DRAFT REPORT OF THE OECD VALIDATION OF THE RAT HERSHBERGER BIOASSAY:

PHASE-2: TESTING OF ANDROGEN AGONISTS, ANDROGEN ANTAGONISTS AND A 5α-REDUCTASE INHIBITOR IN DOSE RESPONSE STUDIES BY MULTIPLE LABORATORIES

FOREWORD

This document provides a description and comprehensive summary of the study results for Phase-2 of the OECD validation of the rat Hershberger bioassay. It contains the background on how the validation study was organised and performed, the standardised protocols used, detailed summaries and statistical analyses of the data, and the conclusions drawn from the studies. Phase-2 consisted of uncoded dose-response studies with two androgen agonists, four androgen antagonists, and a 5α-reductase inhibitor. A single protocol was used for the agonists studies based on direct administration of the agonists and statistically significant increases in the target tissues versus an untreated control. Similarly, a single protocol was used for the antagonists and the 5α-reductase inhibitor based on coadministration with a reference androgen and statistically significant decreases in the target tissues versus a reference androgen only group as the control.

The laboratory-testing portion of this phase was conducted in Japan between January and June 2002 and outside of Japan between November 2002 and June 2003. This document was written by the OECD Secretariat. Comments and input were contributed by the Lead Laboratory, Dr. L. Earl Gray, Jr., (United States Environmental Protection Agency, Research Triangle Park, USA) and members of the Mammalian Validation Management Group, notably Drs. John Ashby (Syngenta, CTL Laboratory, UK), Mike Wade (Health & Welfare, Canada), Gary Timm (US Environmental Protection Agency, Washington, DC), and William Owens (Procter & Gamble, Cincinnati, USA).

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ANNEXES

HERSHBERGER PHASE-2 SUMMARY

i) The OECD has undertaken the validation of the Hershberger bioassay. The studies in this report comprise Phase-2 of the Hershberger validation programme that were conducted in 2002-2003 to demonstrate the Hershberger bioassay's ability in dose response studies to reliably detect strong and weak androgen agonists and antagonists as well as a strong 5α-reductase inhibitor. Combined with the results of Phase-1, these studies further demonstrate the transferability of standardised protocols amongst laboratories and begin to quantitate the inter- and intra-laboratory reproducibility of the assay. These studies were designed and directed by the OECD Task Force on Endocrine Disrupters Testing and Assessment (EDTA), which was established to develop and validate new and improved methods to identify and to assess substances acting through endocrine mechanisms (1). The Hershberger bioassay was one of three *in vivo* assays selected by the EDTA for validation after a review of possible assays (2). (Further information concerning the OECD Endocrine Disruptor testing programme can be found at http://www.oecd.org/EN/document/0,,EN-document-524-nodirectorate-no-24-6685-8,00.html.)

ii) The principle of the rat Hershberger bioassay is that organs and accessory tissues in the male reproductive tract are under the control of androgens, which are necessary to stimulate and to maintain growth of these tissues. If the endogenous source of this hormone is not available, either because of immaturity of the animals or because the animals have been surgically castrated, the animal requires an exogenous androgen source to initiate or restore growth of these tissues. Chemicals that act as agonists may be identified if they cause a statistically significant increase in the weights of the target androgen-dependent tissues, or chemicals may be identified as antagonists if they cause a statistically significant decrease in target tissues when co-administered with a potent androgen. Similarly, several tissues depend upon the local conversion of testosterone to dihydrotestosterone by the enzyme 5α-reductase. The androgen response of those tissues, and not others, are then sensitive to inhibitors of 5α-reductase. Therefore, 5α-reductase inhibitors may be identified if they cause a relative decrease in the weight gain of particular tissues, when co-administered with a potent androgen.

iii) The first phase (Phase-1) of the validation of the rat Hershberger bioassay was supported by the Lead Laboratory from the U.S. EPA. There were 19 participating laboratories from Denmark, France, Germany, Japan, Korea, U.K and U.S. The work was conducted in two phases. Phase-1A studied the dose response to the potent androgen agonist, testosterone propionate (TP) (CASRN 57-82-5). Phase-1B studied the dose response to the potent androgen antagonist, flutamide (FLU) (CASRN 1311-84-7) using two doses of TP as the potent, coadministered androgen. Those TP doses were chosen based upon the preceding Phase-1A studies. Seventeen laboratories contributed to Phase-1A, and seven laboratories to Phase-1B. Despite differences in rat strain used and different levels of experience among the participating laboratories, there was acceptable agreement in both Phase-1A and 1B among laboratories with respect to the magnitudes of the responses at the different dose levels and the doses at which significant responses were obtained (3).

iv) Phase-1A demonstrated the weight increase stimulated by testosterone propionate in five male accessory sex organs and tissues: the ventral prostate (VP), seminal vesicles plus coagulating glands (SVCG), levator ani and bulbocavernosus muscle complex (LABC), glans penis (GLANS), and Cowper's glands (COWS). The same five tissues were mandatory measurements during Phase-2 along with body weight. Additional optional measures were liver, kidney, and adrenal weights and circulating serum levels of testosterone and lutenising hormone. Phase-1B used these same accessory sex organs and tissues in dose response studies with the antagonist, FLU, when co-administered with specified doses of TP. Other parameters examined in Phase-1 included an assessment of weighing certain tissues fresh or after fixation and the response of the dorsolateral prostate weight, which was considered more challenging to dissect (3).

v) All laboratories and all protocols were successful in detecting increases in the weights of the five mandatory accessory sex organs and tissues in response to TP, and in detecting the anti-androgenic effects of

FLU. There was good agreement among laboratories with regard to the dose responses obtained. There was similar agreement in their ability to identify the anti-androgenic effects of FLU. The ability of the five androgen target tissues to detect statistically significant changes in response to androgen agonist and antagonists was similar, and all five mandatory tissues were retained for investigation in Phase-2. The additional tissues provided complimentary toxicological information, but did not respond in an androgen-specific way, but rather to individual characteristics of the test substances. These additional tissues were then measured voluntarily for Phase-2. No effective advantage or disadvantage of tissue fixation was demonstrated. No added value was demonstrated for the dorsolateral prostate, and the dissection and measurement of this tissue was dropped from the Phase-2 studies (3).

vi) Phase-2 of the validation of the rat Hershberger bioassay was conducted in two stages. The first stage involved 7 laboratories from Japan, and the second stage involved 9 laboratories from Denmark, France, Germany, Korea, U.K., and the U.S. This timing was mandated for two reasons 1) budgetary regulations in Japan required that in-life studies begin before the end of the government fiscal year or be lost and 2) the departure and the resulting vacancies of personnel managing the Hershberger programme at the Secretariat. Laboratories from both the public and private sectors participated in both stages of the work. The participating laboratories and principal investigators from both stages are identified in Annex 1. The lead laboratory for the Phase-2 validation study continued to be from United States Environmental Protection Agency, Research Triangle Park, USA.

vii) The Phase-2 work used the potent androgen, testosterone propionate (TP), as the reference agonist at selected doses of 0.2 and 0.4 mg/kg/d. In some studies, the potent antagonist, flutamide (FLU), was used at a dose of 3 mg/kg/d as a reference antagonist to demonstrate the responsiveness of the laboratory animals. The Phase-2 work was conducted with 7 new test substances. Dose response studies were conducted with two additional androgen agonists, 17α-methyl testosterone (MT) and trenbolone (TREN); with four antagonists weaker than flutamide including procymidone (PRO), vinclozolin (VIN), linuron (LIN), and *p,p'*-DDT (DDT); and with a potent 5α-reductase inhibitor, finasteride (FIN). No coded studies were conducted in Phase-2, and no unknown or negative substances were used.

viii) Specific goals of Phase-2 were to:

- evaluate the reproducibility of the protocol for identifying weaker androgen agonists and antagonists;
- evaluate the capability of the protocol to detect and to identify a 5α-reductase inhibitor;
- continue the evaluation of the relative effectiveness of the five sex accessory tissues and glands;
- characterize protocol variability with different test substances; and
- support the reliability and relevance of the Hershberger bioassay as a robust method for the detection of chemicals that may act like either androgen agonists or antagonists and, consequently, may have the potential to interfere with endogenous male hormones; thereby, supporting the recommendation that an OECD Test Guideline for the rat Hershberger bioassay be developed.

ix) Few participating laboratories reported animal deaths during the course of the studies. The causes of these deaths were attributed primarily to gavaging errors and rarely to overt toxicity of any of the test substances.

x) The validation programme successfully achieved the goal of demonstrating the reproducibility of the protocol for detecting weaker androgen agonists. The Hershberger bioassay successfully achieved this goal with MT and TREN. All laboratories were able to detect MT and TREN with all five mandatory tissues achieving statistically significant decreases.

xi) The validation programme successfully achieved the goal of demonstrating the reproducibility of the protocol for detecting weaker androgen antagonists. The Hershberger bioassay successfully achieved this

goal with PRO, VIN, LIN, and DDE. All laboratories were able to detect DDE with all five mandatory tissues achieving statistically significant decreases. All laboratories were able to detect PRO and VIN with four mandatory tissues achieving statistically significant decreases. Although the GP absolute weights were decreased, the GP, however, sometimes failed to achieve statistical significance in all cases. Three of four laboratories were able to detect LIN with at least four of the mandatory tissues achieving statistically significant decreases. The fourth laboratory detected LIN only with the SCVG and only when the more liberal pairwise comparison statistical approach was used. This laboratory had the largest CVs and encountered some apparent difficulties in the dissection of the small unstimulated tissues. This reinforces the need for laboratories to sufficiently train their personnel in the dissection of the small tissues which are sometimes embed in adipose tissues, and for laboratories to demonstrate this proficiency before undertaking the Hershberger bioassay.

xii) The validation programme successfully achieved the goal of demonstrating the reproducibility of the protocol for detecting a 5α-reductase. The Hershberger bioassay successfully achieved this goal with FIN. All laboratories were able to detect FIN with four mandatory tissues achieving statistically significant decreases in all instances. In this case, the lack or low level of activity of a 5α-reductase enzyme in the GP is a plausible reason this tissue did not achieve statistically significant decreases in one laboratory.

xiii) The Phase-2 results suggested some possible protocol refinements. Two laboratories encountered a low rate of incomplete preputial separation, and this impacted the ability to dissect the GP. The basic recommendation made based upon these data is that laboratories should understand particular characteristics of their animal strain and supplier, e.g., if the animals are castrated on pnd 42, whether preputial separation be complete at necropsy. If preputial separation would not be complete, the obvious solution is to delay castration in those particular circumstances by the minimum time needed. This would represent a minimal, but acceptable, change in the protocol. The results of Phase-2 fully supported the previous findings in Phase-1: fixation of the ventral prostate offers no increase in sensitivity and no reduction in CV. It is recommended that no further work be done with ventral prostate fixation, and that this endpoint need not be included in Phase-3.

xiv) The responsiveness and the variability of the five mandatory tissues differed. The three fluid-filled tissues, VP, SVCG, and COWS, responded to sufficient agonist doses by increasing in weight several fold and responded to sufficient antagonist doses with major decreases in weight. However, the coefficients of variation (CV) in these tissues were relatively large. In some laboratories, the CVs were very large. In contrast, the LABC and GP were less responsive to agonist and antagonist doses, but their CVs tended to be lower. As a result, the ability to detect agonists, antagonists, and 5α-reductase inhibitors was approximately the same for the VP, SVCG, COWS, and LABC. However, the GP as the least responsive tissue, sometimes failed to detect weak acting test substances.

xv) The ability of laboratories to perform the protocol also appeared to differ when measured by the CVs of the tissues. The CV values tend to vary amongst laboratories for a given tissue and that the tendency towards a smaller or larger CV continues across all five mandatory tissues. As with the uterotrophic bioassay, the CV differences among laboratories indicates that a validation programme not only characterizes a particular protocol, but also the performance of the participating laboratories. This difference is presumably related to the dissection and the handling of these small and sometimes fluid-filled tissues as the magnitude of the CVs for a given tissue tended to be similar across as many as three experiments within a laboratory. For example, CVs for the VP (38.2, 42.9, and 60.3) and COWS (35.8, 41.4, and 56.5) were among the largest in one laboratory while the CVs for the VP (12.6, 15.5, and 18.4) and the COWS (8.7, 16.7, and 17.7) were among the smallest in another laboratory. This suggests that laboratory personnel training and performance is a major variable in the Hershberger bioassay and may be related to the ability to detect weakly acting substances.

xvi) The protocol itself appears to yield reproducible results. Data from three substances, MT, VIN, and DDE, were available for comparison from laboratories from both stages of work in Phase-2. The VIN doses were identical. The MT doses had been adjusted to avoid possible animal wastage as no response had been observed at the lowest MT doses and to provide for clearly overlapping MT doses with the enhanced TG 407 program where male doses included 10 and 40 mg/kg/d MT. The DDE doses were also adjusted to ensure that a response was observed at the highest dose and to overlap with the TG 407 where male doses were 12.5 – 150 mg/kg/d. All substances were detected by all laboratories, and all five mandatory tissues responded. The dose responses were similar in the magnitude of the response, the shape of the dose response curve, and the doses at which significant responses were observed for the MT agonist and the VIN and DDE antagonists. Further, the studies were conducted using different rat strains, diets, and housing conditions with no indication that these variables influenced the outcome of the assay.

xvii) Phase-1B results indicated that little or no difference was evident between 0.2 mg/kg/d and 0.4 mg/kg/d as the reference dose of TP when coadministered with a dose series of FLU. Phase-2 has then expanded this comparison with dose series of VIN and of DDE. Again, there was no evidence for a difference between the two TP reference doses, particularly, in regards to the ability to detect weaker anitandrogens. Importantly, no obvious difference was evident in sensitivity with the weaker antiandrogen, DDE. Therefore, at this time, both doses appear acceptable for use. It is recommended, however, that studies with the two doses be continued in Phase-3, if it is apparent that different national authorities may desire to use both of the TP reference doses. This would enlarge the data set to support both doses or, alternatively, may yet reveal an important difference.

xviii) The protocol reproducibility was also good for the other substances, TREN, PRO, LIN, and FIN. The magnitude and shape of the dose response curve as well as the doses at which significant responses were observed were again similar for each of the five mandatory tissues across laboratories. Again, the variables such as rat strain and diet did not appear to influence the outcome of the assay.

xix) The need for all five of the mandatory tissues remains open to some question. The VP, SVCG, LABC, and COWS were sufficient in all cases for detection of the test substances in Phase-2. The VP, SVCG, and COWS were on occasion the sole most sensitive endpoint, and the VP, SVCG, and LABC were routinely among the most sensitive endpoints, followed by the COWS. In contrast, the GP did not achieve statistical significance in several instances. The GP may contribute in some cases to identifying the particular mechanistic profile of a chemical, if that is also the intended purpose of conducting a Hershberger assay. However, a Hershberger bioassay alone does not appear to be sufficient to identify a mechanistic profile. Instead, it appears to be an important contributor to the overall weight of evidence, when combined with other information, e.g., androgen receptor binding assays and enzyme inhibition assays. Therefore, further work with the GP should be conducted in Phase-3.

xx) A comparison between the results of the Hershberger bioassay and the findings of developmental and reproductive bioassays was made for six test substances in Tables 40A-F. A similar comparison has been made for the uterotrophic bioassay (64)(65). This comparison strongly supports the toxicological relevance of the Hershberger bioassay in its ability to reliably screen for (anti)androgens. In almost all cases, a correspondence exists between the LOELs observed in Phase-2 and LOELs seen in the developmental and reproductive studies. Final conclusions should, however, await work with coded negative substances.

xxi) Several test substances caused statistically significant changes to the optional tissues (liver, paired adrenals, and paired kidneys). These changes were sporadic, dependent upon the nature of the individual test substance, and were not correlated with changes in the male reproductive tract. As a result, the investigation of these optional tissues should continue to be an option for participating laboratories in Phase-3, but it should be recognized that these changes are not intrinsically (anti)androgenic in mechanism.

xxii) It can be concluded from this second phase of the validation study that the Hershberger protocol is transferable and reliable for identifying androgen agonists, androgen antagonists, and 5α-reductase inhibitors. Proper dissection and handling of several tissues in the male reproductive sometimes remains a challenge for laboratories, apparently resulting in high coefficients of variation that reduce the power of the assay.

xxiii) Based on the success of Phase-2, a third phase of the validation study for the Hershberger protocol is recommended using coded test substances from Phase-2 to assess the reproducibility of the assay results over time. Negative test substances should be included in Phase-3 to quantify the false positive rate of the bioassay.

INTRODUCTION

1. The need to validate the rat Hershberger bioassay arises from the concerns that ambient levels of natural and industrial chemicals may interact with the endocrine system and as a consequence possibly elicit reproductive, developmental, and other adverse effects in humans and wildlife. Current reviews have noted that there is very limited evidence for endocrine disruption in humans, but that local, high level exposures to environmental pollutants have probably resulted in endocrine-related effects in wildlife (4)(5)(6).

2. Androgen were first hypothesized to be necessary for the *in utero* development of the male reproductive tract in the late 1930s (7)(8). Strong support for the hypothesis was first produced in the late 1940s, when fetal rabbits that were surgically castrated prior to development of the male reproductive tract the failed to undergo develop a male phenotype (9)(10). Then, beginning in the 1960s, a series of experiments demonstrated that chemical substances could block the action of testosterone by binding at the androgen receptor (11)(12)(13)(14)(15), the steroidogenic synthetic pathway for testosterone by inhibiting one or more of the enzymes involved (16)(17)(18), and the formation of the more potent dihydrotestosterone agonist from testosterone by inhibiting the 5α-reductase enzyme (19)(20). These mechanisms of action can potentially negatively impact the androgen-dependent tissues of the male reproductive tract, including the anlage from the Wolffian ducts (epididymis, vas deferens, and seminal vesicles), the urogenital sinus (prostate), and the genital tubercle (penis and scrotum). In its severest form, the resulting phenotype is that of a pseudohermaphrodite, including agenesis of several of these tissues. A classical set of internal and external malformations of the male reproductive tract are evident whose occurrence and rate depend on a substance's precise mechanism of action, potency and dose, including epididymal deformities, reduced size of the prostate, and hypospadias. In fact, the ontology of the androgen receptor and the necessary enzymes for steroid synthesis and testosterone conversion to dihydrotestosterone occur coincide with both tissue development and sensitivity to antiandrogens beginning about pnd 14 (21). This period of development and sensitivity extends in the male rat almost to partiution. This phenotype can also be produced in man by genetic mutations leading to defects in the androgen receptor and the biosynthetic enzymes of testosterone and dihydrotestosterone (22)(23)(24). The same phenotype has been produced in several variations of knock-out mice resulting in defects in the androgen receptor and the biosynthesis of androgens (25)(26). Therefore, detection of these hazards is relevant to human health, and a screening bioassay is needed to identify potential toxicants that might act through these various mechanisms.

3. The need then arises for a bioassay to identify potential androgens and antiandrogens that is rapid, efficient, sensitive, and specific. The leading candidate is the surgically castrated male rat or Hershberger bioassay. The surgically castrated male rat assay for androgens has existed in various forms for 70 years (27)(28)(29). This original assay was intended for androgens and was based on the responses of tissues such as VP and SV. The assay was later modified to assess the related myotrophic properties of androgenic chemicals by measuring the LABC and to assess androgen antagonists by measuring the blockade of a coadministered reference androgen (31)(32)(33)(34). The assay has been used primarily in the pharmaceutical industry for work with potent androgen agonists and antagonists. Prior to this time, a standardised protocol has not been available for consideration internationally, e.g., there is no consensus on which of the various tissues of the male reproductive tract to include in a protocol. This current protocol is based on the standardisation and optimisation work performed under OECD auspices in Phase-1 of the Hershberger validation (3).

4. The principle of the rat Hershberger bioassay is that organs and accessory tissues in the male reproductive tract are under the control of androgens, which are necessary to stimulate and to maintain growth. If the endogenous source of this hormone is not available, either because of immaturity of the animals or because the animals have been surgically castrated, the animal requires an exogenous androgen source to initiate or restore growth of these tissues. Chemicals that act as agonists may be identified if they cause a statistically significant increase in the weights of the target androgen-dependent tissues, or chemicals

may be identified as antagonists if they cause a statistically significant decrease in target tissues when co-administered with a potent androgen (see reviews (35)(36)). Similarly, several tissues depend upon the local conversion of testosterone to dihydrotestosterone by the enzyme 5α-reductase. The androgen response of those tissues, and not others, are then sensitive to inhibitors of 5α-reductase. Therefore, 5α-reductase inhibitors may be identified if they cause a relative decrease in the weight gain of particular tissues, when co-administered with a potent androgen. The Hershberger bioassay is included in the OECD conceptual framework for endocrine disrupter testing and assessment as agreed by EDTA6 in June, 2002 (37).

5. The objective for the Hershberger validation programme is to develop and validate a test protocol in order to support the development of a Test Guideline for the detection of chemicals having the potential to act as androgen agonists and antagonists in rats. The Test Guideline, once available, would be intended to be used as one element in an overall testing strategy for the detection and assessment of potential endocrine disrupters.

6. The in-life studies for Phase-1A of the OECD validation study of the Hershberger bioassay, in which 17 laboratories in Europe, Japan, Korea, and the United States participated, occurred from June 2000 through January 2001. The in-life studies for Phase-1B of the OECD validation study of the Hershberger bioassay, in which 7 laboratories from Japan participated, occurred from March 2001 through June 2001. The results showed that the protocol was robust and produced comparable results among laboratories with the reference androgen, testosterone propionate (TP), and with a potent, reference androgen antagonist, flutamide (FLU) (3).

7. The protocol used for Phase-2 of the validation study is largely unchanged from the protocol employed in Phases-1A and 1B. The primary modifications were 1) to cease further work with the dorsolateral prostate and 2) to delay the day of castration until or after pnd 42, as animals in several laboratories in Phase-1 did not undergo preputial separation, the lack of which can compromise the glans penis dissection. The protocol is outlined as follows:

- Agonists. Males entering puberty are surgically castrated on pnd 42 or thereafter. The animals are allowed to recover and the tissues of the male reproductive tract to regress for a minimum of 7 days. Treatment would then preferably begin between pnd 49 and 60. The animals are treated for ten consecutive days, once per day, with the test substance by either s.c. injection or oral gavage. The animals are sacrificed and necropsied 24 hours after the last administration and the target tissues are removed and weighed.
- Antagonists. Males entering puberty are surgically castrated on pnd 42 or thereafter. The animals are allowed to recover and the tissues of the male reproductive tract to regress for a minimum of 7 days. Treatment would then preferably begin between pnd 49 and 60. The animals are treated for ten consecutive days, once per day, with the test substance by either s.c. injection or oral gavage. The animals are coadministered the reference androgen TP via s.c. injection. The animals are sacrificed and necropsied 24 hours after the last administration and the target tissues are removed and weighed.

The protocol is described in more detail in following sections of this document, and the full text of the model protocol used by the laboratories is contained in Annex 2.

8. The Phase-2 validation studies were designed to determine the robustness of the protocol using the dose response of androgen agonists, including a substance that does not undergo conversion to a more potent form due to 5α-reductase conversion (TREN); of androgen antagonists with range of potencies less than FLU; and of a 5α-reductase inhibitor. The selected substances and their CASR-numbers are noted in Table 1. The reference doses of 0.2 and 0.4 mg/kg/d of TP were used by the laboratories in Japan and the laboratories outside of Japan, respectively. Several laboratories also employed a standard dose of 3 mg/kg/d flutamide to demonstrate the effectiveness of a reference antagonists in their laboratory.

Table 1. Chemicals used in Phase-2 of the OECD validation of the Hershberger bioassay

Androgen Receptor Agonists	
Testosterone Propionate [a]	57-85-2
17α-Methyltestosterone	58-18-4
Trenbolone 17βHydroxyestra-4,9,11-trien-3-one	10161-33-8
Androgen Receptor Antagonists	
Vinclozolin	50471-44-8
Procymidone	32809-16-8
Linuron	330-55-2
p,*p'*-DDE 1,1-Dichoro-2,2-bis-(p-chlorophenyl)ethylene	72-55-9
Flutamide [b]	1311-84-7
5α-Reductase Inhibitor	
Finasteride	98319-26-7

[a] Testosterone propionate is the reference androgen agonist, which is coadministered with a suspected antagonist or inhibitor. Standard curves were produced in Phase-1A.

[b] Flutamide is the reference androgen antagonist, which can be coadministered with TP as a positive control antagonist. Standard curves were produced in Phase-1B.

9. The overall goal of the Phase-2 test validation study was to assess the robustness and reproducibility of the Hershberger bioassay in response to test substances acting via androgenic and antiandrogenic mechanisms. The specific goals of Phase-2 were to:

- evaluate the reproducibility of the protocol for identifying weaker androgen agonists and antagonists;
- evaluate the capability of the protocol to detect and to identify a 5α-reductase inhibitor;
- continue the evaluation of the relative effectiveness of the five sex accessory tissues and glands;
- characterize protocol variability with different test substances;
- support the reliability and relevance of the Hershberger bioassay for the detection of chemicals that may act like either androgen agonists or antagonists and, consequently, may have the potential to interfere with endogenous male hormones; and,
- thereby, support a recommendation for the development of an OECD Test Guideline for the Hershberger bioassay.

10. In order to assess inter-laboratory variability, it was recommended that at least three and possibly four laboratories test each chemical during the studies.

TEST VALIDATION

11. *Validation* is a term that refers to the scientific process designed to characterise the operational characteristics and limitations of a test method, and to demonstrate its reliability and relevance for a particular purpose.

12. The Report of the OECD Workshop on Harmonisation of Validation and Acceptance Criteria for Alternative Test Methods (Solna Report) (38) provides the principles of test validation that are followed by OECD. Further practical guidance for the application of the validation and regulatory acceptance principles and criteria were discussed and agreed to by the Stockholm Conference on Validation and Regulatory Acceptance of New and Updated Methods in Hazard Assessment (39). These principles are currently being incorporated into a revised OECD Guidance Document for the Preparation of Test Guidelines (Guidance Document No. 34). The OECD principles are consistent with approaches used in Europe, particularly those of the European Centre for Validation of Alternative Methods (ECVAM) (40) and are consistent with the approaches used in the US as stated by the Interagency Co-ordinating Committee on Validation of Alternative Methods (ICCVAM) (41).

13. In 1998, the Joint Meeting of the OECD Chemicals Group and Committee and Working Party on Chemicals, Pesticides and Biotechnology (the Joint Meeting) decided that the criteria and approaches for the validation of test methods should apply equally to the development of all toxicology tests *in vitro* and *in vivo*, and to tests for ecotoxicological effects. The Joint Meeting agreed that flexibility should be shown in designing validation studies so that they would be appropriate for the specific test and its proposed purpose. Most importantly, all decisions on the extent and design of the validation study should be fully transparent and documented. In principle, a new test is validated for its proposed use by developing a protocol, standardizing it among one or two laboratories, and then testing a number of potent and weakly acting chemicals under code in a number of laboratories, and evaluating the assay's reliability (i.e., reproducibility within and among laboratories) and relevance (i.e., its ability to accurately measure or predict the effect of concern in the species of concern). This approach is represented in Figure 1, which shows how the assessment process of the relevance and reliability of new or significantly revised testing methods for hazard characterisation can be undertaken in a stepwise, yet flexible, manner while still providing the information necessary to address the Solna criteria and principles.

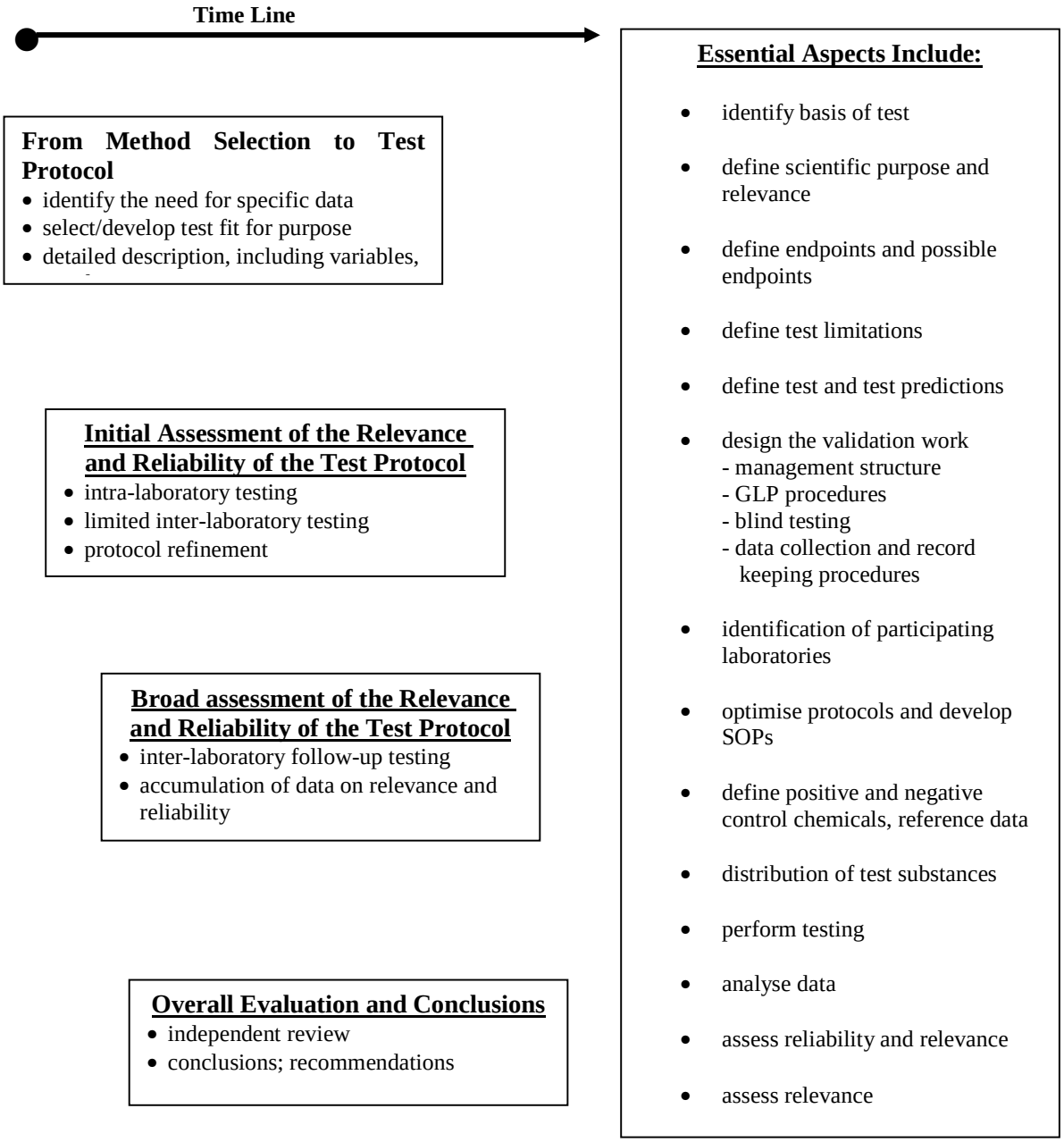
History and organisation of the OECD validation programme for the Hershberger bioassay

14. In early 1998, the National Co-ordinators of the Test Guidelines Programme established a Task Force on Endocrine Disrupters Testing and Assessment (EDTA) to provide a focal point within OECD to consider and recommend priorities for the development of testing and assessment methods for endocrine disrupters (2). Members of EDTA were nominated by Member countries; industry and environmental groups also participated as Invited Experts.

15. The EDTA subsequently established three Validation Management Groups (VMG), one for mammalian test methods, one for ecotoxicology test methods, and one for in vitro or non-animal test methods. The role of each VMGs is to oversee and manage the conduct of the endocrine disrupter test validation studies in its respective area.

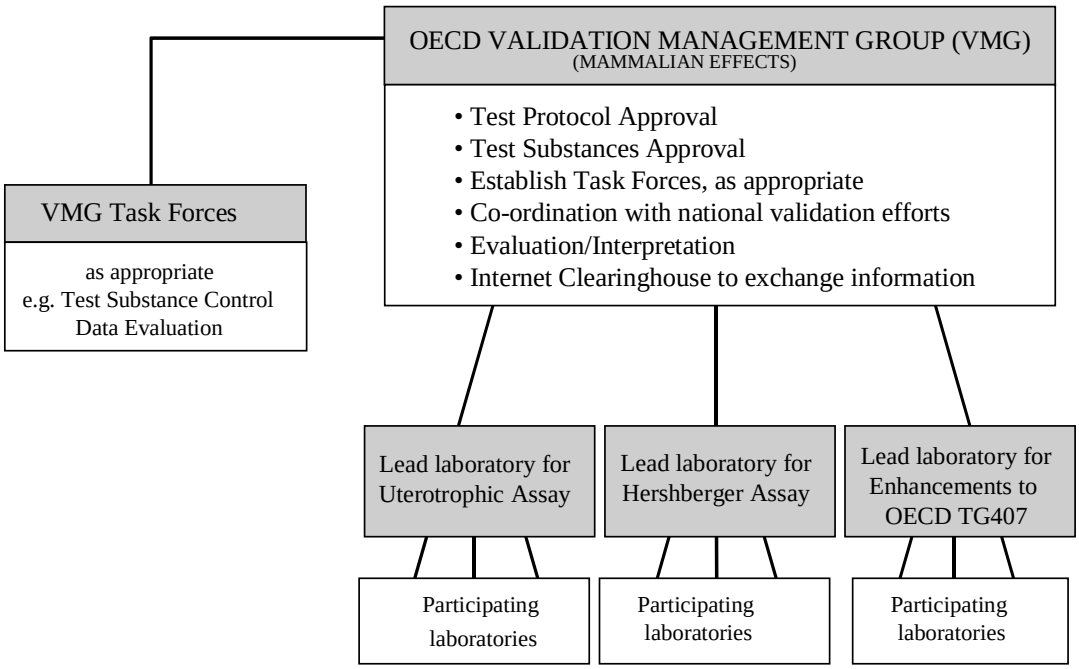
16. A schematic diagram is provided in Figure 2 which describes the role and structure of the OECD VMG-mammalian. The VMG-mammalian is comprised of experts nominated both by Member countries and non-government organisations. The membership contains a balance of experts from disciplines including toxicology, endocrinology, and test method development and validation. The VMGs are charged to provide independent, objective review, to address animal welfare issues, and to consider the requirements for regulatory acceptance of new assays.

Figure 1. Assessment Process of the Relevance and Reliability of New or Significantly Revised Testing Methods for Hazard Characterisation



17. The VMG made the determination to perform the OECD Hershberger validation work in phases, taking into consideration the long history of the assay and its many variants. The measurement of the responses of accessory sex tissues in castrated or immature rats to administered androgens has been in use since 1932 (28). However, the bioassay is commonly attributed to Hershberger *et. al*. (33) who in 1953 published the test results for a number of chemicals. There have been a number of protocols used, which vary with regard to whether sexually immature or castrated rats or mice are used, the number of days dosing and the route, and the tissues examined. The assay has subsequently been accepted and employed by testing laboratories, industry, and regulatory authorities for testing pharmaceuticals for androgenic and anti-androgenic effects.

Figure 2. The role and structure of OECD Validation Management Group (Mammalian)



18. A U.S. EPA research laboratory volunteered as the Lead Laboratory with the responsibilities of addressing day-to-day technical questions from participating laboratories, providing guidance and training to standardize male reproductive tract tissue dissection, summarising and evaluating the data, and preparing recommendations for the next validation phase. The Lead Laboratory, however, did not perform the assay protocols. In addition, an independent statistician was asked to evaluate the results and assess the validity of the statistical procedures used by the Lead Laboratory. The OECD Secretariat provided the overall project co-ordination and has authored the Final Reports.

19. The VMG-mammalian developed protocols for the conduct of the Hershberger assay, designed the each stage or Phase of the validation programme, identified the test substances to be used, and selected the doses of each substance. Expressions of interest were then sought from laboratories wishing to participate in the validation studies. The laboratories that expressed interest were invited to participate in meetings of the VMG, whenever appropriate. The selection of participating laboratories was determined by the willingness of the laboratory to strictly follow the OECD test protocol at their own expense, their willingness to adhere to the projected timeline for completion of the study, and their agreement to provide their data for summary and analysis by the Lead Laboratory and the Secretariat.

20. Phase-1 of the validation procedure was to demonstrate a standardized protocol that would be expected to identify potent androgenic and anti-androgenic substances; Phase-2 was to demonstrate the protocol's variability and inter-laboratory reproducibility with a variety of potent and weakly acting substances, and to determine the relative effectiveness of the different tissues for measuring the effects. The need for subsequent phases for the validation of the assay (e.g., additional substances, coded or blind testing, negative chemicals, and so on) would be determined following the completion and evaluation of the initial phase. A Phase-3 was endorsed at the 4th VMG-mammalian in April, 2003, and was to demonstrate reproducibility over time using coded samples (42).

METHODS

Introduction

21. The Hershberger assay was selected for validation by the OECD following an expert Workshop that was held in Washington, DC in 1998. The expert Workshop noted national recommendations to standardise and validate the Hershberger bioassay as an *in vivo* assay in order to identify possible androgen agonists and antagonists (43) and the OECD's Detailed Review Paper on the appraisal of test methods for sex-hormone disrupting chemicals noting the long history of use of the Hershberger bioassay (2), and the Workshop recommended that the OECD proceed to standardise and validate the Hershberger bioassay. The EDTA then endorsed the standardization and validation of the Hershberger bioassay under the direction of experts in the VMG-mammalian.

22. The precursor of the rodent Hershberger assay was first developed in the 1930s and included various tissues of the male reproductive tract (27) (28) (29), including the ventral prostate, the seminal vesicles with coagulating glands, the Cowper's glands, the glans penis, and preputial glands. The measurement of the levator ani and bulbocavernosus muscles were first investigated in the 1940s (31) (32). After publication of work with an extensive number of compounds by Hershberger *et. al.* in 1953 (33), the assay has subsequently been referred to as the Hershberger assay.

23. The advantages of the Hershberger bioassay include:

- the male reproductive tissues rapidly respond to endogenous androgens and are, thus, relevant targets for assaying exogenous androgens and antiandrogens,
- the biological response is rapid and occurs within 5-10 days of test substance administration,
- the tissue responses can be quantified by the measurement of the tissue weights,
- the dose responses of the tissues can be evaluated statistically,
- the assay can be conducted without specialised facilities, equipment, or techniques.

However, there is a disadvantage in that personnel training and expertise are required because dissection of the male reproductive tract and its small, fluid-filled tissues is difficult.

24. The assay has been used primarily in the pharmaceutical industry for work with potent androgen agonists and antagonists. Prior to this time, a standardised protocol has not been available for consideration internationally, e.g., there is no consensus on which of the various tissues of the male reproductive tract to include in a protocol. A draft standardised protocol was developed by the VMG-mammalian based on the work of an expert meeting in Tokyo in February, 1999 (42). This protocol proposed to assess the responses of five tissues: the ventral prostate (VP), the seminal vesicles and coagulating glands (SVCG), and the levator ani and bulbocavernosus muscles (LABC). Subsequently, the glans penis (GP) and the Cowper's or bulbourethral glands (COWS), and the dorsolateral prostate were suggested by the Lead Laboratory and others and approved by the EDTA (43).

25. In Phase-1A, the dose response of these five tissues to a series of TP doses was assessed and found to be satisfactory and reproducible, while the dorsolateral prostate was found to add no overall value (3). In Phase-1B, the inhibition of this response in all five tissues to selected TP doses as assessed against a series of FLU doses. Again, the response of all five tissues was found to be robust and reproducible across laboratories and in the presence of several variations, e.g., strain of rat, diet, and modest variations in the age of castration. All laboratories and all protocols were successful in detecting increases in the weights of the accessory sex organs and tissues in response to TP, and there was good agreement among laboratories with regard to the dose responses obtained. There was similar robustness, reproducibility, and agreement among laboratories in their ability with regard to the dose responses of the anti-androgenic effects of FLU (3).

26. In Phase-2, the capability of the protocol and the five tissues was to be assessed against additional

androgen agonists, antagonists, and a 5α-reductase inhibitor. In Phase-1, several laboratories encountered some difficulties in the dissection of the glans penis when preputial separation had not occurred in young animals. Therefore, the specified minimum time of castration was increased slightly to pnd 42. No other significant changes were made in the protocol.

Phase-2 Protocol, Laboratories, Test Substances, and Doses

27. The VMG-mammalian then recommended and the EDTA endorsed Phase-2 of the Hershberger validation programme (44). In Phase-2, the capability of the protocol and the five tissues was to be assessed against additional androgen agonists, antagonists, and a 5α-reductase inhibitor. The specific individual objectives of Phase-2 have been described in paragraph 9 of the Introduction. The outline of the protocol is shown in Table 2 and the full model protocol is contained in Annex 2.

28. In addition to the mandatory measurements of the total body weight and the five male reproductive tract tissues, the protocol provided for several optional organ weights and hormone measurements. The optional organ weights included the liver, the paired kidneys, and the paired adrenal glands, and the optional hormonal measurements included serum testosterone and lutenising hormone (LH).

Table 2. Protocol summary for Phase-2

	Factor	**Protocol requirements**
Animals	Species	Rat
	Strain	No preference (not Fischer 344)
	Age at castration	At peripuberty; approx. 6 weeks, bu minimum age of 42 days
	Time after castration	1-2 weeks
	Age at initiation of treatment	7-8 weeks
	Weight at time of treatment	Not specified; should be ± 20%
Animal husbandry	Diet	Laboratory preference
	Bedding	Laboratory preference
	Caging	Laboratory preference
Treatment regimen	Animals per dose group	6
	Vehicle	corn oil
	Volume of administration subcutaneous gavage	 0.5 ml/kg/day 5.0 ml/kg/day
	Dosing regimen (mg/kg/day)	10 consecutive daily administrations
	Sacrifice	24-hrs after last treatment
Measurements	Mandatory weights	Total body weight Ventral prostate (fresh) Seminal vesicle + coagulating glands Levator ani + bulbocavernosus muscles Glans penis Cowper's glands
	Optional weights and measurements	Liver Adrenal gland (paired) weight Kidney (paired) weight Fixed ventral prostate Serum testosterone and LH

29. A total of sixteen laboratories, from Denmark, France, Germany, Japan, Korea, the UK, and the US, volunteered to participate in the Phase-2 studies under the direction of the Lead Laboratory and the

Secretariat. All of the laboratories had previously participated in Phase-1A or 1B. The laboratories were from both the public and private sectors as noted in Table 3. The participating laboratories and lead investigators are identified in Annex 1. As there were differences in the studies in the particular doses of some test substances and in the dose of TP (i.e., 0.2 mg/kg/d and 0.4 mg/kg/d coadministration doses of TP), the studies are first reported separately and then the appropriate results are compared for consistency.

