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Scientific and Regulatory Policy Committee (SRPC) Points to Consider*: Histopathology Evaluation of the Pubertal Development and Thyroid Function Assay (OPPTS 890.1450, OPPTS 890.1500) in Rats to Screen for Endocrine Disruptors

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Abstract

The U.S. Environmental Protection Agency Endocrine Disruptor Screening Program (EDSP) is a multitiered approach to determine the potential for environmental chemicals to alter the endocrine system. The Pubertal Development and Thyroid Function in Intact Juvenile/Peripubertal Female and Male Rats (OPPTS 890.1450, 890.1500) are 2 of the 9 EDSP tier 1 test Guidelines, which

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AUTHOR CONTRIBUTION

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assess upstream mechanistic pathways along with downstream morphological end points including histological evaluation of the kidneys, thyroid, and select male/female reproductive tissues (ovaries, uterus, testes, and epididymides). These assays are part of a battery of *in vivo* and *in vitro* screens used for initial detection of test article endocrine activity. In this Points to Consider article, we describe tissue processing, evaluation, and nomenclature to aid in standardization of assay results across laboratories. Pubertal assay end points addressed include organ weights, estrous cyclicity, clinical pathology, hormonal assays, and histological evaluation. Potential treatment-related findings that may indicate endocrine disruption are reviewed. Additional tissues that may be useful in assessment of endocrine disruption (vagina, mammary glands, and liver) are discussed. This Points to Consider article is intended to provide information for evaluating peripubertal tissues within the context of individual assay end points, the overall pubertal assay, and tier I assays of the EDSP program.

Keywords

EPA; endocrine disruptor screening program; pubertal development; thyroid function; OPPTS 890.1450; OPPTS 890.1500

Background

A working group directed by the Society of Toxicologic Pathology (STP) Scientific and Regulatory Policy Committee was formed to summarize the proceedings of the 2013 Endocrine Disruption Screening Program (EDSP) Workshop on the pathology end points of the male and female pubertal assays hosted by the National Toxicology Program at the National Institute of Environmental Health Sciences (EDSP Workshop; <http://www.niehs.nih.gov/news/newsletter/2013/4/science-review/>). Recommendations described herein are in accordance with the published U.S. Environmental Protection Agency (EPA) guidance documents (EPA 2009a, 2009b) and subsequent peer-reviewed recommendations. As peripubertal animals exhibit subtle morphological changes subject to variation based on numerous intrinsic factors (strain, supplier, and rate of growth; Keenan et al. 2009) and extrinsic factors (nutrition and stress; Everds et al. 2013; Laws et al. 2007), these Points to Consider are put forth in an attempt to assist the study pathologist and EPA reviewers in assay interpretation and improve cross-laboratory harmonization of pathology results (Foster and McIntyre 2002).

Introduction

In 1996, the U.S. Congress passed the Food Quality Protection Act and the Safe Drinking Water Act Amendments, mandating the EPA to develop a regulatory program addressing the ability of environmental chemicals to perturb homeostasis of the endocrine system (Colborn, vom Saal, and Soto 1993; Collin 2006). The EDSP was subsequently developed and validated as a method of identifying chemicals with the potential to alter female (estrogens; E) and/or male (androgens; A) hormone signaling and thyroid function in fish, wildlife, and humans (www.epa.gov/endo). The EDSP is divided into a tier 1 screening battery, which is intended to identify chemicals that have the potential to interact with the endocrine system (Table 1), and tier 2 tests that are intended to confirm endocrine activity, identify adverse

effects in intact animals, and establish a definitive dose–response relationship for risk assessment. The data from tier 1 EDSP assays are interpreted through a weight of evidence (WoE) approach to balance the importance of end points, both within a single assay and between assays within the tier. In this context, tier 1 assays represent a tool for screening compounds requiring definitive testing within tier 2. The spectrum of EDSP assays is intended to provide overlapping data to allow for integrative evaluation of 5 known pathways of endocrine disruption through both mechanistic and apical end points: the estrogen and androgen hormonal pathways, the steroidogenic pathway, and the hypothalamic–pituitary–gonadal (HPG) and hypothalamic–pituitary–thyroid (HPT) axes.

The “pubertal assays” (female OPPTS 890.1450 and male OPPTS 890.1500) or “Guidelines” (EPA 2009a, 2009b) are tier 1 EDSP screening assays (Table 2) that provide *in vivo* mammalian data capable of detecting endocrine modulation via all 5 described pathways (Tables 3 and 4). Within tier 1, the pubertal assays are the only mammalian assessment of the HPG/HPT axes and the only assays performed in a reproductively intact mammalian system. The pathologist is to “evaluate for pathologic abnormalities and potential treatment-related effects” arising from alterations in these axes. The specific criteria required for a valid assay may be found in the Guidelines. Briefly, the pubertal assays utilize weaned Sprague-Dawley (or less commonly Wistar) rats that are administered a test article daily on postnatal day (PND) 22 or 23 until necropsy on PND 42 (43) for females and PND 53 (54) for males (with an additional day allowed for either sex if necessary based on timing restrictions for necropsy completion). Juvenile rats are derived from individually housed, time-mated primiparous females. Treatment groups of 15 nonlittermate F1 pups are treated by oral gavage with vehicle and test article at a minimum of 2 dose levels, plus vehicle control. Dose levels chosen should approach—but not exceed—the maximum tolerated dose (MTD). In the absence of clinical signs, significant changes in clinical pathology parameters, gross or histological signs of toxicity, the MTD may be recognized by a statistically significant ($\alpha = 0.05$) decrement (no more than 10%) in the terminal body weight or body weight gain (Laws et al. 2007) as compared to the concurrent controls. The study director and study pathologist should confirm that these or other signs of systemic toxicity do not confound the interpretation of this assay.

Pubertal development timing is assessed by daily observations for preputial separation in males and vaginal opening in females. The timing of the juvenile and peripubertal periods (PND 22 to PND 30–32) may vary between laboratories and strains of rats (Ojeda and Skinner 2006). End points include organ weights (testes, epididymides, ovaries, uterus, pituitary gland, adrenal glands, liver, kidneys, and thyroid), macroscopic and microscopic (kidney, thyroid gland, testis and epididymis, ovary, and uterus), and measurement of serum thyroxine (T_4), serum thyroid-stimulating hormone (TSH), testosterone (in males), and routine clinical chemistry parameters, including blood urea nitrogen (BUN) and creatinine (CRT). Detailed considerations for each of these end points specific to the male and female assay are discussed subsequently. The female pubertal assay results are presented both by tissue and by estrous cycle, as interpretation is integrally linked to cyclicity, or lack thereof. The male pubertal assay results are presented by organ. Nomenclature presented for both sexes are consistent with terminology in International Harmonization of Nomenclature and

Diagnostic Criteria for Lesions in Rats and Mice (INHAND; D. C. Creasy et al. 2012; Dixon et al. 2014) that contains photomicrographs of lesions described herein.

