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# The mammary gland response to estradiol: Monotonic at the cellular level, non-monotonic at the tissue-level of organization?

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**Abstract**

The role of hormones in mammary gland development has been studied in detail using surgical and genetic models. These studies have indicated roles for estrogen in ductal elongation and terminal end bud formation. However, no comprehensive study has quantified how different doses of estrogen affect morphological parameters of mammary gland development. Additionally, comparisons between the estrogen-responsiveness of the mammary gland and uterus, the model organ for estrogen action are incomplete. In this study, immature mice were ovariectomized and implanted with osmotic pumps releasing one of eight doses of 17β-estradiol for 10 days. As expected from the classical uterotrophic assay, the uterus showed a monotonic dose–response curve for all measured endpoints. In contrast, the mammary gland showed a non-monotonic, inverted-U shaped response to estrogen with regard to morphometric parameters, and a monotonic response with regard to gene expression parameters. These results indicate that estrogen has opposing effects in mammary gland morphogenesis depending on estrogen dose, i.e. low to moderate doses induce terminal end bud formation and ductal elongation while higher doses inhibit these processes. This non-monotonic dose–response in the mammary gland may reflect complex interactions, where estrogen can act on multiple targets either as an agonist or antagonist.


*Keywords:* Terminal end buds; Non-monotonic dose–response; Msx2; Wnt4; Progesterone receptor; Endocrine disruptors

## 1. Introduction

Development of the rodent mammary gland can be separated into five stages: embryonic/fetal, pre-pubertal, pubertal, sexually mature adulthood and pregnancy/lactation (reviewed in [1]). Following birth, the mammary glands grow at about the same rate as the rest of the body. However, at approximately 3 weeks of age, the rate of mammary gland growth increases [2] due to increased estrogen secretion by the ovaries. Administration of estrogens to intact or ovariectomized pre-pubertal animals results in morphological changes similar to those observed at puberty, i.e. formation of terminal end buds (TEBs), and ductal elongation; ovariectomy prior to puberty prevents these changes. Additional studies conducted in female mice and rats that had been ovariectomized, hypophysectomized, and adrenalectomized revealed that in addition to estrogen, growth hormone is also required to restore normal growth of the mammary gland [2]. These results highlight the importance of estrogen in ductal morphogenesis and elongation, but also indicate that estrogen alone cannot complete this development.

The development of knockout mice contributed to our knowledge on the effects of hormones in mammary gland development, by gaining insights into the complex interactions of progesterone, prolactin and estrogen during mammary gland morphogenesis. For example, while studies on the progesterone receptor (PR) knockout mouse have confirmed the role of progesterone in ductal side branching [3,4], recent studies using maspin heterozygous mice, which have a severe deficiency in systemic progesterone, revealed a role for this hormone in ductal elongation [5]. Prolactin, a pituitary hormone, has been found to play a role in alveolar development and lactation [6,7]. Additionally, prolactin receptor knockout

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mice show stunted ductal growth and altered maturation of TEBs, processes previously believed to be regulated by estrogen alone [8,9]. Finally, prolactin and estrogens both regulate expression of PR [10–12]. Thus, these new revelations suggest that the schematic view that one hormone controls one developmental stage is no longer adequate.

Knock-out estrogen receptor (ER)α and ERβ mice (ERαKO and ERβKO, respectively) are available for study [13,14] and their mammary gland phenotypes have been described in detail (see [1,15] for comprehensive reviews). Because no grossly abnormal phenotype is seen in the ERαKO until puberty, it has been assumed that estrogen action is not essential during embryonic/fetal development. Estrogen exposure during fetal life, however, generates significant effects; for instance, altered mammary gland phenotypes are observed in peripubescent and adult females exposed to steroidal estrogens and xenoestrogens (environmental estrogens) during fetal development [16–20]. It is likely that these alterations may be due to either an altered response to ovarian hormones, or to an altered output of ovarian hormones. Hence, determining these dose–response relationships is a necessary step in understanding the effects of perinatal xenoestrogen exposure that manifest at puberty.