Table 3. Laboratories participating in the OECD Hershberger Phase-2 validation study

Country	Laboratory	Number of Laboratories
Denmark	Government	1
France	Industry	1
	Private Contract	1
Germany	Industry	2
Japan	Government	1
	Industry	2
	Private Contract	4
Korea	Government	1
United Kingdom	Industry	1
	Private Contract	1
United States	Government (Lead laboratory)	1
	Industry	1
	Total performing studies	**16**

30. Because the intended purpose of the Hershberger bioassay is the rapid screening of a potentially large number of chemicals, the judgment was that rigorous and detailed standardisation of all of these variables would constrain the ability to widely use the Hershberger in many of the OECD member countries. Therefore, the protocol allowed variations in a number of study conditions, including the choice of rat strain, the laboratory diet, housing and husbandry practices such as the use of cage bedding, the vehicle employed, and within the ranges of permitted by the model protocol for the age of castration and the time period of regression after castration. The specific conditions in each laboratory at the time that the Hershberger Phase-2 studies were conducted have been summarized in Table 4.

Measurements and Data Reporting

31. The mandatory and optional measurements actually performed in each laboratory during the Hershberger Phase-2 studies have been summarized in Tables 5A and 5B.

32. For data submission and records, the Secretariat provided each participating laboratory with a standardized Excel spreadsheet for recording and transmitting the data in a standard format. This standard format provided for rapid e-mail transmission of the results regardless of geographic location to the Secretariat and retransmission to the Lead Laboratory. The spreadsheet contained an initial worksheet for recording the laboratory personnel, parameters such as diet and rat strain as well as the suppliers, protocol variables such as the data of castration and the initiation of treatment, caging practices, bedding, and so on. The spreadsheet then contained two individual worksheets for each test substance. The first worksheet was to record for each dosage group the individual animal number, daily body weights, time of administration,

Table 4. Laboratory parameter and conditions for Phase-2.

Lab	Rat strain	Age at castration	Acclimation time (days)	Vehicle	Age at autopsy	Diet	Animals per cage
1	Wistar rats, CrlGlxBrlHan:Wl	44-46	7	corn oil	61-63	Provimi Kliba SA	1
2	Sprague Dawley	43-45, 47	12, 10-11	0.5% MC	64-67, 67-68	UAR, A04C-10	1
3	SPF-bred Wistar HsdCpb:WU	45-46	12-13	corn oil	67-69	Provimi Kliba SA	3
4	Crl CD® (SD) IGS BR Sprague Dawley	43-44,44-46	12-14	corn oil	69-70	A04 C SAFE	3
5	Brl:WIST Han@Mol outbred	42-45	14-15, 18	corn oil	66-70, 70-73	Proprietary[a]	3
6	Crl:CD(SD)IGS BR	42	14-15	corn oil	66-67	PMI 5002	3
7	CD (SD) IGS BR	42-45	7	corn oil	59-63	SDS, RM1	3
8	Sprague Dawley	42	8	corn oil	60	PMI 5057	3
9	Alpk:APfSD	42-43	9-10	corn oil	62-63	SDS, RM1	3
10	Brl Han: WIST Jcl (GALAS)	41-43	7	corn oil	59-61	MF, Oriental Yeast	3
11	Crj:CD IGS (SD) [b]	40-44	8	corn oil	59-63	MF, Oriental Yeast	1
12	Brl Han: WIST Jcl (GALAS)	40-42	6	corn oil	57-59	MF, Oriental Yeast	3
13	Crj:CD IGS (SD) [b]	41-44	11	corn oil	63-66	MF, Oriental Yeast	1
14	Crj:CD IGS (SD) [c]	43-46	7	corn oil	61-64	MF, Oriental Yeast	2
15	Crj:CD IGS (SD) [c]	41-43	7	corn oil	59-61	MF, Oriental Yeast	3
16	Crj:CD IGS (SD) c[b]	42-44	7	corn oil	60-62	MF, Oriental Yeast	2

MC: Methyl cellulose; UAR: Usine d'Alimentation Rationnelle; SDS: Special Diet Services ; SAFE : Scientific Animal Food & Engineering

[a] Purified (semi synthetic) diet, prepared at laboratory 5

[b] – Atsugi facility in Kanagawa, Japan; [c] – Hino facility in Siga, Japan.

Table 5. The mandatory and optional measurements performed by laboratories in Phase-2.

	Measurements made in Phase-2										
	Mandatory					Optional					
Laboratory (chemicals)	**Ventral prostate**	**Seminal vesicles & coagulating glands**	**LABC muscles**	**Glans penis**	**Cowper's glands**	**Liver**	**Adrenals**	**Kidneys**	**Tissue fixation [a]**	**Serum T**	**Serum LH**
1 (LIN, TREN, VIN)	▲	▲	▲	▲	▲	Y	Y	Y	Y	N	N
2 (FIN, MT, PRO)	▲	▲	▲	▲	▲	Y	Y	Y	Y	Y	Y
3 (DDE, TREN, VIN)	▲	▲	▲	▲	▲	Y	Y	Y	N	N	N
4 (DDE, LIN, MT)	▲	▲	▲	▲	▲	Y	Y	Y	Y	Y [b]	Y [b]
5 (FIN, LIN, VIN)	▲	▲	▲	▲	▲	Y	Y	Y	N	N	N
6 (FIN, LIN, MT)	▲	▲	▲	▲	▲	Y	Y	Y	Y	N	N
7 (PRO, TREN, VIN)	▲	▲	▲	▲	▲						
8 (DDE, MT, PRO)	▲	▲	▲	▲	▲	Y	Y	Y	Y	N	N
9 (DDE, FIN, PRO)	▲	▲	▲	▲	▲	Y	Y	Y	N	N	N
10 (DDE, VIN)	▲	▲	▲	▲	▲	N	N	N	N	N	N
11 (DDE, MT, VIN)	▲	▲	▲	▲	▲	N	N	N	N	N	N
12 (DDE)	▲	▲	▲	▲	▲	N	N	Y	N	N	N
13 (MT, VIN)	▲	▲	▲	▲	▲	Y	Y	Y	N	Y	Y
14 (DDE, VIN)	▲	▲	▲	▲	▲	N	N	N	N	N	N
15 (MT)	▲	▲	▲	▲	▲	Y	N	*	N	N	N
16 (MT, DDE)	▲ [c]	▲ [c]	▲	▲ [c]	▲	Y	Y [c]	Y	Y	N	N

Chemicals: DDE – *p,p'*-DDE; FIN – finasteride; LN – linuron; MT – methyl testosterone; PRO – procymidone; TR – trenbolone; VN - vinclozolin

▲ - Performed mandatory endpoint

Y – Performed optional endpoint; N – Did not perform optional endpoint

* VCZ is Y, and MT is N

[a] Ventral prostate only was fixed and reweighed after fixation.

[b] Hormone analyses were done for DDE, and not MT or LN.

[c] Ventral prostate, seminal vesicles, Cowper's glands and adrenals were fixed in laboratory 16

administration volumes and any clinical signs or observations. The second worksheet provided preset areas to record all mandatory and optional endpoints, group and individual animal identification, dates, entry of preputial separation observations, and so on. These worksheets were adaptable to either androgen agonists or antagonists and 5α-reductase inhibitors. In addition to rapid transmission, the worksheets provided the means to quickly calculate basic means, standard deviations, and coefficients of variation to assist data audits. This proved essential for a rapid assessment of possible entry errors or identification of possible issues by the Secretariat and the Lead Laboratory, e.g., unusually large standard deviations for a group. In addition, the organization and format of the data allowed the rapid extraction into statistical programs by the Secretariat and the Lead Laboratory.

Selection of Test Substances and the Doses

33. The reference chemicals were the same as for Phase-1. For the agonists, the reference group was the vehicle control group. For the antiandrogens, the positive agonist test substance was TP, and it was the TP group that was the stimulated reference against which antagonists and 5α-reductase inhibitors were statistically evaluated. In some laboratories, FLU was voluntarily used as the control antagonist to confirm

the performance of the antagonist assay.

34. Based upon the results of the TP and FLU dose response studies in Phase-1A and 1B, the Lead Laboratory recommended that the standardized protocol should use 0.4 mg TP/kg/d as the reference dose. This TP dose was approximately the ED70 for three of the five androgenic tissues in Phase-1A, so it represented a large dynamic range without reaching a potentially insensitive maximum plateau. This TP dose was as sensitive to FLU as was the 0.2 mg/kg/d TP. In all cases, the FLU effect was larger in the 0.4 mg TP group than in the 0.2 mg TP group for all five tissues when the data from all laboratories was pooled and analyzed. However, the difference between 0.2 and 0.4 mg/kg/d was very modest (see Table 12 (3)). Seven laboratories chose to employ 0.2 mg/kg/d TP, and nine laboratories chose to employ 0.4 mg/kg/d TP.

35. Also based upon the results of Phase-1B, a reference dose of 3 mg/kg/d FLU was chosen. This dose produces a dramatic decrease in the TP response, but was not the maximum in the dose series (see Table 12 (3)). The 3 mg/kg/d FLU dose then allows an assessment of the laboratory response to antagonists is adequately sensitive.

36. The positive compounds nominated by Lead Laboratory and accepted by the VMG-mammalian (3) were:

- methyl testosterone (MT), a potent AR *agonist* active by the oral route and like testosterone propionate, activated by 5α-reductase.
- Trenbolone (TREN), a potent AR *agonist,* which is not activated by 5α-reductase.
- Procymidone (PRO), a weak AR *antagonist.*
- Vinclozolin (VIN), whose metabolites are weak AR *antagonists*.
- Linuron (LIN), a weak AR *antagonist* not requiring metabolism.
- *p,p'*-DDE (DDE), a weak AR *antagonist* not requiring metabolism.
- Finasteride (FIN), a 5α-reductase inhibitor.

37. The basis for selecting the two agonists (MT and TREN) was differences in their activation by 5α-reductase and the hypothesis that the tissues may respond differently to these agonists depending upon the activation by 5α-reductase. This would potentially generate a profile for distinguishing agonists activated by 5α-reductase and distinguishing androgen receptor antagonists from 5α-reductase inhibitors.

38. The basis for selecting the four antagonists was the demonstrated binding affinity of the parent or metabolites to the androgen receptor, the demonstrated activity of the parent or metabolite using *in vitro* assays, activity in previous Hershberger assays, and evidence for biological activity *in utero* inhibiting the development of the male reproductive tract. A summary of these points can be found in Annex 9 of the Hershberger Phase I Report (3).

39. The basis for selecting the 5α-reductase inhibitor (FIN) is similar evidence for its inhibition of the Type 2 5α-reductase activity found primarily in the male reproductive tract and evidence for its biological activity *in utero* inhibiting the development of the male reproductive tract. Again, a summary of these points can be found in Annex 9 of the Hershberger Phase I Report (3).

Dose Selection for Phase-2

40. The Lead Laboratory first made a series of dose proposals for the positive chemicals for Phase-2 in November, 2000. These recommendations were discussed at the 3rd VMG-mammalian meeting in Paris in March, 2001 (45). Meanwhile, for reasons related to the availability of funds only during the current fiscal year ending in March, 2001, the Japanese laboratories were forced to select among the positive chemicals and to make dosing decisions for these substances in order to proceed with their work prior to the VMG-mammalian meeting. Three chemicals were selected; the agonist MT and the antagonists VIN and DDE. The doses selected and employed in Japan were similar, but not identical to, those originally proposed by the

Lead Laboratory. Thee laboratories employed a series of four at either one half or one log intervals with each test substance.

41. In order to make a statistical comparison with that work conducted in the first stage, it was judged necessary to repeat or to at least overlap with one or more of those doses. At the same time, some of the initial MT doses employed in the first stage were clearly non-responsive. Animal welfare considerations were taken into account, and the MT dose series was revised to one-half log intervals in the upper portion of the dose range used in the first stage. Finally, the case of *p,p'*-DDE, the first stage dose series resulted in significant antiandrogen responses only at the highest dose, and it was felt necessary to increase the dose levels of this substance. As a result of a series of expert consultations, the recommended doses for the selected positive compounds in Phase-2 and the rationale for the dose selection are shown in Table 6 for all studies.

Test Chemical Supply

42. The European Chemical Industry Association (CEFIC) has previously supported the uterotrophic validation programme, the enhanced TG 407 validation programme, and Phase-1 of the Hershberger validation programme by providing financial and managerial responsibility for a centralised chemical repository. CEFIC agreed to continue their support for Phase-2 of the Hershberger validation programme. TNO in the Netherlands continued to serve as the centralised chemical repository as it had for other programmes and phases. Chemicals were purchased, donated, or acquired by synthesis. Where chemicals were included in more than one programme, sufficient quantities of test substances, the same batch could be used in parallel or future studies.

Table 6. Selected doses for Phase-2 substances.

Compound	Doses	Rationale
Testosterone propionate	In stage 1: 0.2 mg/kg/d	Selected based on Phase-1 results
	In stage 2: 0.4 mg/kg/d	Selected based on Phase-1 results
Flutamide	3 mg/kg/d	Selected based on Phase-1 results
Methyl testosterone	In stage 1: 0.05, 0.5, 5 and 50 mg/kg/d	Extended dose range with 1 log dose increments.
	In stage 2: 0.5, 2, 10, and 40 mg/kg/d	Achieves adequate coverage of positive dose response range observed in stage 1, and, based on animal welfare concerns, omits the 0.05 mg/kg/d dose, which was clearly unresponsive,.
Trenbolone	0.3, 1.5, 8, and 40 mg/kg/d	New test substance. Doses adapted from the Lead Laboratory's recommendations in order to give 0.5 log dose increments.
Vinclozolin	3, 10, 30, 100 mg/kg/d	Identical dose ranges with 0.5 log dose increments in both stages.
Procymidone	3, 10, 30, 100 mg/kg/d	New test substance with potency similar or slightly greater than vinclozolin. Therefore, the doses are identical to vinclozolin stage 1 dose range with 1 log dose increments.
Linuron	3, 10, 30, 100 mg/kg/d	New test substance with potency somewhat less than vinclozolin. Therefore, the doses are identical to vinclozolin stage 1 dose range with 0.5 log dose increments.
p,p'-DDE	In stage 1: 3, 10, 30, 100 mg/kg/d	In stage 1, this dose range matched the doses selected for vinclozolin with 0.5 log dose increments.
	In stage 2: 5, 16, 50, 160 mg/kg/d	Modest increase in dose range, based on stage 1 data, prediction that *p,p'*-DDE is weakest antagonist, and 0.5 log dose increments.
Finasteride	0.2, 1, 5, 25 mg/kg/d	The dose range was expanded, particularly lowering the first dose from 1 to 0.2 mg/kg/d due to data showing biological effects with finasteride at 0.4 mg/kg/d, from the Lead Laboratory recommendations based on published data and 0.5 log dose increments

43. After the participation of each laboratory was confirmed, the quantities of test substance that would be needed by each laboratory were calculated. Chemicals were shipped in compliance with regulatory and customs requirements of each nation where participating laboratories were located. Shipments were timed to arrive before the study animals in order to avoid wastage, e.g., expiration of the time window for using immature animals. Other details of the substance supply and handling included:

- The amounts of test chemical needed by each laboratory were calculated and weighed into individually coded, opaque vials. Individualised instructions were given to each laboratory, including the volume of test vehicle to be added to provide a test dose solution that could be administered to give the prescribed doses.
- The Lead Laboratory reviewed that the instructions for dissolving, preparing, and making up the solutions for suitability for the test substances. The model protocol for Phase-2 recommended the use of corn oil as most ligands for nuclear receptors such as the androgen receptor have hydrophobic

characteristics and corn oil is widely accepted and used by toxicologists for both s.c. and gavage administration.

- Participating laboratories were asked to ensure that personnel performing the necropsies would not know the identity or doses of the test substances administered to the animals.

Statistical Analyses of the Data

44. The Lead Laboratory calculated means, standard errors, and the coefficients of variation for each endpoint using PROC MEANS on SAS (version 6.08, SAS Institute, Cary, NC, USA). ANOVAs were done using PROC GLM for each laboratory and then the laboratories were pooled for each test substance. Data were then analysed by ANOVA on PROC GLM for each laboratory (with dose as a main effect). Data for each endpoint also were analysed as a two-way ANOVA, with dose and laboratory as main effects, so that the magnitude of the overall dose and laboratory effects, and their interaction, could be determined. The CV for each androgen-dependent organ weight was fairly constant as the means increased, the SD being proportional to the mean. This supported the log transformation of the data to provide for a more valid comparison of the effects of TP on organ weights at lower dosage levels.

45. These analyses also were conducted with body weight as a covariate. The use of body weight covariate adjusts the analysis for experimental variation from several sources, such as, large differences in the size of the rats from laboratory to laboratory, a large component of which appeared to arise from the use of different strains; and differences in the sizes of the rats on study within a laboratory.

46. In addition to means and CVs, the R^2 values for different effects were calculated. An R^2 for an effect was calculated by dividing the sums of squares from the ANOVA for an effect by the total sums of squares in the model. This provides an indication of the strength of the association for an effect with an endpoint. This calculation can be used to compare the robustness of the TP effect across endpoints, the variation from lab to lab, or to what degree the dose-responses vary among laboratories, as indicated by the R^2 for the lab by dose interaction.

47. The Secretariat also conducted a base statistical analysis of the data for the mandatory endpoints. The procedures of the Lead Laboratory are based upon a pairwise t-test comparison of the individual groups with the vehicle control. It was decided to also employ Dunnett's multiple comparison procedure, which is a modification of the t-test. The same estimate of pooled variance is used in both tests, but in Dunnett's test a different critical value is used that takes into account the special case of comparing each treatment group to a single particular control. The number of pairwise comparisons and the correlation of the pairwise comparisons is taken into account in order to control the overall error rate of all comparisons to this single control (correlation exists among the comparisons since each comparison includes the same control group). Since the overall error rate (chance of incorrectly declaring one or more comparisons statistically significant) is addressed in this manner, it is not necessary in the case of the Dunnett's test to have a significant overall ANCOVA F-test in order to control overall error (as is necessary when employing t-tests). Dunnett's procedure was developed specifically for pairwise comparisons of for quantitative and continuous data from multiple treatment groups to a single control, efficiently controls overall error, and dose not rely upon a monotonic dose response (46)(47)(48). In addition, the same Dunnett's approach with body weight as a covariable was used by NIEHS statisticians for the uterotrophic bioassay validation (48)(49). Therefore, it was judged that similar statistics ought to be available for both the Hershberger and the uterotrophic validation programmes.

48. Neither of the two approaches is incorrect. The primary difference in practice is that the ANCOVA F-test followed by a t-test comparison is slightly more liberal in achieving statistical significance. That is, there is an inherent tendency that pairwise comparisons in the t-test approach may achieve statistical significance in some marginal cases where Dunnett's will not. The results of both analyses are reported here

side-by-side in the tables for the mandatory endpoints.

49. For the Dunnett's test, the data were analysed using S-Plus (Insightful Corp., Seattle, WA, USA) both on untransformed data and after A variance-stabilising logarithmic transformation was carried out. In addition to statistical significance, the ratio of the geometric means of the various tissue weights (treated relative to the vehicle control for agonists and the 0.4 mg/kg/d TP reference group for antagonists) after adjusting for the body weight of the individual animals at necropsy along with respective upper and lower 95% confidence levels were also calculated. Outliers were observed (as defined as Studentised Residuals > 4 or < -4), but they were excluded from the statistical analyses in only one case where the residual was exceedingly large, i.e., < -7.

50. In the case of the antagonists, the vehicle control group, if performed by the participating laboratory, was excluded from the analyses (i.e., was not one of the analyzed groups in the Dunnett's approach). A primary assumption of both statistical approaches is that there is constant or nearly constant variance amongst the groups being compared. If the variance of the vehicle control group is much lower than that of the treated groups, the constant variance assumption of the models is violated and the results may be unreasonable.

51. In the case of positive controls (the inclusion of a 0.4 mg/kg/d TP group in an agonist assay or the inclusion of a flutamide co-treatment with 0.4 mg/kg/d TP in an antagonist assay), the positive control was included in the overall Dunnett's multiple comparison.

52. In the cases of the optional organs and tissues and when data were pooled across laboratories for a test substance, the Dunnett's analyses were not performed. Only the pairwise t-test analyses are then reported for those data.

RESULTS OF PHASE-2

53. The results from studies are presented below separately for androgens (agonists), androgen antagonists, and 5α-reductase inhibitors. The results are reported with a section for each individual test substance. For the three test substances (MT, VIN, DDE), there are separate subsection as the dose series differed for MT and the TP dosage was different for VIN and DDE. The results from the sets of studies are then compared in a following third subsection.

54. There was little animal mortality in these studies. There were a total of 241 groups with 1446 animals, and there were a total of 11 mortalities. Half of these mortalities (six) occurred in a single laboratory, where gavage errors by a new technician resulted in all mortalities. In other laboratories, another two animals were lost during test substance administration and a third due to infection at the anaesthetic injection site for the surgical castration. Only two mortalities among the laboratories were attributed to the test substances, one with linuron and one with *p,p'*-DDE.

PHASE-2: ANDROGEN AGONISTS

55. Two androgen agonists were employed as test substances in Phase-2. MT was employed in laboratory studies in eight laboratories with two overlapping dose series. TREN was employed in studies in three laboratories.

17α-Methyl Testosterone

56. Eight laboratories tested two dose series of methyl testosterone (MT) in order to assess the ability of the Hershberger bioassay to detect androgen agonists. There were four MT doses in both series. All laboratories conducted the assigned studies as intended, submitted their laboratory parameters and study data electronically using standardized Excel spreadsheets, audited the study data, and, if necessary, informed the Secretariat of data corrections.

Results of MT studies – dose series 1

57. The results of the individual laboratory studies and the summary results of the accessory organ and tissue weights and the statistical analyses for the MT studies conducted with a dose series of 0.5, 2, 10, and 40 mg/kg/d MT (dose series 1) are reported in Table 7. The Hershberger bioassay successfully and reproducibly detected MT in all four laboratories. The weights of all five male sex accessory tissues increased with the MT doses in a dose-responsive manner. All tissues achieved statistical significance with both statistical techniques in all laboratories at the top MT dose of 40 mg/kg/d.

58. Ventral Prostate (VP). There were statistically significant dose-dependent increases in the weights of the VP in all laboratories with MT. Laboratory 4 achieved statistical significance for the MT-treated VP at 2 mg/kg/d MT, laboratories 2 and 8 at 10 mg/kg/d MT, and laboratory 6 at 10 mg/kg/d MT with the pairwise comparison and at 40 mg/kg/d with the multiple comparison (Table 7). The absolute VP weights in laboratory 6 were less than half those of the other three laboratories. This difference cannot be attributed to modest differences in body weights and is likely due to differences in the dissection procedures and techniques for this tissue. The overall mean CV for the VP ranged from 25 in laboratory 8 to 60 in laboratory 6 (Table 8). This again suggests that the VP may have been somewhat difficult to dissect, and that laboratory technique was an important variable.

59. Seminal Vesicles and Coagulating Glands (SVCG). There were statistically significant dose-dependent increases in the weights of the SVCG in all laboratories with MT. Laboratory 4 achieved statistical significance for the MT-treated SVCG at 2 mg/kg/d MT with the pairwise comparison, laboratories 6 and 8 at 10 mg/kg/d MT, and laboratory 2 at 40 mg/kg/d MT (Table 7). The absolute SVCG weights were similar

among the laboratories. The overall mean CVs for the SVCG ranged from 23-38 (Table 8).

60. Levator ani and Bulbocavernosus muscle complex (LABC). There were statistically significant dose-dependent increases in the weights of the LABC in all laboratories with MT. Laboratories 4, 6 and 8 achieved statistical significance for the MT-treated LABC at 2 mg/kg/d MT and laboratory 2 at 10 mg/kg/d MT (Table 7). The LABC was the most sensitive or among the most sensitive tissues in 3 or the 4 laboratories. The absolute LABC weights differed among the laboratories about 2-fold in both the unstimulated and stimulated state, suggesting possible dissection differences as neither body weights or animal strain appeared related to these data. The overall mean CVs for the LABC were among the lowest of any tissue ranging from 8 to 16 (Table 8).

61. Glans Penis (GP). There were statistically significant dose-dependent increases in the weights of the GP in all laboratories with MT. Laboratory 4 achieved statistical significance for the MT-treated GP at 2 mg/kg/d MT, laboratories 2 and 8 at 10 mg/kg/d, and laboratory 6 at 40 mg/kg/d (Table 7). The absolute values of the GP were similar among laboratories at all doses with the exception of a low mean in laboratory 2 at 2 mg/kg/d. In this particular group, two animals did not achieve preputial separation and the weights for these and another individual appeared to be relatively low. The overall mean CVs for the GP ranged from 12 to 18 (Table 8).

62. Cowper's Glands (COWS). There were statistically significant dose-dependent increases in the weights of the COWS in all laboratories with MT. Laboratories 4, 6 and 8 achieved statistical significance for the MT-treated COWS at 2 mg/kg/d MT and laboratory 2 at 40 mg/kg/d (Table 7). The absolute values of the COWS should be noted, as the weights were similar for laboratories 2, 4 and 8, but the weights in laboratory 6 were only about one-sixth of the others. This suggests a difference among the laboratories in the dissection and tissue handling techniques, as differences in body weights and rat strain could not account for the dissimilar weights. The overall mean CVs for the small, fluid filled COWS ranged from 15 to 26 (Table 8).

63. Overall Review of Mandatory Endpoints. When the data were pooled across the participating laboratories, all five mandatory endpoints achieved statistical significance using the pairwise comparison approach. The VP, LABC, and COWS achieved significance at 2 mg MT/kg/d and the SVCG and GP at 10 mg MT/kg/d (Table 7). The R-square analyses indicate strong overall and treatment relationships, and some relationships for laboratory effects for the VP [29], LABC [33], and COWS [34] (Table 7).

64. Body weights. The initial body weights ranged from approximately 235 to 255 grams among the four laboratories. The body weight gains during the treatment period were approximately 60 to 70 grams. There was no discernable change in body weight gain with increasing doses of MT based on the starting and terminal body weights, and there were no statistically significant changes in any laboratory (Table 7).

65. Optional organ weights. The absolute optional organ weights were all modestly changed by MT administration (Table 9). Liver weights were at least one gram larger at the high MT dose than with the vehicle. This increase in liver weights achieved statistical significance in laboratories 2, 4, and 8 at 40 mg/kg/d MT. The paired adrenal weights were approximately ten milligrams lower at the high MT dose in three of the four laboratories. This decrease achieved statistical significance in laboratories 2, 4, and 6 at 40 mg/kg/d MT. The paired kidney weights were 100 to 300 milligrams larger at the high MT dose. This increase in paired kidney weights achieved statistical significance in labs 4 and 8 at 10 mg/kg/d MT, and in laboratory 6 at 40 mg/kg/d.

Table 7. Body weights, mandatory tissue weights and pooled statistics in methyltestosterone (MT) studies – dose series 1

Lab	Methyl Testosterone (mg/kg/d)	0	0.5	2	10	40
2	Starting Body Wt (g) [a]	240.8 ± 12.42	242.5 ± 10.05	239.5 ± 13.17	238.8 ± 16.18	243.7 ± 9.44
	Terminal Body Wt (g) [a]	300.9 ± 22.51	306.9 ± 14.57	296.8 ± 13.38	303.6 ± 21.42	309.3 ± 11.41
	Ventral prostate (mg)	15.6 ± 5.53	17.5 ± 7.89	21.6 ± 11.34	50.3 ± 25.30 **,^	110.2 ± 34.88 **,^
	Seminal vesicles (mg)	53.4 ± 29.34	55.3 ± 15.86	45.0 ± 17.44	78.3 ± 38.61	142.0 ± 26.74 **,^
	LABC muscles (mg)	123.7 ± 26.73	112.6 ± 13.64	119.2 ± 22.44	159.2 ± 17.09 **,^	262.7 ± 23.12 **,^
	Glans penis (mg)	52.3 ± 6.92	55.2 ± 7.01	44.9 ± 12.72	68.1 ± 16.28 *	79.2 ± 11.35 **,^
	Cowper's glands (mg)	5.9 ± 1.70	4.2 ± 1.05	6.4 ± 2.02	9.9 ± 4.78	19.0 ± 3.62 **,^
4	Starting Body Wt (g)	253.8 ± 12.91	255.3 ± 13.72	261.5 ± 13.56	256.7 ± 12.72	258.0 ± 15.62
	Terminal Body Wt (g)	321.7 ± 16.31	331.6 ± 19.72	334.0 ± 17.69	322.9 ± 15.57	323.3 ± 20.70
	Ventral prostate (mg)	24.8 ± 10.81	26.8 ± 5.97	50.5 ± 28.93 **,^	75.5 ± 9.33 **,^	162.5 ± 58.11 **,^
	Seminal vesicles (mg)	54.0 ± 10.63	64.0 ± 15.04	77.5 ± 15.66 **	110.7 ± 27.48 **,^	246.5 ± 68.36 **,^
	LABC muscles (mg)	213.5 ± 33.78	214.1 ± 26.18	306.5 ± 71.62 **,^	326.6 ± 73.14 **,^	496.1 ± 80.63 **,^
	Glans penis (mg)	50.4 ± 6.71	56.9 ± 8.18	65.1 ± 9.82 **,^	66.6 ± 11.27 **,^	78.9 ± 11.00 **,^
	Cowper's glands (mg)	6.1 ± 1.85	7.2 ± 1.04	10.4 ± 3.58 **,^	13.2 ± 2.89 **,^	23.3 ± 5.97 **,^
6	Starting Body Wt (g)	234.1 ± 4.95	236.9 ± 10.73	234.7 ± 10.15	234.2 ± 14.50	234.3 ± 9.98
	Terminal Body Wt (g)	291.2 ± 7.49	302.0 ± 11.25	298.0 ± 16.33	293.5 ± 23.06	290.7 ± 14.88
	Ventral prostate (mg)	4.9 ± 3.24	4.2 ± 1.55	8.1 ± 4.74	21.1 ± 21.53 *	70.0 ± 26.19 **,^
	Seminal vesicles (mg)	42.1 ± 21.42	43.4 ± 13.94	46.0 ± 13.61	83.9 ± 47.44 *	190.9 ± 39.83 **,^
	LABC muscles (mg)	145.2 ± 37.51	166.2 ± 37.85	188.1 ± 13.02 *	254.5 ± 73.36 **,^	445.9 ± 39.00 **,^
	Glans penis (mg)	60.4 ± 10.43	56.8 ± 12.74	67.2 ± 11.03	73.5 ± 20.31	88.4 ± 15.73 **,^
	Cowper's glands (mg)	0.7 ± 0.29	1.9 ± 1.78	2.3 ± 0.83 **,^	6.9 ± 5.78 **,^	17.4 ± 4.57 **,^
8	Starting Body Wt (g)	250.7 ± 5.49	251.0 ± 7.61	250.3 ± 6.06	249.3 ± 6.89	250.7 ± 9.68
	Terminal Body Wt (g)	319.6 ± 10.64	320.1 ± 15.70	313.5 ± 15.06	324.0 ± 12.37	321.4 ± 12.37
	Ventral prostate (mg)	18.8 ± 6.74	21.1 ± 2.32	24.8 ± 6.87	62.5 ± 14.13 **,^	151.1 ± 38.29 **,^
	Seminal vesicles (mg)	58.0 ± 20.02	62.2 ± 9.32	63.5 ± 14.09	117.0 ± 25.79 **,^	304.9 ± 111.25 **,^
	LABC muscles (mg)	187.7 ± 29.96	214.5 ± 34.69	229.5 ± 38.53 *,^	348.6 ± 57.91 **,^	523.8 ± 40.27 **,^
	Glans penis (mg)	50.5 ± 9.11	56.3 ± 12.43	52.7 ± 12.18	67.8 ± 12.45 *,^	90.3 ± 11.06 **,^
	Cowper's glands (mg)	6.8 ± 1.66	7.8 ± 2.48	11.8 ± 2.62 **,^	16.9 ± 1.91 **,^	29.9 ± 4.58 **,^

	R-square (%)			Overall means and [CV] for tissues at a given dose				
	OVR	TRT	LAB					
Ventral prostate (mg)	77	50	29	16 [62]	14 [58]	26 [83] *	52 [57] **	123 [43] **
Seminal vesicles (mg)	75	67	8.6	52 [40]	56 [28]	58 [34]	98 [39] **	221 [41] **
LABC (mg)	82	53	33	168 [28]	178 [29]	211 [38] **	272 [35] **	432 [26] **
Glans penis (mg)	52	43	4.2	53 [17]	56 [17]	58 [25]	69 [21] **	84 [15] **
Cowper's glands (mg)	77	41	34	5 [59]	5 [55]	8 [57] **	12 [46] **	22 [30] **

OVR - Overall effect; TRT - Effect of treatments; LAB - Effect of laboratory; CV - Coefficient of variation.

*, ** Significantly different from control at P<0.05 and P<0.01, respectively, using T-test pairwise comparisons.

^ Significantly different from control at P<0.05 using two-tailed Dunnett's multiple comparisons of the tissue with body weight adjustment.

[a] The starting body weight is on the first day of substance administration and the terminal body weight is at necropsy approximately 24-hours after the last administration.

Table 8. Coefficients of variation for the mandatory endpoints in the methyltestosterone (MT) studies – dose series 1.

Lab	Methyl Testosterone (mg/kg/d)	0	0.5	2	10	40	MEAN[a]
2	Terminal Body Wt (g)	7.48	4.75	4.51	7.05	3.69	5.50
	Ventral prostate (mg)	35.59	45.24	52.54	50.33	31.67	43.07
	Seminal vesicles (mg)	54.95	28.67	38.75	49.33	18.82	38.11
	LABC muscles (mg)	21.61	12.12	18.84	10.74	8.80	14.42
	Glans penis (mg)	13.25	12.70	28.30	23.90	14.34	18.50
	Cowper's glands (mg)	28.88	24.89	31.44	48.22	19.00	30.49
4	Terminal Body Wt (g)	5.07	5.95	5.30	4.82	6.40	5.51
	Ventral prostate (mg)	43.66	22.23	57.31	12.37	35.75	34.26
	Seminal vesicles (mg)	19.68	23.52	20.21	24.84	27.73	23.19
	LABC muscles (mg)	15.83	12.23	23.37	22.39	16.25	18.02
	Glans penis (mg)	13.32	14.37	15.08	16.93	13.93	14.73
	Cowper's glands (mg)	30.60	14.55	34.41	21.93	25.70	25.44
6	Terminal Body Wt (g)	2.57	3.73	5.48	7.86	5.12	4.95
	Ventral prostate (mg)	65.85	37.23	58.80	102.06	37.44	60.28
	Seminal vesicles (mg)	50.90	32.08	29.58	56.54	20.86	37.99
	LABC muscles (mg)	25.83	22.78	6.92	28.83	8.75	18.62
	Glans penis (mg)	17.28	22.42	16.41	27.62	17.79	20.31
	Cowper's glands (mg)	44.16	91.81	36.37	83.70	26.24	56.46
8	Terminal Body Wt (g)	3.33	4.90	4.80	3.82	3.85	4.14
	Ventral prostate (mg)	35.86	10.98	27.76	22.61	25.35	24.51
	Seminal vesicles (mg)	34.54	14.97	22.18	22.05	36.49	26.05
	LABC muscles (mg)	15.97	16.17	16.79	16.61	7.69	14.65
	Glans penis (mg)	18.05	22.05	23.12	18.36	12.25	18.77
	Cowper's glands (mg)	24.60	31.94	22.26	11.28	15.32	21.08

[a] The overall mean CV, depending upon the laboratory, may also include a testosterone propionate positive control.

Table 9. Optional organ weights in the methyltestosterone (MT) studies – dose series 1.

Lab	Methyl Testosterone (mg/kg/d)	0	0.5	2	10	40
2	Terminal Body Wt (g)	300.9 ± 22.51	306.9 ± 14.57	296.8 ± 13.38	303.6 ± 21.42	309.3 ± 11.41
	Liver (g)	11.6 ± 0.93	11.9 ± 1.09	11.7 ± 0.95	11.8 ± 0.66	13.1 ± 1.04 **
	(relative to bw)	3.86%	3.88%	3.94%	3.89%	4.24%
	Adrenals (mg)	65.9 ± 11.98	61.3 ± 9.85	51.4 ± 7.49 **	62.1 ± 7.68	53.5 ± 7.18 **
	(relative to bw)	0.0219%	0.0200%	0.0173%	0.0205%	0.0173%
	Kidneys (mg)	2062.9 ± 214.41	2127.2 ± 176.65	2071.1 ± 178.22	2126.6 ± 80.26	2194.1 ± 176.12
	(relative to bw)	0.6856%	0.6931%	0.6978%	0.7005%	0.7094%
4	Terminal Body Wt (g)	321.7 ± 16.31	331.6 ± 19.72	334.0 ± 17.69	322.9 ± 15.57	323.3 ± 20.70
	Liver (g)	13.7 ± 0.85	14.4 ± 1.35	15.1 ± 1.14	14.3 ± 0.85	14.9 ± 0.67 *
	(relative to bw)	4.26%	4.34%	4.52%	4.43%	4.61%
	Adrenals (mg)	56.8 ± 5.91	56.2 ± 7.51	58.8 ± 6.03	51.7 ± 6.83	46.7 ± 6.60 *
	(relative to bw)	0.0177%	0.0169%	0.0176%	0.0160%	0.0144%
	Kidneys (mg)	2100.8 ± 76.57	2213.7 ± 265.21	2319.4 ± 213.40	2318.3 ± 168.06 *	2385.4 ± 178.43 **
	(relative to bw)	0.6530%	0.6676%	0.6944%	0.7180%	0.7378%
6	Terminal Body Wt (g)	291.2 ± 7.49	302.0 ± 11.25	298.0 ± 16.33	293.5 ± 23.06	290.7 ± 14.88
	Liver (g)	13.5 ± 1.06	14.7 ± 1.77	14.7 ± 2.20	14.5 ± 1.82	14.5 ± 1.40
	(relative to bw)	4.64%	4.87%	4.93%	4.94%	4.99%
	Adrenals (mg)	55.6 ± 2.39	54.0 ± 8.89	59.9 ± 12.61	53.8 ± 2.91	46.3 ± 4.81 *
	(relative to bw)	0.0191%	0.0179%	0.0201%	0.0183%	0.0159%
	Kidneys (mg)	2326.1 ± 207.46	2434.4 ± 169.78	2461.5 ± 312.41	2542.2 ± 231.14	2566.5 ± 291.1 *
	(relative to bw)	0.7988%	0.8061%	0.8260%	0.8662%	0.8829%
8	Terminal Body Wt (g)	319.6 ± 10.64	320.1 ± 15.70	313.5 ± 15.06	324.0 ± 12.37	321.4 ± 12.37
	Liver (g)	12.9 ± 1.05	13.2 ± 1.10	12.6 ± 1.00	13.7 ± 0.39	14.1 ± 1.10 *
	(relative to bw)	4.04%	4.12%	4.02%	4.23%	4.39%
	Adrenals (mg)	50.6 ± 4.34	51.5 ± 5.14	49.5 ± 9.93	53.0 ± 11.81	47.2 ± 6.67
	(relative to bw)	0.0158%	0.0161%	0.0158%	0.0164%	0.0147%
	Kidneys (mg)	2081.6 ± 134.82	2189.8 ± 182.60	2103.2 ± 138.34	2404.7 ± 203.79 **	2380.9 ± 103.28 **
	(relative to bw)	0.6513%	0.6841%	0.6709%	0.7422%	0.7408%

*, ** Significantly different from control at P<0.05 and P<0.01, respectively, using T-test pairwise comparisons.

Results of Methyl Testosterone studies – dose series 2

66. The results of the individual laboratory studies and the summary results of the accessory organ and tissue weights and the statistical analyses for the MT studies with dose series 2 are reported in Table 10. The doses were 0.05, 0.5, 5, and 50 mg/kg/d MT. First, this Table shows that the Hershberger bioassay successfully and reproducibly detected MT in all four laboratories. The weights of all five male sex accessory tissues increased with increasing MT doses in a dose-responsive manner. All tissues achieved statistical significance in all laboratories at the top MT dose of 40 mg/kg/d.

67. Ventral Prostate (VP). There were statistically significant dose-dependent increases in the weights of the VP in all laboratories with MT. Laboratory 11 achieved statistical significance for the MT-treated VP at 0.5 mg/kg/d MT, laboratories 13 and 15 at 5 mg/kg/d MT, and laboratory 16 at 50 mg/kg/d MT (Table 10). The overall mean CVs of the VP ranged from 10 to 21 (Table 11).

68. Seminal Vesicles and Coagulating Glands (SVCG). There were statistically significant dose-dependent increases in the weights of the SVCG in all laboratories with MT. Laboratories 13 and 15 achieved statistical significance for the MT-treated SVCG at 5 mg/kg/d MT and laboratories 11 and 16 at 50 mg/kg/d MT (Table 10). The overall mean CVs for the SVCG ranged from 8 to 18 (Table 11).

69. Levator ani and Bulbocavernosus muscle complex (LABC). There were statistically significant dose-dependent increases in the weights of the LABC in all laboratories with MT. All laboratories achieved statistical significance for the MT-treated LABC at 5 mg/kg/d MT (Table 10). The overall mean CVs for the LABC ranged from 9 to 12 (Table 11).

70. Glans Penis (GP). There were statistically significant dose-de pendent increases in the weights of the GP in all laboratories with MT. All laboratories achieved statistical significance for the MT-treated GP at 5 mg/kg/d MT based on the pairwise comparison approach (Table 10). The overall mean CVs for the GP ranged from 12 to 18 (Table 11).

71. Cowper's Glands (COWS). There were statistically significant dose-dependent increases in the weights of the COWS in all laboratories with MT. Laboratories 11, 13 and 15 achieved statistical significance for the MT-treated COWS at 5 mg/kg/d MT and laboratory 16 at 50 mg/kg/d (Table 10). The overall mean CVs for the small, fluid filled COWS ranged from 10 to 26 (Table 11).

72. Overall Review of Mandatory Endpoints. When the data were pooled across the participating laboratories, all five mandatory endpoints achieved statistical significance using the pairwise comparison approach at 5 mg MT/kg/d (Table 10). The R-square analyses indicate strong overall and treatment relationships, and one slight relationship for possible laboratory effects for the GP [17] (Table 7).

73. Body and organs weights. The initial body weights ranged from approximately 220 to 260 grams among the four laboratories. The body weight gains during the treatment period were approximately 60 to 70 grams. There was a slight absolute, but not statistically significant, increase in absolute body weights with increasing doses of MT based on the starting and terminal body weights (Table 10). Liver and adrenal weights were measured in two laboratories. There were significant increases in the liver weights and decreases in the adrenal weights at 50 mg MT/kg/d.