The Pubertal Development and Thyroid Function Assay in Female Rats: OPPTS 890.1450

The rat is born at the stage comparable with 150 days of human gestational life. The female postnatal through prepubertal development period can be divided into the following 4 phases: neonatal (birth to PND 7), infantile (PND 8 to PND 21), juvenile (PND 22 to PND 30–32), and peripubertal (PND 30–32 to first ovulation) (Ojeda and Skinner 2006), although the timing of the juvenile and peripubertal periods vary widely between reports, laboratories, and strains of rat. Thus, the pubertal assay specifically tests the endocrine effects of test articles during the juvenile and peripubertal periods, which are the periods of intense maturational changes in the HPG axis. In the female, the peripubertal period culminates with the preovulatory surge of gonadotropins and vaginal opening, events that are followed by the first estrous and initiation of luteal function. The vagina is imperforate before puberty and becomes patent with estrogenic stimulation. Vaginal opening usually occurs on the day after the first preovulatory surge of gonadotropins (Ojeda, Advis, and Andrews 1980; Ojeda and Skinner 2006). The first ovulation in commonly used laboratory rat strains ranges from PND 29 (Picut, Dixon, et al. 2014; Tamura, Abe, and Kogo 2000) to PND 38 (Ojeda, Advis, and Andrews 1980). Perturbation of any of these highly interrelated steps leading to first ovulation can result in clinically apparent changes in the reproductive cycle of the pubertal animal such as delayed vaginal opening, delayed first onset or abnormal estrous cyclicity, and morphologically detectable alterations in the reproductive tissues such as (but not limited to) lack of estrous cyclicity; lack of follicular development in the ovary, uterine myometrial, and/or stromal atrophy or hypertrophy; and uterine and vaginal epithelial atrophy, hypertrophy, or hyperplasia.

The following Points to Consider are recommended:

1. Organ weights: In both the male and female assays, absolute, rather than relative (to body or brain) weights, are the primary data recommended by the test Guideline. The peripubertal period is characterized by intense maturational changes in the HPG axis, marked increases in uterine weight, cyclical accumulation of uterine fluid, and enhanced ovarian responsiveness to gonadotropins (Astwood 1939; Carroll 1945). There are multiple examples of xenobiotics that are associated with uterine weight increases (Cline et al. 2004; Moller et al. 2012) or decreases (Losco et al. 2010; Rodrigues-Junior et al. 2012), many of which are known to perturb the HPG axis or have direct effects on ovarian function. Discerning a spontaneous—or estrous cycle related—organ weight fluctuation from a true test article-related organ weight change often requires a WoE approach including estrous cyclicity, body weight data, and most importantly, histological data. Differences in mean ovarian weights between groups may be more difficult to appreciate due to the inherent smaller size and therefore proportionally higher weight fluctuation of this tissue (Picut et al. 2013).

Normal fluctuations associated with estrous cycle fluid accumulations result in female reproductive tissue organ weight changes (<20%) that may not afford a histological correlate due to tissue blotting (required at necropsy) and processing (Sellers et al. 2007). As such, it is important for the pathologist to be aware of both the estrous cycle stage and organ weights of each animal prior to starting microscopic evaluation. Decreased body weight and reproductive tissue weights in conjunction with a histological appearance suggesting lack of normal cyclicity commonly occur due to stress associated with systemic toxicity (in adult animals) and should not be confused with direct, test article-related endocrine disruption (Everds et al. 2013). Body weight decrements of >6% may result in perturbations of endocrine end points (see Hormone evaluation of thyroid development) and confound interpretation of the assay (Laws et al. 2007).

For these reasons, pubertal organ weight changes should be interpreted with caution. Organ weight changes that are associated with endocrine disruptor compounds generally exhibit both a strong dose-response relationship and concurrent histological findings.

Uterine and ovarian collection techniques demand absolute interanimal consistency to achieve a valid assay as outlined in the Guidelines performance criteria. Figure 1 clarifies the uterine slitting technique and transection points for removing the ovaries and separating the vagina from the uterus and cervix as described in the Guidelines. Meticulous prosector training and scale calibration records are essential to creating reliable data sets (Long, Symanowski, and Roback 1998).

2. Histological evaluation of the female reproductive tract: The female pubertal assay Guideline requires the histological evaluation of the ovaries and uterus; histological evaluation of the oviducts, cervix, and vagina is not required, nor are they validated assay end points. More precise information regarding the normal histological features of the reproductive organs of rats of this age range is available (Picut, Dixon, et al. 2014; Picut, Remick, Asakawa, et al. 2014).
 - a. Ovaries: The primary emphasis of the histological evaluation of the ovary is “to document the lack of follicular development.” Ovarian maturation, responsiveness to gonadotropins (follicle-stimulating hormone [FSH] and luteinizing hormone [LH]), growth hormone, prolactin (PRL), and neural control (via peptidergic and adrenergic nerves) occur throughout the juvenile period. Small (primordial/primary) follicles are gonadotropin-independent phases of follicular development while growing follicles are gonadotropin-dependent (Rajkovic, 2006). The Guideline requires 5 random hematoxylin and eosin (H&E)-stained sections (using the method of Smith et al. 1991) be evaluated for follicular development. Ovarian histology should include an evaluation of the “presence/absence of tertiary/antral follicles, presence/absence of corpora lutea (CL), changes in corpus luteum development, and changes in number of both primary and atretic follicles, in addition to any abnormalities/lesions, such as ovarian atrophy.” Thus, the microscopic evaluation of the ovary should assess follicle development and atresia and

the overall progression of CL through formation, development, and regression. As the reference cited in the Guidelines (Smith et al. 1991) specifically refers to the methods of sectioning the ovary for follicle counting, there was uncertainty as to the necessity for quantitative analysis (i.e., follicle enumeration). Both quantitative and qualitative methods of follicle evaluation were used by EDSP Workshop participants, thus generating a source of interlaboratory variation in the performance of the female pubertal assay. The EPA has since clarified that quantitative methods of follicular evaluation are not required for the female pubertal assay Guideline (EPA 2009a); however, a qualitative evaluation of the number of primordial and atretic follicles is recommended.

Standardization of follicle terminology is essential for interlaboratory harmonization. INHAND terminology is considered adequate for evaluation of toxicological studies and is preferred (Dixon et al. 2014).

i. Follicles

1. **Primordial follicles:** These structures along with the primary growing follicle are normally quite numerous in the ovaries of peripubertal rats (Picut et al. 2013; Dixon et al. 2014). The primordial follicle is defined as an oocyte surrounded by a single, sometimes incomplete, layer of flattened pregranulosa cells at the periphery of the follicle with no cuboidal cells. Although the Guideline requires only the tissue to be examined by H&E staining, it may be advantageous to use adjunct staining techniques that better highlight the primordial and also primary growing follicles (described subsequently). Immunohistochemistry utilizing the proliferating cell nuclear antigen antibody is one such staining method that can assist in the identification of these structures (Picut et al. 2008).
2. **Growing follicles:** For descriptive purposes, growing follicles should be further classified by morphologic appearance as primary, secondary, vesicular, or tertiary follicles. Primary growing follicles have a central oocyte surrounded by either a mixture of flattened pregranulosa cells and plump cuboidal granulosa cells or a single layer of cuboidal granulosa cells. Such follicles often “congregate” in the ovarian medulla but can be difficult to discern from the surrounding interstitial cells in routine H&E sections. Secondary growing follicles contain an ovum surrounded by an eosinophilic zona pellucida with concentric multiple (2 or more) layers of granulosa cells forming a zona granulosa, with or without a final peripheral layer of theca cells. Vesicular growing follicles are secondary growing follicles with fluid-filled spaces that have not coalesced into a single antrum. Tertiary growing follicles

contain all of the structures of a secondary growing follicle plus the development of a single antrum (antral follicles) with or without a cumulous oophorus that forms a stalk extending into the antral cavity. The tertiary classification includes preovulatory follicles (Yuan and Foley 2002).