At puberty, the mammary gland of ERαKO females resembles the wild-type newborn gland, indicating that estrogen action is essential for ductal elongation normally occurring at this time [21,22]. Additionally, the importance of estrogen is highlighted by the mammary glands of the aromatase knockout mouse, which are deficient in the enzyme required to convert testosterone to estradiol; these mice have glands that arrest in pre-pubertal development [23]. Collectively, these studies demonstrate that estrogen and ERα play a significant role during pubertal development of the mammary gland. In contrast, studies of the ERβKO suggest that ERβ does not play a major role in mammary gland morphogenesis. At puberty, mammary glands from mice lacking ERβ appear normal, and seem to undergo normal epithelial growth and development, indicating that ERα is the primary receptor involved in estrogen signaling in the mammary gland [1] during puberty. However, subtle morphological alterations have been detected in lactating mammary glands of ERβKO mice, particularly in the alveoli and secretory epithelium [24] suggesting that ERβ has a role in mammary gland development at pregnancy and lactation.

For the most part, the abovementioned body of research has focused on qualitative alterations in mammary gland morphology in response to the addition or absence of hormones. Yet, quantitative analyses are necessary to determine which responses occur at physiological ranges in a wild-type, normally responding animal, and they are a prerequisite to understanding the hormonal interactions that operate during normal mammary gland morphogenesis.

The aim of this paper was to initiate this line of inquiry by determining the dose–response to estradiol in the mammary gland at puberty. We also compared the sensitivity of the mammary gland with that of the uterus, which is considered the organ of reference to evaluate estrogen action. For this purpose we used mice ovariectomized at 25 days of age and further treated for 10 days with one of eight doses of 17β-estradiol, delivered via subcutaneous osmotic pumps. To complete these studies, we modified the parameters of the standard uterotrophic assay, allowing for steady delivery of 17β-estradiol (instead of daily injections) over a longer period of time. While the standard uterotrophic assay is conducted following 3 days of exposure, we found this time to be insufficient to induce morphological changes in the mammary gland (data not shown). Thus, to compare the response of the mammary gland to the expected response of the uterus, it was necessary to use an extended exposure period. We conducted a pilot study to determine which exposure length would induce a measurable response in the mammary gland; it revealed that 10 days were necessary (data not shown).

## 2. Materials and methods

### 2.1. Animals

Female CD-1 (Charles River) mice were ovariectomized at 25 days of age under isofluorane anesthesia. In brief, a small incision was made on the dorsal skin and the muscle wall above each kidney, the ovary was gently teased out, clamped, tied off, and removed. The muscle wall was closed with silk sutures and the skin was closed with wound clips. A small incision was then made in the dorsal skin above the shoulder blades where an Alzet osmotic pump was placed subcutaneously. The incision was closed using wound clips. Ovariectomized females were maintained in temperature-controlled and light-controlled (14 h light, 10 h dark cycle) conditions in the Tufts University School of Medicine Animal Facility. Food, cages and bedding all tested negligible for estrogenicity by the E-SCREEN assay [25]; water was supplied from glass bottles only. All experimental procedures were approved by the Tufts University—New England Medical Center Animal Research Committee in accordance with the Guide for Care and Use of Laboratory Animals.

### 2.2. Osmotic pumps

Osmotic pumps (Alzet, Model 1002) were filled with solutions delivering 0, 0.25, 0.5, 1, 5, 10 or 50 μg/kg body weight/day 17β-estradiol (Steraloids, Newport, RI) dissolved in 50% dimethyl sulfoxide (DMSO, vehicle control, Sigma) following the manufacturer's recommendations for loading ($n = 5$ animals per dose). Pumps were placed in petri dishes containing Dulbecco's modified medium supplemented with 1.5 M Hepes (1%, pH 7.6) and penicillin (0.25%) and incubated at 37 °C overnight to allow for equilibration. These pumps delivered the intended dose of estradiol for the duration of the treatment.