Comparison of Methyl Testosterone results from the different dose series

74. The MT results with dose series 2 were produced approximately one year earlier than the results with dose series 1. As with other test substance studies in this report, there were modest differences in body weights among the animals in these studies and some apparent absolute weight differences in the tissues that

Table 10. Body weights, mandatory tissue weights and pooled statistics in methyltestosterone (MT) studies – dose series 2

Lab	Methyl Testosterone (mg/kg/d)	0	0.05	0.5	5	50
11	Starting Body Wt (g) [a]	239.0 ± 10.17	238.0 ± 9.96	239.2 ± 6.99	239.4 ± 7.87	237.2 ± 8.83
	Terminal Body Wt (g) [a]	297.6 ± 19.06	291.2 ± 11.74	292.6 ± 10.85	294.6 ± 12.68	299.1 ± 15.28
	Ventral prostate (mg)	12.6 ± 5.57	14.4 ± 5.17	21.4 ± 7.78 ^	45.3 ± 8.79 **,^	128.3 ± 35.38 **,^
	Seminal vesicles (mg)	52.5 ± 12.48	50.2 ± 7.23	48.1 ± 12.96	70.7 ± 21.49	278.5 ± 65.88 **,^
	LABC muscles (mg)	236.3 ± 39.04	218.6 ± 38.49	228.7 ± 19.82	287.3 ± 19.17 **,^	533.8 ± 63.23 **,^
	Glans penis (mg)	48.9 ± 5.69	50.8 ± 3.14	49.9 ± 3.95	55.0 ± 4.48 **	73.3 ± 5.34 **,^
	Cowper's glands (mg)	6.5 ± 2.50	6.8 ± 1.47	7.6 ± 1.80	11.0 ± 2.38 ^	27.2 ± 11.48 **,^
13	Starting Body Wt (g)	260.0 ± 9.66	259.8 ± 8.37	259.3 ± 8.23	259.8 ± 8.77	258.9 ± 8.86
	Terminal Body Wt (g)	309.2 ± 10.91	318.5 ± 16.53	313.4 ± 11.53	315.6 ± 17.62	317.0 ± 10.07
	Ventral prostate (mg)	21.1 ± 4.89	20.4 ± 3.16	18.7 ± 3.24	40.6 ± 14.58 **,^	135.0 ± 18.86 **,^
	Seminal vesicles (mg)	45.2 ± 6.05	43.7 ± 5.12	43.3 ± 4.91	65.7 ± 17.85 ^	248.2 ± 61.78 **,^
	LABC muscles (mg)	192.3 ± 28.59	198.0 ± 19.90	198.1 ± 15.71	253.2 ± 36.02 **,^	460.5 ± 52.65 **,^
	Glans penis (mg)	51.4 ± 2.83	55.6 ± 4.46	53.4 ± 4.83	64.8 ± 5.61 **,^	83.2 ± 6.60 **,^
	Cowper's glands (mg)	6.5 ± 1.54	7.8 ± 1.74	7.6 ± 1.8	10.8 ± 3.58 **,^	25.3 ± 1.12 **,^
15	Starting Body Wt (g)	222.1 ± 7.40	222.5 ± 9.01	225.5 ± 10.45	224.1 ± 8.77	221.1 ± 8.28
	Terminal Body Wt (g)	282.7 ± 17.81	288.6 ± 17.05	293.1 ± 15.21	290.4 ± 13.60	288.7 ± 11.71
	Ventral prostate (mg)	19.3 ± 1.89	22.1 ± 6.81	26.2 ± 4.16	51.1 ± 13.17 **,^	158.2 ± 35.19 **,^
	Seminal vesicles (mg)	44.2 ± 3.75	52.9 ± 23.80	66.2 ± 7.48	108.0 ± 23.28 **,^	312.6 ± 108.93 **,^
	LABC muscles (mg)	201.5 ± 23.25	203.6 ± 27.74	212.6 ± 30.14	254.2 ± 31.82 **,^	482.0 ± 78.02 **,^
	Glans penis (mg)	62.6 ± 4.47	60.8 ± 3.67	61.9 ± 4.66	74.4 ± 10.26 **,^	94.7 ± 11.58 **,^
	Cowper's glands (mg)	8.1 ± 1.39	7.5 ± 2.21	9.5 ± 1.8	11.8 ± 3.10 **,^	22.1 ± 2.72 **,^
16	Starting Body Wt (g)	227.4 ± 15.84	227.4 ± 10.13	227.8 ± 12.14	228.5 ± 15.05	227.3 ± 10.26
	Terminal Body Wt (g)	287.3 ± 16.12	286.0 ± 17.42	281.4 ± 16.92	293.4 ± 19.55	294.2 ± 16.46
	Ventral prostate (mg) [b]	19.2 ± 3.73	20.8 ± 5.08	19.5 ± 3.78	32.3 ± 10.44	150.9 ± 38.65 **,^
	Seminal vesicles (mg)	39.7 ± 4.82	37.3 ± 5.38	34.8 ± 5.67	41.2 ± 6.49	184.4 ± 15.57 **,^
	LABC muscles (mg)	206.3 ± 20.14	199.8 ± 32.16	203.6 ± 28.66	243.8 ± 27.83 **	487.1 ± 26.36 **,^
	Glans penis (mg)	52.8 ± 2.53	53.1 ± 2.90	53.7 ± 1.52	56.8 ± 2.60 *	83.5 ± 3.27 **,^
	Cowper's glands (mg) [b]	6.5 ± 0.65	7.4 ± 0.83	5.8 ± 0.9	7.6 ± 1.58	26.3 ± 5.16 **,^

	R-square value (%)			Overall means and [CV] for tissues at a given dose				
	OVR	TRT	LAB					
Ventral prostate (mg)	92	89	0.6	18 [29]	19 [29]	22 [26]	42 [31] **	143 [23] **
Seminal vesicles (mg)	90	81	3.9	45 [19]	46 [30]	48 [29]	71 [42] **	256 [32] **
LABC (mg)	92	90	7.8	209 [15]	205 [14]	211 [12]	260 [12] **	491 [12] **
Glans penis (mg)	86	68	16.9	54 [13]	55 [9]	55 [11]	63 [16] **	84 [12] **
Cowper's glands (mg)	91	81	0.3	7 [25]	7 [21]	8 [27]	10 [30] **	25 [25] **

OVR - Overall effect; TRT - Effect of treatments; LAB - Effect of laboratory; CV - Coefficient of variation.

*, ** Significantly different from control at P<0.05 and P<0.01, respectively, using T-test pairwise comparisons.

^ Significantly different from control at P<0.05 using two-tailed Dunnett's multiple comparisons of the tissue with body weight adjustment.

[a] The starting body weight is on the first day of substance administration and the terminal body weight is at necropsy approximately 24-hours after the last administration.

[b] The VP and COWS were fixed and then weighed in laboratory 16

Table 11. Coefficients of variation for the mandatory endpoints in the methyltestosterone (MT) studies – dose series 2.

Lab	Methyl Testosterone (mg/kg/d)	0	0.05	0.5	5	50	MEAN[a]
11	Terminal Body Wt (g)	6.40	4.03	3.71	4.30	5.11	4.09
	Ventral prostate (mg)	44.35	35.79	36.33	19.42	27.58	17.83
	Seminal vesicles (mg)	23.77	14.39	26.94	30.38	23.66	17.07
	LABC muscles (mg)	16.52	17.61	8.67	6.67	11.85	9.21
	Glans penis (mg)	11.65	6.18	7.92	8.15	7.29	3.61
	Cowper's glands (mg)	38.30	21.55	23.74	21.58	42.28	25.66
13	Terminal Body Wt (g)	3.53	5.19	3.68	5.58	3.18	4.23
	Ventral prostate (mg)	23.24	15.47	17.33	35.95	13.97	21.19
	Seminal vesicles (mg)	13.39	11.73	11.35	27.18	24.89	17.71
	LABC muscles (mg)	14.87	10.05	7.93	14.22	11.43	11.70
	Glans penis (mg)	5.52	8.02	9.05	8.67	7.93	7.84
	Cowper's glands (mg)	23.83	22.21	23.96	33.17	4.45	21.52
15	Terminal Body Wt (g)	6.30	5.91	5.19	4.68	4.06	6.30
	Ventral prostate (mg)	9.80	30.84	15.90	25.79	22.24	9.80
	Seminal vesicles (mg)	8.49	44.99	11.29	21.56	34.84	8.49
	LABC muscles (mg)	11.54	13.63	14.18	12.51	16.19	11.54
	Glans penis (mg)	7.14	6.04	7.52	13.79	12.22	7.14
	Cowper's glands (mg)	17.26	29.51	18.89	26.24	12.33	17.26
16	Terminal Body Wt (g)	5.61	6.09	6.01	6.66	5.60	5.61
	Ventral prostate (mg)[b]	9.97	11.25	16.36	20.90	19.60	9.97
	Seminal vesicles (mg)	12.15	14.42	16.29	15.74	8.44	12.15
	LABC muscles (mg)	9.77	16.09	14.08	11.41	5.41	9.77
	Glans penis (mg)	4.79	5.46	2.84	4.58	3.92	4.79
	Cowper's glands (mg)[b]	9.97	11.25	16.36	20.90	19.60	9.97

[a] The overall mean CV, depending upon the laboratory, may also include a testosterone propionate positive control.

[b] The VP and COWS were fixed and then weighed in laboratory 16

may be attributed to variations in dissection and handling among the laboratories. In addition, there were strain, diet and other differences among laboratories (see Table 4). The similarity of the results supports the reproducibility of the Hershberger bioassay.

75. To illustrate the consistency of the dose responses, relative increases in tissue weights from MT administration have been analyzed. Figures 3A-E show the relative increase for all tissues from 8 individual laboratories with MT in dose (log x-axis)- response (normal y-axis) plots. The results for the mean relative increase show the excellent reproducibility of the overall dose response both in the dose response and the strength of the response. The lead laboratory has plotted overall mean weights for the mandatory tissues from the studies with the two dose series for the VP, SVCG, LABC, and COWS in Figure 27 of Annex 3, further illustrating the MT dose response reproducibility across laboratories.

76. The primary anomalous findings were the COWS and VP in laboratory 6 (see Figures 3E and 3A, respectively). The absolute mean control COWS weight was 0.7 mg compared to a range of 5.9-8.1 mg in other laboratories. However, the absolute mean weight of this tissue at the high MT dose in laboratory 6 is similar to the other laboratories (see Tables 7 and 10). This leads to a very high relative increase for this tissue in laboratory 6. A similar, but less extreme, case is the VP data from this laboratory. This suggests possible difficulty in dissecting the small, unstimulated tissues compared to other laboratories.

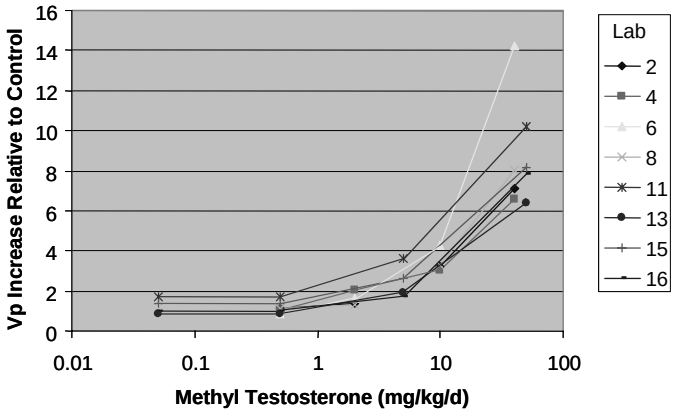


Figure 3A. Relative increase in ventral prostate (VP) mean weights with methyl testosterone (MT) administration in eight laboratories using two dose series (Laboratories 2-8 used 0.5, 2, 10, and 40 mg/kg/d MT and laboratories 11-16 used 0.05, 0.5, 5 and 50 mg/kg/d MT).

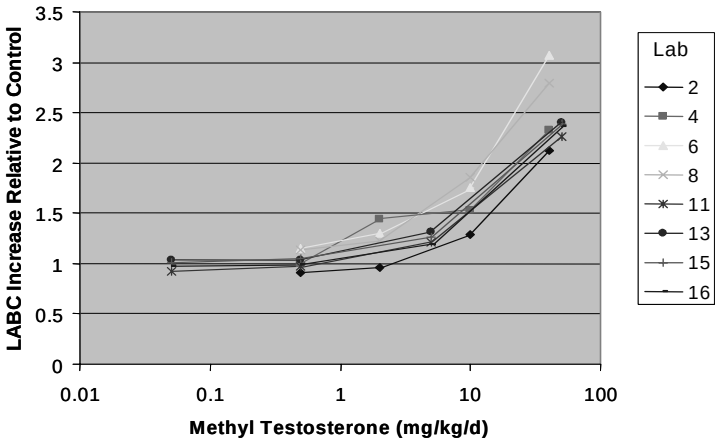


Figure 3B. Relative increase in levator ani and bulbocavernosus (LABC) mean weights with methyl testosterone (MT) administration in eight laboratories using two dose series (Laboratories 2-8 used 0.5, 2, 10, and 40 mg/kg/d MT and laboratories 11-16 used 0.05, 0.5, 5 and 50 mg/kg/d MT).

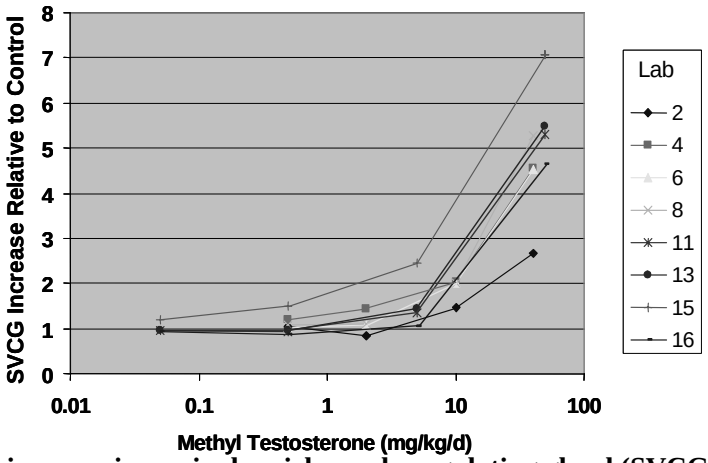


Figure 3C. Relative increase in seminal vesicles and coagulating gland (SVCG) mean weights with methyl testosterone (MT) administration in eight laboratories using two dose series (Laboratories 2-8 used 0.5, 2, 10, and 40 mg/kg/d MT and laboratories 11-16 used 0.05, 0.5, 5 and 50 mg/kg/d MT).

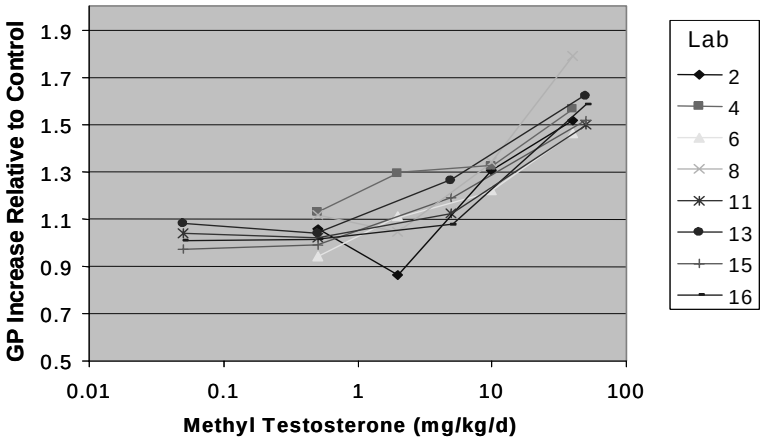


Figure 3D. Relative increase in glans penis (GP) mean weights with methyl testosterone (MT) administration in eight laboratories using two dose series (Laboratories 2-8 used 0.5, 2, 10, and 40 mg/kg/d MT and laboratories 11-16 used 0.05, 0.5, 5 and 50 mg/kg/d MT).

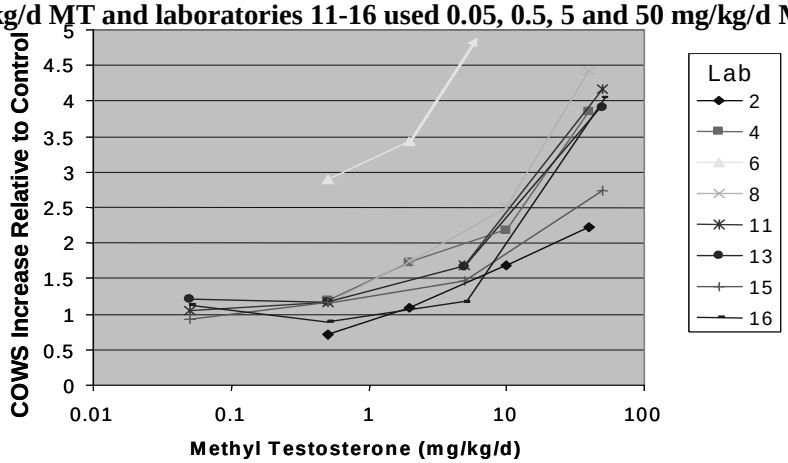


Figure 3E. Relative increase in Cowper's gland (COWS) mean weights with methyl testosterone (MT) administration in eight laboratories using two dose series (Laboratories 2-8 used 0.5, 2, 10, and 40 mg/kg/d MT and laboratories 11-16 used 0.05, 0.5, 5 and 50 mg/kg/d MT). Note that the laboratory 6 COWS values for the last two doses are off the graph scale.

Trenbolone

77. Three laboratories tested four doses of trenbolone (TREN) in order to assess the ability of the Hershberger bioassay to detect other androgen agonists. All three laboratories conducted the assigned studies as intended, submitted their laboratory and study data electronically using standardized Excel spreadsheets, audited the study data, and, if necessary, informed the Secretariat of data corrections. Due to shipment restrictions by the supplier (i.e., TREN could not be reshipped once received), TREN could not be distributed from the central TNO repository. Therefore, arrangements were made with the supplier (Sigma) to ensure that each laboratory could place an order that would be filled from the same lot of TREN.

Results of Trenbolone studies

78. The results of the individual laboratory studies and the summary results of the accessory organ and tissue weights and the statistical analyses for the TREN studies are reported in Table 12. The results indicate that the Hershberger bioassay successfully and reproducibly detected TREN in all laboratories. The absolute weights of all five male sex accessory tissues increased with increasing TREN doses in a dose-responsive manner. All five tissues achieved statistical significance with the pairwise comparison approach, but the SVCG, GP, and COWS failed to achieve significance on one or more occasions with the multiple comparison approach.

79. Ventral Prostate (VP). There were statistically significant dose-dependent increases in the weights of the VP in all laboratories with TREN. Laboratory 7 achieved statistical significance for the TREN-treated VP at 8 mg/kg/d TREN and laboratories 1 and 3 at 40 mg/kg/d (Table 12). The absolute values of the VP was smaller in laboratory 1 compared to the other two laboratories. The overall mean CVs for the VP ranged from 29 to 51 (Table 13).

80. Seminal Vesicles and Coagulating Glands (SVCG). There were dose-dependent increases in the absolute weights of the SVCG in all laboratories with TREN. All laboratories achieved statistical significance at a dose of 40 mg/kg/d TREN using the pairwise comparison, but only laboratory 7 using the multiple comparison (Table 12). The overall mean CVs for the SVCG ranged from 31 to 37, suggesting some contribution to the inability to achieve statistical significance (Table 13).

81. Levator ani and Bulbocavernosus muscle complex (LABC). There were statistically significant dose-dependent increases in the weights of the LABC in all laboratories with TREN. Laboratory 7 achieved statistical significance for the TREN-treated LABC at 8 mg/kg/d TREN and laboratories 1 and 3 at 40 mg/kg/d (Table 12). The overall mean CVs for the LABC ranged from 17 to 19 (Table 13).

82. Glans Penis (GP). There were dose-dependent increases in the absolute weights of the GP in all laboratories with TREN. Laboratory 7 achieved statistical significance at 8 mg/kg/d TREN and laboratories 1 and 3 at 40 mg/kg/d (Table 12). The overall mean CVs for the GP ranged from 9 to 12 (Table 13).

83. Cowper's Glands (COWS). There were dose-dependent increases in the absolute weights of the COWS in all laboratories with TREN. Laboratories 3 and 7 achieved statistical significance with both statistical approaches at a dose of 40 mg/kg/d TREN, and laboratory 1 achieved statistical significance at $p < 0.05$ using the pairwise comparison approach at the same dose (Table 12). The overall mean CVs for the COWS ranged from 22 to 44 (Table 13).

Table 12. Body weights, mandatory tissue weights and pooled statistics in trenbolone (TREN) studies.

Lab	Trenbolone (mg/kg/d)	0	0.3	1.5	8	40
1	Starting Body Wt (g) [a]	192.7 ± 9.37	194.8 ± 7.20	196.3 ± 4.86	194.1 ± 7.90	192.2 ± 8.19
	Terminal Body Wt (g) [a]	207.4 ± 12.71	211.2 ± 14.91	209.4 ± 8.65	198.6 ± 9.22	189.7 ± 10.42*
	Ventral prostate (mg)	15.7 ± 3.14	19.7 ± 5.02	18.8 ± 3.63	19.8 ± 4.27	37.6 ± 11.36 **,^
	Seminal vesicles (mg)	23.2 ± 4.52	24.1 ± 11.50	26.1 ± 8.58	28.1 ± 11.11	58.0 ± 27.23 *
	LABC muscles (mg)	146.4 ± 18.30	137.2 ± 20.79	147.0 ± 26.23	165.1 ± 40.90	262.9 ± 41.55 **,^
	Glans penis (mg)	42.9 ± 3.23	41.9 ± 3.93	44.7 ± 5.32	43.0 ± 6.90	52.2 ± 4.30 **,^
	Cowper's glands (mg)	7.2 ± 2.61	5.8 ± 1.07	6.2 ± 0.99	6.6 ± 1.77	11.4 ± 3.75 *,^
3	Starting Body Wt (g)	201.1 ± 14.62	203.2 ± 11.40	205.9 ± 11.50	210.1 ± 9.24	207.4 ± 15.89
	Terminal Body Wt (g)	238.8 ± 17.47	243.4 ± 10.87	247.6 ± 19.25	236.9 ± 12.20	224.3 ± 23.93
	Ventral prostate (mg)	26.3 ± 13.38	29.9 ± 15.89	32.5 ± 15.29	29.4 ± 16.22	48.3 ± 22.55 *,^
	Seminal vesicles (mg)	63.0 ± 18.53	57.6 ± 9.28	76.6 ± 16.06	67.8 ± 30.94	155.5 ± 71.56 **
	LABC muscles (mg)	195.6 ± 40.07	185.6 ± 36.53	218.7 ± 30.62	223.5 ± 36.83	395.2 ± 50.84 **,^
	Glans penis (mg)	48.1 ± 3.63	48.0 ± 3.94	51.8 ± 5.83	51.3 ± 7.30	69.5 ± 11.34 **,^
	Cowper's glands (mg)	5.7 ± 1.93	5.2 ± 1.26	7.3 ± 1.15	5.5 ± 0.69	11.2 ± 2.43 **,^
7	Starting Body Wt (g)	245.9 ± 14.40	257.2 ± 12.15	248.3 ± 11.92	251.2 ± 13.08	251.8 ± 6.28
	Terminal Body Wt (g)	305.4 ± 20.86	327.3 ± 24.20	312.8 ± 11.86	303.6 ± 30.84	271.2 ± 16.02*
	Ventral prostate (mg)	15.2 ± 5.41	19.5 ± 5.39	16.5 ± 6.11	25.3 ± 5.92 **,^	35.2 ± 6.81 **,^
	Seminal vesicles (mg)	95.4 ± 25.16	76.5 ± 23.20	73.4 ± 18.39	93.5 ± 14.20	170.6 ± 95.48 **,^
	LABC muscles (mg)	233.5 ± 42.05	248.1 ± 57.69	260.3 ± 53.35	321.7 ± 57.23 **,^	477.8 ± 74.51 **,^
	Glans penis (mg)	69.7 ± 5.42	67.9 ± 5.18	65.1 ± 10.25	80.9 ± 5.19 *,^	87.3 ± 5.84 **,^
	Cowper's glands (mg)	8.2 ± 3.52	10.3 ± 6.23	8.0 ± 2.86	10.0 ± 3.41	19.6 ± 9.00 **,^

	R-square (%)			Overall means and [CV] for tissues at a given dose				
	OVR	TRT	LAB					
Ventral prostate (mg)	45	32	11	19 [50]	23 [47]	23 [52]	25 [42] *	40 [33] **
Seminal vesicles (mg)	46	18	57	61 [58]	53 [51]	59 [47]	63 [54]	128 [66] **
LABC (mg)	71	44	37	192 [26]	191 [32]	209 [29]	237 [33] **	379 [28] **
Glans penis (mg)	62	16	66	54 [24]	53 [23]	54 [21]	58 [31]	70 [24] **
Cowper's glands (mg)	46	33	15	7 [63]	7 [59]	7 [27]	7 [39]	14 [48] **

OVR – Overall effect on tissue; TRT - Effect of treatments; LAB - Effect of laboratory; CV - Coefficient of variation.

*, ** Significantly different from control at P<0.05 and P<0.01, respectively, using T-test pairwise comparisons.

^ Significantly different from control at P<0.05 using two-tailed Dunnett's multiple comparisons of the tissue with body weight adjustment.

[a] The starting body weight is on the first day of substance administration and the terminal body weight is at necropsy approximately 24-hours after the last administration.

Table 13. Coefficients of variation for the mandatory endpoints in the trenbolone (TREN) studies

Lab	Trenbolone (mg/kg/d)	0	0.3	1.5	8	40	MEAN[a]
1	Terminal Body Wt (g)	6.13	7.06	4.13	4.64	5.49	5.49
	Ventral prostate (mg)	19.98	25.54	19.34	21.56	30.25	23.34
	Seminal vesicles (mg)	19.44	47.75	32.89	39.52	46.94	37.31
	LABC muscles (mg)	12.50	15.16	17.84	24.77	15.81	17.22
	Glans penis (mg)	7.55	9.38	11.91	16.07	8.25	10.63
	Cowper's glands (mg)	36.55	18.54	16.06	27.00	32.83	26.19
3	Terminal Body Wt (g)	7.32	4.47	7.77	5.15	10.67	7.08
	Ventral prostate (mg)	50.91	53.10	47.12	55.20	46.74	50.61
	Seminal vesicles (mg)	29.41	16.12	20.98	45.62	46.02	31.63
	LABC muscles (mg)	20.48	19.68	14.00	16.48	12.87	16.70
	Glans penis (mg)	7.54	8.21	11.24	14.24	16.32	11.51
	Cowper's glands (mg)	34.09	23.99	15.71	12.51	21.60	21.58
7	Terminal Body Wt (g)	6.83	7.39	3.79	10.16	5.91	6.82
	Ventral prostate (mg)	35.65	27.64	36.96	23.39	19.37	28.60
	Seminal vesicles (mg)	26.36	30.33	25.05	15.18	55.97	30.58
	LABC muscles (mg)	18.01	23.25	20.50	17.79	15.59	19.03
	Glans penis (mg)	7.77	7.62	15.74	6.42	6.68	8.85
	Cowper's glands (mg)	42.82	60.33	35.65	34.06	45.85	43.74

[a] The overall mean CV, depending upon the laboratory, may also include a testosterone propionate positive control.

Table 14. Optional organ weights in trenbolone (TREN) studies

Lab	Trenbolone (mg/kg/d)	0	0.3	1.5	8	40
1	Terminal Body Wt (g)	207.4 ± 12.71	211.2 ± 14.91	209.4 ± 8.65	198.6 ± 9.22	189.7 ± 10.42*
	Liver (g)	6.5 ± 0.52	6.4 ± 0.52	6.6 ± 0.62	6.6 ± 0.35	6.8 ± 0.61
	(relative to bw)	3.13%	3.03%	3.15%	3.32%	3.58%
	Adrenals (mg)	65.2 ± 7.31	66.2 ± 9.90	65.1 ± 7.97	64.3 ± 8.73	66.1 ± 8.04
	(relative to bw)	0.0314%	0.0313%	0.0311%	0.0324%	0.0348%
	Kidneys (mg)	1624.1 ± 109.79	1679.7 ± 166.09	1662.6 ± 107.27	1625.5 ± 95.99	1600.3 ± 94.85
	(relative to bw)	0.7831%	0.7953%	0.7940%	0.8185%	0.8436%
3	Terminal Body Wt (g)	238.8 ± 17.47	243.4 ± 10.87	247.6 ± 19.25	236.9 ± 12.20	224.3 ± 23.93
	Liver (g)	10.4 ± 0.95	10.3 ± 0.76	10.7 ± 1.34	10.6 ± 0.87	10.4 ± 2.26
	(relative to bw)	4.35%	4.24%	4.31%	4.46%	4.61%
	Adrenals (mg)	56.0 ± 7.71	45.9 ± 6.83	48.1 ± 7.63	42.9 ± 7.14	45.0 ± 5.61
	(relative to bw)	0.0235%	0.0189%	0.0194%	0.0181%	0.0201%
	Kidneys (mg)	1600.3 ± 188.99	1523.2 ± 111.96	1485.1 ± 116.91	1582.8 ± 60.08	1501.1 ± 235.58
	(relative to bw)	0.6701%	0.6258%	0.5998%	0.6681%	0.6692%
7	Terminal Body Wt (g)	305.4 ± 20.86	327.3 ± 24.20	312.8 ± 11.86	303.6 ± 30.84	271.2 ± 16.02*
	Liver (g)	14.6 ± 1.68	18.7 ± 3.66	17.5 ± 1.47	17.8 ± 3.29	16.0 ± 1.65
	(relative to bw)	4.78%	5.71%	5.59%	5.86%	5.90%
	Adrenals (mg)	54.6 ± 6.56	54.3 ± 8.84	55.4 ± 7.74	53.1 ± 7.97	55.1 ± 9.37
	(relative to bw)	0.0179%	0.0166%	0.0177%	0.0175%	0.0203%
	Kidneys (mg)	2652.2 ± 236.06	2822.8 ± 267.25	2677.3 ± 208.58	2828.5 ± 382.49	2502.5 ± 128.33
	(relative to bw)	0.8684%	0.8625%	0.8559%	0.9317%	0.9228%

*,** Significantly different from control at P<0.05 and P<0.01, respectively, using T-test pairwise comparisons.

84. Overall Review of Mandatory Endpoints. When the data were pooled across the participating laboratories, all five mandatory endpoints achieved statistical significance using the pairwise comparison approach. The VP and LABC achieved significance at 8 mg TREN/kg/d and the SVCG, GP and COWS at 40 mg TREN/kg/d (Table 12). The R-square analyses indicate moderate overall and treatment relationships, and some relationships for laboratory effects for the SVCG [57], LABC [37], and GP [66] (Table 7).

85. Body weights. The initial body weights for the four labs ranged from approximately 195 to 250 grams among the three laboratories. The body weight gains during the treatment period were reduced with increasing doses of TREN based on the starting and terminal body weights (Table 10). In two laboratories, the body weight gains were reduced by 50%, while in laboratory 1 the terminal body weight mean was below the starting body weight with a loss during treatment of about 2.5 g. The body weight decreases were statistically significant in laboratories 1 and 7 ($p<0.05$) and when the data were pooled ($p<0.01$) at 40 mg/kg/d TREN.

86. Optional organ weights. The absolute optional organ weights were not consistently changed by TREN administration (Table 12). Liver weights were largely unchanged in laboratories 1 and 3 at the high TREN dose and were not significantly different in laboratory 7. The paired adrenal weights and the paired kidney weights were not significantly different with TREN treatment in any laboratory.

PHASE 2: ANDROGEN ANTAGONISTS

87. Four androgen antagonists were employed as test substances in Phase-2. VIN and DDE were employed in eight laboratories each. PRO and LIN were employed in four laboratories each.

Procymidone

88. Four laboratories tested four doses of procymidone (PRO) with coadministration of 0.4 mg/kg/d TP in order to assess the ability of the Hershberger bioassay to detect androgen antagonists. All four laboratories conducted the assigned studies as intended, submitted their laboratory and study data electronically using standardized Excel spreadsheets, audited the study data, and, if necessary, informed the Secretariat of data corrections.

Results of Procymidone studies

89. The results of the individual laboratory studies and the summary results of the accessory organ and tissue weights and the statistical analyses for the PRO studies are reported in Table 15. The Hershberger bioassay successfully and reproducibly detected PRO in all four laboratories. Laboratory 8 performed a second study when gavage errors in the first study resulted in several groups being reduced to 5 animals, and the results of both of these studies are reported and designated 8A for the first study and 8B for the second study. The absolute weights of all five male sex accessory tissues decreased with increasing PRO doses in a dose-responsive manner. The VP, SVCG, LABC and COWS achieved statistically significant decreases in all laboratories, and the GP achieved significance in two of the four laboratories.

90. Ventral Prostate (VP). There were statistically significant dose-dependent decreases in the weights of the VP in all laboratories with PRO. Laboratories 2, 8, and 9 achieved statistically significant decreases for the PRO-treated VP at 10 mg/kg/d PRO (laboratory 2 when the pairwise comparison approach was used), and laboratory 7 achieved significance at 30 mg/kg/d (Table 15). The overall mean CVs for the VP ranged from 12 to 33 (Table 16). The mean CVs differed among laboratories and was 12-13 for laboratories 8 and 9 and 32-33 for laboratories 2 and 7.

91. Seminal Vesicles and Coagulating Glands (SVCG). There were statistically significant dose-dependent decreases in the weights of the SVCG in all laboratories with PRO. Laboratories 8 and 9 achieved statistically significant decreases for the PRO-treated SVCG at 10 mg/kg/d PRO, and both laboratories 2 and 7 achieved significance at 30 mg/kg/d, when the pairwise comparison approach was used for lab 2 data (Table 15). The overall mean CVs for the SVCG ranged from 11 to 30 (Table 16).

92. Levator ani and Bulbocavernosus muscle complex (LABC). There were statistically significant dose-dependent decreases in the weights of the LABC in all laboratories with PRO. Laboratory 9 achieved statistically significant decreases for the PRO-treated LABC at 3 mg/kg/d PRO, when the pairwise comparison approach was used, and laboratories 7 and 8 achieved significance at 30 mg/kg/d, and laboratory 2 at 100 mg/k/d (Table 15). An outlier with a Studentised residual of less than –7 was removed from the 30 mg/kg/d group of laboratory 7 for these statistical analyses. The overall mean CVs for the LABC ranged from 12 to 33 (Table 16).

93. Glans Penis (GP). There were statistically significant dose-dependent decreases in the weights of the GP in two of the four laboratories with PRO. Laboratory 9 achieved statistically significant decreases for the PRO-treated GP at 10 mg/kg/d PRO, when the pairwise comparison approach was used, and laboratory 8 achieved significance in both studies at 30 mg/kg/d. However, labs 2 and 7 did not achieve significance with either statistical approach (Table 15). The overall mean CVs for the VP ranged from 7 to 17 (Table 16).

94. Cowper's Glands (COWS). There were statistically significant dose-dependent decreases in the weights of the COWS in all laboratories with PRO. Laboratory 9 achieved a statistically significant decrease for the PRO-treated COWS at 3 mg/kg/d PRO when the pairwise comparison approach was used, lab study 8B at 10 mg/kg/d, lab 7 at 30 mg/kg/d, and lab 2 and 8A studies achieved significance at 100 mg/kg/d (Table 15). The overall mean CVs for the COWS ranged from 9 to 33 (Table 16).

95. Overall Review of Mandatory Endpoints. When the data were pooled across the participating laboratories, all five mandatory endpoints achieved statistical significance using the pairwise comparison approach. The VP, LABC, and COWS achieved significant decreases at 10 mg PRO/kg/d and the GP at 30 mg MT/kg/d (Table 15). The R-square analyses indicate strong overall and treatment relationships except for the GP, and the R-square indicated some relationships with laboratory effects for the LABC [41] and GP [47] (Table 15).

96. Body weights. The initial body weights ranged from approximately 215 to 250 grams among the four laboratories. The body weight gains during the treatment period were modestly reduced by 10-20 grams with increasing doses of PRO based on the starting and terminal body weights (Table 15). These reductions achieved statistical significance in laboratory 8 at 100 mg/kg/d PRO and in the overall pooled data at 10 mg/kg/d PRO ($p<0.05$) and 30 and 100 mg/kg/d ($p<0.01$).

97. Optional organ weights. The body weight adjusted liver weights were significantly increased in all 4 laboratories (10 mg/kg/d PRO dose in lab 7, 30 mg/kg/d in lab 9, and 100 mg/kg/d in labs 1 and 8) (Table 17). The body weight adjusted paired adrenal weights were significantly increased in laboratories 2, 7, and 8 at the 30 or 100 mg/kg/d PRO dose. Absolute values of the paired adrenals were also increased in laboratory 9, but did not achieve statistical significance. The paired kidney weights were not significantly affected by PRO treatment.

Table 15. Body weights, mandatory tissue weights and pooled statistics in procymidone (PRO) studies

Lab	Testosterone Propionate (mg/kg/d)	0.4	0.4	0.4	0.4	0.4
	Procymidone (mg/kg/d)	0	3	10	30	100
2	Starting Body Wt (g) [a]	218.0 ± 13.59	218.7 ± 7.69	214.7 ± 11.74	222.7 ± 9.67	216.5 ± 14.24
	Terminal Body Wt (g) [a]	307.0 ± 17.57	305.4 ± 6.96	297.0 ± 16.21	301.1 ± 16.27	295.0 ± 16.97
	Ventral prostate (mg)	173.8 ± 32.53	180.2 ± 43.10	150.9 ± 52.33 *	103.3 ± 16.49 **,^	52.7 ± 23.55 **,^
	Seminal vesicles (mg)	356.2 ± 53.09	362.1 ± 46.86	406.3 ± 176.22	215.6 ± 59.20 *	145.7 ± 69.80 **,^
	LABC muscles (mg)	292.6 ± 41.88	339.7 ± 33.72	321.7 ± 79.39	277.9 ± 40.28	204.3 ± 31.79 **,^
	Glans penis (mg)	79.1 ± 4.94	83.2 ± 19.96	85.6 ± 7.75	79.7 ± 10.98	66.6 ± 18.60
	Cowper's glands (mg)	32.7 ± 8.00	30.4 ± 8.32	31.4 ± 8.92	29.1 ± 14.32	15.6 ± 4.96 **,^
7	Starting Body Wt (g)	248.4 ± 3.33	249.3 ± 8.05	234.1 ± 9.13	248.0 ± 11.67	246.2 ± 9.97
	Terminal Body Wt (g)	325.5 ± 20.55	322.8 ± 6.77	304.3 ± 20.97	317.0 ± 23.65	308.8 ± 12.23
	Ventral prostate (mg)	157.4 ± 18.02	140.5 ± 49.23	103.8 ± 33.01	80.4 ± 25.95 **,^	57.9 ± 19.68 **,^
	Seminal vesicles (mg)	485.3 ± 36.90	475.1 ± 105.59	396.6 ± 49.11	224.1 ± 48.65 **,^	205.8 ± 76.43 **,^
	LABC muscles (mg)	541.7 ± 62.63	514.5 ± 45.79	468.0 ± 118.45	324.6 ± 130.67 **,^	333.1 ± 62.28 **,^
	Glans penis (mg)	91.7 ± 15.20	96.9 ± 12.29	85.3 ± 4.43	78.5 ± 8.82	81.2 ± 7.56
	Cowper's glands (mg)	36.8 ± 10.21	34.6 ± 9.05	29.5 ± 9.32	22.3 ± 2.57 **,^	21.5 ± 6.36 **,^
8A [b]	Starting Body Wt (g)	248.7 ± 12.81	248.2 ± 8.95	248.0 ± 11.94	247.8 ± 10.82	248.5 ± 13.24
	Terminal Body Wt (g)	325.1 ± 19.36	319.1 ± 16.43	317.6 ± 7.60	314.8 ± 17.74	312.6 ± 15.87**
	Ventral prostate (mg)	210.4 ± 22.59	206.8 ± 8.64	151.8 ± 31.83 **,^	124.3 ± 13.54 **,^	67.8 ± 10.92 **,^
	Seminal vesicles (mg)	693.9 ± 78.29	612.3 ± 95.93	479.7 ± 41.19 **,^	388.3 ± 64.40 **,^	212.6 ± 56.76 **,^
	LABC muscles (mg)	655.7 ± 80.55	589.4 ± 50.02	537.3 ± 52.33 **,^	441.6 ± 26.28 **,^	318.6 ± 38.01 **,^
	Glans penis (mg)	97.4 ± 8.18	88.0 ± 3.34	88.7 ± 8.81	78.9 ± 5.48 **,^	70.4 ± 11.87 **,^
	Cowper's glands (mg)	50.8 ± 9.69	49.7 ± 4.87	41.5 ± 12.39	41.3 ± 11.35	24.2 ± 4.63 **,^
8B [b]	Starting Body Wt (g)	251.2 ± 6.49	251.2 ± 8.75	249.7 ± 8.68	248.3 ± 6.95	249.2 ± 6.55
	Terminal Body Wt (g)	333.2 ± 17.58	330.6 ± 17.91	329.1 ± 13.81	319.7 ± 11.30	310.9 ± 13.99**
	Ventral prostate (mg)	214.2 ± 19.13	181.9 ± 37.40	140.2 ± 22.69 **,^	136.7 ± 19.89 **,^	68.7 ± 16.64 **,^
	Seminal vesicles (mg)	581.5 ± 125.60	580.1 ± 96.63	498.7 ± 76.13	404.2 ± 29.43 **,^	189.9 ± 53.51 **,^
	LABC muscles (mg)	654.1 ± 58.88	642.2 ± 48.05	571.3 ± 58.51	488.5 ± 42.11 **,^	319.1 ± 43.64 **,^
	Glans penis (mg)	86.8 ± 6.57	82.6 ± 11.94	79.1 ± 6.36	73.8 ± 7.69 **,^	60.7 ± 2.90 **,^
	Cowper's glands (mg)	45.6 ± 6.07	40.7 ± 5.9	34.6 ± 3.63 **,^	29.9 ± 4.32 **,^	19.4 ± 3.29 **,^
9	Starting Body Wt (g)	248.7 ± 12.68	248.0 ± 7.75	247.3 ± 8.98	248.3 ± 8.78	249.2 ± 7.88
	Terminal Body Wt (g)	338.0 ± 19.10	336.2 ± 14.77	335.3 ± 15.56	329.7 ± 8.80	331.5 ± 16.39
	Ventral prostate (mg)	145.8 ± 15.41	138.7 ± 18.71	118.9 ± 17.33 *,^	111.5 ± 16.78 **,^	76.4 ± 9.43 **,^
	Seminal vesicles (mg)	488.8 ± 51.60	445.7 ± 39.53	403.1 ± 30.92 **,^	282.1 ± 27.01 **,^	255.0 ± 21.14 **,^
	LABC muscles (mg)	393.6 ± 30.95	355.0 ± 30.28 *	346.6 ± 29.13 **,^	275.0 ± 25.46 **,^	252.0 ± 18.15 **,^
	Glans penis (mg)	124.1 ± 13.12	115.4 ± 7.76	113.1 ± 7.08 *	108.7 ± 6.45 **,^	107.9 ± 5.18 **,^
	Cowper's glands (mg)	40.1 ± 3.29	35.4 ± 2.55 *	31.6 ± 2.22 **,^	26.1 ± 2.44 **,^	20.8 ± 1.93 **,^

*, ** Significantly different from control at P<0.05 and P<0.01, respectively, using T-test pairwise comparisons.