3. Atretic follicles: These are regressing follicles that are characterized by apoptosis of the granulosa cells and may or may not contain a necrotic ovum. During the juvenile period, the ovary undergoes waves of follicular development and atresia, but follicles do not reach the preovulatory stage, most likely as a result of low gonadotropin levels (FSH and LH; Ojeda and Skinner 2006). Thus, large groups of atretic follicles may be evident in the normal/control juvenile or peripubertal stage, before ovulation has occurred. As such, atretic follicles would be particularly evident in animals that die or are euthanized during the juvenile period, prior to the scheduled Day 42 necropsy. The quantity of atretic follicles may be quite numerous in the first several estrous cycles and may reduce to a more consistent quantity after cyclicity becomes more established. The presence of atretic follicles (congregated within the medulla) is normal within the ovary and most numerous at PND 26. However, an increased number of these structures relative to concurrent controls (at any time point) may be associated with test article-related alterations to the ovary consistent with endocrine disruption (Picut et al. 2013; Dixon et al. 2014).

ii. CL

1. New CL: Postovulatory CLs generally occur during estrous or metestrous and are characterized by small basophilic cells, similar to the follicular granulosa cells. New basophilic CLs may contain a fluid-filled center.
2. Recent CL: These CLs generally occur during proestrous and diestrous of the current estrous cycle and are characterized by large, plump basophilic to eosinophilic cells that may be lightly vacuolated consistent with steroidogenesis. Minimal luteolysis as well as inflammation may be observed in these CLs at proestrous. A small amount of fibrous connective tissue may form at the center of a recent CL.
3. Old CL: These structures are progressing toward a state of quiescence and are characterized by degenerate cells, which are often highly vacuolated with central interstitial fibrous connective tissue. Scattered old CLs are normally present in peripubertal animals but are more common in older animals.

- b. Uterus:** The primary emphasis of the histological evaluation of the uterus is “to document cases of uterine (stromal or myometrial) hyper- or hypotrophy (atrophy) as characterized by changes in uterine horn diameter and myometrial, stromal, or endometrial gland development.” Per the Guideline, uterine examination should include an evaluation of the myometrium, stroma, and endometrial glandular epithelium as primary end points. Variations in the hormonal milieu, particularly estrogen, that normally occur during the juvenile and peripubertal period may result in small but noticeable morphologic changes in the uterus consistent with high or low estrogen levels, ranging from an immature morphology to epithelial and myometrial hypertrophy. These normal variations in the appearance of the peripubertal reproductive tract make identification of test article–related effects challenging. As such, the prepubertal assay microscopic evaluation inherently requires consideration of the terminal estrous stage of each individual. Thus, the evaluation may require subdividing animals within a dose group into their respective estrous stage. Doing so may allow for detection of a group that has an increased number of quiescent tissues (relative to quiescent tissues observed in animals in diestrous) consistent with either impaired or delayed pubertal development (i.e., hypoplasia) that is test article related. Comparison of treated versus concurrent controls, in conjunction with an assessment of the general condition and other findings in an animal, such as body weight, should also be considered to determine whether a smaller uterus is a reflection of systemic stress or negative energy balance. Epithelial changes related to test article endocrine disruption (epithelial apoptosis and alterations in epithelial height) must be differentiated from physiologic epithelial changes such as apoptosis and hypertrophy. Apoptosis is usually associated with sudden decreases in estrogen levels at estrous and early metestrous. Epithelial hypertrophy is characterized by tall columnar epithelium with an increased cytoplasm–nuclear ratio and is usually associated with accompanying mitotic activity due to elevated levels of estrogens during the late diestrous, proestrous, and early estrous phases of the cycle. Physiologic hypertrophy is characterized by orderly changes in the epithelium and concurrent enlargement of the endometrium and myometrium. Changes in one anatomical subcompartment without the other are less likely to be associated with morphological variation, thus potentially indicating a test article–related effect (Moller et al. 2012). Hypoplasia or atrophy due to test substance endocrine disruption is difficult to differentiate from immaturity. Hypoplasia is characterized by a markedly thin endometrium and/or myometrium. Differentiating hypoplasia from atrophy may not be possible based on morphologic criteria; thus, we recommend the use of hypotrophy/atrophy to describe changes in which the reproductive tract tissues are underdeveloped or smaller than normal but for which the mechanism is not clear. Any additional findings in the uterus should be recorded as a single entity and not combined (or “lumped”) with other findings. The Guideline does not specify trimming techniques for

postfixed tissues; however, it is advised that general published recommendations be followed for the uterus (Kittel et al. 2004). Briefly, a cross section of each uterine horn should be collected at approximately the halfway point between the uterine bifurcation and oviduct (Figure 1). In addition, it is recommended that a longitudinal section of the uterine body/cervix is prepared for evaluation. The uterine body cross sections should be consistently obtained either cranial or caudal to the slits made at necropsy to remove fluid prior to weighing, which also requires that the slits not run the entire length of the uterine horn and that they be made routinely in the same place at necropsy for each animal. This is particularly important because part of the microscopic evaluation consists of comparing the cross-sectional thicknesses of the myometrium and endometrial stroma across animals.

- c. Oviduct and cervix: These tissues are hormonally regulated tissues and examination may be warranted on a case-by-case basis (i.e., a macroscopic observation in the intact reproductive tract). Thus, retention of these tissues may be prudent.
- d. Vagina: The vagina is the single most important site to evaluate to determine the estrous stage of an adult rodent. As such, the Guideline recommends daily vaginal smears (including the day of necropsy) to be obtained for determination of estrous cyclicity. The degree to which vaginal lavage alters vaginal histology is unknown. Additionally, test substance-related discrepancies between vaginal cytology and vaginal and uterine morphology have been reported (Andrews et al. 2002) and are expected depending on the time between the last lavage and terminal euthanasia. Despite these caveats, the consensus of those in attendance at the EDSP Workshop was that evaluation of the vagina may afford useful information in the context of endocrine disruption of the female reproductive system. Evaluation may corroborate uterine findings as test article-related influences on pubertal development via perturbations of the endocrine system commonly involve morphological alterations in both tissues (Yuan and Foley 2002); however, exceptions may occur and each tissue should be evaluated in light of the estrous cycle. As evaluation may provide insight into cases of equivocal uterine morphology, it is recommended that the vagina be retained at necropsy.

Other concurrent findings (i.e., neoplasia, inflammation, infectious agents, and/or congenital anomalies) may be observed within reproductive tissues and should be recorded; however, they are unlikely to be associated with test article-related endocrine disruption, given the young age of the animals. Observation of such findings may warrant exclusion of the affected individuals from the study. EDSP Workshop participants felt that microscopic data from control animals should be maintained in searchable databases (along with the literature) to assist in the interpretation if such findings occur (Keenan et al. 2009; Shirota et al. 2013).

3. Concordance of estrous cyclicity data: Microscopic evaluation of female reproductive tissues should be conducted taking into account the final in-life stage of the estrous cycle as determined from the vaginal cytology obtained on the day of necropsy. An awareness of the normal development and morphology of reproductive tissues is essential in order to detect a test article–related alteration to the cycle or to properly ascribe weight changes due to normal estrous cycle fluctuations. It is not required to record the stage of the estrous cycle of each individual in the histological evaluation, as this information is recorded in the cytological data (vaginal lavage) collected on necropsy day. However, if significant discordance in the histological appearance of the uterus and ovary with respect to the stage of the estrous cycle is present, then it is appropriate to record morphological changes indicative of the alterations.