### 2.3. Tissue harvesting

At 35 days of age, animals were weighed and then killed using brief $CO_2$ inhalation followed by decapitation. An incision was made along the skin at the ventral midline and the fourth inguinal mammary glands were dissected from the skin. One mammary gland was immediately immersed in RNA-later (Ambion) and stored at 4 °C following the completion of the dissections. These glands were transferred to RNAse-free Eppendorf tubes and stored at −80 °C. The second mammary gland was spread on a superfrost positive charged glass slide (Fisher) and placed in 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS) (pH 7.4) overnight. A Y-incision was made on the ventral side of the animal, from the vaginal opening to the upper limbs. The uterus was located and gently dissected from the connective tissue and adipose tissue of the abdomen. A cut was made at the cervix to allow removal of the complete uterus from the body cavity. The uterus was cut in half at the cervix, each piece was blotted to remove excess fluids and the collective sections were weighed. One half was immediately placed in RNA-later and treated as described above for the mammary gland. The other half was flushed with 4% paraformaldehyde and immersed in the fixative for 12 h.

### 2.4. Tissue processing

Whole mount mammary glands were dehydrated through a series of alcohols and xylene, treated with toluene to remove fat, rehydrated with another alcohol series, stained with carmine to allow for the visualization of epithelial ducts, and again dehydrated using a series of alcohols and xylene. Whole mounts were immersed in methyl salicilate (Sigma) and sealed in Kpax bags (Fisher), visualized using a Zeiss dissection scope and images were captured with a Zeiss AxioCam digital camera.

Fixed uterine tissue was also processed through a series of alcohols and xylene, infiltrated with paraffin (Fisher) under vacuum, and embedded in paraffin. Five-micrometer cross sections were cut on a microtome (Leica RM2155 Rotary Microtome) and mounted on superfrost positive charged slides (Fisher).

### 2.5. Immunohistochemistry

Sections were treated with xylene to remove paraffin and rehydrated through a series of alcohols and PBS, then microwaved in 10 mM citrate buffer (pH 6) for antigen retrieval. Non-specific binding was blocked for 1 h with normal goat serum in 1.5% milk. Sections were incubated overnight at 4 °C in a humid chamber with rabbit antibody raised against mouse Lactoferrin (C. Teng, NIH) at a concentration of 1:200. Biotinylated goat anti-rabbit IgG (Zymed) was applied to sections at a concentration of 1:200 for 1 h in a humidified chamber at room temperature. Slides were rinsed with PBS and incubated with extravidin-peroxidase complex in 1% BSA (Sigma) for 30 min at room temperature. Expression was visualized by diaminobenzidine (DAB) (Sigma) treatment for 10 min. Sections were then counterstained with Harris' hematoxylin.

### 2.6. Morphometric measurements

#### 2.6.1. Mammary gland whole mounts

Images were captured at 1.25× and 2600 dpi and processed with Axiovision software (version 4.3). This package was used to measure the following parameters: area of terminal end buds, defined as any "club" shaped ductal end $\geq 0.03\,mm^2$ [26]; total number of terminal end buds; total area of the ductal tree as measured by the outermost points of the ductal tree extensions; ductal extension as measured by the furthest point of growth of the leading edge of epithelium from the center of the lymph node. Additional parameters were calculated including number of terminal end buds/$mm^2$ of ductal tree and area of terminal end buds/$mm^2$ of ductal tree.

#### 2.6.2. Cross sections of the uterus

Images were captured at 10× and 3900 dpi and processed with the Axiovision software. This package was used to measure the following parameters: total area of the cross section including the lumen, area of the lumen, area of different uterine structures including myometrium, lamina propria, glands, and luminal epithelium. The volume of each uterine compartment was calculated by correcting the relative area of each compartment by the blotted weight of the uterus. Additionally, the average height of the luminal epithelial cells was calculated by measuring stretches of epithelium totaling 800 μm or greater and measuring the height of every fifth cell within this stretch.

### 2.7. Quantification of lactoferrin expression

Images were collected from two distant areas in each uterine section at 40× and 2600 dpi and the expression of lactoferrin in the cells of the luminal and glandular epithelium was assessed, as previously described [27]. Briefly, the total number of cells in each compartment was counted and each individual cell was determined to be positive or negative for lactoferrin expression. The percentage of lactoferrin positive cells was calculated from these counts.