^ Significantly different from control at P<0.05 using two-tailed Dunnett's multiple comparisons of the tissue with body weight adjustment.

[a] The starting body weight is on the first day of substance administration and the terminal body weight is at necropsy approximately 24-hours after the last administration.

[b] Laboratory 8 performed two studies as several mortalities occurred reducing group size in the first study due to gavage errors.

Table 15 continued. Body weights, mandatory tissue weights and pooled statistics in procymidone (PRO) studies

Lab	Testosterone Propionate (mg/kg/d)			0.4	0.4	0.4	0.4	0.4
	Procymidone (mg/kg/d)			0	3	10	30	100
	R-square (%)			Overall means and [CV] for tissues at a given dose				
	OVR	TRT	LAB					
Ventral prostate (mg)	76	67	8	180 [19]	170 [25]	132 [28] **	111 [24] **	65 [28] **
Seminal vesicles (mg)	78	61	14	521 [26]	495 [24]	434 [23] *	303 [31] **	202 [32] **
LABC (mg)	67	33	41	508 [31]	488 [27]	441 [28] *	362 [30] **	285 [22] **
Glans penis (mg)	37	16	47	96 [19]	93 [18]	91 [15]	84 [18] **	77 [25] **
Cowper's glands (mg)	63	49	13	41 [24]	38 [24]	33 [25] **	30 [34] **	20 [25] **

OVR – Overall effect on tissue; TRT - Effect of treatments; LAB - Effect of laboratory; CV - Coefficient of variation.

*, ** Significantly different from control at P<0.05 and P<0.01, respectively, using T-test pairwise comparisons.

^ Significantly different from control at P<0.05 using two-tailed Dunnett's multiple comparisons of the tissue with body weight adjustment.

[a] The starting body weight is on the first day of substance administration and the terminal body weight is at necropsy approximately 24-hours after the last administration.

Table 16. Coefficients of variation of the mandatory endpoints in the procymidone (PRO) studies

Lab	Testosterone Prop. (mg/kg/d)	0.4	0.4	0.4	0.4	0.4	MEAN[a]
	Procymidone (mg/kg/d)	0	3	10	30	100	
2	Terminal Body Wt (g)	5.72	2.28	5.46	5.40	5.75	5.03
	Ventral prostate (mg)	18.72	23.92	34.68	15.96	44.67	31.79
	Seminal vesicles (mg)	14.91	12.94	43.38	27.46	47.92	30.03
	LABC muscles (mg)	14.32	9.93	24.68	14.50	15.56	17.73
	Glans penis (mg)	6.25	23.99	9.05	13.78	27.92	16.81
	Cowper's glands (mg)	27.36	27.36	28.44	49.26	31.82	32.90
7	Terminal Body Wt (g)	5.96	5.15	2.39	5.64	5.08	5.28
	Ventral prostate (mg)	10.74	4.18	20.97	10.90	16.10	32.63
	Seminal vesicles (mg)	11.28	15.67	8.59	16.59	26.70	23.15
	LABC muscles (mg)	12.28	8.49	9.74	5.95	11.93	24.04
	Glans penis (mg)	8.40	3.79	9.93	6.95	16.86	11.36
	Cowper's glands (mg)	9.80	9.80	29.86	27.46	19.15	25.67
8A[b]	Terminal Body Wt (g)	5.96	5.15	2.39	5.64	5.08	4.87
	Ventral prostate (mg)	10.74	4.18	20.97	10.90	16.10	12.32
	Seminal vesicles (mg)	11.28	15.67	8.59	16.59	26.70	15.07
	LABC muscles (mg)	12.28	8.49	9.74	5.95	11.93	9.76
	Glans penis (mg)	8.40	3.79	9.93	6.95	16.86	8.78
	Cowper's glands (mg)	9.80	9.80	29.86	27.46	19.15	22.63
8B[b]	Terminal Body Wt (g)	5.28	5.42	4.20	3.53	4.50	4.83
	Ventral prostate (mg)	8.93	20.56	16.19	14.55	24.25	16.40
	Seminal vesicles (mg)	21.60	16.66	15.27	7.28	28.18	16.93
	LABC muscles (mg)	9.00	7.48	10.24	8.62	13.68	10.06
	Glans penis (mg)	7.57	14.44	8.04	10.42	4.78	9.93
	Cowper's glands (mg)	14.38	14.38	10.47	14.44	16.95	15.45
9	Terminal Body Wt (g)	5.65	4.39	4.64	2.67	4.94	4.53
	Ventral prostate (mg)	10.57	13.49	14.58	15.04	12.35	12.54
	Seminal vesicles (mg)	10.56	8.87	7.67	9.57	8.29	10.79
	LABC muscles (mg)	7.86	8.53	8.41	9.26	7.20	9.29
	Glans penis (mg)	10.58	6.73	6.27	5.93	4.80	6.62
	Cowper's glands (mg)	8.22	7.01	7.01	9.35	9.26	8.68

[a] The overall mean CV, depending upon the laboratory, may also include the vehicle control and a flutamide positive control.

[b] Laboratory 8 performed two studies as several mortalities occurred reducing group size in the first study due to gavage errors.

Table 17. Optional organ weights in the procymidone (PRO) studies.

Lab	Testosterone Prop. (mg/kg/d)	0.4	0.4	0.4	0.4	0.4
	Procymidone (mg/kg/d)	**0**	**3**	**10**	**30**	**100**
2	Terminal Body Wt (g)	307.0 ± 17.57	305.4 ± 6.96	297.0 ± 16.21	301.1 ± 16.27	295.0 ± 16.97
	Liver (g)	13.4 ± 0.78	13.1 ± 0.75	12.5 ± 0.98	13.8 ± 1.60	13.9 ± 0.84 *
	(relative to bw)	4.36%	4.29%	4.21%	4.58%	4.71%
	Adrenals (mg)	54.0 ± 9.04	63.4 ± 14.12	61.9 ± 9.86	66.2 ± 6.26 *	80.6 ± 13.53 **
	(relative to bw)	0.0176%	0.0208%	0.0208%	0.0220%	0.0273%
	Kidneys (mg)	2226.6 ± 199.91	2165.5 ± 129.50	2138.6 ± 100.53	2165.2 ± 107.41	2082.7 ± 95.33
	(relative to bw)	0.725%	0.709%	0.720%	0.719%	0.706%
7	Terminal Body Wt (g)	325.5 ± 20.55	322.8 ± 6.77	304.3 ± 20.97	317.0 ± 23.65	308.8 ± 12.23
	Liver (g)	16.3 ± 1.67	17.4 ± 1.87	17.9 ± 2.00 **	18.7 ± 2.57 **	20.2 ± 0.70 **
	(relative to bw)	5.01%	5.39%	5.88%	5.90%	6.54%
	Adrenals (mg)	57.8 ± 5.32	57.7 ± 7.25	61.8 ± 7.78	66.3 ± 7.22 *	64.9 ± 7.07
	(relative to bw)	0.0178%	0.0179%	0.0203%	0.0209%	0.0210%
	Kidneys (mg)	2818.3 ± 170.89	2705.2 ± 136.19	2659.2 ± 142.98	2759.5 ± 263.11	2722.6 ± 232.62
	(relative to bw)	0.866%	0.838%	0.874%	0.871%	0.882%
8A [a]	Terminal Body Wt (g)	325.1 ± 19.36	319.1 ± 16.43	317.6 ± 7.60	314.8 ± 17.74	312.6 ± 15.87**
	Liver (g)	13.9 ± 1.28	13.6 ± 1.21	13.3 ± 0.84	13.3 ± 0.55	14.5 ± 1.19 **
	(relative to bw)	4.28%	4.26%	4.19%	4.22%	4.64%
	Adrenals (mg)	51.2 ± 6.41	48.5 ± 6.28	58.6 ± 7.38	60.2 ± 5.85	62.3 ± 6.01
	(relative to bw)	0.0157%	0.0152%	0.0185%	0.0191%	0.0199%
	Kidneys (mg)	2198.4 ± 186.19	2179.1 ± 197.50	2072.8 ± 129.05	2134.1 ± 193.25	2003.3 ± 194.90
	(relative to bw)	0.676%	0.683%	0.653%	0.678%	0.641%
8B [a]	Terminal Body Wt (g)	333.2 ± 17.58	330.6 ± 17.91	329.1 ± 13.81	319.7 ± 11.30	310.9 ± 13.99**
	Liver (g)	13.1 ± 1.1	13.5 ± 1.0	13.2 ± 0.5	13.3 ± 0.7	13.7 ± 1.3 *
	(relative to bw)	3.94%	4.07%	4.00%	4.16%	4.40%
	Adrenals (mg)	57.1 ± 5.6	50.6 ± 5.4	54.4 ± 7.2	57.5 ± 8.2	69.7 ± 9.6 **
	(relative to bw)	0.0171%	0.0153%	0.0165%	0.0180%	0.0224%
	Kidneys (mg)	2270.8 ± 168.9	2334.1 ± 169.1	2121.8 ± 106.0	2145.6 ± 183.3	2036.5 ± 60.8
	(relative to bw)	0.681%	0.706%	0.645%	0.671%	0.655%
9	Terminal Body Wt (g)	338.0 ± 19.10	336.2 ± 14.77	335.3 ± 15.56	329.7 ± 8.80	331.5 ± 16.39
	Liver (g)	17.2 ± 1.33	16.3 ± 0.91	16.8 ± 0.84	18.0 ± 1.07 **	18.4 ± 1.73 **
	(relative to bw)	5.09%	4.85%	5.01%	5.46%	5.55%
	Adrenals (mg)	56.6 ± 4.70	56.7 ± 5.20	53.5 ± 10.49	61.3 ± 14.05	67.2 ± 11.72
	(relative to bw)	0.0167%	0.0169%	0.0160%	0.0186%	0.0203%
	Kidneys (mg)	2398.6 ± 169.06	2525.8 ± 138.74	2394.0 ± 111.39	2529.0 ± 98.65	2483.6 ± 166.40
	(relative to bw)	0.710%	0.751%	0.714%	0.767%	0.749%

*, ** Significantly different from control at P<0.05 and P<0.01, respectively, using T-test pairwise comparisons.

[a] Laboratory 8 performed two studies as several mortalities occurred reducing group size in the first study due to gavage errors.

Vinclozolin

98. Eight laboratories tested the same doses of vinclozolin (VIN) in order to assess the ability of the Hershberger bioassay to detect androgen antagonists. Four laboratories performed their studies using coadministration of 0.2 mg/kg/d TP, and four laboratories performed their studies using coadministration of 0.4 mg/kg/d TP. All laboratories conducted the assigned studies as intended, submitted their laboratory and study data electronically using standardized Excel spreadsheets, audited the study data, and, if necessary, informed the Secretariat of data corrections.

Results of Vinclozolin studies using 0.4 mg/kg/d TP coadministration

99. The summary results of the accessory organ and tissue weights and the statistical analyses for the VIN studies conducted with 0.4 mg/kg/d TP coadministration are reported in Table 18. The results show that the Hershberger bioassay successfully and reproducibly detected VIN in all laboratories. The weights of all five male sex accessory tissues decreased with increasing VIN doses in a dose-responsive manner and achieved statistical significance.

100. Ventral Prostate (VP). There were statistically significant dose-dependent decreases in VP weights in all laboratories with VIN. Labs 1, 3, and 7 achieved a statistically significant decrease for the VIN-treated VP at 30 mg/kg/d using the pairwise comparison approach. Lab 5 achieved significance at 100 mg/kg/d (Table 18). The overall mean CVs for the VP ranged from 22 to 26 (Table 19).

101. Seminal Vesicles and Coagulating Glands (SVCG). There were statistically significant dose-dependent decreases in the weights of the SVCG in all laboratories with VIN. All laboratories achieved statistically significant decreases for the VIN-treated SVCG at 30 mg/kg/d VIN when using the pairwise comparison approach (Table 18). The overall mean CVs for the SVCG ranged from 19 to 28 (Table 19).

102. Levator ani and Bulbocavernosus muscle complex (LABC). There were statistically significant dose-dependent decreases in the weights of the LABC in all laboratories with VIN. All laboratories achieved a statistically significant decrease for the VIN-treated LABC at 30 mg/kg/d VIN with both the pairwise and multiple comparison approaches (Table 18). The overall mean CVs for the LABC ranged from 10 to 15 (Table 19).

103. Glans Penis (GP). There were statistically significant dose-dependent decreases in the weights of the GP in three of four laboratories with VIN. Laboratory 1 achieved a statistically significant decrease for the VIN-treated GP at 30 mg/kg/d, and labs 3 and 7 achieved significance at 100 mg/kg/d (Table 18). The absolute decrease in GP weight in lab 5 was similar, but did not achieve statistical significance using either statistical approach. However, this laboratory did not dissect and weight the GP in cases where preputial separation was judged to be incomplete; this reduced the group size in some cases and, therefore, the power for the high VIN doses (see notations for lab 5 in Table 18). The overall mean CVs for the GP ranged from 9 to 12 (Table 19).

104. Cowper's Glands (COWS). There were statistically significant dose-dependent decreases in the weights of the COWS in all laboratories with VIN. Laboratory 1 achieved a statistically significant decrease for the VIN-treated COWS at 30 mg/kg/d. Labs 3, 5 and 7 achieved significance at 100 mg/kg/d (Table 18). The overall mean CVs for the COWS ranged from 16 to 30 (Table 19).

Table 18. Body weights, mandatory tissue weights and pooled statistics in vinclozolin (VIN) studies with 0.4 mg/kg/d TP

Lab	Testosterone Propionate (mg/kg/d)	0.4	0.4	0.4	0.4	0.4
	Vinclozolin (mg/kg/d)	0	3	10	30	100
1	Starting Body Wt (g) [a]	205.2 ± 11.42	212.6 ± 11.97	209.6 ± 11.11	208.9 ± 14.78	206.4 ± 12.95
	Terminal Body Wt (g) [a]	219.9 ± 11.37	226.9 ± 14.96	223.4 ± 16.59	221.0 ± 14.55	219.0 ± 12.18
	Ventral prostate (mg)	106.5 ± 19.82	103.7 ± 25.01	94.5 ± 12.57	79.0 ± 16.38 *	32.2 ± 8.04 **,^
	Seminal vesicles (mg)	216.7 ± 58.54	261.7 ± 100.28	201.7 ± 39.71	143.3 ± 43.20 *	45.0 ± 10.49 **,^
	LABC muscles (mg)	310.0 ± 22.80	336.7 ± 56.45	295.0 ± 42.78	235.0 ± 31.46 **,^	160.0 ± 14.14 **,^
	Glans penis (mg)	66.0 ± 4.29	66.0 ± 4.98	58.3 ± 6.02	55.3 ± 7.06 **,^	42.8 ± 5.49 **,^
	Cowper's glands (mg)	21.7 ± 1.97	25.2 ± 7.19	18.7 ± 3.93	15.0 ± 2.19 **,^	10.7 ± 2.58 **,^
3	Starting Body Wt (g)	249.6 ± 15.42	245.6 ± 15.95	244.2 ± 13.94	241.5 ± 16.72	244.1 ± 14.68
	Terminal Body Wt (g)	309.0 ± 17.55	287.7 ± 25.08	299.8 ± 16.81	294.7 ± 16.73	297.9 ± 18.83
	Ventral prostate (mg)	179.4 ± 25.28	182.7 ± 55.77	172.2 ± 37.86	130.3 ± 22.47 *	83.2 ± 26.24 **,^
	Seminal vesicles (mg)	814.4 ± 130.88	913.8 ± 151.31	764.3 ± 98.40	532.9 ± 117.17 *,^	347.5 ± 49.45 **,^
	LABC muscles (mg)	653.4 ± 81.23	699.5 ± 80.61	633.8 ± 57.25	483.3 ± 23.62 **,^	369.3 ± 23.73 **,^
	Glans penis (mg)	90.7 ± 7.83	89.5 ± 11.60	84.2 ± 3.08	91.8 ± 11.31	73.8 ± 6.94 **,^
	Cowper's glands (mg)	31.7 ± 4.36	36.4 ± 7.65	33.4 ± 4.04	29.8 ± 2.43	20.7 ± 2.88 **,^
5	Starting Body Wt (g)	234.2 ± 9.56	238.7 ± 15.90	234.7 ± 12.50	230.8 ± 13.04	234.0 ± 15.49
	Terminal Body Wt (g)	290.3 ± 15.06	293.3 ± 18.57	291.2 ± 16.74	281.2 ± 12.64	281.2 ± 21.20
	Ventral prostate (mg)	131.8 ± 35.65	123.7 ± 52.05	126.3 ± 35.30	110.2 ± 19.75	50.3 ± 9.87 **,^
	Seminal vesicles (mg)	546.8 ± 106.80	463.9 ± 55.52	491.0 ± 79.51	341.3 ± 109.94 **,^	146.5 ± 29.32 **,^
	LABC muscles (mg)	467.0 ± 53.65	396.4 ± 45.83	429.5 ± 60.06	352.3 ± 59.31 **,^	256.7 ± 29.00 **,^
	Glans penis (mg) [b]	89.5 ± 13.58	86.9 ± 8.46	87.1 ± 7.72	86.5 ± 7.96 (5)	75.1 ± 6.69 (2)
	Cowper's glands (mg)	34.3 ± 8.62	27.2 ± 5.64	32.9 ± 7.01	25.7 ± 3.83	16.4 ± 5.37 **,^
7	Starting Body Wt (g)	253.7 ± 5.04	261.1 ± 15.25	259.1 ± 11.96	251.9 ± 7.37	257.4 ± 7.09
	Terminal Body Wt (g)	326.1 ± 11.41	337.3 ± 24.42	343.1 ± 17.71	328.6 ± 14.28	330.1 ± 9.03
	Ventral prostate (mg)	161.1 ± 28.20	131.4 ± 31.05	130.6 ± 23.82	92.9 ± 18.57 **,^	50.7 ± 23.49 **,^
	Seminal vesicles (mg)	420.3 ± 81.32	433.7 ± 49.81	397.7 ± 121.51	280.5 ± 55.20 **,^	135.2 ± 47.47 **,^
	LABC muscles (mg)	548.4 ± 68.92	501.3 ± 61.75	511.8 ± 94.43	376.8 ± 24.11 **,^	282.4 ± 40.42 **,^
	Glans penis (mg)	91.3 ± 6.57	91.6 ± 13.75	94.3 ± 10.24	86.7 ± 7.63	74.2 ± 11.33 **,^
	Cowper's glands (mg)	36.7 ± 3.80	36.2 ± 2.77	34.7 ± 8.76	42.2 ± 33.77	18.2 ± 4.91 **,^

	R-square (%)			Overall means and [CV] for tissues at a given dose				
	OVR	TRT	LAB					
Ventral prostate (mg)	71	57	18	145 [27]	135 [37]	131 [30]	103 [26] **	54 [47] **
Seminal vesicles (mg)	81	40	48	487 [49]	518 [51]	464 [48]	325 [51] **	169 [70] **
LABC (mg)	76	37	50	495 [28]	471 [33]	468 [30]	361 [27] **	267 [30] **
Glans penis (mg)	43	16	62	84 [16]	84 [17]	81 [19]	80 [21]	65 [26] **
Cowper's glands (mg)	63	37	32	31 [25]	31 [25]	30 [30]	28 [67] **	17 [33] **

OVR – Overall effect on tissue; TRT - Effect of treatments; LAB - Effect of laboratory; CV - Coefficient of variation.

*, ** Significantly different from control at P<0.05 and P<0.01, respectively, using T-test pairwise comparisons.

^ Significantly different from control at P<0.05 using two-tailed Dunnett's multiple comparisons of the tissue with body weight adjustment.

[a] The starting body weight is on the first day of substance administration and the terminal body weight is at necropsy approximately 24-hours after the last administration.

[b] If preputial separation was incomplete, this lab did not dissect and weigh that individual GP. Actual numbers per group are in parenthesis, if the group size was decreased as a result.

Table 19. Coefficients of variation for the mandatory endpoints in the vinclozolin (VIN) studies with 0.4 mg/kg/d TP

Lab	Testosterone Prop. (mg/kg/d)	0.4	0.4	0.4	0.4	0.4	MEAN[a]
	Vinclozolin (mg/kg/d)	0	3	10	30	100	
1	Terminal Body Wt (g)	5.17	6.59	7.43	6.58	5.56	6.27
	Ventral prostate (mg)	18.61	24.12	13.30	20.74	24.98	22.35
	Seminal vesicles (mg)	27.02	38.32	19.69	30.14	23.31	28.05
	LABC muscles (mg)	7.36	16.77	14.50	13.39	8.84	12.70
	Glans penis (mg)	6.50	7.55	10.32	12.76	12.82	11.91
	Cowper's glands (mg)	28.59	28.59	21.07	14.61	24.21	21.14
3	Terminal Body Wt (g)	5.68	8.20	5.61	5.68	6.32	6.38
	Ventral prostate (mg)	14.09	30.53	21.98	17.24	31.53	26.32
	Seminal vesicles (mg)	16.07	16.56	12.87	21.98	14.23	19.47
	LABC muscles (mg)	12.43	11.52	9.03	4.89	6.42	9.79
	Glans penis (mg)	8.63	12.96	3.66	12.32	9.41	9.69
	Cowper's glands (mg)	17.13	21.03	12.09	8.18	13.92	16.33
5	Terminal Body Wt (g)	5.19	6.33	5.75	4.50	7.54	5.61
	Ventral prostate (mg)	27.04	42.07	27.95	17.93	19.61	25.86
	Seminal vesicles (mg)	19.53	11.97	16.19	32.21	20.02	20.00
	LABC muscles (mg)	11.49	11.56	13.98	16.84	11.30	15.35
	Glans penis (mg)	15.18	9.73	8.86	9.20	8.91	9.35
	Cowper's glands (mg)	27.12	20.71	21.26	14.89	32.83	24.13
7	Terminal Body Wt (g)	3.50	7.24	5.16	4.34	2.73	4.75
	Ventral prostate (mg)	17.51	23.62	18.25	19.99	46.33	23.79
	Seminal vesicles (mg)	19.35	11.48	30.55	19.68	35.12	24.87
	LABC muscles (mg)	12.57	12.32	18.45	6.40	14.31	12.33
	Glans penis (mg)	7.19	15.02	10.86	8.80	15.27	11.70
	Cowper's glands (mg)	7.66	7.66	25.28	80.11	27.07	30.13

[a] The overall mean CV, depending upon the laboratory, may also include the vehicle control and a flutamide positive control.

Table 20. Optional organ weights in the vinclozolin (VIN) studies with 0.4 mg/kg/d TP

Lab	Testosterone Prop. (mg/kg/d)	0.4	0.4	0.4	0.4	0.4
	Vinclozolin (mg/kg/d)	**0**	**3**	**10**	**30**	**100**
1	Terminal Body Wt (g)	219.9 ± 11.37	226.9 ± 14.96	223.4 ± 16.59	221.0 ± 14.55	219.0 ± 12.18
	Liver (g)	6.6 ± 0.29	6.7 ± 0.53	6.7 ± 0.77	6.7 ± 0.94	7.2 ± 0.48 **
	(relative to bw)	3.00%	2.95%	3.00%	3.03%	3.29%
	Adrenals (mg)	69.3 ± 12.79	63.7 ± 9.33	65.0 ± 8.51	77.5 ± 17.81	95.0 ± 12.90 **
	(relative to bw)	0.0315%	0.0281%	0.0291%	0.0351%	0.0434%
	Kidneys (mg)	1681.7 ± 75.74	1748.3 ± 116.86	1645.0 ± 67.45	1680.0 ± 233.84	1756.7 ± 122.58
	(relative to bw)	0.7648%	0.7705%	0.7363%	0.7602%	0.8021%
3	Terminal Body Wt (g)	309.0 ± 17.55	283.8 ± 25.08	299.8 ± 16.81	294.7 ± 16.73	297.9 ± 18.83
	Liver (g)	13.2 ± 1.71	13.0 ± 1.98	13.0 ± 0.916	13.7 ± 0.901 **	14.0 ± 1.39 **
	(relative to bw)	4.27%	4.58%	4.33%	4.66%	4.69%
	Adrenals (mg)	49.4 ± 5.93	53.8 ± 8.10	59.7 ± 7.78 **	62.7 ± 8.21 **	62.3 ± 13.85 **
	(relative to bw)	0.0160%	0.0190%	0.0199%	0.0213%	0.0209%
	Kidneys (mg)	1895.9 ± 179.90	1898.0 ± 180.58	1855.6 ± 211.91	1888.3 ± 133.50	1856.0 ± 202.25
	(relative to bw)	0.6136%	0.6688%	0.6189%	0.6408%	0.6230%
5	Terminal Body Wt (g)	290.3 ± 15.06	293.3 ± 18.57	291.2 ± 16.74	281.2 ± 12.64	281.2 ± 21.20
	Liver (g)	10.4 ± 1.10	10.5 ± 1.02	10.7 ± 1.10	11.1 ± 0.70 *	12.1 ± 1.11 **
	(relative to bw)	3.58%	3.58%	3.67%	3.95%	4.30%
	Adrenals (mg)	60.1 ± 6.50	63.9 ± 9.04	60.6 ± 8.88	69.0 ± 8.55 *	86.8 ± 19.01 **
	(relative to bw)	0.0207%	0.0218%	0.0208%	0.0245%	0.0309%
	Kidneys (mg)	1763.2 ± 84.13	1811.0 ± 117.23	1886.0 ± 144.75	1740.3 ± 79.55	1793.8 ± 125.04
	(relative to bw)	0.6074%	0.6175%	0.6477%	0.6189%	0.6379%
7	Terminal Body Wt (g)	326.1 ± 11.41	337.3 ± 24.42	343.1 ± 17.71	328.6 ± 14.28	330.1 ± 9.03
	Liver (g)	15.9 ± 1.43	17.4 ± 0.93	17.9 ± 1.99	17.9 ± 1.69 **	18.2 ± 1.15 **
	(relative to bw)	4.88%	5.16%	5.22%	5.45%	5.51%
	Adrenals (mg)	59.5 ± 6.92	56.8 ± 9.52	63.7 ± 11.01	63.5 ± 5.87	71.1 ± 7.51 *
	(relative to bw)	0.0182%	0.0168%	0.0186%	0.0193%	0.0215%
	Kidneys (mg)	2720.4 ± 166.57	2691.8 ± 270.86	2950.0 ± 262.69	2709.8 ± 199.39	2712.1 ± 222.35
	(relative to bw)	0.8342%	0.7980%	0.8598%	0.8247%	0.8216%

*, ** Significantly different from control at P<0.05 and P<0.01, respectively, using T-test pairwise comparisons.

105. Overall Review of Mandatory Endpoints. When the data were pooled across the laboratories, all five mandatory endpoints achieved statistical significance using the pairwise comparison approach. The VP, SVCG, LABC, and COWS achieved significance at 30 mg/kg/d and the GP at 100 mg/kg/d (Table 18). The R-square analyses indicate moderate to strong overall relationships. There were moderate treatment relationships with the tissue responses except for the GP, and some relationships for laboratory effects for the SCVG [48], LABC [50], GP [62], and COWS [32] (Table 18).

106. Body weights. The initial mean body weights ranged from approximately 205 to 255 grams among the four laboratories. The body weight gains during the treatment period were largely unchanged with increasing doses of VIN based on the starting and terminal body weights in laboratories 1 and 7 and with slight decreases in laboratories 3 and 5 (Table 18). The latter did not achieve statistical significance.

107. Optional organ weights. The absolute organ weights of the liver and the adrenals were increased by VIN administration (Table 20). With body weight adjustments, both the liver and adrenal weights were significantly increased at the high VIN dose in all laboratories, and one or both organs were significantly increased at lower VIN doses in laboratories 3, 5, and 7. The paired kidney weights were not affected by VIN treatment in any laboratory.

Results of Vinclozolin studies using 0.2 mg/kg/d TP coadministration

108. The summary results of the accessory organ and tissue weights and the statistical analyses for the VIN studies conducted using 0.2 mg/kg/d TP coadministration are reported in Table 21. First, this Table shows that the Hershberger bioassay successfully and reproducibly detected VIN in all laboratories. The weights of all five male sex accessory tissues decreased with increasing VIN doses in a dose-responsive manner and achieved statistical significance at the 100 mg/kg/d dose.

109. Ventral Prostate (VP). There were statistically significant dose-dependent decreases in the weights of the VP in all labs with VIN. Labs 10, 13 and 14 achieved significant decreases for the VIN-treated VP at 10 mg/kg/d with the pairwise comparison approach, and lab 11 achieved significance at 100 mg/kg/d (Table 21). The overall mean CVs for the VP ranged from 20 to 34 (Table 22).

110. Seminal Vesicles and Coagulating Glands (SVCG). There were statistically significant dose-dependent decreases in the weights of the SVCG in all lab with VIN. Laboratory 14 achieved a statistically significant decrease for the VIN-treated SVCG at 3 mg/kg/d with the pairwise comparison approach, and laboratories 10, 11, and 13 achieved significance at 30 mg/kg/d (Table 21). The overall mean CVs for the SVCG ranged from 17 to 32 (Table 22).

111. Levator ani and Bulbocavernosus muscle complex (LABC). There were statistically significant dose-dependent decreases in the weights of the LABC in all laboratories with VIN. Laboratory 13 achieved a statistically significant decrease for the VIN-treated LABC at 10 mg/kg/d, and laboratories 10, 11 and 14 achieved significance at 30 mg/kg/d (Table 21). The overall mean CVs for the LABC ranged from 9 to 20 (Table 22).

112. Glans Penis (GP). There were statistically significant dose-dependent decreases in the weights of the GP in all labs with VIN. Laboratory 13 achieved a statistically significant decrease for the VIN-treated GP at 10 mg/kg/d, and labs 10, 11 and 14 achieved significance at 30 mg/kg/d using the pairwise comparison approach (Table 21). The overall mean CVs for the GP ranged from 5 to 13 (Table 22).

Table 21. Body weights, mandatory tissue weights and pooled statistics in Vinclozolin (VIN) studies with 0.2 mg/kg/d TP

Lab	Testosterone Propionate (mg/kg/d)	0.2	0.2	0.2	0.2	0.2
	Vinclozolin (mg/kg/d)	0	3	10	30	100
10	Starting Body Wt (g) [a]	232.8 ± 6.77	233.1 ± 7.93	232.9 ± 9.63	232.9 ± 7.07	231.4 ± 6.73
	Terminal Body Wt (g) [a]	291.2 ± 6.73	289.9 ± 6.69	286.2 ± 12.68	286.3 ± 14.62	289.2 ± 11.56
	Ventral prostate (mg)	106.4 ± 14.26	98.9 ± 21.14	84.1 ± 13.72 **	75.3 ± 1.31 **	38.9 ± 13.18 **,^
	Seminal vesicles (mg)	216.7 ± 38.21	221.6 ± 16.72	168.4 ± 42.77	116.2 ± 20.78 **,^	47.3 ± 15.09 **,^
	LABC muscles (mg)	361.3 ± 46.54	320.6 ± 41.20	323.9 ± 19.29	268.0 ± 23.87 **,^	181.8 ± 34.78 **,^
	Glans penis (mg)	70.1 ± 3.88	69.9 ± 1.98	67.3 ± 3.97	64.7 ± 4.48 **	51.4 ± 6.59 **,^
	Cowper's glands (mg)	20.8 ± 1.29	21.1 ± 5.3	19.5 ± 3.49	15.1 ± 2.87 **,^	7.4 ± 1.75 **,^
11	Starting Body Wt (g)	247.6 ± 8.96	246.7 ± 8.10	245.2 ± 10.72	246.2 ± 5.91	245.9 ± 8.33
	Terminal Body Wt (g)	326.8 ± 11.23	327.7 ± 9.75	320.8 ± 15.11	319.6 ± 14.03	319.1 ± 17.51
	Ventral prostate (mg)	97.2 ± 42.65	111.6 ± 18.27	105.1 ± 18.69	79.4 ± 14.81	34.1 ± 10.86 **,^
	Seminal vesicles (mg)	361.7 ± 75.94	335.9 ± 54.90	321.0 ± 34.82	210.8 ± 56.27 **,^	71.8 ± 16.77 **,^
	LABC muscles (mg)	537.7 ± 90.89	500.5 ± 80.02	485.2 ± 69.83	416.0 ± 53.61 **,^	275.2 ± 25.42 **,^
	Glans penis (mg)	81.7 ± 8.86	75.9 ± 6.08	73.7 ± 5.55	69.8 ± 7.25 **,^	58.5 ± 5.12 **,^
	Cowper's glands (mg)	28.0 ± 6.21	26.8 ± 3.06	21.1 ± 5.65 **	20.1 ± 2.39 **	11.2 ± 1.94 **,^
13	Starting Body Wt (g)	273.4 ± 10.42	273.6 ± 10.41	273.3 ± 10.78	274.8 ± 10.48	273.3 ± 11.13
	Terminal Body Wt (g)	338.4 ± 17.57	344.7 ± 12.17	340.3 ± 16.34	347.4 ± 18.84	334.0 ± 13.12
	Ventral prostate (mg)	136.6 ± 33.74	118.8 ± 13.20	91.3 ± 22.93 **,^	60.7 ± 8.20 **,^	36.4 ± 9.93 **,^
	Seminal vesicles (mg)	393.5 ± 51.83	358.5 ± 47.52	248.7 ± 45.50 **,^	174.5 ± 31.98 **,^	60.7 ± 13.00 **,^
	LABC muscles (mg)	533.9 ± 25.92	511.5 ± 25.01	441.9 ± 33.29 **,^	381.8 ± 50.41 **,^	257.8 ± 51.50 **,^
	Glans penis (mg)	91.1 ± 5.70	88.9 ± 4.42	79.8 ± 4.48 **,^	76.8 ± 4.37 **,^	64.0 ± 2.90 **,^
	Cowper's glands (mg)	32.7 ± 5.98	32.7 ± 5.1	24.3 ± 6.53 **,^	20.2 ± 3.07 **,^	12.4 ± 2.51 **,^
14	Starting Body Wt (g)	257.8 ± 7.79	257.7 ± 4.50	256.1 ± 6.72	258.2 ± 7.25	258.7 ± 4.60
	Terminal Body Wt (g)	340.6 ± 12.23	337.0 ± 8.56	338.8 ± 5.18	333.5 ± 9.43	335.3 ± 8.76
	Ventral prostate (mg) [b]	183.6 ± 21.96	149.7 ± 16.28 **	136.7 ± 14.60 **,^	98.2 ± 10.79 **,^	51.0 ± 10.33 **,^
	Seminal vesicles (mg)	420.8 ± 92.86	458.7 ± 102.65	344.3 ± 46.63	247.7 ± 69.88 **,^	96.4 ± 16.13 **,^
	LABC muscles (mg)	590.4 ± 52.57	608.8 ± 95.73	529.3 ± 52.23	430.7 ± 32.79 **,^	308.6 ± 31.24 **,^
	Glans penis (mg)	76.4 ± 7.60	78.0 ± 11.24	77.7 ± 4.42	70.2 ± 5.66 *	52.7 ± 2.56 **,^
	Cowper's glands (mg) [b]	38.6 ± 4.22	36.0 ± 7.4	32.9 ± 5.13	25.9 ± 5.57 **,^	16.2 ± 5.65 **,^

	R-square (%)			Overall means and [CV] for tissues at a given dose				
	OVR	TRT	LAB					
Ventral prostate (mg)	81	60	16	131 [34]	120 [21] **	104 [25] **	78 [21] **	69 [34] **
Seminal vesicles (mg)	91	64	19	348 [29]	344 [30]	271 [30] **	187 [36] **	69 [34] **
LABC (mg)	83	50	34	506 [21]	485 [25]	445 [20] **	374 [20] **	256 [23] **
Glans penis (mg)	75	53	23	80 [13]	78 [12]	75 [9] *	70 [10] **	57 [12] **
Cowper's glands (mg)	78	51	26	30 [27]	29 [26]	25 [30] **	20 [25] **	12 [38] **

OVR – Overall effect on tissue; TRT - Effect of treatments; LAB - Effect of laboratory; CV - Coefficient of variation.

*, ** Significantly different from control at P<0.05 and P<0.01, respectively, using T-test pairwise comparisons.

^ Significantly different from control at P<0.05 using two-tailed Dunnett's multiple comparisons of the tissue with body weight adjustment.

[a] The starting body weight is on the first day of substance administration and the terminal body weight is at necropsy approximately 24-hours after the last administration.

[b] The VP and COWS were fixed and then weighed in laboratory 16

Table 22. Coefficients of variation for the mandatory endpoints in the vinclozolin (VIN) studies with 0.2 mg/kg/d TP.

Lab	Methyl Testosterone (mg/kg/d)	0.2	0.2	0.2	0.2	0.2	MEAN[a]
	Vinclozolin (mg/kg/d)	0	3	10	30	100	
10	Terminal Body Wt (g)	3.48	2.31	2.31	4.43	5.10	4.00
	Ventral prostate (mg)	20.35	13.40	21.38	16.32	1.74	33.91
	Seminal vesicles (mg)	15.98	17.63	7.55	25.39	17.88	31.95
	LABC muscles (mg)	12.38	12.88	12.85	5.96	8.91	19.13
	Glans penis (mg)	7.15	5.54	2.83	5.90	6.93	12.82
	Cowper's glands (mg)	18.91	6.18	24.85	17.95	19.04	23.65
11	Terminal Body Wt (g)	4.97	3.44	2.98	4.71	4.39	5.49
	Ventral prostate (mg)	27.68	43.90	16.36	17.79	18.66	31.84
	Seminal vesicles (mg)	21.65	20.99	16.35	10.85	26.70	23.34
	LABC muscles (mg)	7.87	16.90	15.99	14.39	12.89	9.24
	Glans penis (mg)	4.67	10.84	8.00	7.54	10.39	8.75
	Cowper's glands (mg)	29.66	11.41	11.41	26.71	11.87	17.31
13	Terminal Body Wt (g)	4.82	5.19	3.53	4.80	5.42	3.93
	Ventral prostate (mg)	11.81	24.71	11.11	25.13	13.52	27.29
	Seminal vesicles (mg)	11.66	13.17	13.26	18.30	18.33	21.40
	LABC muscles (mg)	8.12	4.86	4.89	7.53	13.20	19.97
	Glans penis (mg)	4.77	6.26	4.97	5.61	5.69	4.53
	Cowper's glands (mg)	14.47	18.29	15.54	26.82	15.24	20.22
14	Terminal Body Wt (g)	3.40	3.59	2.54	1.53	2.83	2.61
	Ventral prostate (mg)	25.51	11.96	10.88	10.68	10.99	20.25
	Seminal vesicles (mg)	15.01	22.07	22.38	13.54	28.22	16.73
	LABC muscles (mg)	8.42	8.90	15.72	9.87	7.61	10.12
	Glans penis (mg)	11.00	9.95	14.40	5.68	8.05	4.85
	Cowper's glands (mg)	31.70	10.91	20.53	15.59	21.54	34.83

[a] The overall mean CV, depending upon the laboratory, may also include a vehicle control group and a flutamide positive control group.

113. Cowper's Glands (COWS). There were statistically significant dose-dependent decreases in the weights of the COWS in all laboratories with VIN. Labs 11 and 13 achieved statistically significant decreases for the VIN-treated COWS at 10 mg/kg/d, and laboratories 10 and 14 achieved significance at 30 mg/kg/d (Table 21). The overall mean CVs for the COWS ranged from 17 to 35 (Table 22).

114. Body weights. The initial mean body weights ranged from approximately 230 to 275 grams among the four laboratories. The body weight gains during the treatment period were largely unchanged with increasing doses of VIN based on the starting and terminal body weights (Table 21).

Comparison of Vinclozolin results with different TP coadministration doses

115. The VIN results using 0.2 mg/kg/d TP coadministered dose were produced approximately one year earlier than the results using 0.4 mg/kg/d TP. Despite modest differences in body weights, strain, diet and other differences among laboratories (see Table 4), the results are similar, supporting the robustness of the bioassay.

116. To illustrate the consistency of the dose response of these tissues, the relative decreases in tissue weights in response to VIN doses have been analyzed together for both 0.2 and 0.4 mg/kg/d TP. Figures 4A-E show the relative decrease for the five tissues in the eight laboratories with increasing VIN doses and using the group administered only TP as the control. The dose response results for the mean relative decrease show the good reproducibility of the overall dose response with VIN.

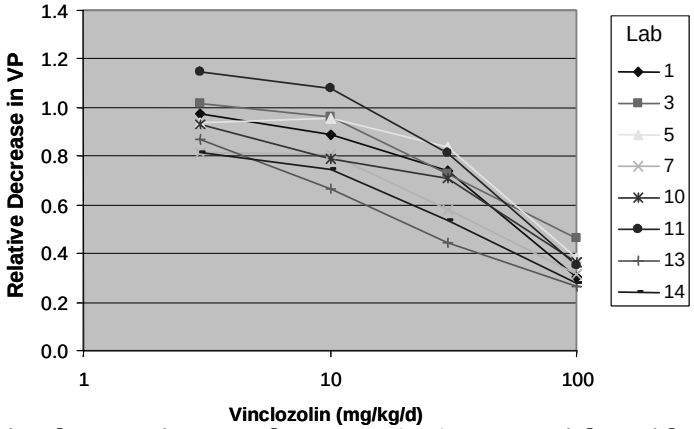


Figure 4A. Relative decrease in ventral prostate (VP) mean weights with VIN doses in eight laboratories. Labs 1 through 7 used 0.4 mg/kg/d TP and labs 10 through 14 used 0.2 mg/kg/d TP.

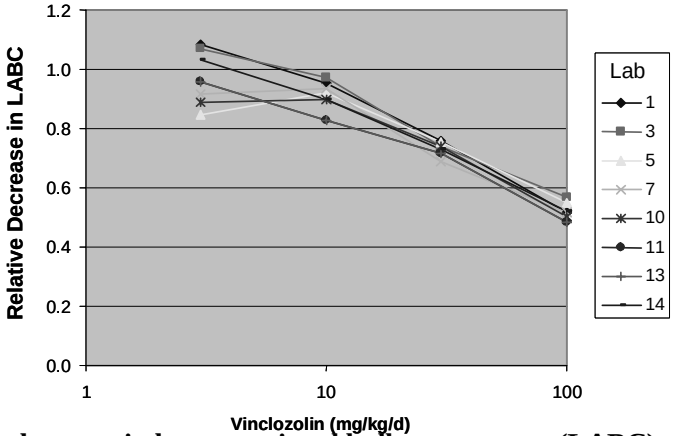


Figure 4B. Relative decrease in levator ani and bulbocavernosus (LABC) mean weights with VIN doses in eight labs. Labs 1 through 7 used 0.4 mg/kg/d TP and labs 10 through 14 used 0.2 mg/kg/d TP.