The morphological variations that occur during the normal 4 to 5 day estrous cycle of the postpubescent rat have been previously described (Dixon et al. 2014; Westwood 2008) as have the normal morphologies of the developing juvenile and peripubescent rat (Picut et al. 2014). The key histological features of female reproductive tissues are summarized here by stage, with the understanding that vaginal cytology and/or histopathology data may require multiple stages to be grouped/analyzed together:

a. Immature

- i.** Ovary: Mixes of new and recent CL are expected in the peripubertal individual. Decrements in CL numbers may be associated with test article–related alterations to the ovary consistent with endocrine disruption. Increased numbers of animals (when compared with controls) with an immature morphology may indicate a test article–related delay in maturation (developmental delay).
- ii.** Uterus: The uterine lumen is comprised of a single layer of low, cuboidal epithelium with small nuclei and scant, nonvacuolated cytoplasm. The myometrium has condensed and orderly nuclei arranged in intersecting patterns with scant cytoplasm and indistinct cell borders. Mitotic figures, endometrial glandular epithelium, and stromal cells are uncommon in prepubescent rats but may appear along with scattered epithelial vacuolar degeneration and leukocyte infiltration as the animals mature.
- iii.** Cervix: The epithelium is comprised of 3 to 5 cell layers of stratum germinativum (i.e., a single cell layer stratum basale with overlying stratum spinosum) while the stratum corneum and stratum mucification are generally absent. Similar to the uterus, scant leukocyte infiltrates, necrotic cells, and mitotic figures can be observed with maturity.

b. Diestrous

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- i. Ovary: New CLs are present and may be observed (based on section).
 - ii. Uterus: Uterine endometrial morphology during estrous is characterized by progressive degeneration and necrosis of both the glandular and luminal endometrial epithelium accompanied by an infiltrate of leukocytes. Uterine lumen dilation may be observed in early estrous but disappears by mid-estrous.
 - iii. Cervix: The stratum mucification will slough in early estrous. The cervix will have its maximum thickness of stratum corneum during estrus and separation (i.e., shedding) from the stratum granulosum will begin to occur.
 - iv. Vagina: The superficial layer of the epithelium is composed of acellular keratin or cornified flakes that may be sloughed into the lumen. The full epithelium is 6 to 8 cells thick.
- e. e. Metestrous

Although it is described separately here for completeness sake, this stage is not recorded in the cytology data or estrous cycle data at necropsy.

- i. Ovary: New and recent CLs may be present during this stage.
 - ii. Uterus: The uterus continues to exhibit degeneration and necrosis with leukocyte infiltration in metestrous. Mitotic figures in the endometrium will become more apparent.
 - iii. Cervix: The defining characteristic of metestrous will be seen in the cervix with complete separation of the stratum corneum and concurrent loss of the stratum granulosum via regression in the height of the stratum germinativum.
 - iv. Vagina: The superficial layer of the epithelium is predominantly apoptotic, noncornified cells. The full epithelium in early metestrous will be approximately 6 cells thick and reduce to 4 cells over this stage. Leukocytes are often apparent within the epithelium and lumen.
4. Hormone evaluation of female pubertal development: Female hormonal measurements from assays such as the radioimmunoassay, immunoradiometric assay, and enzyme-linked immunosorbent assays are suggested in the guidance but not required. These recommendations are consistent with a recent STP Scientific and Regulatory Policy paper that states “Diagnosing female reproductive hormone disturbances can be complicated by many factors, including estrous/menstrual cyclicity, diurnal variation, and age- and stress-related factors. Thus, female reproductive hormonal measurements should not generally be included in first-tier toxicity studies of standard design with groups of unsynchronized intact female animals. Rather, appropriately designed and statistically powered investigative studies are recommended in order to properly identify ovarian and/or pituitary hormone changes and bridge these effects to mechanistic evaluations and safety assessments” (Andersson et al. 2013).

The Pubertal Development and Thyroid Function Assay in Male Rats: OPPTS 890.1500

In the male rat, preputial separation (the separation of the prepuce from the glans penis) is an androgen-dependent external sign of sexual development (Korenbrod, Huhtaniemi, and Weiner 1977). At the time of sexual development, spermatogenesis is initiated by gonadotropin-releasing hormone (GnRH), with subsequent increases in FSH and LH. Spermatogenesis (and the development of Sertoli cells) is enhanced by FSH and LH, which promote Leydig cell secretion of testosterone into the testes and blood. Spermatogenesis is maintained through the “androgen environment,” which implies a combination of circulating levels of testosterone and/or dihydrotestosterone (DHT) and their activity through the androgen receptor (AR). Reduction in the androgen environment, due to lowered hormone levels or reduced AR activity, can occur either secondary to a central depressive effect on GnRH and/or LH release, through a direct effect on steroidogenesis, and through increased catabolism and excretion of testosterone (Chapin and Creasy 2012).

1. Organ weights: Decrements in body weight gain and/or decreased food intake may result in decreased testosterone production via decreased GnRH release and/or a developmental delay (Bergendahl, Perheentupa, and Huhtaniemi 1989). However, similar to body weight issues presented in female Organ Weights, these effects should not be mistaken for a direct chemical effect on androgen balance or environment.

Reduced weight of accessory sex organs (ASO), prostate, and seminal vesicles (including secretory contents) is the most consistent indicator of the androgen environment (Chapin and Creasy 2012). Much of the weight of the rodent ventral prostate, and most of the weight of the seminal vesicles (>70%), is a result of secretory activity governed by the androgen environment; therefore, organ weights should include secretory contents. When pronounced, reduction in secretory content may be observed histologically, but organ weight analysis is a more precise indicator of effects on the ASO. Sperm and fluid from the testes constitute approximately 50% the weight of the epididymis (Brooks 1979), but the structure and function of the epididymal epithelium is also androgen dependent and will show atrophy and ductal contraction, resulting in weight loss following decreased testosterone influence. Epididymal weights can also be influenced by changes in estrogen balance, since estrogens play a role in contractility and fluid resorption in the efferent and epididymal ductules (Joseph, Shur, and Hess 2011).

2. Histological evaluation of male reproductive tissues: The male pubertal assay Guideline requires the histological evaluation of a single testis and associated epididymis; histological evaluation of the ASO is not required and are not validated assay end points.
 - a. Testis: The Guideline indicates that the testis and epididymis should be evaluated from the same side and that one (either the right or left) should be chosen consistently unless a gross lesion necessitates collection of the contralateral organ. The selection of right or left should be standardized

within a laboratory and, if possible, throughout the program. Either cross sections or longitudinal sections of testis may be used for evaluation; however, selection should be standardized within a laboratory. If cross sections are used, consistently obtaining tissue from the upper 1/3 of the testis will optimize inclusion of the rete testis and standardize vasculature within the section examined. The Guideline does not identify inclusion of rete testis in histological sections.

Formalin is not an acceptable fixative for the testis. The Guideline states that testis and epididymis are placed in Bouin's fixative overnight (no longer than 24 hr) and then transferred to 70% ethanol pending histological processing. Individual laboratories have run trials to validate procedures allowing for fixation of up to 72 hr in Bouin's. Although not recommended by the Guidelines, modified Davidson's solution has been demonstrated to be a satisfactory fixative for testis and may be considered as a substitute in situations where local laboratory requirements constrain the use of Bouin's fixative (Latendresse et al. 2002). If modified Davidson's solution is used, the testes can be transferred into 10% neutral-buffered formalin after at least 48 hr of fixation.

General guidance for histological evaluation of the testis and epididymis is given in the EPA Health Effects Test Guideline OPPTS 870.3800. This guidance is consistent with practices recommended by the STP (D. M. Creasy 2003; Lanning et al. 2002).

Pathologists interpreting pubertal assays should have sufficient experience to confidently identify all cell populations and the spermatogenic stages of the cycle. This expertise is essential because the morphologic changes that characterize endocrine imbalance in the testis is both stage-specific and cell-specific and is relatively subtle. The histological features of spermatogenic stages in the rat are well documented (Hess 1999; Hess et al. 1990; Russell 1990). Although periodic acid-Schiff staining of rodent testes is a recommended practice in studies of 28 or fewer days in life duration (in order to accentuate acrosomes and facilitate spermatogenic staging; Lanning et al. 2002), it is not specified in the Guideline. The consensus of EDSP Workshop participants holds that evaluation of H&E-stained sections should be adequate for the testis evaluation in pubertal assays if the pathologist has an adequate familiarity with spermatogenic staging.