### 2.8. RNA isolation

RNA from the mammary glands was isolated using a combination of the TRIzol and column purification methods according to the manufacturer's instructions. In brief, mammary glands stored at −80 °C were weighed and homogenized in the appropriate volume of TRIzol (Invitrogen Inc., Carlsberg, CA) and centrifuged to remove cell debris. The supernatant was subjected to chloroform extraction and the colorless aqueous phase obtained after centrifugation was

mixed with 70% ethanol and loaded on an RNeasy mini column (Qiagen Inc., Valencia, CA) to complete RNA isolation. The quantity of RNA was determined by O.D. readings taken at 260 nm and the quality was determined by the ratio of O.D. read at 260/280 nm.

### 2.9. Quantitative real time RT-PCR

Two micrograms of total RNA was reverse transcribed with oligo (dT) primers using Superscript II (Invitrogen Inc., Carlsberg, CA). Three microliters of the cDNA solution was subjected to Real Time PCR using the Quantitect SYBR Green PCR kit (Qiagen Inc.,) on a Stratagene MX4000 machine (Stratagene, La Jolla, CA). The PCR was performed as described previously [20]. The primer pairs used for the PCR were as follows—Msx2: forward primer, GGAAGACCAGATGGACCAGA; reverse primer, TCTGTATCAAGTGGCCCTGTC; Wnt4: forward primer, AATGCACAGTTCAAGCCACA; reverse primer, AGCCGTCAATGGCTTTAGAT; PR: forward primer, GAATTGATCAAGGCAATTGGT; reverse primer, TTCACAAGATCATGCAAGCTG; L19: forward primer, ATCGCCAATGCCAACTCC; reverse primer, TCATCCTTCTCATCCAGGTCA.

The PCR program consisted of 45 cycles of denaturation at 95 °C for 15 s, annealing at 59 °C for 30 s and extension at 72 °C for 30 s. The PCR products were detected using the SYBR Green dye normalized to 6-carboxy-X-rhodamine dye. The Ct (threshold cycle) value of every sample was normalized with that of L19, a housekeeping gene. Gene expression was calculated using the 'Comparative Quantitation' method in the Stratagene MX4000 software to determine fold-change of the unknown samples with respect to a pre-designated calibrator sample. In these experiments the sample with no estradiol treatment was chosen as the calibrator. PCR for each sample was repeated three times and the average fold-change value was reported. The purity and specificity of the PCR products was confirmed by the dissociation curve set up at the end of each run.

### 2.10. Statistical analysis

All calculated parameters and statistical significance were determined using SPSS statistical software. To determine statistical differences between the responses at each dose, we used non-parametric Mann–Whitney *U*-tests as well as parametric *t*-tests when data were normally distributed. All figures are graphed as mean ± S.E.M.

## 3. Results

### 3.1. Determinations of monotonic and non-monotonic responses to estradiol

For each parameter, we calculated the following statistical relationships: (1) the lowest dose that caused a statistical difference in sensitivity for each parameter, this is referred to as the lowest observable effect level (LOEL) dose, (2) the doses at which a plateau in response occurred or the dose where the highest response is achieved, this is reported as the dose of maximal response and (3) half-maximal doses, described as the amount of estradiol that induces half of the maximal response observed for each parameter.

In order to use objective methods to determine which responses to estradiol could be considered non-monotonic, first it was determined whether the maximal response of a given parameter occurred at one of the intermediate doses administered. Additionally, it was also determined whether the peak response could be statistically distinguished from the response at the highest dose administered. When both of these criteria were met, the parameter of interest was considered to exhibit a non-monotonic response to estradiol. If these criteria were not met, the response of the parameter was considered to be monotonic. Finally, curve-fitting analysis was performed for each parameter examined to determine whether the response to estradiol best conformed to a sigmoidal (monotonic) curve, or to a polynomial (non-monotonic) curve. To do this, best fits ($R$, correlative values) were determined using model curves. The androgen dose–response curve of LNCaP LNO cells was the model for a non-monotonic curve, and the androgen dose–response curve of LNCaP FGC cells was the model for a monotonic response [28].

### 3.2. Uterine responses to estradiol

Wet weight of the uterus represents the classical end point for assessing estrogenicity. As expected, it displayed a monotonic dose–response curve. The maximal response to estradiol reached a plateau between 10 and 50 μg/kg per day E2 and the LOEL was 0.25 μg/kg per day E2, the lowest dose we administered (Fig. 1A), indicating that this parameter is a highly sensitive measure. A similar pattern was observed when uterine weight was corrected by body weight.