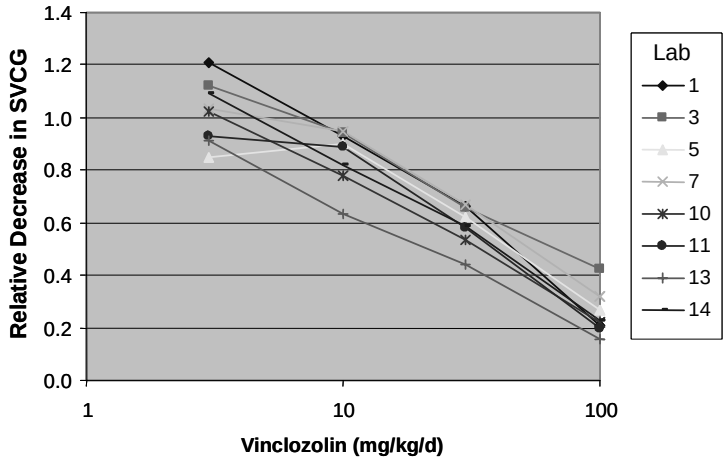


Figure 4C. Relative decrease in seminal vesicles and coagulating gland (SVCG) mean weights with VIN doses in eight laboratories. Labs 1 through 7 used 0.4 mg/kg/d TP and labs 10 through 14 used 0.2 mg/kg/d TP.

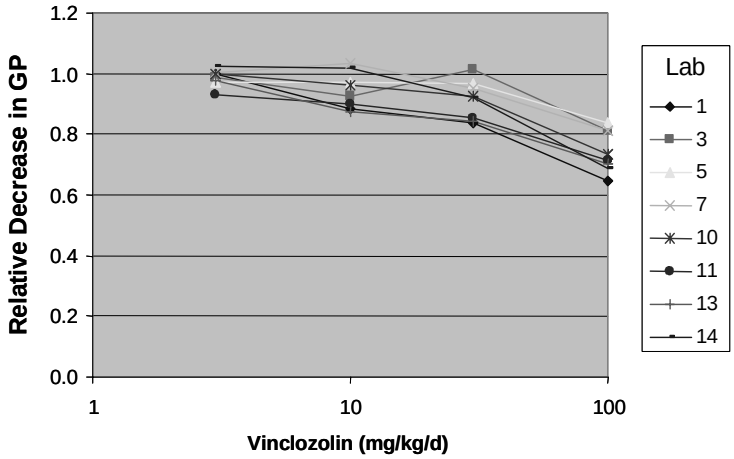


Figure 4D. Relative decrease in glans penis (GP) mean weights with VIN doses in eight laboratories. Labs 1 through 7 used 0.4 mg/kg/d TP and labs 10 through 14 used 0.2 mg/kg/d TP.

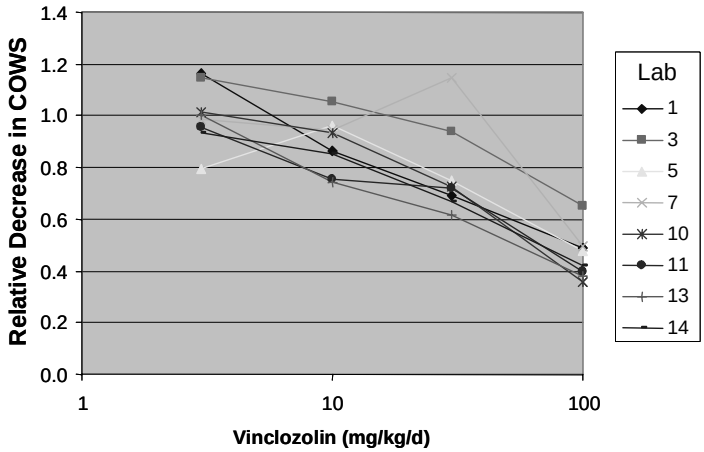


Figure 4E. Relative decrease in Cowper's gland (COWS) mean weights with VIN doses in eight laboratories. Labs 1 through 7 used 0.4 mg/kg/d TP and labs 10 through 14 used 0.2 mg/kg/d TP.

Linuron

117. Four laboratories tested four doses of LIN with coadministration of 0.4 mg/kg/d TP in order to assess the ability of the Hershberger bioassay to detect androgen antagonists. All four laboratories conducted the assigned studies as intended, submitted their laboratory and study data electronically using standardized Excel spreadsheets, audited the study data, and, if necessary, informed the Secretariat of data corrections.

Results of Linuron studies

118. The summary results of the accessory organ and tissue weights and the statistical analyses for the LIN studies are reported in Table 23. The results show that the Hershberger bioassay successfully detected LIN in three of the four laboratories. The VP, LABC, and COWS achieved statistical significance in three of the four laboratories, the SVCG in two of the four laboratories, and the GP in one of the four laboratories. In laboratory 6, which did not detect statistically significant changes with linuron, decreases in absolute weights were not observed in the GP and COWS at the top dose, and the absolute decreases in the VP, SVCG, and LABC were more modest when compared to the other laboratories.

119. Ventral Prostate (VP). There were statistically significant dose-dependent decreases in the weights of the VP in three of four laboratories with LIN. Laboratory 5 achieved a statistically significant decrease for the LIN-treated VP at 30 mg/kg/d using the pairwise comparison approach, and labs 1 and 4 achieved significance at 100 mg/kg/d. In laboratory 6, the VP did not achieve statistical significance (Table 23). The overall mean CVs for the VP ranged from 18 to 43 (Table 24).

120. Seminal Vesicles and Coagulating Glands (SVCG). There were statistically significant dose-dependent decreases in the weights of the SVCG in all laboratories with LIN. In two cases, the achievement of statistical significance depended upon the pairwise comparison statistical approach. Labs 1 and 5 achieved statistically significant decreases for the LIN-treated SVCG at 30 mg/kg/d LIN, and laboratories 4 and 6 achieved significance 100 mg/kg/d (Table 23). The overall mean CVs for the SVCG ranged from 19 to 27 (Table 24).

121. Levator ani and Bulbocavernosus muscle complex (LABC). There were statistically significant dose-dependent decreases in the weights of the LABC in three of four laboratories with LIN. Laboratory 5 achieved a statistically significant decrease for the LIN-treated LABC at 30 mg/kg/d, and labs 1 and 4 achieved significance at 100 mg/kg/d. In lab 6, the LABC did not achieve statistical significance (Table 23). The overall mean CVs for the LABC ranged from 11 to 14 (Table 24).

122. Glans Penis (GP). There were statistically significant dose-dependent decreases in the weights of the GP in only one of four laboratories with LIN. Laboratory 1 achieved a statistically significant decrease for the LIN-treated GP at 100 mg/kg/d. In laboratories 4, 5 and 6, the GP did not achieve statistical significance (Table 23). However, laboratory 5 did not dissect and weight the GP in cases where preputial separation was judged to be incomplete; this reduced the group size in some cases and, therefore, the power for the high LIN dose (see notations for lab 5 in Table 23). The overall mean CVs for the GP ranged from 10 to 15 (Table 24).

123. Cowper's Glands (COWS). There were statistically significant dose-dependent decreases in the weights of the COWS in three of four laboratories with LIN. Labs 1, 4 and 5 achieved a statistically significant decrease for the LIN-treated COWS at 100 mg/kg/d. In lab 6, the COWS did not achieve statistical significance (Table 23). The overall mean CVs for the SVCG ranged from 15 to 41 (Table 24).

Table 23. Body weights, mandatory tissue weights and pooled statistics in linuron (LIN) studies

Lab	Testosterone Propionate (mg/kg/d)	0.4	0.4	0.4	0.4	0.4
	Linuron (mg/kg/d)	0	3	10	30	100
1	Starting Body Wt (g) [a]	205.2 ± 11.42	209.1 ± 9.41	209.6 ± 13.18	202.7 ± 9.69	206.5 ± 9.94
	Terminal Body Wt (g) [a]	219.9 ± 11.37	221.7 ± 17.58	218.9 ± 16.63	218.2 ± 11.13	203.1 ± 11.04*
	Ventral prostate (mg)	106.5 ± 19.82	113.3 ± 16.66	122.5 ± 23.95	89.0 ± 20.10	60.7 ± 10.93 **,^
	Seminal vesicles (mg)	216.7 ± 58.54	293.3 ± 54.28	265.0 ± 41.83	191.7 ± 35.45 *	101.7 ± 11.69 **,^
	LABC muscles (mg)	310.0 ± 22.80	368.3 ± 32.51	333.3 ± 49.67	286.7 ± 48.03	188.3 ± 44.46 **,^
	Glans penis (mg)	66.0 ± 4.29	68.0 ± 8.53	65.8 ± 6.91	61.5 ± 5.89	55.2 ± 4.79 **,^
	Cowper's glands (mg)	21.7 ± 1.97	30.2 ± 7.28	24.2 ± 2.14	23.3 ± 5.72	15.2 ± 1.47 **,^
4	Starting Body Wt (g)	275.8 ± 11.58	279.2 ± 11.21	274.7 ± 12.19	276.2 ± 12.30	275.3 ± 12.08
	Terminal Body Wt (g)	372.3 ± 14.74	364.7 ± 15.59	355.7 ± 17.42	351.8 ± 14.84*	330.9 ± 12.86**
	Ventral prostate (mg)	167.1 ± 34.58	192.8 ± 35.16	203.2 ± 33.54	184.4 ± 38.47	99.0 ± 26.74 **,^
	Seminal vesicles (mg)	484.6 ± 65.58	568.5 ± 172.20	536.1 ± 79.17	541.5 ± 103.25	293.4 ± 72.09 **
	LABC muscles (mg)	651.3 ± 87.10	654.6 ± 93.90	631.1 ± 52.24	571.5 ± 54.51	438.0 ± 40.11 **,^
	Glans penis (mg)	91.2 ± 5.90	94.4 ± 14.34	102.4 ± 11.32	101.1 ± 4.80	89.5 ± 16.77
	Cowper's glands (mg)	43.1 ± 7.41	37.8 ± 7.14	43.8 ± 6.61	40.5 ± 4.38	31.6 ± 5.68 **,^
5	Starting Body Wt (g)	237.3 ± 15.37	245.3 ± 24.62	234.7 ± 19.24	238.5 ± 13.92	241.8 ± 14.20
	Terminal Body Wt (g)	281.2 ± 18.73	284.0 ± 22.71	280.0 ± 21.48	277.2 ± 18.17	264.2 ± 10.85
	Ventral prostate (mg)	114.2 ± 22.22	111.6 ± 14.95	111.6 ± 16.92	83.7 ± 21.10 *	46.2 ± 11.28 **,^
	Seminal vesicles (mg)	436.5 ± 97.17	388.6 ± 64.12	339.1 ± 71.55	247.9 ± 44.07 **,^	140.6 ± 40.20 **,^
	LABC muscles (mg)	377.5 ± 41.51	351.0 ± 7.38	349.5 ± 56.18	297.3 ± 23.22 **,^	158.3 ± 33.15 **,^
	Glans penis (mg) [b]	73.3 ± 6.81	75.0 ± 8.39	73.8 ± 6.60	79.0 ± 8.19	64.1 ± 3.98 (3)
	Cowper's glands (mg)	24.9 ± 4.73	23.1 ± 4.04	24.6 ± 6.12	19.5 ± 4.04	12.2 ± 4.20 **,^
6	Starting Body Wt (g)	231.4 ± 4.47	231.9 ± 8.09	228.5 ± 9.31	232.4 ± 10.65	228.9 ± 9.59
	Terminal Body Wt (g)	306.5 ± 9.96	310.5 ± 12.69	305.5 ± 8.63	302.3 ± 13.13	291.8 ± 17.62
	Ventral prostate (mg)	83.3 ± 33.97	109.5 ± 51.89	115.2 ± 25.07	109.8 ± 25.32	67.9 ± 18.94
	Seminal vesicles (mg)	371.7 ± 137.19	412.3 ± 92.28	394.7 ± 82.92	323.1 ± 44.27	255.6 ± 58.67 *
	LABC muscles (mg)	477.7 ± 50.98	485.5 ± 59.16	492.3 ± 30.27	496.1 ± 42.24	427.7 ± 42.40
	Glans penis (mg)	95.7 ± 12.07	93.4 ± 11.77	85.1 ± 13.88	97.9 ± 4.91	92.7 ± 19.05
	Cowper's glands (mg)	24.1 ± 5.52	25.3 ± 2.93	24.6 ± 3.46	23.4 ± 5.54	24.6 ± 8.61

	R-square (%)			Overall means and [CV] for tissues at a given dose				
	OVR	TRT	LAB					
Ventral prostate (mg)	59	32	33	118 [35]	132 [36]	139 [33]	117 [45]	69 [47] **
Seminal vesicles (mg)	65	35	49	377 [31]	416 [34]	383 [32]	326 [45] *	200 [47] **
LABC (mg)	63	22	60	454 [31]	465 [29]	450 [30]	413 [32] **	309 [44] **
Glans penis (mg)	26	3.8	66	82 [18]	83 [19]	82 [21]	85 [20]	77 [28] *
Cowper's glands (mg)	43	16	49	28 [35]	29 [27]	30 [33]	27 [36]	21 [44] **

OVR – Overall effect on tissue; TRT - Effect of treatments; LAB - Effect of laboratory; CV - Coefficient of variation.

*, ** Significantly different from control at P<0.05 and P<0.01, respectively, using T-test pairwise comparisons.

^ Significantly different from control at P<0.05 using two-tailed Dunnett's multiple comparisons of the tissue with body weight adjustment.

[a] The starting body weight is on the first day of substance administration and the terminal body weight is at necropsy approximately 24-hours after the last administration.

[b] If preputial separation was incomplete, this lab did not dissect and weigh that individual GP. Actual numbers per group are in parenthesis, if the group size was decreased as a result.

Table 24. Coefficients of variation for the mandatory endpoints in the linuron (LIN) studies

Lab	Testosterone Prop. (mg/kg/d)	0.4	0.4	0.4	0.4	0.4	MEAN[a]
	Linuron (mg/kg/d)	0	3	10	30	100	
1	Terminal Body Wt (g)	5.17	7.93	7.60	5.10	5.43	6.26
	Ventral prostate (mg)	18.61	14.70	19.55	22.58	18.02	20.97
	Seminal vesicles (mg)	27.02	18.51	15.79	18.50	11.50	20.19
	LABC muscles (mg)	7.36	8.83	14.90	16.75	23.61	14.47
	Glans penis (mg)	6.50	12.55	10.50	9.58	8.69	11.55
	Cowper's glands (mg)	24.13	24.13	8.84	24.49	9.71	16.85
4	Terminal Body Wt (g)	3.96	4.28	4.90	4.22	3.89	4.25
	Ventral prostate (mg)	20.69	18.24	16.51	20.86	27.00	20.66
	Seminal vesicles (mg)	13.53	30.29	14.77	19.07	24.57	20.45
	LABC muscles (mg)	13.37	14.35	8.28	9.54	9.16	10.94
	Glans penis (mg)	6.48	15.19	11.06	4.75	18.74	11.24
	Cowper's glands (mg)	17.20	15.07	15.07	10.82	18.00	15.23
5	Terminal Body Wt (g)	6.66	8.00	7.67	6.56	4.11	6.65
	Ventral prostate (mg)	19.46	13.39	15.17	25.22	24.44	17.81
	Seminal vesicles (mg)	22.26	16.50	21.10	17.78	28.60	19.14
	LABC muscles (mg)	11.00	2.10	16.07	7.81	20.94	11.10
	Glans penis (mg)	9.29	11.19	8.94	10.37	6.20	10.23
	Cowper's glands (mg)	26.08	17.50	24.84	20.76	34.57	27.74
6	Terminal Body Wt (g)	3.25	4.09	2.82	4.34	6.04	3.82
	Ventral prostate (mg)	40.76	47.39	21.75	23.06	27.90	42.92
	Seminal vesicles (mg)	36.91	22.38	21.01	13.70	22.95	27.07
	LABC muscles (mg)	10.67	12.19	6.15	8.52	9.91	12.46
	Glans penis (mg)	12.62	12.60	16.30	5.02	20.55	14.86
	Cowper's glands (mg)	64.94	11.60	14.04	23.72	35.04	41.37

[a] The overall mean CV, depending upon the laboratory, may also include the vehicle control and a flutamide positive control.

Table 25. Optional organ weights in the linuron (LIN) studies.

Lab	Testosterone Prop. (mg/kg/d)	0.4	0.4	0.4	0.4	0.4
	Linuron (mg/kg/d)	**0**	**3**	**10**	**30**	**100**
1	Terminal Body Wt (g)	219.9 ± 11.37	221.7 ± 17.58	218.9 ± 16.63	218.2 ± 11.13	203.1 ± 11.04
	Liver (g)	6.6 ± 0.29	6.4 ± 0.44	6.7 ± 0.78	6.5 ± 0.48	6.3 ± 0.67
	(relative to bw)	3.00%	2.89%	3.06%	2.98%	3.10%
	Adrenals (mg)	69.3 ± 12.79	60.3 ± 7.17	67.0 ± 16.96	68.5 ± 7.31	63.7 ± 15.07
	(relative to bw)	0.0315%	0.0272%	0.0306%	0.0314%	0.0314%
	Kidneys (mg)	1681.7 ± 75.74	1761.7 ± 132.73	1665.0 ± 115.89	1633.3 ± 77.11	1626.7 ± 139.67
	(relative to bw)	0.7648%	0.7946%	0.7606%	0.7485%	0.8009%
4	Terminal Body Wt (g)	372.3 ± 14.74	364.7 ± 15.59	355.7 ± 17.42	351.8 ± 14.84	330.9 ± 12.86
	Liver (g)	15.7 ± 1.34	15.6 ± 1.54	14.7 ± 1.30	13.9 ± 1.27	15.7 ± 1.34
	(relative to bw)	4.22%	4.28%	4.13%	3.95%	4.74%
	Adrenals (mg)	64.6 ± 11.20	58.3 ± 7.30	57.3 ± 4.89	64.1 ± 4.68	64.6 ± 11.20
	(relative to bw)	0.0174%	0.0160%	0.0161%	0.0182%	0.0195%
	Kidneys (mg)	2573.5 ± 145.73	2522.5 ± 125.99	2345.0 ± 183.04	2404.1 ± 77.97	2573.5 ± 145.73
	(relative to bw)	0.6912%	0.6917%	0.6593%	0.6834%	0.7777%
5	Terminal Body Wt (g)	281.2 ± 18.73	284.0 ± 22.71	280.0 ± 21.48	277.2 ± 18.17	264.2 ± 10.85
	Liver (g)	9.9 ± 1.49	11.0 ± 1.32	10.3 ± 1.21	9.6 ± 1.06	9.0 ± 0.92
	(relative to bw)	3.52%	3.87%	3.68%	3.46%	3.41%
	Adrenals (mg)	57.2 ± 11.04	63.5 ± 9.80	47.5 ± 8.66	60.4 ± 14.94	66.1 ± 10.22
	(relative to bw)	0.0203%	0.0224%	0.0170%	0.0218%	0.0250%
	Kidneys (mg)	1607.0 ± 199.61	1615.3 ± 171.69	1558.4 ± 131.98	1563.7 ± 95.03	1539.7 ± 96.96
	(relative to bw)	0.5715%	0.5688%	0.5566%	0.5641%	0.5828%
6	Terminal Body Wt (g)	306.5 ± 9.96	310.5 ± 12.69	305.5 ± 8.63	302.3 ± 13.13	291.8 ± 17.62
	Liver (g)	15.1 ± 2.021	17.3 ± 2.42	16.9 ± 1.33	14.9 ± 1.89	15.8 ± 2.34
	(relative to bw)	4.93%	5.57%	5.53%	4.93%	5.41%
	Adrenals (mg)	57.9 ± 6.5	64.9 ± 8.82	62.7 ± 18.42	59.1 ± 8.30	63.9 ± 7.82
	(relative to bw)	0.0189%	0.0209%	0.0205%	0.0196%	0.0219%
	Kidneys (mg)	2622.7 ± 288.08	2768.1 ± 87.85	2674.7 ± 197.14	2639.9 ± 73.07	2699.5 ± 309.99
	(relative to bw)	0.8557%	0.8915%	0.8755%	0.8733%	0.9251%

*, ** Significantly different from control at P<0.05 and P<0.01, respectively, using T-test pairwise comparisons.

124. Overall Review of Mandatory Endpoints. When the data were pooled across the labs, all five mandatory endpoints achieved statistical significance using the pairwise comparison approach. The SCVG and LABC achieved significance at 30 mg LIN/kg/d, and the VP, GP, and COWS achieved significance at 100 mg/kg/d (Table 23), although the GP attained significance only at $p < 0.05$. The R-square analyses indicate moderate overall relationships, treatment relationships were modest for the VP, SCVG, and LABC and increasingly weak for the COWS [16] and GP [3.8], and there were relationships for laboratory effects for all tissues that were probably accentuated by the modest to weak treatment relationships (Table 23).

125. Body weights. The initial body weights ranged from approximately 205 to 275 grams among the four laboratories. The absolute body weight gains during the treatment period were reduced at the high doses LIN based on the starting and terminal body weights (Table 23). Significant decreases occurred at 30 mg/kg/d in lab 4 and 100 mg/kg/d in lab 1. The decrease was statistically significant in the pooled data at 100 mg/kg/d ($p<0.01$).

126. Optional organ weights. The absolute optional organ weights were largely unchanged by LIN administration (Table 25). Increasing relative organ weights appeared to parallel decreased body weights, so no specific organ effects were attributed to LIN, and no statistically significant changes were observed.

p,p'-DDE

127. Nine laboratories tested four doses of *p,p'*-DDE (DDE) in order to assess the ability of the Hershberger bioassay to detect androgen antagonists. Five laboratories performed the studies using coadministration with 0.2 mg/kg/d TP, and four laboratories performed the studies using coadministration with 0.4 mg/kg/d TP. All laboratories conducted the assigned studies as intended, submitted their laboratory and study data electronically using standardized Excel spreadsheets, audited the study data, and, if necessary, informed the Secretariat of data corrections.

Results of *p,p'*-DDE studies using 0.4 mg/kg/d TP coadministration

128. The summary results of the accessory organ and tissue weights and the statistical analyses for the DDE studies conducted using 0.4 mg/kg/d TP coadministration are reported in Table 26. The results show that the Hershberger bioassay successfully and reproducibly detected DDE in three of the four laboratories. The absolute weights of all five male sex accessory tissues decreased with increasing DDE doses in a dose-responsive manner. The SVCG and GP did not achieve statistical significance in one of the four laboratories.

129. Ventral Prostate (VP). There were statistically significant dose-dependent decreases in the weights of the VP in all four laboratories with DDE. Laboratory 8 achieved a statistically significant decrease for the DDE-treated VP at 50 mg/kg/d DDE with both statistical methods, and lab 3 and 9 using the pairwise comparison approach. In laboratory 9, the VP was significantly decreased at 160 mg/kg/d (Table 26). The overall mean CVs for the VP ranged from 15 to 35 (Table 27).

130. Seminal Vesicles and Coagulating Glands (SVCG). There were statistically significant dose-dependent decreases in the weights of the SVCG in all laboratories with DDE. Labs 8 and 9 achieved a statistically significant decrease for the DDE-treated SVCG at 50 mg/kg/d DDE, lab 3 achieved significance at 160 mg/kg/d with both statistical approaches, and lab 4 at the same dose only when using the pairwise comparison approach (Table 26). The overall mean CVs for the SVCG ranged from 13 to 32 (Table 27).

Table 26. Body weights, mandatory tissue weights and pooled statistics in *p,p'*-DDE (DDE) studies using 0.4 mg/kg/d TP.

Lab	Testosterone Propionate (mg/kg/d)	0.4	0.4	0.4	0.4	0.4
	***p,p'*-DDE (mg/kg/d)**	**0**	**5**	**16**	**50**	**160**
3	Starting Body Wt (g) [a]	202.6 ± 13.22	205.2 ± 18.74	210.7 ± 16.70	205.6 ± 13.89	209.2 ± 12.48
	Terminal Body Wt (g) [a]	259.3 ± 20.92	261.1 ± 34.32	273.5 ± 20.22	257.1 ± 21.56	228.5 ± 32.18
	Ventral prostate (mg)	152.7 ± 16.11	156.1 ± 32.23	147.2 ± 19.51	94.3 ± 21.81 *	40.1 ± 22.69 **,^
	Seminal vesicles (mg)	539.4 ± 77.45	601.4 ± 133.48	523.8 ± 85.71	401.9 ± 97.74	127.9 ± 54.21 **,^
	LABC muscles (mg)	528.8 ± 83.97	498.4 ± 75.97	451.3 ± 27.73	369.9 ± 40.58 **,^	194.2 ± 36.01 **,^
	Glans penis (mg)	80.2 ± 10.13	80.9 ± 9.92	76.4 ± 6.14	73.6 ± 7.84	53.8 ± 8.18 **,^
	Cowper's glands (mg)	29.3 ± 5.06	27.4 ± 1.68	23.5 ± 4.3	17.3 ± 2.54 **,^	10.7 ± 2.89 **,^
4	Starting Body Wt (g)	278.2 ± 12.07	278.0 ± 11.75	280.2 ± 19.85	278.2 ± 13.56	279.0 ± 15.56
	Terminal Body Wt (g)	377.9 ± 19.85	364.4 ± 11.71	376.6 ± 38.44	356.8 ± 23.13	333.7 ± 28.80*
	Ventral prostate (mg)	183.8 ± 81.46	166.1 ± 22.94	202.0 ± 48.74	159.3 ± 34.67	90.9 ± 27.43 **,^
	Seminal vesicles (mg)	551.2 ± 179.45	599.5 ± 185.33	594.2 ± 121.37	483.8 ± 194.37	310.0 ± 109.74 **
	LABC muscles (mg)	635.2 ± 72.45	616.5 ± 72.95	619.1 ± 47.67	525.5 ± 57.59 **	338.5 ± 43.53 **,^
	Glans penis (mg)	101.4 ± 6.34	100.9 ± 17.35	107.1 ± 7.16	100.2 ± 13.81	83.8 ± 13.63 *
	Cowper's glands (mg)	45.3 ± 6.66	45.7 ± 5.80	42.8 ± 5.41	33.2 ± 8.24 **,^	28.0 ± 5.98 **,^
8	Starting Body Wt (g)	248.7 ± 12.81	247.7 ± 12.20	248.5 ± 13.68	248.3 ± 12.28	248.6 ± 14.56
	Terminal Body Wt (g)	325.1 ± 19.36	325.9 ± 13.00	321.0 ± 17.19	322.1 ± 20.40	299.3 ± 24.69*
	Ventral prostate (mg)	210.4 ± 22.59	200.4 ± 24.02	195.9 ± 25.96	163.4 ± 25.67 *,^	78.0 ± 21.64 **,^
	Seminal vesicles (mg)	693.9 ± 78.29	590.2 ± 107.61	701.2 ± 131.03	460.8 ± 73.67 **,^	214.5 ± 45.64 **,^
	LABC muscles (mg)	655.7 ± 80.55	579.8 ± 29.46	606.2 ± 54.61	509.8 ± 37.96 *,^	341.0 ± 108.73 **,^
	Glans penis (mg)	97.4 ± 8.18	84.5 ± 3.70 *	92.7 ± 9.70	87.0 ± 6.20	68.4 ± 12.63 **,^
	Cowper's glands (mg)	50.8 ± 9.69	51.6 ± 10.14	46.1 ± 5.53	40.8 ± 9.59 *	27.0 ± 4.53 **,^
9	Starting Body Wt (g)	244.2 ± 10.65	245.8 ± 11.58	245.3 ± 9.56	244.8 ± 11.03	244.0 ± 14.03
	Terminal Body Wt (g)	335.3 ± 17.83	327.7 ± 15.53	326.8 ± 18.03	337.5 ± 15.41	317.8 ± 30.10
	Ventral prostate (mg)	144.2 ± 23.18	123.6 ± 13.05	123.0 ± 37.41	105.4 ± 10.88 *	71.4 ± 8.21 **,^
	Seminal vesicles (mg)	474.9 ± 37.42	423.8 ± 73.33	396.2 ± 78.63	382.8 ± 36.77 *,^	229.2 ± 26.02 **,^
	LABC muscles (mg)	372.0 ± 28.74	372.6 ± 43.20	340.4 ± 26.08	314.5 ± 29.16 **,^	231.2 ± 34.65 **,^
	Glans penis (mg)	110.6 ± 4.53	111.0 ± 10.63	112.0 ± 9.57	105.7 ± 4.75	88.3 ± 7.40 **,^
	Cowper's glands (mg)	36.7 ± 2.91	33.8 ± 5.32	31.4 ± 7.17	29.4 ± 4.11 *	19.1 ± 2.38 **,^

	R-square (%)			Overall means and [CV] for tissues at a given dose				
	OVR	**TRT**	**LAB**					
Ventral prostate (mg)	68	54	15	173 [29]	160 [25]	162 [30]	122 [38] **	64 [46] **
Seminal vesicles (mg)	73	65	7.1	565 [24]	545 [28]	550 [27]	394 [38] **	193 [50] **
LABC (mg)	81	49	36	548 [24]	506 [24]	498 [27] *	409 [31] **	268 [36] **
Glans penis (mg)	56	27	18	97 [14]	95 [18]	95 [15]	87 [15] *	71 [22] **
Cowper's glands (mg)	70	37	48	41 [26]	39 [31]	35 [30] **	28 [42] **	19 [47] **

TRT - Effect of treatments; LAB - Effect of laboratory; CV - Coefficient of variation.

[T] Significantly different from control at P<0.05 using. T-test pairwise comparisons.

[D] Significantly different from control at P<0.05 using two-tailed Dunnett's multiple comparisons of the tissue with body weight adjustment.

[a] The starting body weight is on the first day of substance administration and the terminal body weight is at necropsy approximately 24-hours after the last administration.

Table 27. Coefficients of variation for the mandatory endpoints in the *p,p'*-DDE (DDE) studies using 0.4 mg/kg/d TP

Lab	Testosterone Prop. (mg/kg/d)	0.4	0.4	0.4	0.4	0.4	MEAN[a]
	***p,p'*-DDE (mg/kg/d)**	**0**	**5**	**16**	**50**	**160**	
3	Terminal Body Wt (g)	8.07	13.15	7.39	8.39	14.08	9.74
	Ventral prostate (mg)	10.55	20.65	13.25	23.14	56.61	34.54
	Seminal vesicles (mg)	14.36	22.20	16.36	24.32	42.40	26.74
	LABC muscles (mg)	15.88	15.24	6.14	10.97	18.54	12.83
	Glans penis (mg)	12.64	12.26	8.03	10.68	15.21	14.11
	Cowper's glands (mg)	6.12	6.12	18.33	14.69	26.96	16.89
4	Terminal Body Wt (g)	5.25	3.21	10.21	6.48	8.58	6.75
	Ventral prostate (mg)	44.32	13.82	24.13	21.77	30.17	26.84
	Seminal vesicles (mg)	32.55	30.92	20.43	40.17	35.41	31.90
	LABC muscles (mg)	11.41	11.83	7.70	10.96	12.86	10.95
	Glans penis (mg)	6.26	17.19	6.68	13.78	16.26	12.03
	Cowper's glands (mg)	14.72	12.62	12.62	24.84	21.39	17.24
8	Terminal Body Wt (g)	5.96	3.99	5.36	6.33	8.25	5.82
	Ventral prostate (mg)	10.74	11.99	13.25	15.71	27.76	15.08
	Seminal vesicles (mg)	11.28	18.23	18.69	15.99	21.28	16.18
	LABC muscles (mg)	12.28	5.08	9.01	7.44	31.89	12.65
	Glans penis (mg)	8.40	4.37	10.46	7.13	18.46	9.26
	Cowper's glands (mg)	19.67	19.67	11.99	23.53	16.79	21.89
9	Terminal Body Wt (g)	5.32	4.74	5.52	4.57	9.47	5.80
	Ventral prostate (mg)	16.07	10.56	30.41	10.32	11.49	18.36
	Seminal vesicles (mg)	7.88	17.30	19.85	9.61	11.35	13.09
	LABC muscles (mg)	7.73	11.59	7.66	9.27	14.99	11.33
	Glans penis (mg)	4.10	9.58	8.54	4.49	8.38	6.65
	Cowper's glands (mg)	7.93	22.86	22.86	13.99	12.45	17.67

[a] The overall mean CV, depending upon the laboratory, may also include the vehicle control and a flutamide positive control.

Table 28. Optional organ weights in the *p,p'*-DDE (DDE) studies using 0.4 mg/kg/d TP.

Lab	Testosterone Prop. (mg/kg/d)	0.4	0.4	0.4	0.4	0.4
	***p,p'*-DDE (mg/kg/d)**	**0**	**5**	**16**	**50**	**160**
3	Terminal Body Wt (g)	259.3 ± 20.92	261.1 ± 34.32	273.5 ± 20.22	257.1 ± 21.56	228.5 ± 32.18
	Liver (g)	12.2 ± 0.99	12.9 ± 2.31	15.5 ± 1.51 **	16.7 ± 2.26 **	18.0 ± 2.89 **
	(relative to bw)	4.71%	4.95%	5.67%	6.51%	7.86%
	Adrenals (mg)	44.8 ± 7.27	43.2 ± 7.98	44.5 ± 3.89	45.6 ± 3.60	51.0 ± 8.4 **
	(relative to bw)	0.0173%	0.0165%	0.0163%	0.0177%	0.0223%
	Kidneys (mg)	1736.2 ± 106.59	1756.0 ± 259.19	1781.4 ± 197.78	1781.4 ± 161.22	1679.0 ± 241.0
	(relative to bw)	0.6703%	0.6725%	0.6514%	0.6929%	0.7348%
4	Terminal Body Wt (g)	377.9 ± 19.85	364.4 ± 11.71	376.6 ± 38.44	356.8 ± 23.13	333.7 ± 28.80*
	Liver (g)	15.7 ± 1.18	15.5 ± 0.72	18.2 ± 2.56 **	19.6 ± 1.54 **	23.1 ± 2.44 **
	(relative to bw)	4.15%	4.25%	4.83%	5.49%	6.88%
	Adrenals (mg)	57.2 ± 7.79	56.9 ± 8.91	56.1 ± 5.31	52.2 ± 5.29	59.3 ± 7.34
	(relative to bw)	0.0151%	0.0156%	0.0149%	0.0146%	0.0177%
	Kidneys (mg)	2265.1 ± 163.56	2232.5 ± 80.55	2306.4 ± 230.42	2217.2 ± 167.79	2214.5 ± 261.35
	(relative to bw)	0.5994%	0.6127%	0.6124%	0.6214%	0.6597%
8	Terminal Body Wt (g)	325.1 ± 19.36	325.9 ± 13.00	321.0 ± 17.19	322.1 ± 20.40	299.3 ± 24.69*
	Liver (g)	13.9 ± 1.28	14.7 ± 0.76	16.0 ± 1.16 **	19.0 ± 0.74 **	20.6 ± 2.99 **
	(relative to bw)	4.28%	4.51%	4.98%	5.90%	6.88%
	Adrenals (mg)	51.2 ± 6.41	59.0 ± 3.70	55.3 ± 5.84	50.5 ± 9.42	55.6 ± 10.99
	(relative to bw)	0.0157%	0.0181%	0.0172%	0.0157%	0.0186%
	Kidneys (mg)	2198.4 ± 186.19	2174.7 ± 105.99	2142.4 ± 118.00	2236.5 ± 125.02	2112.7 ± 151.36
	(relative to bw)	0.6762%	0.6673%	0.6674%	0.6943%	0.7059%
9	Terminal Body Wt (g)	335.3 ± 17.83	327.7 ± 15.53	326.8 ± 18.03	337.5 ± 15.41	317.8 ± 30.10
	Liver (g)	16.0 ± 1.60	17.2 ± 1.19 *	18.6 ± 1.33 **	23.1 ± 1.26 **	25.7 ± 3.35 **
	(relative to bw)	4.77%	5.25%	5.69%	6.84%	8.09%
	Adrenals (mg)	54.5 ± 7.56	57.9 ± 6.00	60.4 ± 4.45	65.2 ± 4.11	58.7 ± 6.28
	(relative to bw)	0.0163%	0.0177%	0.0185%	0.0193%	0.0185%
	Kidneys (mg)	2349.0 ± 96.38	2392.4 ± 118.63	2443.5 ± 172.32	2560.1 ± 157.59	2506.4 ± 226.47
	(relative to bw)	0.7006%	0.7301%	0.7477%	0.7585%	0.7887%

*, ** Significantly different from control at P<0.05 and P<0.01, respectively, using T-test pairwise comparisons.

131. Levator ani and Bulbocavernosus muscle complex (LABC). There were statistically significant dose-dependent decreases in the weights of the LABC in all laboratories with DDE. All laboratories achieved statistically significant decreases for the DDE-treated LABC at 50 mg/kg/d (Table 26). The overall mean CVs for the LABC ranged from 11 to 13 (Table 27).

132. Glans Penis (GP). There were statistically significant dose-dependent decreases in the weights of the GP in all laboratories with DDE. Labs 3, 8 and 9 achieved a statistically significant decrease for the DDE-treated GP at 160 mg/kg/d with both statistical approaches, and lab 4 achieved significance only with pairwise comparisons (Table 26). The overall mean CVs for the GP ranged from 7 to 14 (Table 27).

133. Cowper's Glands (COWS). There were statistically significant dose-dependent decreases in the weights of the COWS in all laboratories with DDE. Labs 3 and 4 achieved a statistically significant decrease for the DDE-treated COWS at 50 mg/kg/d with both statistical approaches, and labs 8 and 9 achieved significance at 50 mg/kg/d only with pairwise comparisons (Table 26). The overall mean CVs for the COWS ranged from 17 to 22 (Table 27).

134. Overall Review of Mandatory Endpoints. When the data were pooled across the participating laboratories, all five mandatory endpoints achieved statistical significance using the pairwise comparison approach. The LABC and COWS achieved significance at 16 mg DDE/kg/d, and the VP, SVCG, and GP achieved significance at 50 mg DDE/kg/d (Table 26). The R-square analyses indicate overall and treatment relationships exist for all tissues, and some relationships for laboratory effects exist for the LABC [36] and COWS [48] (Table 26).

135. Body weights. The initial body weights ranged from approximately 205 to 280 grams among the four laboratories. The body weight gains during the treatment period were reduced with increasing doses of DDE based on the starting and terminal body weights. The changes in body weights achieved statistical significance at 160 mg/kg/d DDE in labs 4 and 8 ($p<0.05$) (Table 26) and in the overall pooled data at the same dose ($p<0.01$).

136. Optional organ weights. The absolute liver organ weights were changed by DDE administration (Table 27). The liver weights were dramatically increased by DDE in all laboratories, achieving statistical significance at 5 mg/kg/d in lab 9 and at 16 mg/kg/d in the other three laboratories. The paired adrenal weights were significantly increased only in lab 3 at the high DDE dose. The paired kidney weights were not significantly changed by DDE administration.

Results of *p,p'*-DDE studies using 0.2 mg/kg/d TP coadministration

137. The summary results of the accessory tissue weights and the statistical analyses for the DDE studies conducted using 0.2 mg/kg/d TP coadministration are reported in Table 29. The results show that the Hershberger bioassay successfully and reproducibly detected DDE in all laboratories. All five sex accessory tissue weights decreased with increasing DDE doses and achieved statistical significance in all labs.

138. Ventral Prostate (VP). There were statistically significant dose-dependent decreases in the weights of the VP in three of four laboratories with DDE. Labs 10, 11, and 16 achieved statistically significant decreases for the DDE-treated VP at 30 mg/kg/d, and labs 12 and 14 achieved significance at 100 mg/kg/d (Table 29). The overall mean CVs for the VP ranged from 15 to 31 (Table 30).

139. Seminal Vesicles and Coagulating Glands (SVCG). There were statistically significant dose-dependent decreases in the weights of the SVCG in all laboratories with DDE. Labs 10, 11, 12 and 15 achieved significant decreases for the DDE-treated SVCG at 30 mg/kg/d, and lab 13 achieved significance at 100 mg/kg/d (Table 29). The overall mean CVs for the SVCG ranged from 16 to 24 (Table 30).