Detailed and specific guidance on the histopathologic changes that would be expected with different types of endocrine disruption in the male reproductive tract is provided in an OECD Guidance document available and may be downloaded on line (D. M. Creasy 2008) and has been recently reviewed (Chapin and Creasy 2012). More precise information regarding the normal histological features of the reproductive organs of rats of this age range are available (Picut, Remick, Rijk, et al. 2014). There are a number of

ways that endocrine regulation of the male reproductive tract can be disturbed:

1. Inhibition of testosterone biosynthesis,
2. androgen/LH receptor antagonism,
3. gonadotropin or PRL inhibition,
4. inhibition of testosterone metabolism to estradiol or DHT,
5. increased testosterone clearance.

Each of these different disruptive pathways results in a different profile of changes in the weight and/or morphology of the testis, epididymis, and ASOs. When evaluating the male reproductive tract for evidence of endocrine disruption, it is critical to integrate organ weight changes in all of the reproductive tissues with histopathologic changes seen in the testis and epididymis. In general, changes that will be seen in the rat testis in response to short-term endocrine disruption are not striking upon routine histological examination, therefore, particularly close attention must be given to any evidence of degeneration of pachytene spermatocytes, step 7 spermatids and step 19 spermatids of stage VII/VIII tubules (Chapin and Creasy 2012; Russell and Clermont 1977). Unlike many other species, rat spermatogenesis can be maintained at qualitatively normal levels until testicular testosterone levels are significantly reduced (Chapin and Creasy 2012). Even then, the changes in the testes are relatively subtle. For a detailed review of these changes and for the different profiles of change with different pathways of endocrine disruption, see D. M. Creasy (2008) and Chapin and Creasy (2012).

The characteristic lesion for reduced testosterone levels in the rat testis is the presence of occasional degenerating pachytene spermatocytes and round spermatids in stage VII/VIII tubules and sometimes degeneration of occasional dividing spermatocytes in stage XIV tubules. This may be accompanied by a small amount of spermatid retention (the persistence of step 19 spermatids into stage IX–XII tubules). If testosterone synthesis is severely reduced it may be possible to see atrophy of the Leydig cells, but this can be difficult to detect without using morphometric methodology. Reduced weight of the ASOs invariably accompanies reduced testosterone levels and will be a more sensitive end point than the spermatogenic changes. It should be used to confirm any conclusions drawn from the histopathologic evaluation. Severe reductions in testosterone levels will result in a progressive degeneration, depletion of elongating spermatids in all stages of the spermatogenic cycle, and degeneration and sloughing of more round spermatids into the epididymis. With these severe reductions in testosterone, tubular vacuolation (stage VIII) and reduced tubular diameter due to reduced fluid production can also be seen.

The characteristic lesion for AR inhibition/antagonism in the rat testis is Leydig cell hypertrophy/hyperplasia in the presence of normal spermatogenesis. In this case, ASO weight will again be significantly decreased and will be the most sensitive end point for the hormonal disturbance, but this is due to AR inhibition in the presence of normal or increased levels of testosterone produced by the Leydig cells. These 2 examples demonstrate the importance of using the detailed histopathologic changes in spermatogenesis and Leydig cells in conjunction with organ weight changes to identify endocrine disruption.

As illustrated previously, the main cell types impacted by endocrine disruption are the Leydig cell (either atrophy or hypertrophy), occasional Stage VII/VIII pachytene spermatocytes and round spermatids (degeneration), and occasional Stage XIV dividing spermatocytes. Degeneration and depletion of elongating spermatids can be seen, but only in cases of severe testosterone depletion. Endocrine disruption does not generally produce morphological changes in the Sertoli cell and does not produce degeneration or depletion of other germ cell types, so if generalized germ cell degeneration, tubular atrophy, or Sertoli cell injury is observed, the cause is likely to be through a mechanism other than endocrine disruption.

The examples provided are the typical responses of the adult rat to endocrine disruption. The Pubertal Assay requires dosing of males from PND 22/23 (prepubertal) through to PND 53/54 (peripubertal). Most of the major postnatal development of the testis and epididymis has already been completed by PND 22, so although endocrine disruption can have major effects on organ development during *in utero* and early postnatal exposure, it is unlikely to result in significant developmental abnormalities when dosing commences on PND 22. However, the testis, epididymis, and neuroendocrine pathways are undergoing a number of important maturational events during this time, which could be impacted by endocrine disruption. The most obvious is a delay in puberty (i.e., maturation of spermatogenesis and spermiation). By PND 53, rats should have tubules with a full complement of elongating spermatids and an epididymis that has significant numbers of sperm throughout its length, but the cauda will generally have relatively few sperm or not be fully expanded. Decreased numbers of elongating spermatids in the testes and decreased numbers of sperm in the epididymis accompanied by sloughed testicular germ cells could indicate an endocrine-mediated delay in puberty.

- b. Epididymis:** The epididymis is a complex, androgen-dependent organ (Chapin and Creasy 2012) that plays an important role in sperm maturation, transport, concentration, protection, and storage (for review, see Robaire, Hinton, and Orgebin-Crist 2006). Although histological characteristics have been used to subdivide the epididymis into as many as 12 distinct regions (Robaire, Hinton, and Orgebin-Crist 2006), it is customarily divided into the following 4 gross anatomical regions: the initial segment, head (caput), body

(corpus), and tail (cauda). The Guideline indicates that histological evaluation of the epididymis should include the caput, corpus, and cauda regions, which may be accomplished by preparation of longitudinal sections of the epididymis. For adequate interpretation of test article effects, it is important that the specific region of occurrence (caput, corpus, or cauda) of lesions is noted. Epididymal tubules are lined by 6 distinct cell types: principal cells, apical cells, narrow (pencil) cells, clear cells, basal cells, and halo cells. Of these, the Guideline specifically refers to a reduction in the number of clear cells (absence of clear cells in the cauda epididymal epithelium) as a histological change in specific relevance in pubertal assays. Disappearance of the clear cells in the cauda epididymis was reported after 4 days of treatment with chloroethyl methanesulfonate or ethane dimethane sulfonate but was considered to be independent of any androgen-mediated effects (Klinefelter et al. 1994; Klinefelter et al. 1990). Clear cells endocytose cytoplasmic droplets released by maturing spermatozoa as they traverse the epididymal duct. Clear cells are present in the epididymal caput, corpus, and cauda, with region-specific endocytic activity; those within the cauda region have particularly pronounced endocytic activity. Clear cells become abnormally large and filled with lysosomes as part of various pathologic processes. Although the more common pathologic observation relative to clear cells is an increase in the number and size of the cells, the Guidance suggestion is reasonable when one considers that clear cell populations develop during the prepubertal period; thus, a decrement in the clear cell population could be a subtle indication of an interruption or hindrance in the development of the epididymis.

As previously discussed, the testis and epididymis evaluated should be from the same side unless otherwise precluded by gross abnormalities. Histological evaluation of the epididymis should include the luminal contents as well as the epithelial and stromal structure of the organ. Though it is not typically possible to perform a precise estimate of spermatozoa numbers or identify morphologic alterations in individual spermatozoa, it is possible to detect atypical populations of degenerative cells, which commonly originate in the testes. Pathological accumulations of degenerative cells must be distinguished from background accumulations of similar cells that are commonly present in the epididymis of rats at the time spermatogenesis is first initiated. Reduced testosterone levels (experimental castration) result in reduced epididymal sperm counts via a wave of apoptosis (Ezer and Robaire 2002; O'Connor et al. 1998).

