurring in different studies could explain dissimilar effects on endocrine endpoints along with other plausible explanations like differences in reduction of body weight gain and different degrees of stress experienced by the test animals.

In summary, this analysis represents an attempt to systematically gather, analyze and weigh all evidence currently available related to potential endocrine activity of MTBE. Several investigators (including one of the authors) have conducted experiments in rodent and fish models and found some hormone concentrations and other endocrine-related endpoints changed with high doses of MTBE; however, one must remember that hormone concentrations alone, or isolated reports of other changes that do not show dose response or are not confirmed by multiple other studies, should never be assumed to demonstrate an endocrine system effect. To reach scientifically justified conclusions based on the totality of *in vitro* and *in vivo* evidence, this WoE procedure involved a semi-quantitative relevance weighting of each endpoint for each hypothesis and a systematic consideration of each endpoint in a variety of different study designs. The totality of the evidence thus far does not support a direct effect on the endocrine system in terms of the hypotheses tested. A weight of evidence approach is therefore essential for revealing potential endocrine effects of MTBE.

Conflict of interest

Dr. de Peyster reports personal fees from the European Fuel Oxygenates Association during the conduct of the study; personal fees from the European Fuel Oxygenates Association outside the submitted work; and consultation assistance provided on several occasions to one of the members of the European Fuel Oxygenates Association (Lyondell Basell). Dr. Mihaich reports personal fees from the European Fuel Oxygenates Association and the Asian Clean Fuels Association during the conduct of the study; and personal fees from the Endocrine Policy Forum, the European Fuel Oxygenates Association, the Asian Clean Fuels Association, and various other clients outside the submitted work.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.yrtph.2014.04.017.

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