Table 29. Body weights, mandatory tissue weights and pooled statistics in *p,p'*-DDE (DDE) studies using 0.2 mg/kg/d TP

Lab	Testosterone Propionate (mg/kg/d)	0.2	0.2	0.2	0.2	0.2
	***p,p'*-DDE (mg/kg/d)**	**0**	**3**	**10**	**30**	**100**
10	Starting Body Wt (g) [a]	220.8 ± 8.33	219.8 ± 8.72	219.9 ± 7.31	220.5 ± 8.99	224.8 ± 6.10
	Terminal Body Wt (g) [a]	276.6 ± 11.71	270.2 ± 9.94	272.6 ± 8.38	274.7 ± 11.41	273.0 ± 8.01
	Ventral prostate (mg)	106.2 ± 13.20	89.8 ± 6.28 **	100.0 ± 18.09	71.7 ± 13.49 **,^	52.4 ± 8.47 **,^
	Seminal vesicles (mg)	225.7 ± 49.00	219.4 ± 29.11	202.4 ± 13.19	164.5 ± 35.71 **,^	75.1 ± 11.69 **,^
	LABC muscles (mg)	300.3 ± 31.26	305.7 ± 22.67	290.3 ± 33.57	309.0 ± 59.86	209.9 ± 16.29 **,^
	Glans penis (mg)	67.0 ± 4.45	62.3 ± 5.13	66.3 ± 3.02	65.0 ± 5.42	56.6 ± 7.47 **,^
	Cowper's glands (mg)	21.0 ± 2.35	21.5 ± 3.1	19.8 ± 1.28	15.4 ± 2.93 **,^	11.0 ± 2.18 **,^
11	Starting Body Wt (g)	237.6 ± 7.92	237.1 ± 8.75	237.9 ± 9.29	238.9 ± 9.06	237.3 ± 9.78
	Terminal Body Wt (g)	313.4 ± 16.57	313.4 ± 11.52	313.0 ± 12.67	317.3 ± 14.54	303.3 ± 17.14
	Ventral prostate (mg)	137.8 ± 33.84	125.7 ± 26.44	128.8 ± 22.07	93.6 ± 26.49 **	51.1 ± 16.36 **,^
	Seminal vesicles (mg)	387.2 ± 63.24	272.3 ± 21.77 **	377.0 ± 47.69	256.0 ± 68.18 **,^	88.3 ± 42.45 **,^
	LABC muscles (mg)	549.9 ± 35.88	521.9 ± 63.63	519.4 ± 57.96	458.5 ± 59.68 **,^	300.4 ± 31.12 **,^
	Glans penis (mg)	73.3 ± 2.72	76.5 ± 3.90	73.5 ± 2.72	73.6 ± 3.46	63.0 ± 3.04 **,^
	Cowper's glands (mg)	27.2 ± 7.21	21.8 ± 4.43	28.3 ± 4.77	23.7 ± 7.17	17.4 ± 3.96 **,^
12	Starting Body Wt (g)	170.9 ± 6.87	171.4 ± 3.47	171.1 ± 5.77	170.5 ± 4.25	172.0 ± 4.98
	Terminal Body Wt (g)	240.9 ± 7.31	241.4 ± 6.78	241.4 ± 9.46	238.0 ± 6.45	235.8 ± 8.61
	Ventral prostate (mg)	90.6 ± 16.56	79.1 ± 7.84	88.1 ± 14.65	89.6 ± 12.10	56.5 ± 7.03 **,^
	Seminal vesicles (mg)	282.8 ± 33.32	246.2 ± 48.56	240.0 ± 27.39	231.6 ± 39.18 **	152.0 ± 31.77 **,^
	LABC muscles (mg)	435.7 ± 74.78	430.3 ± 52.45	407.0 ± 14.95	408.2 ± 55.67	311.9 ± 28.81 **,^
	Glans penis (mg)	65.5 ± 8.62	64.9 ± 3.66	63.8 ± 5.80	66.9 ± 6.69	52.0 ± 10.93 **,^
	Cowper's glands (mg)	26.2 ± 4.04	26.7 ± 4.9	25.4 ± 2.77	25.9 ± 3.46	17.0 ± 2.17 **,^
14	Starting Body Wt (g)	244.3 ± 6.97	243.2 ± 7.46	242.6 ± 4.73	244.9 ± 5.38	245.1 ± 6.86
	Terminal Body Wt (g)	319.4 ± 14.69	326.9 ± 10.90	322.9 ± 6.93	323.6 ± 7.73	307.3 ± 12.49
	Ventral prostate (mg)	153.3 ± 20.50	158.4 ± 40.49	165.9 ± 43.29	141.8 ± 16.57	77.6 ± 27.64 **,^
	Seminal vesicles (mg)	371.1 ± 56.95	432.6 ± 47.60	411.8 ± 60.35	326.1 ± 81.75	148.1 ± 47.07 **,^
	LABC muscles (mg)	518.7 ± 11.70	574.3 ± 39.93 **	547.0 ± 38.67	490.9 ± 54.06	291.3 ± 31.25 **,^
	Glans penis (mg)	77.3 ± 3.89	75.9 ± 4.87	76.5 ± 5.63	74.0 ± 4.29	60.8 ± 2.10 **,^
	Cowper's glands (mg)	33.9 ± 6.42	33.8 ± 5.6	32.8 ± 4.31	32.6 ± 9.05	20.2 ± 5.79 **,^
16	Starting Body Wt (g)	224.6 ± 10.24	223.8 ± 9.60	223.6 ± 12.35	222.5 ± 9.98	224.0 ± 9.93
	Terminal Body Wt (g)	292.7 ± 23.38	291.0 ± 15.15	290.4 ± 18.64	293.2 ± 15.55	289.4 ± 12.62
	Ventral prostate (mg) [b]	115.0 ± 18.8	101.3 ± 27.6	103.3 ± 16.5	74.7 ± 18.3 **,^	48.7 ± 12.2 **,^
	Seminal vesicles (mg)	237.2 ± 27.21	219.5 ± 41.02	251.4 ± 48.84	156.8 ± 33.24 **,^	82.7 ± 23.07 **,^
	LABC muscles (mg)	495.5 ± 79.30	496.6 ± 30.97	450.9 ± 48.10	395.1 ± 30.79 **,^	301.5 ± 42.63 **,^
	Glans penis (mg)	81.0 ± 6.76	80.1 ± 3.27	77.6 ± 4.85	76.2 ± 5.02	66.7 ± 6.76 **,^
	Cowper's glands (mg) [b]	30.2 ± 4.55	28.1 ± 5.1	25.5 ± 4.43	24.2 ± 6.02 **	12.3 ± 3.13 **,^

Table 29 continued. Body weights, mandatory tissue weights and pooled statistics in *p,p'*-DDE (DDE) studies using 0.2 mg/kg/d TP

Lab	Testosterone Propionate (mg/kg/d)	0.2	0.2	0.2	0.2	0.2
	***p,p'*-DDE (mg/kg/d)**	**0**	**3**	**10**	**30**	**100**
	R-square (%)			**Overall means and [CV] for tissues at a given dose**		
	OVR	**TRT**	**LAB**					
Ventral prostate (mg)	63	36	32	121 [25]	111 [34]	117 [31]	94 [33] **	57 [32] **
Seminal vesicles (mg)	45	45	31	301 [27]	278 [32] **	297 [31]	227 [36] **	109 [42] **
LABC (mg)	69	37	42	460 [22]	466 [22]	443 [23]	412 [19] **	283 [17] **
Glans penis (mg)	54	30	37	73 [11]	72 [11]	72 [10]	71 [9]	60 [14] **
Cowper's glands (mg)	56	33	29	28 [24]	26 [24]	26 [21]	24 [33] **	16 [31] **

OVR – Overall effect on tissue; TRT - Effect of treatments; LAB - Effect of laboratory; CV - Coefficient of variation.

*, ** Significantly different from control at P<0.05 and P<0.01, respectively, using T-test pairwise comparisons.

^ Significantly different from control at P<0.05 using two-tailed Dunnett's multiple comparisons of the tissue with body weight adjustment.

[a] The starting body weight is on the first day of substance administration and the terminal body weight is at necropsy approximately 24-hours after the last administration.

[b] The VP and COWS were fixed and then weighed in laboratory 16

Table 30. Coefficients of variation for the mandatory endpoints in the *p,p'*-DDE (DDE) studies using 0.2 mg/kg/d TP.

Lab	Methyl Testosterone (mg/kg/d)	0.2	0.2	0.2	0.2	0.2	MEAN[a]
	***p,p'*-DDE (mg/kg/d)**	**0**	**3**	**10**	**30**	**100**	
10	Terminal Body Wt (g)	4.23	3.68	3.08	4.15	2.94	3.78
	Ventral prostate (mg)	12.43	6.99	18.10	18.82	16.16	14.53
	Seminal vesicles (mg)	21.72	13.27	6.51	21.71	15.56	16.19
	LABC muscles (mg)	10.41	7.42	11.57	19.38	7.76	12.37
	Glans penis (mg)	6.64	8.23	4.56	8.33	13.21	8.20
	Cowper's glands (mg)	11.19	14.51	6.47	19.09	19.85	14.91
11	Terminal Body Wt (g)	5.29	3.68	4.05	4.58	5.65	4.73
	Ventral prostate (mg)	24.57	21.04	17.13	28.30	32.01	30.99
	Seminal vesicles (mg)	16.33	8.00	12.65	26.64	48.09	24.17
	LABC muscles (mg)	6.53	12.19	11.16	13.02	10.36	10.82
	Glans penis (mg)	3.72	5.10	3.70	4.71	4.82	5.38
	Cowper's glands (mg)	20.28	20.28	16.85	30.24	22.77	22.07
12	Terminal Body Wt (g)	3.03	2.81	3.92	2.71	3.65	3.46
	Ventral prostate (mg)	18.28	9.91	16.63	13.51	12.45	21.76
	Seminal vesicles (mg)	11.78	19.73	11.41	16.92	20.90	16.71
	LABC muscles (mg)	17.16	12.19	3.67	13.64	9.24	12.36
	Glans penis (mg)	13.15	5.63	9.08	10.01	21.05	15.53
	Cowper's glands (mg)	15.41	18.31	10.92	13.38	12.75	16.16
14	Terminal Body Wt (g)	4.60	3.33	2.15	2.39	4.06	3.25
	Ventral prostate (mg)	13.38	25.56	26.09	11.68	35.61	22.39
	Seminal vesicles (mg)	15.35	11.00	14.65	25.07	31.79	17.39
	LABC muscles (mg)	2.26	6.95	7.07	11.01	10.73	7.88
	Glans penis (mg)	5.03	6.41	7.36	5.79	3.46	6.26
	Cowper's glands (mg)	18.92	16.45	13.14	27.74	28.66	21.07
16	Terminal Body Wt (g)	7.99	5.21	6.42	5.30	4.36	5.90
	Ventral prostate (mg)[b]	16.34	27.29	15.93	24.46	25.11	19.65
	Seminal vesicles (mg)	11.47	18.69	19.43	21.20	27.89	18.18
	LABC muscles (mg)	16.00	6.24	10.67	7.79	14.14	11.50
	Glans penis (mg)	8.35	4.08	6.24	6.59	10.14	7.00
	Cowper's glands (mg)[b]	15.09	18.25	17.38	24.85	25.54	21.57

[a] The overall mean CV, depending upon the laboratory, may also include a vehicle control group and a flutamide positive control group.

[b] The ventral prostate and Cowper's glands were first fixed and then weighed in laboratory 16

140. Levator ani and Bulbocavernosus muscle complex (LABC). There were statistically significant dose-dependent decreases in the LABC weight in all labs with DDE. Labs 11 and 16 achieved statistically significant decreases for the DDE-treated LABC at 30 mg/kg/d, and labs 10, 12 and 14 achieved significance at 100 mg/kg/d (Table 29). The overall mean CVs for the LABC ranged from 8 to 12 (Table 30).

141. Glans Penis (GP). There were statistically significant dose-dependent decreases in the GP weight in all laboratories with DDE. All five laboratories achieved a statistically significant decrease for the DDE-treated GP at 100 mg/kg/d (Table 23). The overall mean CVs for the GP ranged from 5 to 16 (Table 30).

142. Cowper's Glands (COWS). There were statistically significant dose-dependent decreases in the COWS weight in all laboratories with DDE. Laboratories 10 and 16 achieved statistically significant decreases for the DDE-treated COWS at 30 mg/kg/d, and labs 11, 12, and 14 achieved significance at 100 mg/kg/d (Table 29). The overall mean CVs for the SVCG ranged from 15 to 22 (Table 304).

143. Overall Review of Mandatory Endpoints. When the data were pooled across the participating laboratories, all five mandatory endpoints achieved statistical significance using the pairwise comparison approach. The statistical significance for the SVCG at 3 mg DDE/kg/d is judged to be spurious where labs 10 and 11 had significant decreases and lab 14 displayed a significant increase in this particular tissue at this dose. This suggests some possible variability in the TP baseline. The significance was achieved using the pairwise comparison technique and did not occur with the multiple comparisons Dunnett's technique. The VP, SCVG, LABC and COWS all achieved significance at 30 mg/kg/d, and the GP achieved significance at 100 mg DDE/kg/d (Table 29). The R-square analyses indicated moderate to strong overall relationships and treatment relationships and indicated laboratory effects for all tissues (Table 29).

144. Body weights. The initial body weights for the five labs ranged from approximately 170 to 245 grams. The body weight gains during the treatment period were slightly reduced at the high doses DDE based on the starting and terminal body weights (Table 29). This is in contrast to the labs with the other dose series (Table 26). The difference appears to be related to the high DDE dose which was 160 mg/kg/d in the affected dose series and lower at 100 mg/kg/d in the unaffected dose series.

Comparison of *p,p'*-DDE results with different TP coadministration doses

145. The DDE results using 0.2 mg/kg/d TP coadministered dose were produced approximately one year earlier than the results using 0.4 mg/kg/d TP. As with other test substance studies in this report, there were modest differences in body weights among the animals in these studies and some apparent absolute weight differences in the tissues that may be attributed to variations in dissection and handling among the laboratories. In addition, there were strain, diet and other differences among laboratories (see Table 4). However, the results were similar with the weak DDE antagonist, supporting the reproducibility and robustness of the Hershberger bioassay.

146. To illustrate the consistency of the dose response of these tissues, the relative decreases in tissue weights in response to DDE administration have been analyzed. Figures 5A-E show the relative decrease for the five tissues in the nine laboratories with increasing DDE doses coadministered with TP and using the group administered only TP as the control. The results for the mean relative decrease show the excellent reproducibility of the overall dose response.

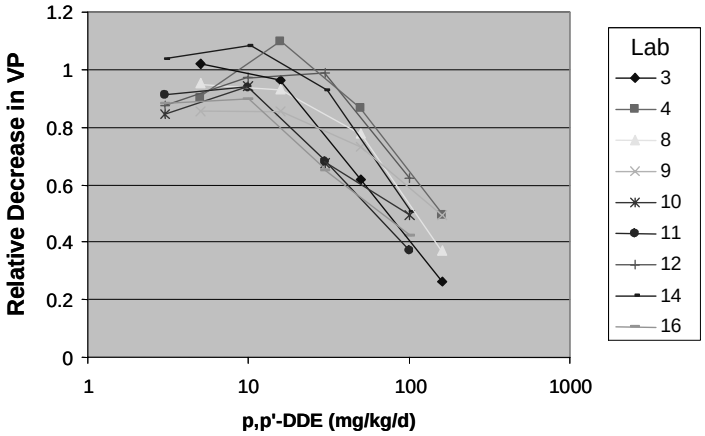


Figure 5A. Relative decrease in ventral prostate (VP) mean weights with DDE doses in nine laboratories. Labs 3 through 9 used 0.4 mg/kg/d TP and labs 10 through 16 used 0.2 mg/kg/d TP.

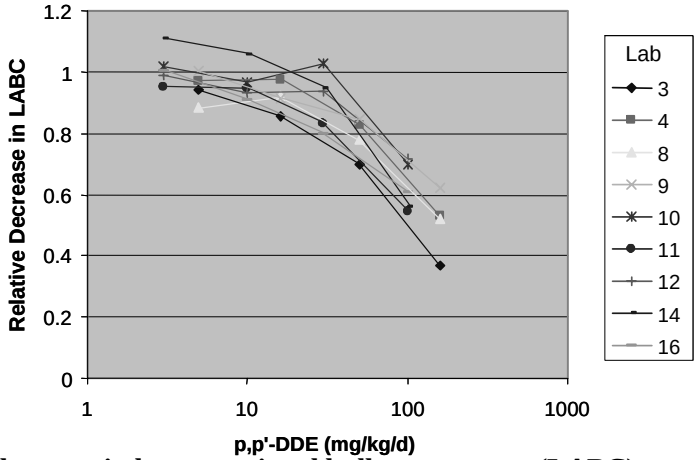


Figure 5B. Relative decrease in levator ani and bulbocavernosus (LABC) mean weights with DDE doses in nine laboratories. Labs 3 through 9 used 0.4 mg/kg/d TP and labs 10 through 16 used 0.2 mg/kg/d TP.

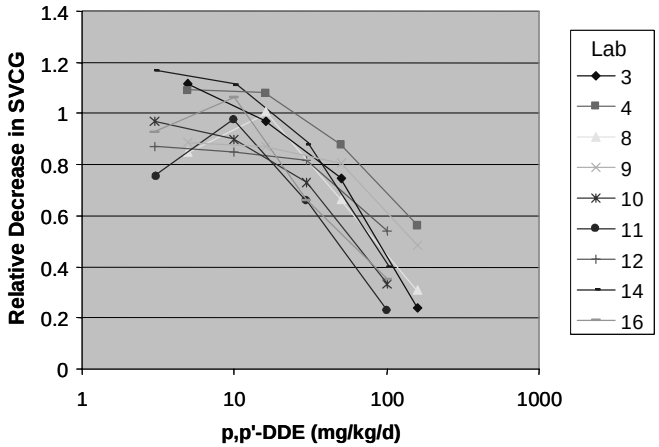


Figure 5C. Relative decrease in seminal vesicles and coagulating gland (SVCG) mean weights with DDE doses in nine laboratories. Labs 3 through 9 used 0.4 mg/kg/d TP and labs 10 through 16 used 0.2 mg/kg/d TP.

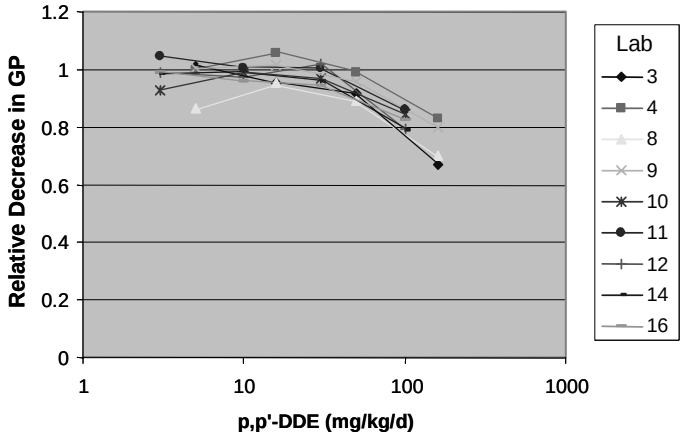


Figure 5D. Relative decrease in glans penis (GP) mean weights with DDE doses in nine laboratories. Labs 3 through 9 used 0.4 mg/kg/d TP and labs 10 through 16 used 0.2 mg/kg/d TP.

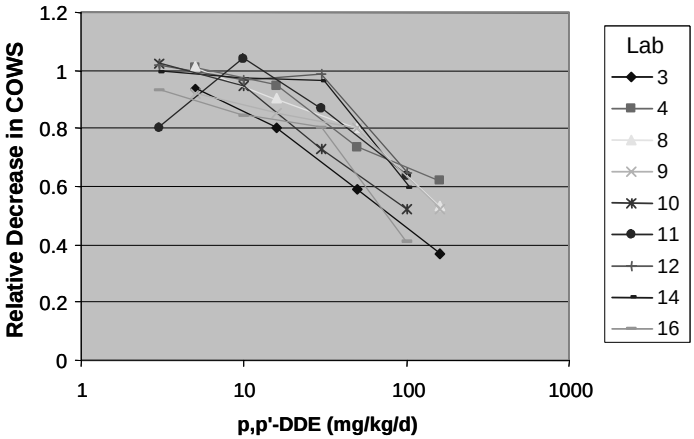


Figure 5E. Relative decrease in Cowper's gland (COWS) mean weights with DDE doses in nine laboratories. Labs 3 through 9 used 0.4 mg/kg/d TP and labs 10 through 16 used 0.2 mg/kg/d TP.

PHASE 2: 5α-REDUCTASE INHIBITORS

147. FIN was employed as a 5α-reductase inhibitor test substance in Phase-2 laboratory studies.

Finasteride

148. Four laboratories tested four doses of finasteride (FIN) with coadministration of 0.4 mg/kg/d TP in order to assess the ability of the Hershberger bioassay to detect 5α-reductase inhibitors. All four laboratories conducted the assigned studies as intended, submitted their laboratory and study data electronically using standardized Excel spreadsheets, audited the study data, and, if necessary, informed the Secretariat of data corrections.

Results of Finasteride studies

149. The results of the accessory organ and tissue weights and the statistical analyses for the FIN studies are reported in Table 31. FIN proved to be very potent; in three laboratories a NOEL was not observed in

one or more tissues (VP, SCVG, LABC, COWS), indicating that, although the original suggested doses for Phase-2 had been reduced from the original recommendation of the Lead Laboratory, this reduction of nearly one order of magnitude had not been sufficient.

150. The results show that the Hershberger bioassay successfully and reproducibly detected FIN in all laboratories. The weights of all five sex accessory tissues decreased with increasing FIN doses in a dose-responsive manner, and the decreases in four tissues were consistently statistically significant. However, the GP did not achieve significance in either laboratory 2 or 6.

151. Ventral Prostate (VP). There were statistically significant dose-dependent decreases in the weights of the VP in all laboratories with FIN. Laboratories 5 and 9 achieved a statistically significant decrease for the FIN-treated GP at the lowest 0.2 mg/kg/d, and labs 2 and 6 achieved significance at 1 mg/kg/d (Table 31). The overall mean CVs for the VP ranged from 16 to 32 (Table 32).

152. Seminal Vesicles and Coagulating Glands (SVCG). There were statistically significant dose-dependent decreases in the weights of the SVCG in all laboratories with FIN. Labs 5, 6 and 9 achieved a statistically significant decrease for the FIN-treated GP at the lowest 0.2 mg/kg/d, and lab 2 achieved significance at 1 mg/kg/d (Table 31). The overall mean CVs for the SVCG ranged from 13 to 46 (Table 32).

153. Levator ani and Bulbocavernosus muscle complex (LABC). There were statistically significant dose-dependent decreases in the weights of the LABC in all laboratories with FIN. Labs 5 and 9 achieved a statistically significant decrease for the FIN-treated GP at 1 mg/kg/d with both statistical approaches, lab 2 achieved significance at 1 mg/kg/d with pairwise comparisons, and lab 6 achieved significance at 5 mg/kg/d (Table 31). The overall mean CVs for the LABC ranged from 9 to 14 (Table 32).

154. Glans Penis (GP). There were statistically significant dose-dependent decreases in the weights of the GP in two of four laboratories with FIN. Laboratories 5 and 9 achieved a statistically significant decrease for the FIN-treated GP at 0.2 mg/kg/d with pairwise comparisons. In contrast, laboratory 6 achieved a statistically significant decrease at 5 mg/kg/d, but not at 25 mg/kg/d, so this event was judged not to be a dose response. In laboratory 2, FIN-treatment did not significantly reduce the GP weight at any dose (Table 31). Laboratory 5, as noted previously, did not dissect and weight the GP in cases where preputial separation was judged to be incomplete; this reduced the group size in some cases (see notations for lab 5 in Table 31). The overall mean CVs for the GP ranged from 8 to 12 (Table 32).

155. Cowper's Glands (COWS). There were statistically significant dose-dependent decreases in the weights of the COWS in all laboratories with FIN. Laboratory 5 achieved a statistically significant decrease for the FIN-treated GP at the lowest dose of 0.2 mg/kg/d, lab 2 achieved significance at 1 mg/kg/d laboratories, and 6 and 9 achieved significance at 5 mg/kg/d, and (Table 31). The overall mean CVs for the COWS ranged from 17 to 39 (Table 32).

156. Overall Review of Mandatory Endpoints. When the data were pooled across the participating laboratories, all five mandatory endpoints achieved statistical significance. All tissues achieved significance at 0.2 mg FIN/kg/d using pairwise comparisons, indicating that a NOEL was not observed. This was despite the reduction in the lowest dose by five-fold from the original recommendation of 1 mg/kg/d for the lowest dose in the series (Table 31). The R-square analyses indicate strong overall relationships; strong treatment relationships for the VP, SVCG, and COWS; and some relationships for laboratory effects for the LABC [60] and GP [49] (Table 31). This treatment relationship is consistent with the expected sensitivity of the VP, SVCG, and COWS to FIN as these tissues have the 5α-reductase, while the LABC and GP do not.

Table 31. Body weights, mandatory tissue weights and pooled statistics in finasteride (FIN) studies

Lab	Testosterone Propionate (mg/kg/d)	0.4	0.4	0.4	0.4	0.4
	Finasteride (mg/kg/d)	0	0.2	1	5	25
2	Starting Body Wt (g) [a]	211.5 ± 9.09	212.2 ± 10.65	208.8 ± 4.26	209.3 ± 6.89	211.8 ± 6.43
	Terminal Body Wt (g) [a]	301.2 ± 20.90	287.9 ± 18.69	288.5 ± 12.64	283.9 ± 21.19	293.2 ± 6.79
	Ventral prostate (mg)	133.1 ± 36.20	115.5 ± 21.60	67.4 ± 27.29 **,^	54.0 ± 16.10 **,^	42.7 ± 12.53 **,^
	Seminal vesicles (mg)	316.2 ± 107.89	186.0 ± 49.63	89.1 ± 57.74 **,^	102.3 ± 39.28 **,^	62.8 ± 50.88 **,^
	LABC muscles (mg)	301.1 ± 36.40	286.1 ± 20.89	254.6 ± 11.60 *	254.5 ± 19.09 *	247.6 ± 46.75 **,^
	Glans penis (mg)	79.7 ± 6.44	72.7 ± 7.13	72.2 ± 8.96	69.7 ± 8.53	70.2 ± 16.11
	Cowper's glands (mg)	28.8 ± 8.72	24.4 ± 2.68	15.8 ± 10.24 *	14.7 ± 4.71 *	10.3 ± 4.69 **,^
5	Starting Body Wt (g)	221.7 ± 13.03	222.7 ± 11.06	227.5 ± 9.85	228.3 ± 14.54	226.3 ± 8.04
	Terminal Body Wt (g)	272.8 ± 21.48	273.5 ± 17.24	278.0 ± 22.53	276.0 ± 22.92	275.0 ± 15.10
	Ventral prostate (mg)	155.1 ± 25.55	62.2 ± 14.14 **,^	58.2 ± 16.80 **,^	49.4 ± 38.22 **,^	36.8 ± 19.67 **,^
	Seminal vesicles (mg)	576.1 ± 96.66	166.4 ± 30.55 **,^	137.9 ± 30.00 **,^	114.7 ± 19.79 **,^	97.9 ± 16.83 **,^
	LABC muscles (mg)	463.9 ± 50.41	317.1 ± 40.87 **,^	336.8 ± 55.83 **,^	303.5 ± 58.30 **,^	242.7 ± 35.32 **,^
	Glans penis (mg) [b]	84.4 ± 6.03	71.9 ± 4.83 **	68.3 ± 4.92 **,^	70.9 ± 5.65 ** (4)	61.5 ± 5.82 **,^ (3)
	Cowper's glands (mg)	34.8 ± 4.84	21.3 ± 3.40 **,^	18.4 ± 2.55 **,^	15.3 ± 3.03 **,^	8.5 ± 2.36 **,^
6	Starting Body Wt (g)	240.4 ± 11.52	242.2 ± 10.10	240.8 ± 10.88	241.0 ± 6.14	238.0 ± 8.34
	Terminal Body Wt (g)	311.9 ± 18.24	316.7 ± 12.13	317.3 ± 14.90	313.9 ± 10.83	314.3 ± 13.60
	Ventral prostate (mg)	116.9 ± 27.88	73.2 ± 15.81	57.4 ± 27.09 **,^	53.4 ± 14.30 **,^	29.8 ± 13.44 **,^
	Seminal vesicles (mg)	425.5 ± 54.25	192.7 ± 58.04 **,^	212.5 ± 48.29 **,^	132.1 ± 27.73 **,^	139.6 ± 56.40 **,^
	LABC muscles (mg)	512.1 ± 70.15	455.5 ± 55.74	542.1 ± 48.95	408.7 ± 53.47 **,^	473.3 ± 33.73
	Glans penis (mg)	97.4 ± 8.64	90.2 ± 8.95	100.9 ± 11.04	85.1 ± 3.71 **	99.5 ± 3.71
	Cowper's glands (mg)	26.4 ± 4.54	22.5 ± 5.10	26.6 ± 7.22	16.2 ± 6.07 **,^	15.0 ± 4.05 **,^
9	Starting Body Wt (g)	249.7 ± 9.81	255.2 ± 10.70	251.2 ± 8.66	252.8 ± 6.27	254.3 ± 7.06
	Terminal Body Wt (g)	322.3 ± 13.08	333.5 ± 14.10	330.3 ± 11.38	338.2 ± 14.47	334.3 ± 13.44
	Ventral prostate (mg)	122.0 ± 25.06	79.0 ± 11.56 **,^	72.6 ± 11.29 **,^	58.8 ± 7.48 **,^	45.1 ± 8.50 **,^
	Seminal vesicles (mg)	428.9 ± 45.19	274.6 ± 54.49 **,^	222.7 ± 14.82 **,^	172.2 ± 28.02 **,^	140.3 ± 14.50 **,^
	LABC muscles (mg)	385.8 ± 37.60	362.9 ± 31.72	304.5 ± 24.86 **,^	301.2 ± 15.61 **,^	301.8 ± 23.52 **,^
	Glans penis (mg)	106.6 ± 16.62	92.5 ± 6.44 *	90.9 ± 5.74 *	84.8 ± 4.43 **,^	82.1 ± 6.39 **,^
	Cowper's glands (mg)	33.1 ± 4.32	31.2 ± 4.67	27.3 ± 3.94	20.5 ± 4.51 **,^	16.0 ± 3.47 **,^

	R-square (%)			Overall means and [CV] for tissues at a given dose				
	OVR	TRT	LAB					
Ventral prostate (mg)	71	61	3.5	132 [23]	83 [31] **	64 [33] **	54 [37] **	39 [38] **
Seminal vesicles (mg)	82	61	15	437 [26]	205 [30] **	166 [41] **	131 [30] **	110 [45] **
LABC (mg)	56	15	60	416 [23]	355 [21] **	359 [33] **	318 [22] **	316 [32] **
Glans penis (mg)	44	12	49	92 [16]	82 [14] **	83 [19] **	78 [12] **	81 [21] **
Cowper's glands (mg)	64	46	10	31 [21]	25 [22] *	22 [37] **	17 [30] **	12 [38] **

OVR – Overall effect on tissue; TRT - Effect of treatments; LAB - Effect of laboratory; CV - Coefficient of variation.

*,** Significantly different from control at P<0.05 and P<0.01, respectively, using T-test pairwise comparisons.

^ Significantly different from control at P<0.05 using two-tailed Dunnett's multiple comparisons of the tissue with body weight adjustment.

[a] The starting body weight is on the first day of substance administration and the terminal body weight is at necropsy approximately 24-hours after the last administration.

[b] If preputial separation was incomplete, this lab did not dissect and weigh that individual GP. Actual numbers per group are in parenthesis, if the group size was decreased as a result.

Table 32. Coefficients of variation for the mandatory endpoints in the finasteride (FIN) studies

Lab	Testosterone Prop. (mg/kg/d)	0.4	0.4	0.4	0.4	0.4	MEAN[a]
	Finasteride (mg/kg/d)	0	0.2	1	5	25	
2	Terminal Body Wt (g)	6.94	6.49	4.38	7.46	2.32	4.94
	Ventral prostate (mg)	27.21	18.71	40.50	29.82	29.34	29.32
	Seminal vesicles (mg)	34.13	26.68	64.80	38.39	80.97	46.40
	LABC muscles (mg)	12.09	7.30	4.56	7.50	18.88	11.53
	Glans penis (mg)	8.08	9.81	12.41	12.24	22.96	12.33
	Cowper's glands (mg)	10.99	10.99	64.88	32.12	45.71	38.76
5	Terminal Body Wt (g)	7.87	6.30	8.10	8.31	5.49	7.24
	Ventral prostate (mg)	16.47	22.73	28.86	77.39	53.48	31.56
	Seminal vesicles (mg)	16.78	18.36	21.76	17.26	17.20	15.94
	LABC muscles (mg)	10.87	12.89	16.58	19.21	14.56	14.11
	Glans penis (mg)	7.15	6.72	7.21	7.96	9.46	9.82
	Cowper's glands (mg)	41.19	15.94	13.88	19.80	27.90	25.26
6	Terminal Body Wt (g)	5.85	3.83	4.69	3.45	4.33	4.53
	Ventral prostate (mg)	23.85	21.58	47.22	26.78	45.16	38.22
	Seminal vesicles (mg)	12.75	30.11	22.73	20.99	40.42	28.14
	LABC muscles (mg)	13.70	12.24	9.03	13.08	7.13	12.59
	Glans penis (mg)	8.87	9.92	10.95	4.36	3.73	8.53
	Cowper's glands (mg)	38.76	22.62	27.19	37.46	27.90	35.78
9	Terminal Body Wt (g)	4.06	4.23	3.44	4.28	4.02	4.13
	Ventral prostate (mg)	20.53	14.64	15.54	12.73	18.84	15.50
	Seminal vesicles (mg)	10.54	19.84	6.66	16.27	10.33	12.61
	LABC muscles (mg)	9.75	8.74	8.16	5.18	7.80	9.47
	Glans penis (mg)	15.59	6.96	6.31	5.22	7.79	8.33
	Cowper's glands (mg)	13.06	14.99	14.47	22.03	21.72	16.88

[a] The overall mean CV, depending upon the laboratory, may also include the vehicle control and a flutamide positive control.

Table 33. Optional organ weights in the finasteride (FIN) studies.

Lab	Testoster. Prop. (mg/kg/d)	0.4	0.4	0.4	0.4	0.4
	Finasteride (mg/kg/d)	**0**	**0.2**	**1**	**5**	**25**
2	Terminal Body Wt (g)	301.2 ± 20.90	287.9 ± 18.69	288.5 ± 12.64	283.9 ± 21.19	293.2 ± 6.79
	Liver (g)	12.2 ± 1.03	11.5 ± 1.59	11.6 ± 1.52	11.3 ± 1.01	12.5 ± 0.70
	(relative to bw)	4.05%	3.99%	4.02%	3.98%	4.26%
	Adrenals (mg)	55.3 ± 7.75	54.8 ± 9.64	45.7 ± 12.18	55.8 ± 4.38	52.3 ± 11.30
	(relative to bw)	0.0184%	0.0190%	0.0158%	0.0197%	0.0178%
	Kidneys (mg)	2194.7 ± 149.79	1947.8 ± 185.24	1988.0 ± 138.86	1961.7 ± 211.31	2013.3 ± 109.20
	(relative to bw)	0.7287%	0.6766%	0.6891%	0.6910%	0.6867%
5	Terminal Body Wt (g)	272.8 ± 21.48	273.5 ± 17.24	278.0 ± 22.53	276.0 ± 22.92	275.0 ± 15.10
	Liver (g)	10.8 ± 1.85	10.6 ± 1.41	10.7 ± 1.86	10.5 ± 1.13	10.8 ± 1.23
	(relative to bw)	3.96%	3.88%	3.85%	3.80%	3.93%
	Adrenals (mg)	57.6 ± 10.52	58.5 ± 7.09	52.8 ± 5.85	52.5 ± 4.64	52.7 ± 11.18
	(relative to bw)	0.0211%	0.0214%	0.0190%	0.0190%	0.0192%
	Kidneys (mg)	1724.7 ± 120.74	1718.0 ± 115.96	1680.7 ± 185.77	1750.9 ± 126.18	1669.3 ± 182.22
	(relative to bw)	0.6322%	0.6282%	0.6046%	0.6344%	0.6070%
6	Terminal Body Wt (g)	311.9 ± 18.24	316.7 ± 12.13	317.3 ± 14.90	313.9 ± 10.83	314.3 ± 13.60
	Liver (g)	15.8 ± 1.65	16.5 ± 1.98	16.6 ± 1.66	16.7 ± 1.98	16.7 ± 1.96
	(relative to bw)	5.07%	5.21%	5.23%	5.32%	5.31%
	Adrenals (mg)	59.3 ± 9.62	57.4 ± 6.53	53.6 ± 13.68	53.7 ± 7.92	51.7 ± 6.13
	(relative to bw)	0.0190%	0.0181%	0.0169%	0.0171%	0.0164%
	Kidneys (mg)	2688.9 ± 246.29	2666.2 ± 236.50	2751.9 ± 155.57	2643.7 ± 178.06	2716.7 ± 272.94
	(relative to bw)	0.8621%	0.8419%	0.8673%	0.8422%	0.8644%
9	Terminal Body Wt (g)	322.3 ± 13.08	333.5 ± 14.10	330.3 ± 11.38	338.2 ± 14.47	334.3 ± 13.44
	Liver (g)	15.7 ± 1.06	16.9 ± 1.11	16.7 ± 1.21	17.4 ± 1.23	17.4 ± 0.73
	(relative to bw)	4.87%	5.07%	5.06%	5.14%	5.20%
	Adrenals (mg)	57.1 ± 6.19	54.7 ± 5.63	50.2 ± 3.16	50.5 ± 3.54	53.6 ± 2.83
	(relative to bw)	0.0177%	0.0164%	0.0152%	0.0149%	0.0160%
	Kidneys (mg)	2423.1 ± 188.53	2488.1 ± 195.63	2423.4 ± 179.53	2496.5 ± 161.99	2494.0 ± 190.43
	(relative to bw)	0.7518%	0.7461%	0.7337%	0.7382%	0.7460%

*, ** Significantly different from control at P<0.05 and P<0.01, respectively, using T-test pairwise comparisons.

157. Body weights. The initial body weights for the four labs ranged from approximately 210 to 255 grams. Coadministration of testosterone propionate and finasteride had no discernable impact on body weights and body weight gains during the administration period in any of the 4 laboratories (Table 31.)

158. Optional organ weights. Administration of testosterone propionate and finasteride did not significantly change any of the optional organ weights in any laboratory (Table 33).

PROTOCOL CONDITIONS AND PROCEDURES

159. Several protocol conditions and procedures are reviewed below, including the occurrence or lack thereof of preputial separation, a comparison of weighing the ventral prostate fresh and after fixation, a comparison of the differences in mandatory tissue variability and the impact of laboratory performance on variability, a comparison of the performance of the five mandatory tissues, and a review of the two TP reference doses used (i.e., 0.2 and 0.4 mg/kg/d) in the antagonist studies.

Preputial Separation

160. The model protocol (Annex 2) calls for timing the castration so that the animals are still sexually immature, but are still highly likely to achieve preputial separation during the recovery period and administration of the test substance. Preputial separation has been considered necessary for proper dissection of the glans penis and achievement of lower coefficient of variations.

161. The model protocol also calls for the recording of the state of preputial separation at necropsy. The degree of preputial separation recorded in the nine laboratories during the 27 studies is shown in Table 34. These data indicate 100 % preputial separation was observed in 7 of the 9 laboratories. In laboratories 2 and 5, about 30% of the animals did not achieve preputial separation. This did impact the results for the glans penis in laboratory 5, because, in those individual animals with incomplete preputial separation, this laboratory did not dissect and weigh the glans penis.

162. This reduced the group size in some cases as the instances of incomplete preputial separation tended to be treatment related. In the LIN studies of laboratory 5, this included 5 of 6 animals in the vehicle group, 0 of 6 in the 0.4 mg/kg/d TP group, 4 of 6 in the FLU control group (3 mg/kg/d FLU with TP), and 2 of 6 in the high dose LIN group coadministered with TP, and none in the three lower dose LIN groups coadministered with TP. In the VIN studies, this included 4 of 6 animals in the vehicle group, 0 of 6 in the 0.4 mg/kg/d TP group, 3 of 6 in the FLU control group (3 mg/kg/d FLU with TP), 1 of 6 in the 30 mg/kg/d VIN group coadministered with TP, 4 of 6 in the 100 mg/kg/d VIN group coadministered with TP, and none in the two lower dose VIN groups coadministered with TP. In the FIN studies, this included 4 of 6 animals in the vehicle group, 0 of 6 in the 0.4 mg/kg/d TP group, 1 of 6 in the FLU control group (3 mg/kg/d FLU with TP), 1 of 6 in the 5 mg/kg/d FIN group coadministered with TP, 3 of 6 in the 25 mg/kg/d FIN group coadministered with TP, and none in the two lower dose FIN groups coadministered with TP.

163. In laboratory 2, similar patterns were observed in the FIN and PRO studies. Interestingly, with MT, this agonist appeared not to accelerate preputial separation. The rates of incomplete preputial separation in this study were 1 of 6 in the vehicle control, 0 of 6 in the 0.5 mg/kg/d MT group, 2 of 6 in the 2 mg/kg/d MT group, 3 of 6 in the 10 mg/kg/d MT group, and 1 of 6 in the 40 mg/kg/d MT group. EARL – ANY COMMENT ON PLASUBILITY OR MECHANISM HERE??.

Table 34. Preputial separation recorded at necropsy during Phase-2 Hershberger studies.

Lab	Substance #1	Preputial Separation	Substance #2	Preputial Separation	Substance #3	Preputial Separation
1	Linuron	100%	Trenbolone	100%	Vinclozolin	100%
2	Finasteride	66.7%	Methyl Testosterone	76.7%	Procymidone	66.7%
3	DDE	100%	Trenbolone	100%	Vinclozolin	100%
4	DDE	100%	Linuron	100%	Methyl Testosterone	100%
5	Finasteride	78%	Linuron	73.2%	Vinclozolin	71.4%
6	Finasteride	100%	Methyl Testosterone	100%	Linuron	100%
7	Procymidone	100%	Trenbolone	100%	Vinclozolin	100%
8	DDE	100%	Methyl Testosterone	100%	Procymidone	100%
9	DDE	100%	Finasteride	100%	Procymidone	100%

164. In laboratory 2, where the GP was dissected despite incomplete preputial separation, the CVs of this tissue were somewhat higher than other laboratories, but so were several other mandatory tissues. This observation and the other data in this section leads to several recommendations:

- Each laboratory should have data on their particular strain and animal supplier as to when to expect preputial separation.
- In the circumstances where animals have a late arrival to sexual maturity, the timing of castration may need to be delayed for a few days beyond pnd 42.
- If incomplete preputial separation is observed, it should be recorded. However, the dissection should proceed and the GP should be weighed to maintain animal numbers and overall power, accepting the modest diminishment in power of a slightly higher CV.

Possible Alternative Procedures: Fixation of the Ventral Prostate

165. Fixation has been proposed to reduce the variability of the ventral prostate weights and thereby improving sensitivity and the detection of changes in the ventral prostate (50). Six of the nine laboratories compared the weights of the ventral prostate when weighed fresh and when weighed after 24-hours of fixation. Data is available from a minimum of two laboratories for each test substance used in Phase-2 to examine this hypothesis.

166. The comparison of the potential benefit of fixation was tested in two ways: by a comparison of the coefficients of variation of the fresh and fixed ventral prostate weights and by a comparison of statistical significance achieved with the fresh and fixed ventral prostate. These data are presented for the androgen agonists MT and TREN in Table 35A, for the 5α-reductase inhibitor FIN in Table 35B, and for the androgen antagonists PRO, VIN, LIN, and DDE in Table 35C.

167. The coefficients of variation between the fresh and fixed ventral prostate weights were consistently similar across all classes of test substances and the individual test substances for a given laboratory (Tables 35A, 35B, and 35C). This similarity of the coefficient of variation within a laboratory reinforces the finding that laboratory procedures and techniques have a major impact on the performance of the assay. This similarity of the coefficient of variation also suggests that the individual tissue weights and their distribution before and after fixation as might be expected if one posits that any major losses of tissue fluid during dissection and handling occur prior to fresh weighing or fixation.

Table 35A. Comparison of fresh and fixed ventral prostate data for androgen agonists: means, standard deviations, coefficients of variations, and statistical significance.