Selected histological findings that may be associated with test article-related alterations to the epididymides include atrophy, single cell necrosis/apoptosis, and reduction in sperm count. Epididymal atrophy (Atrophy, ductal) is generally caused by decreased androgen support or decreased fluid and androgen output from the testis. The primary diagnostic feature is generalized or segmental narrowing of ductal lumina with normal-appearing

or slightly lower epithelial cell height. Intraductal folding of epithelium may be present. When segmental, the ductal atrophy is most commonly observed at the corpus–caudal junction. Epididymal atrophy is commonly associated with reduction or absence of sperm in the affected region. Single cell necrosis or apoptosis (Single cell necrosis, epithelial) may be caused by a decreased androgen environment and efferent duct occlusion. The primary histological features are epithelial cells with decreased size, shrunken and sometimes eosinophilic cytoplasm, and condensed or fragmented nuclei. The changes are most commonly seen in the initial segment or proximal caput epididymis, particularly when related to androgen deficit. Reduction in sperm content (Reduced sperm, luminal) is generally the result of reduced sperm output by the testis due to germ cell injury or decreased androgen support. The primary diagnostic feature is a reduction in sperm, which may be most pronounced in one epididymal region. Reduced luminal sperm may be accompanied by an increased amount of luminal cellular debris, particularly when the reduced sperm content is due to germ cell degeneration. At the time of evaluation of tissues in the pubertal assay (PND 53/54), spermatozoa are generally present throughout the epididymis, but relatively few and still increasing in number in the cauda, therefore considerable variability in the amount of intraluminal spermatozoa may be expected. The epididymis of a rat from a pubertal assay would not be expected to contain the full complement of spermatozoa that are seen in a rat from, for example, a typical 28-day toxicity study where the animals are approximately 70 days of age at necropsy.

- c. Accessory sex organs: Histological evaluation of the ASO is not required; however, test article–related histological alterations may occur within the ASO epithelia. Such alterations, when they occur, are subtle, consisting primarily of reductions in the height of acinar epithelial cells.
3. Hormonal evaluation of male pubertal development: Testosterone plays a pivotal role in overall testicular function (D. M. Creasy 1999), both directly and through feedback to the hypothalamic–pituitary unit to influence levels of FSH and LH (Ojeda and Ramirez 1972). In the rat, testosterone and other testicular steroids are secreted by the testes at an early age (Resko, Feder, and Goy 1968). Intratesticular testosterone levels required to maintain spermatogenesis at maximal efficiency may be as much as 100× higher than circulating testosterone levels (Chapin and Creasy 2012). Determination of serum testosterone levels can be conducted using radioimmunoassay, immunoradiometric assay, enzyme-linked immunosorbent assay, or time-resolved immunofluorescent procedures, but because of the very high variability (coefficient of variation) for this pulsatile hormone in the rat, it is essential to have adequate numbers of animals to provide statistical relevance of the results. This generally means using >20 animals/group (Chapin and Creasy 2012).

Thyroid Gland Function

Thyroid gland function has been clearly demonstrated to play a significant role in the development of the male and female reproductive tract. Experimental hypothyroidism leads to delayed sexual maturation with atrophy of the testis and accessory sex glands, reduced levels of gonadotropins and testosterone, severe inhibition of gametogenesis (Valle et al. 1985), and delay of the first ovulation due to absence of the LH surge (Tamura et al. 1998). Sertoli cell structure and function is critical to spermatogenesis, and thyroid hormone is known to control the onset and pattern of Sertoli cell function (Stoker et al. 2000). Administration of triiodothyronine (T_3) to newborn rats causes enlargement of the testis with increased numbers of Sertoli cells and spermatogenic cells, as well as an increase in testis size (Jannini et al. 1993). Conversely, reproductive hormones as well as gonadal development and function can affect thyroid gland development (Banu, Govindarajulu, and Aruldas 2002). There is a positive correlation between thyroid gland growth and serum estrogen/testosterone levels; thyroid follicular cells of immature rats have receptors for estradiol and testosterone. Androgens are known to sensitize thyroid follicular cells to TSH effects (Banu, Govindarajulu, and Aruldas 2002). This sensitization, coupled with the higher circulating TSH levels in male rats (Capen 1997), may be the basis for greater follicular cell height that is commonly observed in male rats. Thyroid gland weight coupled with thyroid hormone analyses and histological evaluation are the most reliable end points for identifying thyrotoxicants in a short-term screening battery (O'Connor et al. 1999). Thyroid-related end points of the EPA pubertal assays therefore include thyroid gland weights (with parathyroid), histological evaluation of the thyroid glands, analysis of serum TSH, T_4 , and (optionally) T_3 levels.

1. Organ weights: Thyroid gland weight, which is commonly increased with thyrotoxicants, can be a useful indicator of thyroid-related perturbations; however, a prolonged period (i.e., 2–4 weeks) of chemical exposure (Marty, Crissman, and Carney 1999; O'Connor et al. 1999) may be required prior to manifestation of weight changes. For this reason, the male assay (~4.5 weeks of exposure) may be more sensitive to weight changes than the female assay (~3 weeks of exposure).

Great care must be given to technical considerations in weighing thyroid glands, as failure to collect the entire gland or inclusion of extraneous tissues can have a marked effect on the recorded weight. The pubertal assay Guidelines recommend postfixation weighing of thyroid glands, which is necessary for careful dissection. Proper microdissection instruments and use of a dissecting microscope for thyroid gland dissection contribute to accurate collection of thyroid gland weights.

2. Histological evaluation of the thyroid gland: Histological evaluation of the thyroid glands is considered to be the most reliable indicator of thyrotoxicosis; however, interpretation is largely subjective. In an effort to increase reproducibility and interlaboratory concordance, the Guidelines included specific parameters for histological evaluation. Measurements of follicular colloid content and follicular epithelial cell height are to be made from a minimum of 2 sections of each lobe. Each parameter is scored on a 1 to 5 scale, typically identified as C1 to C5 for colloid content and F1 to F5 for follicular epithelial cell height. It is important to

recognize the grading scales progress in opposite directions, that is, F1 is “normal” follicular epithelial cell height and C5 is normal colloid content. Colloid content is commonly decreased with increasing epithelial cell height, therefore, a “typical” pattern of scores would be F1C5, F2C4, F3C3, F4C2, and F5C1. However, this linkage of the scored parameters is not inviolate. The Guidelines contain photomicrographs that illustrate the various follicular cell height and colloid area grades; however, the study pathologist is “encouraged to establish a range that is appropriate to the particular study being evaluated.” If this guidance is interpreted to mean separate grading criteria are applied by each pathologist to each study, the practice would hinder ability to repeat results and adversely impact the establishment of historical control values within the laboratory. It is therefore suggested that each laboratory should establish grading criteria that will be applied uniformly by all pathologists across studies. Any required exception to this uniform grading practice should be noted in the narrative pathology report. It is also suggested that uniform grading criteria should be applied equally to males and females, as opposed to separate grading criteria for males versus females. As a result, it would be expected that follicular cell epithelial height would be slightly higher in males than females. Although the Guidelines require only scoring of the above-mentioned parameters, it is assumed standard operating procedures in the laboratory will require the pathologist to record any additional histological observations in the thyroid glands.

There is substantial variation in follicular cell height and colloid area in different regions of the same section of thyroid gland. The pathologist should examine the entire section and develop a subjective impression of these features, rather than attempting a quasi-morphometric approach based on a few selected thyroid follicles. Importantly, although there is a substantial delay between initiation of toxicity and histological manifestation of follicular epithelial cell responses, reduction in follicular colloid may occur within a few days of insult (Capen 1997).

Of note, spontaneous thyroid gland dysplasia of Wistar rats (Weber et al. 2009) could pose a problem in interpretation of thyroid-related data from pubertal assays in this strain. An effort is underway to eliminate spontaneous thyroid gland dysplasia from the Wistar strain, but the condition remains a consideration if outlier data are encountered.