Lab	MT (mg/kg/d)	0	0.5	2	10	40
2	Ventral prostate – fresh (mg)	15.6 ± 5.53 [36] [a]	17.5 ± 7.89 [45]	21.6 ± 11.34 [53]	50.3 ± 25.30 [D] [50]	110.2 ± 34.88 [D] [32]
	Ventral prostate – fixed (mg)	22.7 ± 18.74 [83]	20.2 ± 8.35 [41]	27.0 ± 18.41 [68]	74.3 ± 33.73 [D] [45]	141.8 ± 48.86 [D] [34]
4	Ventral prostate – fresh (mg)	24.8 ± 10.81 [44]	26.8 ± 5.97 [22]	50.5 ± 28.93 [D] [57]	75.5 ± 9.33 [D] [12]	162.5 ± 58.11 [D] [36]
	Ventral prostate – fixed (mg)	31.8 ± 13.47 [42]	33.6 ± 6.27 [19]	66.7 ± 38.91 [D] [58]	103.3 ± 13.77 [D] [13]	212.0 ± 72.10 [D] [34]
6	Ventral prostate – fresh (mg)	4.9 ± 3.24 [66]	4.2 ± 1.55 [37]	8.1 ± 4.74 [59]	21.1 ± 21.53 [102]	70.0 ± 26.19 [D] [37]
	Ventral prostate – fixed (mg)	9.4 ± 4.40 [47]	17.6 ± 14.70 [83]	19.2 ± 9.41 [49]	42.4 ± 40.16 [D] [95]	112.1 ± 57.61 [D] [51]
8	Ventral prostate – fresh (mg)	18.8 ± 6.74 [36]	21.1 ± 2.32 [11]	24.8 ± 6.87 [28]	62.5 ± 14.13 [D] [23]	151.1 ± 38.29 [D] [25]
	Ventral prostate – fixed (mg)	21.3 ± 3.78 [18]	26.1 ± 4.07 [16]	31.1 ± 10.55 [D] [34]	78.8 ± 16.55 [D] [21]	177.5 ± 47.02 [D] [27]
Lab	**Trenbolone (mg/kg/d)**	**0**	**0.3**	**1.5**	**8**	**40**
1	Ventral prostate – fresh (mg)	15.7 ± 3.14 [20]	19.7 ± 5.02 [26]	18.8 ± 3.63 [19]	19.8 ± 4.27 [22]	37.6 ± 11.36 [D] [30]
	Ventral prostate – fixed (mg)	22.9 ± 2.09 [9]	22.7 ± 5.99 [26]	23.8 ± 3.98 [17]	23.4 ± 5.35 [23]	49.9 ± 15.08 [D] [30]
7	Ventral prostate – fresh (mg)	15.2 ± 5.41 [36]	19.5 ± 5.39 [28]	16.5 ± 6.11 [37]	25.3 ± 5.92 [D] [23]	35.2 ± 6.81 [D] [19]
	Ventral prostate – fixed (mg)	20.8 ± 6.62 [32]	23.4 ± 6.30 [27]	20.3 ± 7.11 [35]	30.8 ± 7.11 [23]	42.9 ± 7.95 [D] [19]

[a] Mean ± standard deviation [coefficient of variation]

[D] Significantly different from control at P<0.05 using two-tailed Dunnett's multiple comparisons of the tissue with body weight adjustment.

Table 35B. Comparison of fresh and fixed ventral prostate data for 5α-reductase inhibitors: means, standard deviations, coefficients of variations, and statistical significance.

Lab	Testosterone Prop. (mg/kg/d)	0.4	0.4	0.4	0.4	0.4
	Finasteride (mg/kg/d)	0	0.2	1	5	25
2	Ventral prostate – fresh (mg)	133.1 ± 36.20 [27] [a]	115.5 ± 21.60 [19]	67.4 ± 27.29 [D] [41]	54.0 ± 16.10 [D] [30]	42.7 ± 12.53 [D] [29]
	Ventral prostate – fixed (mg)	180.6 ± 55.38 [31]	152.5 ± 30.62 [20]	93.7 ± 37.46 [D] [40]	73.7 ± 16.21 [D] [22]	62.0 ± 10.91 [D] [18]
6	Ventral prostate – fresh (mg)	116.9 ± 27.88 [24]	73.2 ± 15.81 [22]	57.4 ± 27.09 [D] [47]	53.4 ± 14.30 [D] [27]	29.8 ± 13.44 [D] [45]
	Ventral prostate – fixed (mg)	193.7 ± 54.06 [28]	123.8 ± 25.01 [20]	86.9 ± 37.21 [D] [43]	79.4 ± 21.58 [D] [27]	52.7 ± 22.51 [D] [43]

[a] Mean ± standard deviation [coefficient of variation]

[D] Significantly different from control at P<0.05 using two-tailed Dunnett's multiple comparisons of the tissue with body weight adjustment.

Table 35C. Comparison of fresh and fixed ventral prostate data for androgen antagonists: means, standard deviations, coefficients of variations, and statistical significance.

Lab	Testosterone Prop. (mg/kg/d)	0.4	0.4	0.4	0.4	0.4
	Procymidone (mg/kg/d)	**0**	**3**	**10**	**30**	**100**
2	Ventral prostate – fresh (mg)	173.8 ± 32.53 [19] [a]	180.2 ± 43.10 [24]	150.9 ± 52.33 [35]	103.3 ± 16.49 [D] [16]	52.7 ± 23.55 [D] [45]
	Ventral prostate – fixed (mg)	257.3 ± 43.78 [17]	259.7 ± 69.15 [27]	217.7 ± 66.42 [31]	146.8 ± 28.12 [D] [19]	82.3 ± 30.43 [D] [37]
7	Ventral prostate – fresh (mg)	157.4 ± 18.02 [11]	140.5 ± 49.23 [35]	103.8 ± 33.01 [32]	80.4 ± 25.95 [D] [32]	57.9 ± 19.68 [D] [34]
	Ventral prostate – fixed (mg)	200.0 ± 21.73 [11]	173.2 ± 61.29 [35]	125.7 ± 43.15 [34]	100.4 ± 33.37 [D] [33]	71.0 ± 22.51 [D] [32]
8	Ventral prostate – fresh (mg)	210.4 ± 22.59 [11]	206.8 ± 8.64 [4]	151.8 ± 31.83 [D] [21]	124.3 ± 13.54 [D] [11]	67.8 ± 10.92 [D] [16]
	Ventral prostate – fixed (mg)	255.0 ± 28.03 [11]	269.3 ± 28.81 [11]	198.0 ± 21.52 [D] [12]	185.4 ± 20.41 [D] [11]	95.2 ± 16.27 [D] [17]
	Vinclozolin (mg/kg/d)	**0**	**3**	**10**	**30**	**100**
1	Ventral prostate – fresh (mg)	106.5 ± 19.82 [19]	103.7 ± 25.01 [24]	94.5 ± 12.57 [13]	79.0 ± 16.38 [21]	32.2 ± 8.04 [D] [25]
	Ventral prostate – fixed (mg)	120.2 ± 18.31 [15]	123.2 ± 35.11 [29]	104.2 ± 16.76 [16]	91.8 ± 17.22 [19]	38.2 ± 10.87 [D] [28]
7	Ventral prostate – fresh (mg)	161.1 ± 28.20 [18]	131.4 ± 31.05 [24]	130.6 ± 23.82 [18]	92.9 ± 18.57 [D] [20]	50.7 ± 23.49 [D] [46]
	Ventral prostate – fixed (mg)	193.8 ± 32.70 [17]	155.5 ± 37.23 [24]	166.2 ± 22.66 [14]	118.5 ± 20.98 [D] [18]	67.2 ± 29.39 [D] [44]
	Linuron (mg/kg/d)	**0**	**3**	**10**	**30**	**100**
1	Ventral prostate – fresh (mg)	106.5 ± 19.82 [19]	113.3 ± 16.66 [15]	122.5 ± 23.95 [20]	89.0 ± 20.10 [23]	60.7 ± 10.93 [D] [18]
	Ventral prostate – fixed (mg)	120.2 ± 18.31 [15]	125.3 ± 14.65 [12]	137.8 ± 21.24 [15]	125.8 ± 51.68 [41]	72.0 ± 12.18 [D] [17]
4	Ventral prostate – fresh (mg)	167.1 ± 34.58 [21]	192.8 ± 35.16 [18]	203.2 ± 33.54 [17]	184.4 ± 38.47 [21]	99.0 ± 26.74 [D] [27]
	Ventral prostate – fixed (mg)	217.4 ± 36.28 [17]	269.0 ± 44.91 [17]	277.6 ± 46.80 [17]	255.0 ± 61.63 [24]	128.8 ± 29.54 [D] [23]
6	Ventral prostate – fresh (mg)	83.3 ± 33.97 [41]	109.5 ± 51.89 [41]	115.2 ± 25.07 [22]	109.8 ± 25.32 [23]	67.9 ± 18.94 [28]
	Ventral prostate – fixed (mg)	122.0 ± 53.45 [44]	173.9 ± 86.56 [50]	192.3 ± 37.71 [20]	165.6 ± 30.24 [18]	117.9 ± 23.43 [20]
	***p,p'*-DDE (mg/kg/d)**	**0**	**5**	**16**	**50**	**160**
4	Ventral prostate – fresh (mg)	183.8 ± 81.46 [44]	166.1 ± 22.94 [14]	202.0 ± 48.74 [24]	159.3 ± 34.67 [22]	90.9 ± 27.43 [D] [30]
	Ventral prostate – fixed (mg)	258.6 ± 113.43 [44]	232.3 ± 39.20 [17]	287.3 ± 76.88 [27]	233.1 ± 66.34 [28]	121.1 ± 36.27 [D] [30]
8	Ventral prostate – fresh (mg)	210.4 ± 22.59 [11]	200.4 ± 24.02 [12]	195.9 ± 25.96 [13]	163.4 ± 25.67 [D] [16]	78.0 ± 21.64 [D] [28]
	Ventral prostate – fixed (mg)	254.5 ± 27.84 [11]	285.5 ± 33.67 [12]	273.2 ± 27.47 [10]	237.9 ± 43.53 [18]	110.5 ± 30.76 [D] [28]

[a] Mean ± standard deviation [coefficient of variation]

[D] Significantly different from control at P<0.05 using two-tailed Dunnett's multiple comparisons of the tissue with body weight adjustment.

168. The statistical significance of the fresh and fixed ventral prostate was achieved at the same dose in 14 of the 18 studies. In two studies, the fixed ventral prostate achieved statistical significance at one dose unit lower than the fresh ventral prostate. These studies were both with the more potent androgen agonist MT in laboratories 6 and 8 (Table 35A). In two other studies, the fresh ventral prostate achieved statistical significance at one dose unit lower than the fixed ventral prostate. These studies were with the weak androgen agonist TREN in laboratory 7 (Table 35A) and the weak androgen antagonist DDE in laboratory 8 (Table 35C). The limited differences between the procedures in achieving statistical significance and the equal number of instances in which one technique achieved statistical significance at a lower dose were consistent with the similarity in the coefficient of variation comparison. The conclusion is that fixation of the ventral prostate provides no significant benefit in sensitivity particularly taking into account the additional work the day after necropsy.

169. Although histopathology was not used in these studies, it is an endpoint in many other toxicity studies. Thus, the impact of fixation on preserving the integrity of the VP for histopathological examination was not tested in these studies. The negative results here for tissue weight should not be extrapolated to the need to preserve the integrity of the VP for procedures such as histopathology.

Coefficient of Variation – Mandatory tissue variability and laboratory performance

170. In the previous section, the CV within and among labs for the fresh and fixed VP suggested that laboratories procedures and techniques have a major impact on the performance of the assay. This analysis is expanded in Table 36 to include the mean CV for all five mandatory tissues in all laboratories.

171. Table 36 supports two findings. First, there were apparent differences in the variability as indicated by CV values among the five mandatory tissues. The LABC and GP have lower CVs, suggesting that these tissues may be somewhat easier to consistently dissect and handle. This would appear to correspond to the general absence of fluid in these solid tissues. In contrast, the fluid-filled tissues such as the VP, SVCG, and COWS generally have greater and more variable CVs from laboratory to laboratory than the LABC and GP in Table 36. These unstimulated tissues in some cases are not only physically small, but embedded in adipose tissue increasing the difficulty of dissection.

172. Second, there were indeed differences in the variability (CV values for a given tissue) among labs. Table 36 is arranged so that the results are also grouped by individual laboratory. This arrangement suggests that individual laboratories were relatively consistent internally in the CVs for the five tissues from experiment to experiment. The CVs in the Table also suggests that certain laboratories were more proficient overall than others, e.g., compare the CVs of laboratories 8 and 9 to those of 2 and 6 for the all mandatory tissues. This further reinforces the finding that laboratory procedures and techniques themselves have a major impact on the performance of the assay. This range of lab performance and general consistency within a lab was also observed with the uterotrophic bioassay (49), indicating that validation programmes characterize not only the performance of particular assays, but also characterize the performance of the individual participating labs.

Table 36. Mean coefficients of variation for the five mandatory tissues in each Phase-2 study.

Lab – Test Substance	LABC	GP	VP	SVCG	COWS
1 – TREN	17.2	10.6	23.3	37.3	26.2
1 – VIN	12.7	11.9	22.4	28.1	21.1
1 – LIN	14.5	11.6	21.0	20.2	16.9
2 – MT	14.4	18.5	43.1	38.1	30.5
2 – PRO	17.7	16.8	31.8	30.3	32.9
2 – FIN	11.5	12.3	29.3	46.4	38.8

Table 36 continued. Mean coefficients of variation for the five mandatory tissues in each Phase-2 study.

Lab – Test Substance	LABC	GP	VP	SVCG	COWS
3 – TREN	16.7	11.5	50.6	31.6	21.6
3 – VIN	9.8	9.7	26.3	19.5	16.3
3 – DDE	12.8	14.1	34.5	26.7	16.9
4 – MT	18.0	14.7	34.3	23.2	25.4
4 – LIN	10.9	11.2	20.7	20.5	15.2
4 – DDE	11.0	12.0	26.8	31.9	17.2
5 – VIN	15.4	9.4	25.9	20.0	24.1
5 – LIN	11.1	10.2	17.8	19.1	27.7
5 – FIN	14.1	9.8	31.6	15.9	25.3
6 – MT	18.6	20.3	60.3	38.0	56.5
6 – LIN	12.5	14.9	42.9	27.1	41.4
6 – FIN	12.6	8.5	38.2	28.1	35.8
7 – TREN	19.0	8.9	28.6	30.6	43.7
7 – PRO	24.0	11.4	32.6	23.2	25.7
7 – VIN	12.3	11.7	23.8	24.9	30.1
8 – MT	14.7	18.8	24.5	26.1	21.1
8 – PRO - A	9.8	8.8	12.3	15.1	22.6
8 – PRO - B	10.1	9.9	16.4	16.9	15.5
8 –DDE	12.7	9.3	15.1	16.2	21.9
9 – PRO	9.3	6.6	12.6	10.8	8.7
9 – DDE	11.3	6.7	18.4	13.1	17.7
9 - FIN	9.5	8.3	15.5	12.6	16.7
10 – VIN	12.8	19.1	33.9	32.0	23.7
10 – DDE	12.4	8.2	14.5	16.2	14.9
11 – MT	9.2	3.6	17.8	17.1	25.7
11 – VIN	9.2	8.8	31.8	23.3	17.3
11 – DDE	10.8	5.4	31.0	24.2	22.1
12 – DDE	12.4	15.5	21.8	16.7	16.2
13 – MT	11.7	7.8	21.2	17.7	21.5
13 - VIN	20.0	4.5	27.3	21.4	20.2
14 – VIN	10.1	4.9	20.3	16.7	34.8
14 – DDE	7.9	6.3	22.4	17.4	21.1
15 – MT	11.5	7.1	8.8	8.5	17.3
16 – MT	9.8	4.8	10.0	12.2	10.0
16 - DDE	11.5	7.0	19.7	18.2	21.6

Endpoint Responsiveness and Value

173. The Phase-1 report concluded that the need for all five mandatory tissues remained to be tested against weaker (anti)androgens and differences in metabolism. There are two approaches that can be used to assess the value of each mandatory endpoint. In the first approach, the ability of the tissue to reach statistical significance with test substances of varying mechanisms and to be the most sensitive tissue is analyzed. In the second approach, the R-square for a tissue and the effect of test substances of varying mechanisms can be analyzed. While the R-square is not a direct statistical metric, it certainly illustrates whether a strong relationship exists between the substance and a change in the tissue has occurred. Both approaches should be mindful of the different uses to which the results can be applied, ranging from the simple identification of a possible hazard to contributing to the diagnosis of a substance's mechanism of action based on the differential response profile among the tissues.

174. Table 37 compares, for each test substance and laboratory, whether a tissue achieved statistical

significance in the doses used in these studies, whether the more conservative Dunnett's approach achieved statistical significance, the times a tissue was among the most sensitive endpoints in a laboratory (the lowest LOEL), and the times a tissue was the sole most sensitive endpoint. The Table is arranged by androgen, antagonists, and the 5α-reductase mechanisms, and includes studies from both stages. The LABC and GP with lower relative response, but lower CVs, are grouped in the first two columns. The VP, SVCG, and COWS with the higher relative response, but higher CVs, are grouped in the three right columns.

175. If the need for the Hershberger bioassay is simply to identify potential hazard, Table 37 suggests that the battery of the VP, SVCG, COWS, and LABC are sufficient for detection of all mechanisms. The GP was not of significant value in the ability to grossly detect substances. However, it should be noted that in two instances, the apparent failure of the GP to detect a compound, is confounded. These two instances involve laboratory 5, the incomplete preputial separation in some animals, and diminished group sizes at the high doses of VIN and LIN.

176. Table 38 approaches the problem from a somewhat different perspective. Rather than fulfilling the simple purpose of detection of several possible mechanisms, would the Hershberger be used as discriminating among or helping to discriminate among mechanisms of action. That is, does the response of the individual tissues and their overall profile generated provide any additional insights into questions of mechanism? In Table 38, the overall R-square of the tissue response is reviewed and are ranked. With the agonists, the LABC, VP, SVCG, and COWS have high R-square values with MT in all studies from both stages. However, with the weaker TREN, which is not metabolised by the 5α-reductase, the relationship of the GP to the response is higher and those of tissues containing the 5α-reductase (VP, SVCG, and COWS) were lower. With the antagonists, the values for the LABC, VP, SVCG, and COWS were consistently high and the values for the GP were consistently lower. For the 5α-reductase, the three 5α-reductase containing tissues (VP, SVCG, and COWS) were higher, while the LABS and GP were lower. This suggests some utility in the battery of tissue providing some indication of a metabolic profile for the test substances and a possible role for the GP endpoint.

Table 37. Summary of the LOEL dose performance of the five mandatory tissues in each Phase-2 study.

Test Substance - Lab		VP	SVCG	LABC	GP	COWS
ANDROGEN AGONISTS						
MT	2	10	40	10	10	40
MT	4	2	2	2	2	2
MT	6	10	10	2	40	2
MT	8	10	10	2	10	2
MT	11	**0.5** [c]	50	5	5	5
MT	13	5	5	5	5	5
MT	15	5	5	5	5	5
MT	16	50	50	5	5	50
TREN	1	40	40	40	40	40
TREN	3	40	40 [a]	40	40	40
TREN	7	8	40 [a]	8	8	40
ANDROGEN ANTAGONISTS						
PRO	2	**10** [c]	30	100	- -	100
PRO	7	30	30	30	- -	30
PRO	8 A	10	10	10	30	100
PRO	8 B	10	30	30	30	10
PRO	9	10	10	3	10	3
VIN	1	30	30	30	30	30
VIN	3	30	30	30	100	100
VIN	5	100	30	30	- - [d]	100
VIN	7	30	30	30	100	100
VIN	10	**10** [c]	30	30	30	30
VIN	11	100	30	30	30	**10** [c]
VIN	13	10	10	10	10	10
VIN	14	**3** [c]	30	30	30	30
LIN	1	100	**30** [c]	100	100	100
LIN	4	100	100 [a]	100	- -	100
LIN	5	30	30	30	- - [d]	100
LIN	6	- -	**100** [a, c]	- -	- -	- -
DDE	3	50	160	50	160	50
DDE	4	160	160 [a]	50	160 [a]	50
DDE	8	50	50	50	160	50
DDE	9	50	50	50	160	50
DDE	10	30	30	100	100	30
DDE	11	30	30	30	100	100
DDE	12	100	30	100	100	100
DDE	14	100	100	100	100	100
DDE	16	30	30	30	100	30
5α-REDUCTASE INHIBITORS						
FIN	2	1	1	1	- -	1
FIN	5	0.2	0.2	0.2	0.2	0.2
FIN	6	1	**0.2** [c]	5	5 - 25 [a,b]	5
FIN	9	0.2	0.2	1	0.2	5
One among most sensitive endpoints		30	27	29	13	23
Sole most sensitive endpoint		4	3	- -	- -	1
Times not achieving statistical significance by either approach		1	0	1	7 [b]	1
Times not achieving statistical significance by Dunnett's		1	5	1	9	1

[a] Highest dose in series and did not achieve statistical significance using Dunnett's multiple comparison approach.

[b] The 5 mg/kg/d dose achieved significance using the pairwise comparison approach only, the higher 25 mg/kg/d dose was not significant for the glans penis. If this point was judged spurious, then the times the glans penis did not achieve statistical significance would rise to 8 instances.

[c] Where a tissue is the sole most sensitive endpoint in a study the value is in boldface to assist the reader.

[d] Potential loss of statistical power should be recognized; animals with incomplete preputial separation were not dissected.

Table 38. R-Square for test substance response of the five mandatory tissues in each Phase-2 study.

Chemical	Lab	VP	SV	LABC	GP	COWS	Lab Mean
R2 ANDROGENS							
MT	2	68	64	84	57	69	68
MT	4	83	86	76	55	80	76
MT	6	67	66	80	37	73	65
MT	8	91	85	89	59	87	82
Tissue Mean [Rank]		77 [2]	75 [4]	82 [1]	52 [5]	77 [2]	73
MT	11	89	90	92	82	71	85
MT	13	95	90	92	87	93	91
MT	15	92	82	88	77	86	85
MT	16	91	98	95	97	92	95
Tissue Mean [Rank]		89 [1]	81 [3]	90 [1]	68 [5]	81 [3]	90
TREN	1	64	31	68	65	42	54
TREN	3	18	57	77	60	58	54
TREN	7	53	51	69	61	39	55
Tissue Mean [Rank]		45 [3]	46 [3]	71 [1]	62 [2]	46 [3]	54
R2 ANTIANDROGENS - ANTAGONISTS							
PRO	2	74	59	57	23	50	53
PRO	7	62	77	40	34	44	51
PRO	8	88	84	88	57	68	71
PRO	9	78	90	83	34	90	75
Tissue Mean [Rank]		76 [2]	78 [1]	67 [3]	37 [5]	63 [4]	63
VIN	1	84	85	84	75	72	80
VIN	3	63	72	65	44	69	63
VIN	5	62	86	75	14	61	91
VIN	7	74	80	80	38	49	64
Tissue Mean [Rank]		71 [3]	81 [1]	76 [2]	43 [5]	63 [4]	67
VIN	10	77	86	79	74	75	81
VIN	11	62	84	69	62	71	75
VIN	13	77	89	83	83	72	87
VIN	14	91	79	82	69	71	83
Tissue Mean [Rank]		60 [2]	64 [1]	50 [4]	53 [3]	51 [4]	82
LIN	1	67	80	73	41	67	66
LIN	4	67	61	67	20	38	51
LIN	5	74	77	86	29	64	66
LIN	6	26	41	27	12	2.5	22
Tissue Mean [Rank]		59 [3]	65 [1]	63 [2]	26 [5]	43 [4]	46
DDE	3	79	85	89	67	82	80
DDE	4	47	39	83	31	61	52
DDE	8	86	89	74	62	66	75
DDE	9	60	79	78	65	72	71
Tissue Mean [Rank]		68 [4]	73 [2]	81 [1]	56 [5]	70 [3]	70
DDE	10	75	79	56	38	76	65
DDE	11	65	84	79	72	37	67
DDE	12	57	62	49	38	55	52
DDE	14	55	78	89	71	45	68
DDE	16	65	78	72	51	67	67
Tissue Mean [Rank]		36 [2]	45 [1]	37 [2]	30 [5]	33 [4]	64
OVERALL R2 Stage 2		**68 [3]**	**74 [1]**	**71 [2]**	**40 [5]**	**60 [4]**	

Table 38 continued. R-Square for test substance response of the five mandatory tissues in each Phase-2 study.

R2 ANTIANDROGENS - 5 ALPHA REDUCTASE INHIBITORS							
FIN	2	70	70	37	13	44	47
FIN	5	69	93	72	69	88	78
FIN	6	64	75	47	42	49	55
FIN	9	82	90	67	50	76	73
Tissue Mean [Rank]		71 [2]	82 [1]	56 [4]	44 [5]	64 [3]	63

177. Such a putative profile may not be consistently clear, e.g., the LABC and GP R-square values were relatively high in laboratory 5 in the finasteride studies. In addition, there are more direct analyses for androgen receptor binding and 5α-reductase metabolism and inhibition. Therefore, it is assumed that the profile of mandatory endpoints generated by a Hershberger would be interpreted as part of the overall weight of the evidence and not necessarily as the primary evidence, but rather as the supporting evidence, for mechanistic studies.

178. Another means to review the GP data is to examine the treatment related R-square for this endpoint. These values have been extracted into Table 39. The summary indicates a relatively poor association with response in a number of cases. This should be true with FIN, which is the 5α-reductase inhibitor, but the low value with TREN brings the interpretation of the overall R-square value with this compound into question.

Table 39. Comparison of GP treatment R-square values across Phase-2 test substances

Test Substance	Treatment R-square
Methyl Testosterone – series 1	43
Methyl Testosterone – series 2	68
Trenbolone	16
Procymidone	16
Vinclozolin – 0.4 mg/kg/d TP	16
Vinclozolin – 0.2 mg/kg/d TP	53
Linuron	3.8
DDE – 0.4 mg/kg/d TP	27
DDE – 0.2 mg/kg/d TP	30
Finasteride	12

179. Therefore, while the GP may not be essential to the detection of any particular mechanism, the question then remains open that it may have some utility in assisting a discrimination among mechanisms, particularly when combined with other data.

Testosterone propionate dosage for antiandrogen control groups

180. In Phase 1B, the reference TP doses of 0.2 mg/kg/d and 0.4 mg/kg/d had both been coadministered to the same series of FLU doses (3). In those studies, the overall performance of the two TP reference doses had been equivalent in their ability to provide a clear dose response in all five mandatory tissues with FLU. Neither dose appeared to have truly superior qualities over the other in any respect. While the 0.4 mg/kg/d TP dose provided an overall larger absolute increase in the baseline weights against which the antiandrogen could act, the lower absolute increase from the 0.2 mg/kg/d TP dose offered a less robust response that might provide slightly greater sensitivity against a weak

antiandrogen.

181. In Phase-2, the TP doses were presented with a somewhat weaker antiandrogen VIN and a very weak antiandrogen DDE. This presents the opportunity to reevaluate whether these TP reference doses remain equivalent against a broad range of antiandrogen potencies or whether one TP reference dose displays discernable advantages over the other.

182. The Lead Laboratory has performed several comparisons of the 0.2 and 0.4 mg/kg/d TP reference doses using the data from VIN (see Section II A 3 of Annex 3) and DDE (Section II C 3 of Annex 3).

183. As the VIN dose series was identical, a direct statistical comparison could be made between the TP reference doses. The results of the analysis based on VIN dose * TP dose (termed protocol by the Lead Laboratory) did not achieve significance for any tissue. The P values were 0.57 (VP), 0.45 (LABC), 0.15 (COWS) and 0.07 (SCVG).

184. A comparison of the LOELs for both VIN and DDE was conducted to assess whether either of the two TP reference doses would be more sensitive (produce a consistently lower LOEL). In this regard, the 0.2 mg/kg/d TP dose has a theoretical advantage.

Positive TP control group variability

185. One characteristic of the positive TP control group that should be taken into account and analyzed is its inherent variability. Whether a 0.2 or a 0.4 mg/kg/d dose is used, both doses are in the midst of an ascending dose response curve prior to a maximum plateau. A review of the anitandrogen data in Tables 15, 18, 21, 23, 26 and 29 will indicate that, on a number of occasions, tissues from the TP + test substance groups were sometimes <u>larger</u> in absolute terms than the TP only dose. This strongly suggests group-to-group variability based only on the TP dose. Further, on four occasions, isolated events of statistical significance occurred (see Tables 26 and 29) using the pairwise statistical approach, where three groups were significantly decreased and one group was significantly increased. All four events were in the two DDE dose series. This raises the possibility of both false positives, but also of false negatives. For example, in lab 6 in Table 23, in addition to the size of the CVs, absolute weights of the VP are higher than the positive control at 3 treatment doses, SVCG at 2 treatment doses, LABC at 3 treatment doses, GP at 1 treatment dose, and COWS at 3 treatment doses. This raises legitimate concerns about the TP positive control values in this case as contributing to the absence of detection. Therefore, the combined TP data from Phases-1, -2, and -3, when complete, should be pooled and analyzed for the contribution that TP group tissue weight variability may contribute to false positive and negative events.

TOXICOLOGICAL RELEVANCE AND REPRODUCIBILITY

Correspondence of Hershberger Phase-2 results with developmental and reproductive results

186. An important question is whether the Hershberger bioassay can reasonably indicate the occurrence of (anti)androgen effects in developmental and reproductive assays. That is, what is the apparent toxicological relevance or correspondence of the Hershberger bioassay results? To address this important question, available studies with the test substances used in Phase-2 have been compiled and extracted for LOELs. No data were discovered for methyl testosterone. In Tables 40A-F, brief summary comparisons are made between the Hershberger Phase-2 results and the available data.

Table 40A. Comparison of TREN Hershberger Phase 2 results with developmental and reproductive results

Hershberger Bioassay	Developmental and/or Reproductive Bioassay (51)
VP: 8-40 mg/kg/d	By sc administration – increased female anogenital distance ≥ 0.5 mg/kg/d By sc administration – reduced female areolas retention ≥ 2 mg/kg/d By sc administration – reduced female nipple retention ≥ 0.5 mg/kg/d
SVCG: 40 mg/kg/d	
LABC: 8-40 mg/kg/d	
GP: 8-40 mg/kg/d	
COWS: 40 mg/kg/d	
	No frank malformations observed
Comments: The route of administration differences should be taken into account. The authors performed both oral and sc Hershberger studies. By gavage, LABC and SVCG were significant at 10 mg/kg/d trenbolone via po and VP, COWS, GP at 50 mg/kg/d trenbolone via po. By injection, LABC was significant at 0.05 μg/kg/d sc; other tissues at 0.20 μg/kg/d sc.	

Table 40B. Comparison of PRO Hershberger Phase 2 results with developmental and reproductive results

Hershberger Bioassay	Developmental and/or Reproductive Bioassay (51)(52)
VP: 10-30 mg/kg/d	Anogenital distance: 25 mg/kg/d Retained nipples: 50 mg/kg/d
SVCG: 10-30 mg/kg/d	
LABC: 3-100 mg/kg/d	Malformations (hypospadias) incidence at ≥ 50 mg/kg/d Decreased adult sex accessory tissue wts at ≥ 100 mg/kg/d Histopath lesions in adult sex accessory tissue at ≥ 50 mg/kg/d
GP: 10 mg/kg/d – Non-detect	
COWS: 3-100 mg/kg/d	
Comments: There is general correspondence between the Hershberger results and those studies with *in utero* exposure to procymidone.	

Table 40C. Comparison of VIN Hershberger Phase 2 results with developmental and reproductive results

Hershberger Bioassay	Developmental and/or Reproductive Bioassay (53)(54)
VP: 10-100 mg/kg/d	Anogenital distance reduced ≥ 3.125 mg/kg/d Nipple retention in pnd 14 males increased ≥ 50 mg/kg/d Nipple retention in pubertal males increased ≥ 50 mg/kg/d
SVCG: 10-30 mg/kg/d	
LABC: 10-100 mg/kg/d	Malformations (hypospadias) incidence at ≥ 50 mg/kg/d Decreased adult VP wts at ≥ 50 mg/kg/d Reduced sperm count at 100 mg/kg/d
GP: 10-100 mg/kg/d	
COWS: 10-100 mg/kg/d	
Comments: There is a general correspondence between the Hershberger results and the developmental assays (53)(54). However, a multi-generation study did not reproduce the Anogenital distance findings at 3.125 -15 mg/kg/d (55),.	

Table 40D. Comparison of LIN Hershberger Phase 2 results with developmental and reproductive results

Hershberger Bioassay	Developmental and/or Reproductive Bioassay (51)(56)(57)(58)
VP: 30-Nondetect mg/kg/d	Anogenital distance not statistically significant up to 50 mg/kg/d Nipple retention in pnd 13 males increased 50 mg/kg/d Nipple retention in pubertal males increased ≥ 50 mg/kg/d
SVCG: 30-100 mg/kg/d	
LABC: 30-Nondetect mg/kg/d	Malformations (epididymis) incidence observable at ≥ 25 mg/kg/d Decreased adult dorsolateral prostate wts at 50 mg/kg/d Histological abnormalities in male repro tract ≥ 25 mg/kg/d
GP: 100-Nondetect mg/kg/d	
COWS: 100-Nondetect mg/kg/d	
Comments: There is a general correspondence between the Hershberger results and the developmental assays.	

Table 40E. Comparison of DDE Hershberger Phase 2 results with developmental and reproductive results

Hershberger Bioassay	Developmental and/or Reproductive Bioassay (51)(59)(60)
VP: 30-160 mg/kg/d	Anogenital distance reduced in Long Evans and not SD rats 100 mg/kg/d
SVCG: 30-160 mg/kg/d	Nipple retention in pnd 13 males increased 10 mg/kg/d SD rats; 100 mg/kg/d Long Evans rats
LABC: 30-100 mg/kg/d	Malformations (hypospadias) low incidence at 100 mg/kg/d in one study but not observed in second study
GP: 100-160 mg/kg/d	
COWS: 30-100 mg/kg/d	Decreased adult VP wts at 200 mg/kg/d
Comments: There is a general correspondence between the Hershberger results and the developmental assays.	

Table 40F. Comparison of FIN Hershberger Phase 2 results with developmental and reproductive results

Hershberger Bioassay	Developmental and/or Reproductive Bioassay (19)(20)(61)(62)(63)
VP: 0.2-1 mg/kg/d	Anogenital distance reduced pnd 1 ≥ 0.01 mg/kg/d
SVCG: 0.2-1 mg/kg/d	Nipple retention in pnd 13 males increased ≥ 0.01 mg/kg/d Nipple retention in adult males increased ≥ 0.1 mg/kg/d
LABC: 0.2-5 mg/kg/d	Malformations (multiple tissues) significant at ≥ 10 mg/kg/d
GP: 0.2-Nondetect mg/kg/d	Decreased adult LABC wts at ≥ 1 mg/kg/d
COWS: 0.2-5 mg/kg/d	Decreased adult VP and COWS wts at ≥ 10 mg/kg/d
Comments: 0.2 mg/kg/d was the lowest dose; the data of Bowman et al. were not available when the doses were selected.	

187. The brief summary comparisons of the Hershberger Phase-2 results with data available from various developmental and reproductive data published in the literature (Tables 40A-F) show a high level of correspondence when the route of administration is similar. In the case of trenbolone (Table 40A), there are route of administration considerations. The investigators performed two Hershberger bioassays themselves using very similar protocol conditions. The sc Hershberger corresponded well to the developmental data after exposure *in utero* with sc administration to the dams shown in Table 40A. In parallel, the investigators' gavage Hershberger data corresponded well to those generated in Phase-2. There were no developmental or reproductive data for methyl testosterone discovered.

188. In conclusion, this brief comparison lends support to the toxicological relevance of the Hershberger bioassay to predict possible hazards from *in utero* exposure to (anti)androgens.

Overall reproducibility of the Hershberger bioassay in Phase-2

189. The reproducibility in Phase-2 of the Hershberger validation programme can be assessed by comparing the F values for the chemical effect (the dose response) versus the F values for the chemical * lab effect. The chemical F value is typically from one to two orders of magnitude larger that the chemical * lab F value as compared in Tables 41A and 41B. This demonstrates that chemical dose was the far stronger effect.

Table 41A. Comparison of chemical F values across Phase-2 test substances and tissues

Test Substance	Test Substance * Laboratory R-square				
	VP	SVCG	LABC	GP	COWS
Methyl Testosterone – series 1	68	73	110	23	61
Methyl Testosterone – series 2	265	176	274	125	127
Trenbolone	11.1	14	45	19.2	13.6
Procymidone	101	95	11	16	46
Vinclozolin – 0.4 mg/kg/d TP	62	107	78.5	20.9	34.4
Vinclozolin – 0.2 mg/kg/d TP	102	264	118	70	88
Linuron	27	49	58	3.5*	15
DDE – 0.4 mg/kg/d TP	57	74	109	29	62
DDE – 0.2 mg/kg/d TP	43	97	83	31	31
Finasteride	51	90	24	10	33

* P NOT < 0.0001; if no asterisk then P was < 0.0001

Table 41B. Comparison of chemical * lab F values across Phase-2 test substances and tissues

Test Substance	Test Substance * Laboratory R-square				
	VP	SVCG	LABC	GP	COWS
Methyl Testosterone – series 1	0.9	0.8	1.6	1.4	4.1 **
Methyl Testosterone – series 2	1.1	2.4 **	0.4	0.3	0.2
Trenbolone	0.5	0.5	0.5	1.9 *	0.9
Procymidone	2.2 *	2.5 *	2.6 *	1.7	1.1
Vinclozolin – 0.4 mg/kg/d TP	0.8	2.2 *	0.8	1.6	1.3
Vinclozolin – 0.2 mg/kg/d TP	1.8	1.0	0.5	1.1	0.8
Linuron	1.5	3.0 **	6.2 **	1.3	2.9 **
DDE – 0.4 mg/kg/d TP	3.0 *	4.0 **	2.9 **	1.0	1.9 *
DDE – 0.2 mg/kg/d TP	1.8	5.0 **	3.8 **	0.7	1.2
Finasteride	1.5	3.6 **	5.3 **	1.9 *	2.1 *

* P < 0.05; ** P < 0.01

190. The F values for the chemical * lab effect indicate that significant differences were observed in 20 of 50 cases. As the analysis is based on absolute weight values, this is not unexpected. There are two possible factors. One is the body size of the rats, and these did vary from lab to lab due to the use of different strains, but even similar strains showed some differences (all laboratories in the MT dose series 2 used the same strain from the same supplier). A second factor may be differences in the dissection technique among laboratories, yielding different weights for certain tissues (e.g., the substantial difference in COWS absolute weights in laboratory 6 in the MT dose series 1 studies and the LABC absolute weight differences among labs evident in Table 23 with LIN).

DISCUSSION

191. The rat Hershberger bioassay has been recommended for use as a screening assay to identify substances potentially acting through (anti)androgen mechanisms, including androgen agonists, androgen antagonists, and 5α-reductase inhibitors. This validation study was conducted in 2002-2003 to demonstrate the ability of the Hershberger bioassay to reliably detect androgen agonists, androgen antagonists, and 5α-reductase inhibitors in dose response studies; to demonstrate the transferability of standardised protocols amongst laboratories; and to quantify the inter-laboratory reproducibility of the bioassay in the dose response studies. The results of this validation study are intended to support the

development of an OECD Test Guideline for the uterotrophic bioassay.

192. The first phase (Phase-1) of the validation of the rat Hershberger bioassay was conducted in two phases. Phase-1A studied the dose response to the potent androgen agonist, testosterone propionate (TP) (CASRN 57-82-5). Phase-1B studied the dose response to the potent androgen antagonist, flutamide (FLU) (CASRN 1311-84-7) using two doses of TP as the potent, coadministered androgen. Those TP doses were chosen based upon the preceding Phase-1A studies. Seventeen laboratories contributed to Phase-1A, and seven laboratories to Phase-1B. Despite differences in rat strain used and different levels of experience among the participating laboratories, there was acceptable agreement in both Phase-1A and 1B among laboratories with respect to the magnitudes of the responses at the different dose levels and the doses at which significant responses were obtained (3). In Phase-1A, TP reproducibly stimulated weight increase in five male accessory sex organs and tissues: the ventral prostate (VP), seminal vesicles plus coagulating glands (SVCG), levator ani and bulbocavernosus muscle complex (LABC), glans penis (GLANS), and Cowper's glands (COWS). In Phase-1B, FLU reproducibly antagonized the weight increase in these same accessory sex organs and tissues in dose response studies, when co-administered with specified doses of TP.

Demonstration of the ability to detect other androgen agonists

193. In Phase 1A, the Hershberger bioassay detected the strong androgen agonist TP and the dose response was reproducible among laboratories (3). One of the specific goals of Phase-2 was to evaluate the reproducibility of the protocol for detecting weaker androgen agonists. The Hershberger bioassay successfully achieved this goal with MT and TREN. All laboratories were able to detect MT and TREN with all five mandatory tissues achieving statistically significant decreases.

Demonstration of the ability to detect weak androgen antagonists

194. In Phase 1B, the Hershberger bioassay detected the strong androgen antagonist FLU and the dose response was reproducible among laboratories (3). One of the specific goals of Phase-2 was to evaluate the reproducibility of the protocol for detecting weaker androgen antagonists. The Hershberger bioassay successfully achieved this goal with PRO, VIN, LIN, and DDE. All laboratories were able to detect DDE with all five mandatory tissues achieving statistically significant decreases. All laboratories were able to detect PRO and VIN with four mandatory tissues achieving statistically significant decreases. Although the GP absolute weights were decreased, the GP, however, sometimes failed to achieve statistical significance in all cases. Three of four laboratories were able to detect LIN with at least four of the mandatory tissues achieving statistically significant decreases. The fourth laboratory detected LIN only with the SCVG and only when the more liberal pairwise comparison statistical approach was used. This laboratory had the largest CVs and encountered some apparent difficulties in the dissection of the small, unstimulated tissues. This reinforces the need for laboratories to sufficiently train their personnel in the dissection of the small tissues which are sometimes embed in adipose tissues, and for laboratories to demonstrate this proficiency before undertaking the Hershberger bioassay.

Demonstration of the ability to detect weak a strong 5α-reductase inhibitor

195. Several of the male reproductive tract tissues require an active 5α-reductase enzyme in order to covert testosterone to dihydrotestosterone and thereby amplify the androgen signal.. A final specific goal of Phase-2 was to evaluate the reproducibility of the protocol for detecting a strong 5α-reductase inhibitor and possibly to assist in the identification of this mechanism with a differential response profile among the mandatory tissues. The Hershberger bioassay successfully achieved this goal with FIN. All laboratories were able to detect FIN with four mandatory tissues achieving statistically significant

decreases in all instances. In this case, the lack or low level of activity of a 5α-reductase enzyme in the GP is a plausible reason this tissue did not achieve statistically significant decreases in one laboratory.

Evaluation of protocol conditions

196. The experience in Phase-2 continues to indicate that the Hershberger protocol is robust. There are several aspects of the protocol that deserve discussion.

Timing and preputial separation

197. The dissection of the GP requires relatively complete preputial separation, which, in turn, is dependent upon the maturation rate of the set of animals. Based on the data generated in Phase-1 and Phase-2, the large majority of animals do achieve preputial separation by necropsy, when castration occurs on about pnd 42. However, in Phase-2, two of the laboratories continued to display incomplete preputial separation at necropsy. The animals with incomplete preputial separation occurred in thee types of groups: the vehicle control, FLU positive reference groups, and those groups administered high doses of antiandrogens. In one laboratory, the GP was not dissected from those animals with incomplete preputial separation, and this appeared to have reduced the statistical power of the assay.

198. The basic recommendation made based upon these data is that laboratories should understand particular characteristics of their animal strain and supplier, e.g., if the animals are castrated on pnd 42, whether preputial separation be complete at necropsy. Given that this will depend upon the particular sexual maturation rate, the obvious solution is to delay castration in those particular circumstances by the minimum time needed to ensure that preputial separation would be complete. It is presumed that the window of sensitivity will be similar in these cases, as this, too, depends upon the rate of sexual maturation. This would represent a minimal, but acceptable, change in the protocol.

199. In addition, those laboratories encountering animals with incomplete preputial separation should proceed with GP dissection. The data from the second laboratory appeared acceptable, and a small increase in CV is preferable to the significant loss of power due to a reduced number of animals.