3. Hormone evaluation of thyroid development: Thyroid endocrine function of the rat is maturing through PND 29 (Choksi et al. 2003) and is fully mature by the termination of pubertal assays. T₄ reaches adult level by PND 16 while T₃ reaches adult level by PND 30. Serum TSH levels demonstrate a bimodal pattern during postnatal development. There is an early peak during the first 2 weeks of life followed by a second elevation from PND 30 to 50 that coincides with puberty and a return to normal adult levels by PND 60 (Goldman et al. 2000; Simpkins et al. 1976). T₄ is subject to rapid changes in response to stress, changes in body weight and body weight gain, and estrous stage among other factors (Dohler, Wong, and von zur Muhlen 1979). Circulating T₄ levels are typically decreased with thyrotoxicants, with the response often occurring promptly after chemical exposure

(i.e., 3 days). However, use of serum T₄ levels for detection of thyrotoxicants is complicated by circadian rhythms of production, as well as the relatively short serum half-life (12–24 hr) of T₄ in the rat. There is marked interanimal variability in T₄, as evidenced by the coefficients of variation for performance criteria in the Guidelines.

Circulating TSH levels are typically increased in response to T₄ decrement that occur with thyrotoxicants; this response is often more pronounced in males. In a classical scenario of chemically mediated thyrotoxicosis, one would expect to see decreased T₄ levels with a subsequent responsive elevation in TSH levels, followed by histological evidence of follicular epithelial hypertrophy in response to the elevated TSH levels. Unfortunately, this coordinated pattern of changes is not dependably present, as numerous examples of “paradoxical” increases in T₄ have been observed. A paradoxical increase in T₄ could occur from sudden release of T₄ stored in colloid, but this paradoxical increase would be short lived. Some of the unexpected observations may be associated with technical considerations or unanticipated biorhythms. For these reasons, thyroid gland weights and histopathology represent more sustained changes in the HPT axis. As discussed, body weight decrements adversely affect assay end points. Studies suggest that subtle body weight changes (i.e., >6% decrement in final body weight of males) may result in thyroid gland perturbations, in particular lower serum T₄ levels (Laws et al. 2007). The possibility of body weight-associated changes in the thyroid gland, rather than direct endocrine disruption, should be considered when there is >6% change in body weight in male rats.

Additional Considerations for Endocrine-mediated Pathology End Points for Both Male and Female Assays: Mammary Gland

While the Guidelines do not recommend evaluation of the mammary gland in the male or female rat, mammary tissue constitutes a potentially sensitive end point for evaluating the direct or indirect effects of endocrine-disrupting compounds. For this reason, EDSP Workshop participants agreed that examination of mammary tissue may provide corroboratory information in the context of endocrine disruption.

1. Histological evaluation of the mammary gland: Mammary growth in the male and female rat during puberty is dependent on normal gonadal function; therefore, development and function are controlled by the same hormonal systems under evaluation in the pubertal assays. Perturbations of these systems are commonly associated with morphological alterations the mammary gland (Lucas et al. 2007; Yuan and Foley 2002).

Mammary glands of rats exhibit sexual dimorphism (Cardy 1991). In females, PRL, progesterone, and estrogen are primarily responsible for the growth of the mammary gland during puberty. Growth is characterized by differentiation of the epithelium into terminal end bud units, elongation and branching of the ducts, and hypertrophy of the fat pad. The onset of ovarian cyclicity is a critical cue for mammary gland ductal growth (Hovey et al. 2011) in the female. Mammary tissue

of young male rats is generally more florid than in females of the same age and consists of lobular groups of alveolar cells that lack obvious tubular or ductal orientation. The cells are generally characterized by abundant, foamy, and eosinophilic cytoplasm containing distinct, variably sized vacuoles. Alveolar lumens are mostly indistinct but may contain evidence of secretory activity. The lobular architecture of the male rat mammary gland is maintained by the presence of androgens (Sourla et al. 1998) and will exhibit apoptosis with androgen withdrawal and atrophy with either sustained low androgen levels, androgen antagonism, or estrogenic influence (Toyoda et al. 2000; W. G. Foster et al. 2004; Lucas et al. 2007; Wang et al. 2006; You et al. 2002). The morphologic shift is sometimes termed “feminization” rather than atrophy. The term feminization is somewhat inaccurate and possibly confusing, as it implies the morphologic alteration in the mammary gland of the male rat is due to female hormone influence, as opposed to the true pathogenesis that may be based on either female hormone influence or a decrement in male hormone influence. The term “dysplasia” is defined in a broad sense as altered tissue development, thus is a technically accurate descriptor for the mammary gland alteration, but dysplasia as it is commonly used has connotations of precancerous change that are unwarranted in this situation. A descriptive term such as “glandular alteration” is preferable. This change in the male mammary gland is considered to be a very sensitive indicator and is typically diffuse; therefore, special procedures (whole mounts, etc.) are not necessary to detect the alteration. Imbalance of mammotrophic hormones like PRL, estrogens, and androgens may result in the male and female rat mammary gland converting to the morphologic appearance expected of the opposite sex (Rudmann et al. 2012). In the female, masculinization or virilization may be observed when the normal female morphology (tubulo-alveolar) becomes more male-like (lobuloalveolar). This alteration in females includes an increased number of large basophilic alveolar epithelial cells with vacuolated cytoplasm in irregular nests and/or pseudostratified appearance. As with the equivalent alterations in males, diagnostic terminology should be descriptive rather than interpretative.

Other morphological findings in the mammary gland, such as fibroadenomas or other tumors are possible but extremely unlikely in the young animals used for this assay. These other findings are more likely to be spontaneous and less likely to be associated with test article-related endocrine disruption. A comprehensive listing of morphological diagnoses of the rodent mammary gland is reviewed elsewhere (Rudmann et al. 2012). A routine section of mammary gland that includes the skin and nipple (if possible) is sufficient for descriptions of endocrine-related alterations (Ruehl-Fehlert et al. 2003).

Nonendocrine Pathology End Points for Both Male and Female Assays

Valid pubertal assay results require the animals to generally tolerate dosing and not be unduly stressed during the study as stress alone can produce nonspecific endocrine and/or reproductive system dysfunction (Everds et al. 2013). It is incumbent upon the study pathologist to rule out stress-related changes in these studies prior to concluding that test

article-related endocrine disruption is present. For this purpose, the Guidelines require evaluation of clinical chemistry parameters (including BUN and CRT), kidney (weights and histopathology), liver (weights), and gross lesions (histopathology). Significant deviations of blood chemistry values from treated versus control animals, even in the absence of statistical significance, histological alterations, or clinical observations, may be an indication that the MTD was exceeded. Particular attention should be given to clinical pathology alterations that could indicate internal organ toxicity, as opposed to alterations that indicate minor perturbations in homeostasis. Histological evaluation of the kidney for evidence of systemic toxicity may similarly indicate that the MTD was exceeded. A complete listing of kidney pathology findings indicative of overt toxicity are reviewed elsewhere (Khan et al. 2002). Evaluation of other known test compound target organs may be considered for the same purpose. A recent summary publication indicates that the liver is the most common site of xenobiotic injury (Horner et al. 2013), suggesting that alterations in hepatic-specific serum analytes and histological evaluation of the liver may be useful in determination of systemic toxicity. A complete listing of liver pathology findings that are indicative of overt toxicity are reviewed elsewhere (Cattley and Popp 2002).

Conclusions

In conclusion, the pubertal assay specifically tests the endocrine effects of environmental chemicals during the juvenile and peripubertal periods of development, during which time, the HPG axis is undergoing intense maturational changes. Pathologists should consider normal biological variation when evaluating and interpreting pubertal assays and refrain from “overinterpretation” of subtle differences in morphological features. What we present herein is intended to educate the study pathologist both in evaluating peripubertal tissues and in providing a deeper understanding of the impact of histological findings in the overall context of the pubertal assays. EDSP tier 1 tests are intended to be viewed collectively, using a WoE process to examine the extent of complementarity not just within a particular test but across the battery. As such, the pubertal assays end points serve as 2 of the collective 11 indicators of the potential for a chemical to act on endocrine-mediated processes.

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Abbreviations

AR	androgen receptor
ASO	accessory sex organs
BUN	blood urea nitrogen

CL	corpora lutea
CRT	creatinine
DHT	dihydrotestosterone
EDSP	endocrine disruption screening program
EPA	Environmental Protection Agency
FSH	follicle-stimulating hormone
GnRH	gonadotropin-releasing hormone
H&E	hematoxylin and eosin
HPT	hypothalamic–pituitary–thyroid
INHAND	International Harmonization of Nomenclature and Diagnostic Criteria for Lesions in Rats and Mice
LH	luteinizing hormone
MTD	maximum tolerated dose
PND	postnatal day
PRL	prolactin
STP	Society of Toxicologic Pathology
T₃	triiodothyronin
T₄	serum thyroxine
TSH	thyroid-stimulating hormone
WoE	weight of evidence

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FIGURE 1.

Removal and Preparation of the Uterine and Ovarian Tissues for Weight Measurement and Histology Adapted from Environmental Protection Agency (EPA 2009a): To remove the uterus and ovaries, make a medial incision approximately 5-inch long on the ventral aspect of the rat from the vaginal opening toward the head. Locate the vagina ventral to the urinary bladder. Locate the uterine horns and ovaries bilaterally and detach from the dorsal abdominal wall. Detach the uterus and vagina from the body by incising the vaginal wall (A). Detach the ovaries by cutting between the oviducts and the ends of the uterine horns (B). Remove the vagina by cutting just caudal to the uterine cervix (C). Trim fat and connective tissues; slit the uterine horns and remove fluid (blotting). Cut between each oviduct and ovary (D), remove the ovarian bursa and trim away the ovarian fat. Obtain weights of the trimmed, blotted uterus and ovaries (paired). Cross sections of uterus for histopathology should be consistently taken anterior to slits made for draining fluid at necropsy (=).

TABLE 1

EPA OPPTS 890 test guideline series for the endocrine disruptor screening program.

Assay	Species	Sex	Type	End point of compound effect
1100 Amphibian metamorphosis	<i>Xenopus</i>	M/F	<i>In vivo</i>	Body development, weight, thyroid histology
1150 Androgen receptor binding	Rat	M	<i>In vitro</i>	Receptor binding in prostate
1200 Aromatase	Human	F	<i>Enzyme</i>	Inhibition of enzyme
1250 Estrogen receptor binding	Rat	F	<i>In vitro</i>	Receptor binding in uterus
1300 Estrogen receptor transcript	Human	F	<i>In vitro</i>	Activation of genes in HeLa cells
1350 Fish short-term reproduction	Fathead Minnow	M/F	<i>In vivo</i>	Fecundity, fertilization, gross and histopathology of gonads, vitellogenin, steroids
1400 Hershberger (castrated)	Rat	M	<i>In vivo</i>	Sex organ weight and hormones
1450 Female pubertal/thyroid	Rat	F	<i>In vivo</i>	Weight, histology, hormone, estrous, and clinical pathology
1500 Male pubertal/thyroid	Rat	M	<i>In vivo</i>	Weight, histology, hormone, and clinical pathology
1550 Steroidogenesis	Human	M/F	<i>In vitro</i>	Steroid production in cell culture
1600 Uterotrophic (ovariectomized)	Rat	F	<i>In vivo</i>	Sex organ weight and histology

Source: Adapted from the EPA Validation of a Test Method for Assessment of Pubertal Development and Thyroid Function in Juvenile Female/Male Rats as a Potential Screen in the Endocrine Disruptor Screening Program Tier-1 Battery.

Note. F = female; M = male; EPA = Environmental Protection Agency.

TABLE 2

Assay end points in the pubertal development and thyroid function in intact juvenile/peripubertal female and male rats (OPPTS 890.1450 or 890.1500, respectively).

End point	Female	Male
Growth	Daily body weight	Daily body weight
Puberty	Age and body weight at vaginal opening	Age and body weight at preputial separation
Estrous cyclicity	Age of first vaginal estrous after opening Length of cycle Percentage of animals cycling Percentage of animals cycling regularly	Not applicable
Organ weights	Uterus, blotted Ovaries, paired Thyroid, postfixation Liver Kidneys, paired Pituitary Adrenal glands, paired	Seminal vesicle plus coagulating glands, with and without fluid Ventral prostate Dorsolateral prostate LABC Epididymides, left and right Testes, left and right Thyroid, postfixation Liver Kidneys, paired Pituitary Adrenal glands, paired
Histology	Uterus Ovary Thyroid Kidney	Epididymidis, 1 Testis, 1 Thyroid Kidney
Hormones	T ₄ , total Serum thyroid- stimulating hormone	Serum testosterone, total T ₄ , total Serum thyroid-stimulating hormone
Clinical chemistry	Standard blood panel include CRT/BUN	Standard blood panel include CRT/BUN

Source: Adapted from the EPA Validation of a Test Method for Assessment of Pubertal Development and Thyroid Function in Juvenile Female/Male Rats as a Potential Screen in the Endocrine Disruptor Screening Program Tier-1 Battery and EDSP Test Guidelines OPPTS 890.1500 Pubertal Development and Thyroid Function in Intact Juvenile/Peripubertal Male Rats.

Note: LABC = levator ani/bulbocavernosus muscle complex; T₄ = serum thyroxine; CRT = creatinine; BUN = blood urea nitrogen; EPA = Environmental Protection Agency.

TABLE 3

Potential changes indicative of different modes of action that may^a be observed in the female pubertal protocol.

Estrogen agonist	Inhibition of steroidogenesis	Disruption of hypothalamic–pituitary–gonadal axis	Thyrototoxicants
Early vaginal opening Pseudoprecocious puberty Reduced body weight at vaginal opening Altered organ histology Possible persistent estrous Reduced ovarian weight Increased uterine weight	Delayed vaginal opening Delayed first estrous Reduced uterine weight Altered organ histology	Alteration in vaginal opening timing Alteration in cyclicity Altered organ histology	Decreased T ₄ Alteration in TSH Alteration in thyroid weight Changes in liver weight or enzyme profile

Source: Adapted from the EPA Validation of a Test Method for Assessment of Pubertal Development and Thyroid Function in Juvenile Female Rats as a Potential Screen in the Endocrine Disruptor Screening Program Tier-1 Battery and EDSP Test Guidelines OPPTS 890.1450 Pubertal Development and Thyroid Function in Intact Juvenile/Peripubertal Female Rats.

Note: T₄ = serum thyroxine; TSH = thyroid-stimulating hormone; EPA = Environmental Protection Agency.

^aNot all end points may be observed depending on test article potency and mode of action.

TABLE 4

Potential changes indicative of different modes of action that may^a be observed in the male pubertal protocol.

Androgen antagonist	Steroidogenesis inhibitor or HPG suppression	Hypothyroidism
Increased age of puberty Atrophy of ventral prostate, seminal vesicles, LABC, and epididymides Increased testosterone	Increased age of puberty Atrophy of ventral prostate, seminal vesicles, LABC, and epididymides Decreased testosterone or no effect	Decreased T ₄ Increased TSH Increased thyroid weight Increased follicular cell height; decreased colloid area Increased liver weight

Source: Adapted from the EPA Validation of a Test Method for Assessment of Pubertal Development and Thyroid Function in Juvenile Female/Male Rats as a Potential Screen in the Endocrine Disruptor Screening Program Tier-1 Battery and EDSP Test Guidelines OPPTS 890.1500 Pubertal Development and Thyroid Function in Intact Juvenile/Peripubertal Male Rats.

Note: T₄ = serum thyroxine; LABC = levator ani/bulbocavernosus muscle complex; HPG = hypothalamic–pituitary–gonadal; EPA = Environmental Protection Agency.

^aNot all end points may be observed depending on test article potency and mode of action.