Fixation of the ventral prostate

200. The results of Phase-2 fully support the previous findings in Phase-1: fixation of the ventral prostate offers no increase in sensitivity and no reduction in CV. It is recommended that no further work be done with ventral prostate fixation, and that this endpoint need not be included in Phase-3.

TP reference doses

201. Phase-1B results indicated that little or no difference was evident between 0.2 mg/kg/d and 0.4 mg/kg/d as the reference dose of TP when coadministered with a dose series of FLU. Phase-2 has expanded this comparison with dose series of VIN and of DDE. Again, there was no evidence for a difference between the two TP reference doses, particularly, in regards to the ability to detect weaker anitandrogens. Importantly, no obvious difference was evident in sensitivity with the weaker antiandrogen, DDE. Therefore, at this time, both doses appear acceptable for use. It is recommended, however, that studies with the two doses be continued, if it is apparent that different national authorities may desire to both of the TP reference doses. This would enlarge the data set to support both or, alternatively, may yet reveal an important difference.

202. In addition, viability in absolute and relative weights was observed in the TP dose groups and the lower test substance treatment groups in several cases. Tissues from the TP + test substance groups were sometimes larger in absolute terms than the TP only dose, and there were isolated events of statistical significance, both negative and positive decreases, on four occasions. In the case of lab 6 with linuron (see Table 23), low tissue weight values in the TP positive control group may have contributed to

the absence of detectable decreases in this study. This suggests some risk exists for the variability in the positive controls to lead to false positives and false negatives. This possibility should be evaluated at the end of the validation program by pooling Phase-1, -2, and -3 data and the use of statistical simulations.

Tissue sensitivity and sources of variability

CVs among tissues and laboratories

203. In addition to animal number and the degree of change in an endpoint, the coefficient of variation in the measurement is one of the determining factors in the power of an assay. The results of Phase-1 and Phase-2 show that the five mandatory tissues appear to have different inherent CV values. Those of the LABC and GP are lower than for the VP, SCVG, and COWS. The tissues differ in two important respects in the unstimulated state: the former tend to be larger and the latter both contain fluid and may be embedded in adipose tissue posing further difficulty in dissection. Therefore, the observed differences in CV values are entirely plausible.

204. When this difference in CV is combined with the relative increase or decrease in a given tissue, this appears to explain the basic sensitivity of a tissue. For example, the low CV observed with the LABC and its intermediate relative response appear to make this tissue among the more reliable against various agonists, antagonists, and even 5α-reductase inhibitors. The high relative response of the VP, SCVG, and COWS appears to be somewhat compromised by their higher CVs, but these tissues appear to perform in the same class as the LABC (see Tables 37 and 38). The GP despite its low CV is apparently hampered by the low relative response of this tissue. Finally, the Phase-2 data also indicate that the particular value of a tissue may vary depending on the mode of action of a particular test substance.

205. In addition, the data set from both Phases also indicates the CV values tend to vary amongst laboratories for a given tissue and that the tendency towards a smaller or larger CV continues across all five mandatory tissues. As with the uterotrophic bioassay, the CV differences among laboratories indicates that a validation programme not only characterizes a particular protocol, but also the participating laboratories. This point concerning laboratory skill and its relationship with CV is discussed further in the following section.

Laboratory skill

206. The combination of CV values and some evident issues with large differences in the absolute weights of tissues like the COWS and VP, suggests that a major factor in the performance of the Hershberger bioassay is the dissecting skill and consistency of the laboratory staff. This proficiency is assumed to come both from previous training and from care exercised during the necropsy. Clearly, the publication of the Lead Laboratory's procedures for dissection, which were shared with the laboratories and which includes detailed photographs of the dissection procedures, is a priority as it appears to be essential to assist with laboratory training and consistency. Just as important, the data in the Phase-1 and Phase-2 reports provide a basis to perform studies with the studied test substances and to judge the current proficiency of a new laboratory and its ability to generate acceptable data.

Evaluation of male reproductive accessory tissue endpoints

207. As noted above, the VP, SCVG, LABC, and COWS tissues appear to constitute a robust battery for the detection of androgen agonists, androgen antagonists, and 5α-reductase inhibitors when used by trained laboratories. It remains open to question, however, how well and consistently these tissues can contribute to the generation of tissue response profiles that can consistently discriminate different mechanisms or metabolic characteristics of a test substance. The data provide both some supporting evidence as well as cautions in this respect.

208. The performance of the GP was the weakest of the five mandatory tissues. The GP was never the sole most sensitive tissue (the only tissue to establish a LOEL with statistical significance regardless of statistical approach). As for the GP being one of several most sensitive tissue, this occurred only 13 times in 41 assays, and this compares to rates of 30 (LABC), 29 (SCVG), 30 (VP), and 23 (COWS) in other tissues. Further, the GP on several occasions failed to reach statistical significance, although the impact of loss of power due to incomplete preputial separation in one laboratory should be taken into account.

209. The current data set on all tissues, including the GP, can be enlarged in Phase-3. Including the GP is not a significant effort. Therefore, it is recommended that the GP continue to be used at least through Phase-3.

Toxicological relevance of the Hershberger bioassay

210. A comparison between the results of the Hershberger bioassay and the findings of developmental and reproductive bioassays was made for six test substances in Tables 40A-F. A similar comparison has been made for the uterotrophic bioassay (64)(65). This comparison strongly supports the toxicological relevance of the Hershberger bioassay in its ability to predict androgen- and antiandrogen-related findings, including frank malformations in the male reproductive tract (e.g., hypospadias and epididymal deformities). In almost all cases, the LOELs observed in Phase-2 are similar to the LOELs seen in the developmental and reproductive studies. Therefore, the predictivity of the Hershberger bioassay based on the current data appears to be excellent. Final conclusions should, however, await work with coded negative substances.

CONCLUSIONS

211. The following conclusions are made based upon the results of Phase-2 and in combination with the previous results of Phase-1:

1. The Hershberger bioassay has successfully demonstrated reliability and reproducibility in dose response studies to detect androgen agonists, antagonists, and a 5α-reductase inhibitor.
2. Four mandatory male sex accessory tissues and glands have proven robust and complementary in this successful detection: the ventral prostate, the seminal vesicles and coagulating glands, the levator ani and bulbocavernosus muscles, and the Cowper's glands. Although the value of the glans penis is open to some question, it would be premature to cease work on this tissue.
3. The dissection and tissue handling (e.g., trimming) capabilities of individual laboratories appears to differ. This conclusion is reached based on differences in CVs and, in a few instances, large and unexplained differences in the absolute weights of tissues. Both appear related to small, unstimulated tissues that may also be embedded in adipose tissue, and laboratories would need skilled and experienced personnel. Therefore, the role of personnel training and experiences should be reemphasized, and the excellent dissection description provided by the Lead Laboratory should be published in the literature as a guide.
4. The fixation of the ventral prostate before weighing has not demonstrated any value in the ability or sensitivity to detect the test substances. Work on this protocol option should cease.
5. In two laboratories, a low rate of incomplete preputial separation was observed, and this impairs the ability to dissect the glans penis. The protocol should be modified so that laboratories whose particular strain or animal supplier may result in a slower rate of sexual maturation may delay castration for a minimal time beyond pnd 42 in order to achieve complete preputial separation.
6. These Phase-2 data in the peripubertal surgically castrated male provide an additional body of data other than Phase-1 reference compounds against which to compare the proposed alternative animal model, the stimulated weanling. In particular, the Phase-2 body of data includes a weak agonist,

trenbolone, and two weak antagonists, linuron and *p,p'*-DDE, to compare the capabilities and performance of the models.

RECOMMENDATIONS

212. The following recommendations are offered:

1. The current plans to the use of coded doses in inter-laboratory studies in a Phase-3 of the Hershberger validation programme should proceed with the surgical castrate.
2. The current plans to study the use of the stimulated weanling as an alternative to the surgical castrate should proceed.
3. The training of laboratory personnel for the five mandatory tissues should be reemphasized to the participating laboratories.
4. Minor changes in the protocol should be made:

    - the provision should be made to delay the timing of castration in laboratories with slower rates of sexual maturation in their animals in order to achieve complete preputial separation.
    - the option to fix the ventral prostate prior to weighing should be deleted.

REFERENCES

1. OECD. (1998). Report of the First Meeting of the OECD Endocrine Disrupter Testing and Assessment (EDTA) Task Force, 10^{th}-11^{th} March 1998, ENV/MC/CHEM/RA(98)5.

2. OECD. (1998). Detailed review paper on the appraisal of test methods for sex hormone disrupting chemicals. OECD Monograph No 21.

3. OECD. (2002). Final OECD report of the initial work towards the validation of the rat Hershberger assay. Phase 1. Androgenic response to testosterone propionate and anti-androgenic effects of flutamide. ENV/JM/TG/EDTA(2002)1/REV2/ADD1.

4. Crisp, TM, Clegg ED., Cooper, RL, Wood, WP, Anderson, DG, Baetcke, KP, Hoffmann, JL, Morrow, MS, Rodier, DJ, Schaeffer, JE, Touart, LW, Zeeman, MG, and Patel, YM. (1998). Environmental endocrine disruption: An effects assessment and analysis. Environ. Health Perspect. 106(Suppl. 1):11-56.

5. CSTEE. (1999). CSTEE Opinion on Human and Wildlife Health Effects of Endocrine Disrupting Chemicals, with Emphasis on Wildlife and on Ecotoxicology Test Methods. Report of the Working Group on Endocrine Disrupters of the Scientific Committee on Toxicity, Ecotoxicity and the Environment (CSTEE) of DG XXIV, Consumer Policy and Consumer Health Protection. Brussels, Belgium.

6. IPCS. (2002). Global Assessment of the State-of-the-Science of Endocrine Disruptors. T. Damstra, S. Barlow, A. Bergman, R. Kavlock, G. Van der Kraack, ed. International Programme on Chemical Safety. WHO/PCS/ECD/02.2 Geneva, Switzerland.

7. Weisner BP. (1934). The post-natal development of the genital organs in the albino rat. With a discussion of a new theory of sexual differentiation. I. Introduction. *J. Obstet. Gynaec. Brit. Emp.* 41:867-922.

8. Weisner BP. (1935). The post-natal development of the genital organs in the albino rat. With a discussion of a new theory of sexual differentiation. VI. Effects of sex hormones in the heteronomous sex. *J. Obstet. Gynaec. Brit. Emp.* 41:8-78.

9. Jost A. (1947). Recherches sur la différenciation de l'embryon de Lapin. III. Rôle des gonads foetales dans la différenciation seuelle. Somatique. *Arch. Anat. Microscop. Morphol. Exptl.* 36:271-289.

10. Jost A. (1953). Problems of fetal endocrinology: The gonadal and hypophyseal hormones. *Rec. Prog. Horm. Res.* 8:379-402.

11. Hamada H, Neumann F, Junkmann K. (1963). *Acta Endocrinol. (Copenhagen)* 44:380-.

12. Neumann F, Wilger W, Kramer M. (1966). Development of a vagina in male rats by inhibiting androgen receptors with an anti-androgen during the critical phase of organogenesis. *Endocrinology* 78:628-632.

13. Neuman F, von Berswordt-Wallrabe R, Elger W, Steinbeck H, Han JD, Kramer M. (1970). Aspects of androgen-dependent events as studied by antiandrogens. *Rec. Prog. Horm. Res.* B26:337-410.

14. Neri R, Florance K, Koziol P, van Cleave S. (1972). A biological profile of a nonsteroidal antiandrogen, SCH 13521 (4'-nitro-3'-trifluoromethylisobutyranilide) *Endocrinology* 91:427-437.

15. Viguier-Martinez MC, de Reviers MTH, Barenton B, Perreau C. (1983). Effect of a non-steroidal antiandrogen, Flutamide, on the hypothalamo-pituitary axis, genital tract and testis in growing male rats: Endocrinological and histological data. *Acta Endocrinol.* 102:299-306.

16. Bloch E, Lew M, Klein M. 1971. Studies on the inhibition of fetal androgen formation: Inhibition of testosterone synthesis in rat and rabbit fetal testes with observations on reproductive tract development. *Endocrinology* 89:16-31.

17. Goldman AS. (1971). Production of hypospadias in the rat by selective inhibition of fetal testicular 17α-hydroxylase and C_{17-26}-lyase. *Endocrinology* 88:527-531.

18. Goldman AS, Eavey RD, Baker MK. (1976). Production of male pseudohermaphroditism in rats by two new inhibitors of steroid 17α-hydroxylase and C_{17-26}-lyase. *J. Endocrinol.* 71:289-297.

19. Imperato-McGinley, J., Binieda, Z., Arthur A, Miniberg DT, Vaughan ED, Jr, Quimby FW. (1985). The development of a male pseudohermaphrodite rats using an inhibitor of the enzyme 5α-reductase. *Endocrinology* 116:807-812.

20. Imperato-McGinley J, Sanchez RS, Spencer JR, Yee B**,** Vaughan ED. (1992). Comparison of the effects of 5α-reductase inhibitor Finasteride and the antiandrogen Flutamide on prostate and genital differentiation: Dose-response studies. *Endocrinology* 131:1149-1156.

21. Cunha GR, Cooke PS, Bigsby R, Brody JR. 1992. Ontogeny of sex steroid receptors in mammals. In: Nuclear hormone receptors. Molecular mechanisms, cellular functions, clinical abnormalities. Parker, MG, ed. Academic Press, pp235-268.

22. Imperato-McGinley J, Peterson RE. (1976). Male pseudohermaphoroditism: The complexities of male phenotypic development. *Am. J. Med.* 61:251-272.

23. Peterson RE, Imperato-McGinley J, Gautier T, Stural E. (1977). Male pseudoherma-phrodism due to steroid 5α-reductase deficiency. *Am. J. Med.* 62:170-191.

24. Wilson JD, Griffin JE, Russell DW. (1993). Steroid 5α-reductase deficiency. *Endocr. Rev.* 14:577-593.

25. Yeh SY, Tsai MY, Xu QQ, Mu XM, Lardy H, Huang KE, Lin H, Yeh SD, Altuwaijri S, Zhou XC, Xing LP, Boyce BF, Hung MC, Zhang S, Gan L, Chang CS. (2002). Generation and characterization of androgen receptor knockout (ARKO) mice: An in vivo model for the study of androgen functions in selective tissues *PNAS* 99:13498-13503.

26. Sato T, Kawano H, Kato S. (2002). Study of androgen action in bone by analysis of androgen-receptor deficient mice. *J. Bone Min. Metab.* 20:326-330

27. Callow RK, Deanesly R. (1935). Effect of androsterone and of male hormone concentrate on the accessory reproductive organs of castrated rats, mice and guinea-pigs. *Biochem. J.* 29:1424-1445.

28. Korenchevsky V. (1932). The assay of testicular hormone preparations. Biochem. J. 26:413-422.

29. Korenchevsky V, Dennison M, Schalit R. (1932). The response of castrated male rats to the injection of the testicular hormone. *Biochem. J.* 26:1306-1314.

31. Eisenberg E, Gordan GS. (1950). The levator ani muscle of the rat as an index of myotrophic activity of steroidal hormones. *J. Pharmacol. Exp. Therap.* 99:38-44.

32. Eisenberg E, Gordan GS, Elliott HW. (1949). Testosterone and tissue respiration of the castrate male rat with a possible test for mytrophic activity. *Endocrincology* 45:113-119.

33. Hershberger L, Shipley E, Meyer R. (1953). Myotrophic activity of 19-nortestosterone and other steroids determined by modified levator ani muscle method. *Proc Soc Exp Biol Med.* 83:175-180.

34. Peets EA, Henson MF, Neri R. (1973). On the mechanism of the antiandrogenic action of flutamide (α-α-α-trifluoro-2-methyl-4'-nitro-m-propionotoluidide) in the rat. *Endocrinology* 94:532-540.

35. Dorfman RI. (1969) Androgens and anabolic agents. In: *Methods in Hormone Research, volume IIA*. Ed.: RI Dorfman. Academic Press, New York. pp 151-220.

36. Dorfman RI. (1969) Antiandrogens. In: *Methods in Hormone Research, volume IIA*. Ed.: RI Dorfman. Academic Press, New York. pp 221-249.

37. OECD. (2002). Summary record of the sixth meeting of the Task Force on Endocrine Disrupters Testing and Assessment (EDTA 6). ENV/JM/TG/EDTA/M(2002)2.

38. OECD. (1996). Final Report of the OECD Workshop on the Harmonisation of Validation and Acceptance Criteria for Alternative Toxicological Test Methods (Solna Report) as presented to the Seventh Meeting of the National Co-ordinators of the Test Guidelines Programme, 18th-19th September 1996. ENV/MC/CHEM/TG(96)9.

39. OECD. (2002). Report of the Stockholm Conference on Validation and Regulatory Acceptance of New and Updated Methods in Hazard Assessment Document. ENV/JM/TG/M(2002)2.

40. ECVAM. (1995). Practical Aspects of the Validation of Toxicity Test Procedures: The Report and Recommendations of ECVAM Workshop No. 5. ATLA 23: 129-147.

41. ICCVAM (Intra-agency Coordinating Committee on the Validation of Alternative Methods). 1997. Validation and Regulatory Acceptance of Toxicological Test Methods. A report of the Ad Hoc Interagency Coordinating Committee on the Validation of Alternative Methods, NIH Report No. 97-3981, National Institute of Environmental Health Sciences, Research Triangle Park, NC, March, 1997.

42. OECD. (1999). Final record of the OECD Validation Management Committee for the Screening and Testing of Endocrine Disrupting Substances – Mammalian Effects. ENV/JM/TG/EDTA(99)1/FINAL.

43. OECD. (2000). Summary record of the fourth meeting of the Task Force on Endocrine Disrupters (EDTA 4). OECD document ENV/JM/TG/EDTA/M(2000)2.

43. EPA. (1998). Endocrine Disrupter Screening and Testing Advisory Committee (EDSTAC) Final Report EPA/743/R-98/003. [http://www.epa.gov/scipoly/oscpendo/history/finalrpt.htm]

44. OECD. (2001). Summary record of the fifth meeting of the Task Force on Endocrine Disrupters (EDTA 5). OECD document ENV/JM/TG/EDTA/M(2001)3.

45. OECD. (2001). Summary record of the third meeting of the OECD Validation Management Group on Screening and Testing of Endocrine Disrupters for Mammalian Effects (VMG 3). OECD document ENV/JM/TG/EDTA/M(2001)1\REV1.

46. Dunnett CW. (1955). A multiple comparison procedure for comparing several treatments with a control. *J. Amer. Statistical Assoc.* 50:1096-1121.

47. Dunnett CW. (1964). New tables for multiple comparisons with a control. *Biometrics* 20:482-491.

48. Hsu JC. (1996). *Multiple Comparisons: Theory and Methods.* London: Chapman and Hall.

48. OECD. (2001). Final Report of the Phase 1 of the Validation Study of the Uterotrophic Assay. ENV/JM/TG/EDTA (2001)1/REV1.

49. OECD. (2003). OECD Report of the Validation of the Rat Uterotrophic Bioassay: Phase 2:. Testing of Potent and Weak Oestrogen Agonists by Multiple Laboratories. ENV/JM/TG/EDTA(2003)1.

50. Yamada T, Sunami O, Kunimatsu T, Kmita Y, Okuno Y, Seki T, Nakatsuka I, Matsuo M. (2001). Dissection and weighing of accessory sex glands after formalin fixation, and a 5-day assay using young mature rats are reliable and feasible in the Hershberger assay. Toxicology 162:103-119.

51. Gray LE, Jr, Wolf C, Lambright C, Mann P, Price M, Cooper RL, Ostby J. (1999). Administration of potentially antiandrogenic pesticides (procymidone, linuron, iprodione, chlozolinate, *p,p´*-DDE, and ketoconazole) and toxic substances (dibutyl- and diethylhexyl phthalate, PCB 169, and ethane dimethane sulphonate) during sexual differentiation produces diverse profiles of reproductive malformations in the male rat. *Toxicol. Ind. Health* 15:94-118.

52. Ostby J, Kelce WR, Lambright C, Wolf C, Mann P, Gray LE, Jr. (1999). The fungicide procymidone alters sexual differentiation in the male rat by acting as an androgen-receptor antagonist *in vivo* and *in vitr*o. *Toxicol. Ind. Health* 15:80-93.

53. Gray LE, Jr, Ostby JS, Kelce WR. (1994). Developmental effects of an environmental antiandrogen: The fungicide vinclozolin alters sex differentiation of the male rat. *Toxicol. Appl. Pharmacol.* 129:46–52.

54. Monosson E, Kelce WR, Lambright CR., Ostby J, Gray LE, Jr. (1999). Peripubertal exposure to the antiandrogenic fungicide, vinclozolin, delays puberty, inhibits the development of androgen-dependent tissues, and alters androgen receptor function in the male rat. *Toxicol. Ind. Health* 15:65-79.

55. Hellwig J, van Ravenzwaay B, Mayer M, Gembardt C. (2000). Pre- and postnatal oral toxicity of Vinclozolin in Wistar and Long-Evans rats. *Reg. Toxicol. Pharmacol*. **32:**42-50.

56. McIntyre BS, Barlow NJ, Wallace DG, Maness SC, Gaido KW, Foster PMD. (2000). Effects of in utero exposure to linuron on androgen-dependent reproductive development in the male Crl:CD(SD)BR rat. *Toxicol. Appl. Pharmacol.* 167:87-99.

57. McIntyre BS, Barlow NJ, Foster PMD. (2002). Male rats exposed to linuron in utero exhibit permanent changes in anogenital distance, nipple retention, and epididymal malformations that result in subsequent testicular atrophy. *Toxicol. Sci.* 65:62-70.

58. McIntyre BS, Barlow NJ, Sar M, Wallace DG, Foster PM. (2002). Effects of *in utero* linuron exposure on rat Wolffian duct development. *Reprod. Toxicol.* 16:131-139.

59. Kelce WR., Stone CR, Laws SC, Gray LE, Kemppainen JA, Wilson EM. (1995). Persistent DDT metabolite *p,p'*-DDE is a potent androgen receptor antagonist. *Nature* 375:581-585.

60. You L, Casanova M, Archibeque-Engle S, Sar M, Fan LQ, Heck HA. (1998). Impaired male sexual development in perinatal Sprague-Dawley and Long-Evans hooded rats exposed in utero and lactationally to *p,p´*-DDE. *Toxicol. Sci.* 45:162-73.

61. Bowman CJ, Barlow NJ, Turner KJ, Wallace DG, Foster PMD. (2003). Effects of *in utero* exposure to Finasteride on androgen-dependent reproductive development in the male rat. *Toxicol. Sci.* 74:393-406.

62. Clark RL., Antonello JM, Grossman SJ, Wise LD, Anderson C, Bagdon WJ., Prahalada S, MacDonald JS, Robertson RT. (1990). External genitalia abnormalities in male rats exposed *in utero* to finasteride, a 5α-reductase inhibitor. *Teratology* 42:91–100.

63. Imperato-McGinley J, Binieda Z, Gedney J, Vaughan ED, Jr. (1986). Nipple differentiation in fetal male rats treated with an inhibitor of the enzyme 5α-reductase: Definition of selective role for dihydrotestosterone. *Endocrinology* 118:132-137.

64. OECD. (2002). EHS Monograph No.38 in the Series on Testing and Assessment: Detailed Background Review of the Uterotrophic Bioassay.

65. Owens JW, Ashby J. (2002). Critical review and evaluation of the uterotrophic bioassay for the identification of possible estrogen agonists and antagonists: In support of the validation of the OECD uterotrophic protocols for the laboratory rodent. Crit. Rev. Toxicol. 32:445-520.

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Annex 1

Participating Laboratories – Phase-2 Hershberger Validation Program

This Information is Only Available to Government Representatives of OECD Member Countries

There were seven participating laboratories in stage 1 and nine participating laboratories in stage 2. Their participation was voluntary. In some laboratories, the work receives financial support from their national authorities or from CEFIC. In other cases, the laboratories were bearing the financial costs themselves. This support and assistance to the OECD is sincerely appreciated and such contributions should be recognized by the VMG-mammalian

Annex 2
Protocol Outline for Phase-2 of the Hershberger Validation Program Outside of Japan

This protocol was contained in a document distributed to each of the participating laboratories as the basis for writing their individual laboratory protocols.

The intended use and purpose of the protocol is to identify potential androgen agonists and antagonists using an in vivo anima model that will incorporate metabolism, distribution, and excretion characteristics while measuring biological responses in the target tissues of the male reproductive tract.

Androgen agonists

1. Biological activity consistent with androgen agonists is tested by administering a test substance to immature castrated rats for 10 consecutive days. The positive control for the tissue responses is TP. The vehicle is the negative control. The weights of the sex accessory tissues of the test chemical groups are compared to the vehicle group for a statistically significant increase in weight.

Androgen antagonists

2. Biological activity consistent with androgen antagonists and 5-alpha reductase inhibitors is tested by administering the test substance to immature castrated rats for 10 consecutive days together with a reference androgen agonist, TP. TP is converted to a more potent from, DHT, by 5-alpha reductase. Administration of TP alone is the negative control. The weights of the sex accessory tissues after co-administration of the test chemical and the reference androgen TP together are compared with the weights of tissues of the reference androgen TP alone for a statistically significant decrease in weight.

DESCRIPTION OF METHOD/PREPARATIONS FOR THE TEST

Animal Species and Strain

16. This protocol allows laboratories to select the strain of rat to be used in the validation of the assay. The selection should be the strain used historically by the participating laboratory, but should not include strains like the Fisher 344 rat. The Fisher 344 rat has a different timing of sexual development compared to other more commonly used strains such as Sprague Dawley or Wistar strains. Where the screening assay may be preliminary to a repeated dose oral study, a reproductive and developmental study, or a long-term study, preferably animals from the same strain and source should be used in all studies. If a laboratory is planning to use an unusual rat strain, or one unique to their own facility, they should determine whether the sexual development criteria noted under the section, *INITIAL CONSIDERATIONS AND PRINCIPLE OF THE ASSAY*, are met.

Castration

17. After an initial acclimatisation period to ensure that the animals are health and thriving, the animals are castrated under anesthesia by placing an incision in the scrotum and removing both testes and epididymes with ligation of blood vessels and seminal ducts. After confirming that no bleeding is occurring, the scrotum should be closed with suture or autoclips. If castrated animals are purchased from an animal supplier, the age of animals and stage of sexual maturity should be assured by the supplier. The time between castration and initiation of dosing will be counted as part of the acclimatisation period. In Phase 1, four laboratories did not observe preputial separation in over two thirds or more of the control group animals. In all these cases, the animals were castrated before 42 days of age or postnatal day (pnd) 42 (6). Therefore, animals shall be castrated on pnd 42 or thereafter, not before.

Acclimatisation after castration

18. Healthy young animals should be acclimatised to the laboratory conditions for a minimum of 7 days following castration. Animals will be observed daily, and any animals with evidence of disease or physical abnormalities will be removed. The treatment with initiation of dosing (on study) may

commence as early as pnd 49 days of age, but not later than pnd 60. Age at necropsy should not be greater than pnd 70. This flexibility allows a laboratory to schedule the experimental work efficiently.

Housing and feeding conditions

19. Temperature in the experimental animal room should be 22 °C (±3°). The relative humidity should be 50 to 60%, but should not exceed limits of 30 to 70% except during room cleaning. Lighting should be artificial, the photoperiod being 12 hours light, 12 hours dark.

20. Laboratories participating in the validation should use the laboratory diet normally used in their chemical testing work. In phase 1, no effects or variability were observed that were attributable to the diet. The diet used will be recorded and a sample of the laboratory diet will be retained for possible future analysis. Both diet and drinking water will be supplied *ad libitum*.

21. Animals should be caged in groups of no more than 3 similarly treated rats per cage, giving a minimum of 2 cages of 3 rats/cage per treatment group. Three animals or less per cage will avoid crowding and associated stress that may interfere with the hormonal control of the development of the sex accessory tissue. Individual housing of animals is permitted. Cages should be thoroughly cleaned to remove possible contaminants and arranged in such a way that possible effects due to cage placement are minimised.

22. Each animal will be identified individually (e.g., ear mark or tag). The method of identification will be recorded.

Body Weight and the selection of animals for the study

23. Increasing differences in body weight may be a source of variability in the weight of tissues of interest (especially the liver) within and among groups of animals. This variability may potentially reduce the assay sensitivity, possibly leading to false positives or false negatives. Therefore, variations in body weight should be both experimentally and statistically controlled, and the statistical analysis should be done both with and without body weight as a covariate. As toxicity may also impact the body weight, the body weight on the first day of administration should be used as the covariate in these cases, not the necropsy body weight.

24. Experimental control of body weight is accomplished in two steps. The first step involves selection of animals with relatively small variation in body weight for the study cohort from the larger population of animals that have been supplied. Unusually small or large animals should be avoided and not placed in the study cohort. A reasonable level of body weight variation within the study cohort should be tolerated. Here, ±20% of the mean body weight for the cohort population is judged to be reasonable (e.g. 175g ± 35g). The second step involves the assignment of animals to different treatment groups (n = 6) by a randomised complete block approach. Under this approach animals are randomly assigned to treatment groups so that each group has the same mean and standard deviation in weight at the beginning of the study. The procedure used for block randomization should be recorded.

Non-routine health and safety requirements

25. The test substances are known as possible reproductive and developmental toxicants and therefore appropriate precautions should be taken to protect personnel during the validation work, e.g. necessary training, labeling and storage procedures, and protective handling procedures during dose preparation and dose administration.

26. Appropriate precautions such as wearing protective gloves, protective clothing and eye protection will be taken when handling the animals, diets, cages, and wastes (e.g. remaining test solutions, faeces, and carcasses). Waste disposal will be in accordance with good practice and existing regulations.

PROCEDURE - VALIDATION PHASE 2

27. The following procedure is focused on the initial validation work where the only test substances used are a reference androgen agonist and a reference antagonist.

Reference substances and vehicle

28. The reference androgen agonist will be Testosterone Propionate (TP), CAS No 57-85-2. The reference androgen antagonist will be Flutamide (FT), CAS No 1311-84-7.

29. All participating laboratories should use a vehicle, such as stripped corn oil, that is not easily disposed to potential microbial degradation of the vehicle or the reference and test substances. If the dosing samples are not made daily, care should be taken to preserve and to avoid contamination and spoilage of the samples.

Test substances in phase 2 of the Hershberger validation

30. The test substances for Phase-2 dose response studies will be:

17α-Methyl Testosterone	CAS No 58-18-4
Trenbolone (17β-Hydroxyestra-4,9,11-trien-3-one)	CAS No 10161-33-8
Vinclozolin	CAS No 50471-44-8
Procymidone	CAS No 32809-16-8
Linuron	CAS No 330-55-2
p,p'-DDE (4,4'Dichlorodiphenyldichloroethylene)	CAS No 72-55-9
Finasteride	CAS No 98319-26-7

31. Ideally, three substances will be assigned to each laboratory with four prescribed doses of each test substance. However, due to resource constraints, some laboratories may be assigned less than three chemicals. A minimum of three laboratories will analyze the response of each test substance.

Test groups in phase 2 of the Hershberger validation

32. 6 animals of the same age and cohort will be used for the vehicle, TP, and any other control group and for each treatment or test substance group.

33. The response of the sex accessory tissues and glands to two agonists, methyl testosterone and trenbolone, will be studied. This work will involve four test groups for each agonist having a prescribed does of the test substance and one vehicle control group.

34. The response of the sex accessory tissues and glands to four antagonists, vinclozolin, procymidone, linuron, and p,p'-DDE, and one 5-alpha reductase inhibitor, finasteride, will be studied. This work will involve four test groups for each antagonist or inhibitor and the coadministration of a dose of 0.4 mg/kg/d of the reference agonist TP to each group. Each test substance will have a prescribed dose for each of its groups. The negative control group will be the reference dose of 0.4 mg/kg/d TP.

Doses in phase 2 of the Hershberger validation

35. All participating laboratories will use the same dose levels. The following table provides the requirements. Ideally, each chemical will have its own unique control group and, where appropriate, a negative control. However, some laboratories may choose to study more than one chemical at a time. In these cases, a single control group and negative control group may be used in order to reduce the number of animals. For those studies of the agonist trenbolone, a TP control group is needed to compare the response profile of the target tissues against the trenbolone profile. In these studies, a dose of ____ mg/kg/d TP should be used in this comparative control group. Additionally, participating laboratories may choose to run an additional, optional control group in antagonists studies using 0.4 mg/kg/d TP and ___ mg/kg/d Flutamide to demonstrate the full response of their system to a potent antagonist. Examples are outlined in the following table:

	Agonist response	Antagonist response
Group A Vehicle Control	Vehicle	Vehicle
Group B Negative Control	Provided by vehicle control (no additional group needed for agonist)	TP 0.4 mg/kg/d
Group C	Test substance at prescribed dose 1[a] in a series of mg/kg/day doses for that substance	TP 0.4 mg/kg/d Test substance at prescribed dose 1[a] in a series of mg/kg/day doses for that substance
Group D	Test substance at prescribed dose 2[a] in a series of mg/kg/day doses for that substance	TP 0.4 mg/kg/d Test substance at prescribed dose 2[a] in a series of mg/kg/day doses for that substance
Group E	Test substance at prescribed dose 3[a] in a series of mg/kg/day doses for that substance	TP 0.4 mg/kg/d Test substance at prescribed dose 3[a] in a series of mg/kg/day doses for that substance
Group F	Test substance at prescribed dose 4[a] in a series of mg/kg/day doses for that substance	TP 0.4 mg/kg/d Test substance at prescribed dose 4[a] in a series of mg/kg/day doses for that substance

[a] The doses of each test substance will be prescribed in order to have comparable data for the analysis of variability among labs.

36. The selected routes of administration and doses for the test substances in phase 2 are:

17α-Methyl Testosterone	per os	0.5, 2, 10, and 40 mg/kg/d
Trenbolone	per os	0.3, 1.5, 8, and 40 mg/kg/d
Vinclozolin	per os	3, 10, 30, and 100 mg/kg/d
Procymidone	per os	3, 10, 30, and 100 mg/kg/d
Linuron	per os	3, 10, 30, and 100 mg/kg/d
p,p'-DDE	per os	5, 16, 50, and 160 mg/kg/d
Finasteride	per os	0.2, 1, 5, and 25 mg/kg/d

In addition, for those laboratories with trenbolone as a test substance, an optional study is to perform a parallel subcutaneous administration, at doses of 0.015, 0.0.62, 0.250 and 1 mg/kg/d, in order to demonstrate dose response differences in the routes of administration.

Administration of doses

37. TP will be administered by s.c injection as in phase 1.

38. All test substances in phase 2 will be administered by oral gavage.

39. S.c. injections will be on the dorsal surface of the animal after shaving or trimming of fur. Multiple injections sites may be used. The maximum limit on the volume administered per animal is approximately 0.5 ml/kg body weight per day.

40. Oral gavage will be the delivery of the test substance in vehicle by means such as intubation with an oral gavage syringe. The maximum limit on the volume administered per animal will be 5 ml/kg/day.

41. The animals will be dosed in the same manner and time sequence for ten consecutive days at approximately 24 hour intervals. The dosage level will be adjusted daily based on the concurrent daily measures of body weight. The volume of dose and time that it is administered will be recorded on each day of exposure.

Good Laboratory Practice

42. Work will be conducted according to the principles of Good Laboratory Practice (OECD Good Laboratory Practice and Compliance Monitoring (7). In particular, data will have a full audit trail and be retained on file. Data will be collected in a manner that will allow independent peer review. Calibration data for all balances used should be determined a part of the study and written records maintained.

OBSERVATIONS

Clinical observations

43. Animals will be evaluated at least once daily for mortality, morbidity, and signs of injury as well as general appearance and signs of toxicity. Any animals in poor health will be identified for further monitoring.

44. Any animal found dead will be removed and disposed of without further data analysis. Any mortality of animals prior to necropsy will be included in the study record together with any apparent reasons for mortality.

Body weight and food consumption

45. Individual body weights will be recorded prior to start of treatment (to the nearest 0.1g), on each day of administration period and prior to necropsy. Group means and standard deviations will be calculated.

46. Food consumption should be generally observed and any significant changes recorded.

Necropsy

47. Approximately 24 hours after the last administration of the test substance, the rats will be euthanized and exsanquinated according to the normal procedures of the participating laboratory, and necropsy carried out. The method of humane killing will be recorded in the laboratory report.

48. The order in which the animals are necropsied will be designed such that one animal from each of the groups is necropsied in a random fashion before necropsy of the second animal from each group. In this way, all the animals in the same treatment group are not necropsied at once.

49. The five sex accessory tissues (VP, SV, LABC, COW, GP) are mandatory measurements. The sex accessory tissues will be excised, carefully trimmed of excess adhering tissue and fat, and their fresh (unfixed) weights determined. Each tissue should be handled with particular care to avoid the loss of fluids and to avoid desiccation, which may introduce significant errors and variability by decreasing the recorded weights.

50. Several of the tissues may be very small or difficult to dissect, and this will introduce variability. Therefore, it is important that persons carrying out the dissection of the sex accessory tissues are familiar with standard dissection procedures for these tissues. A standard operating procedure (SOP) manual for dissection has been provided by the Lead Laboratory for phase 1. This manual will remain the SOP reference for phase 2. Careful training according to the SOP guide will minimise a potential source of variation in the study. Ideally the same prosector should be responsible for the weighing a given tissue to

eliminate inter-individual differences in tissue processing. If this is not possible, the necropsy should be designed such that each prosector weighs a given tissue from all treatment groups as opposed to one individual weighing all tissues from a control group, while someone else is responsible for the treated groups.

51. Each sex accessory tissues will be weighed without blotting to the nearest 0.1 mg, and the weights recorded for each animal.

52. As an optional exercise, the impact of fixation on the ventral prostate may be studied. In these cases, after excision and weighing of the fresh ventral prostate, the tissue will be fixed for 24 hours in 10% neutral buffered formalin (4% formaldehyde) and weighed again approximately 24 hours later and the weight recorded to the nearest 0.1 mg for each animal.

53. Liver, paired kidney, and paired adrenal weights are optional measurements. Again, tissues should be trimmed free of any adhering fascia and fat. The liver will be weighted to the nearest 0.1 g, and the paired kidneys and paired adrenals will be weighted to the nearest 0.1 mg. All weights will be recorded for each animal.

54. The serum hormones LH and T are optional measurements. In those cases, the rats will be anesthetised prior to necropsy and blood taken by cardiac puncture, and the method of anesthesia should be chosen with care so that it does not affect hormone measurement. The method of serum preparation, the source of radioimmunoassay or other measurement kits, the analytical procedures, and the results should be recorded. LH levels should be reported as ng per ml of serum, and T should also be reported as ng per ml of serum.

55. If the evaluation of each chemical requires necropsy of more animals than is reasonable for a single day, necropsy may be staggered on two consecutive days. In this case the work could be divided so that necropsy of 3 animals per treatment per day (1 cage) takes place on the first day with the dosing and necropsy being delayed by one day in the second half of the animals.

56. Carcasses will be disposed of in an appropriate manner following necropsy.

REPORTING

Data

57. Data will be reported individually (i.e. body weight, accessory sex tissue weights, optional measurements and other responses and observations) and for each group of animals (means and standard deviations). The data will be summarised in tabular form. The data will show the number of animals at the start of the test, the number of animals found dead during the test or found the test number of animals found showing signs of toxicity, a description of the signs of toxicity observed, including time of onset, duration and severity.

58. To assist data reporting and compilation, a standardised electronic spreadsheet will be used by participating laboratories to report and transmit data during the validation work to the Secretariat so that it may be easily exchanged and compiled with the Lead Laboratory and independent statisticians. This spreadsheet will be provided by the OECD Secretariat.

Test report

59. The test report must include the following information:

Laboratory identification

- Name of laboratory, location
- Principal investigator and other personnel and their roles in the study
- Dates study began and ended

Test substance:

- Physical nature and, where relevant, physicochemical properties;
- Identification data and source
- Purity

Vehicle:

Test animals and procedures:

- Species/strain used;
- Source or supplier of animals, including full address;
- Number, age and sex of animals;
- Housing conditions (temperature, lighting, and so on), diet used, lot of diet, source of diet, bedding and source of bedding;
- Caging conditions and number of animals per cage;
- Age at castration and time of acclimitisation after castration;
- Individual weights of animals at the start of the study (to nearest 0.1 g);
- Randomization process and a record of the assignment to vehicle, reference, and test substance groups;
- Mean and standard deviation of the body weights for each group at the start of the study;
- Necropsy procedures, including means of exsanguination and any anesthesia; and
- If serum analyses are performed, the RIA procedure, source of RIA kits, procedure for scintillation counting, and standardization.

Results:

- Daily observations during administration, including:
  - Daily body weights (to the nearest 0.1 g),
  - Clinical signs (if any),
  - TP treatment (Yes or No),
  - Test substance treatment (Yes or No),
  - Dose level and volume administered each day,
  - Time of dosing each day, and
  - Notes on food consumption or measurement of actual food consumption each day.
- On the day of necropsy, individual necropsy data on each animal including absolute sex accessory tissue weights, liver and body weights including the following:
  - Date of necropsy,
  - Animal ID,
  - Home Cage Number or ID,
  - Prosector,
  - Time of day necropsy performed,
  - Animal age, and
  - Order of animal exsanguination and dissection at necropsy,
- Weights of all five mandatory sex accessory tissues and glands.
  - Ventral prostate (fresh weight is mandatory and a second weight after 24 hours of fixation is optional – to the nearest 0.1 mg in both cases),
  - Seminal vesicles plus coagulating glands, including fluid (fresh weight – paired, to nearest 0.1 mg),
  - Levator ani plus bulbocavernosus muscle (fresh weight - to nearest 0.1 mg),
  - Glans penis (fresh weight to nearest 0.1 mg), and

- Cowper's glands (fresh weight – paired, to nearest 0.1 mg).
- Weights of optional tissues, if performed.
  - Liver (optional – to nearest 0.1 g),
  - Kidney (optional – paired, to nearest 0.1 mg), and
  - Adrenal (optional – paired, to nearest 0.1 mg).
- Analyses of serum hormones, if performed.
  - Serum LH (optional – ng per ml of serum), and
  - Serum T (optional – ng per ml of serum)

- General remarks and comments

Discussion

Conclusions

Interpretation of results

60. Statistical comparisons in individual laboratories will be made for the different sex accessory by analysis of variance. For androgen agonism, the test substance groups will be compared to the vehicle control. A statistically significant increase in tissue weight will be considered a positive androgen agonist result. For androgen antagonism, the test substance with co-administered reference androgen groups will be compared to the reference androgen control. A statistically significant decrease in tissue weight will be considered a positive antagonist result. If more than one set of comparisons is required, all comparisons will be conducted separately for each test group against its control.

Annex 3
Report of the Lead Laboratory on Phase-2

For technical reasons this annex was not able to be incorporated into the main document. Annex 3 therefore exists as a separate document [ENV/JM/TG/EDTA(2003)5/ANN].