To determine whether other end points would respond to estrogen treatment in a similar way, we examined the uteri on a compartmental and cellular level. Monotonic dose–response curves were obtained for the relative area covered by glands, glandular epithelium, and the myometrium (Table 1). Monotonic dose–response curves were also obtained for the absolute volume of myometrium, lamina propria, luminal epithelium and glandular epithelium. However, the LOEL observed in each of these parameters varied from 0.5 to 2.5 μg/kg per day, indicating that wet weight is the most sensitive measure of estradiol exposure for this organ (Table 2).

Another well-established method to measure the response of the uterus to estradiol is the increase in height of the uterine epithelial cells. The height of the uterine epithelium displayed a monotonic dose–response curve with a maximal response at 50 μg/kg per day. The LOEL was 5 μg/kg per day (Fig. 1B). We also found that the expression of lactoferrin, an estrogen-induced protein, in the uterus followed a monotonic pattern

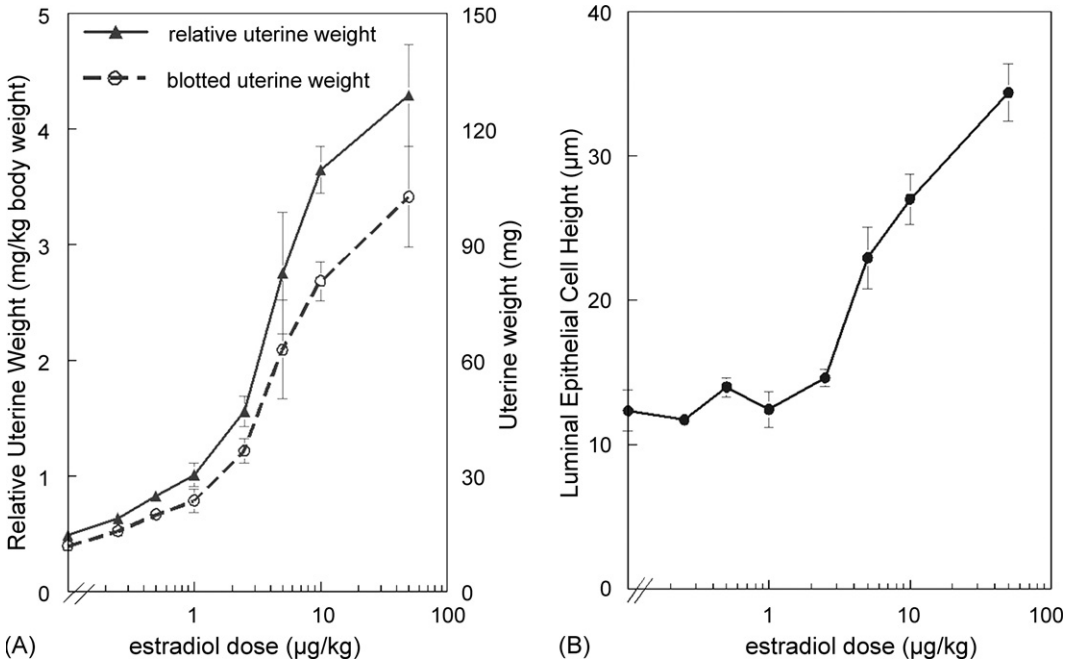


Fig. 1. Responses of the uterus to estrogen at several levels of tissue organization: (A) uterine weight (open circle, dashed line) and uterine weight corrected by animal body weight (closed triangle, solid line); (B) height of cells composing the luminal epithelium.

Table 1
Relative area of uterine compartments

| Parameter | LOEL (μg/kg) | Half-maximal dose (μg/kg) | Plateau in response (μg/kg) | Fold-change |
|---|---|---|---|---|
| Glands | 5 | 6.5 | 50 | 5.6 |
| Glandular epithelium | 5 | 6.2 | 50 | 4.9 |
| Myometrium | 2.5 | 1.25 | 5–50 | 1.9 |

Table 2
Absolute volume of uterine compartments

| Parameter | LOEL (μg/kg) | Half-maximal dose (μg/kg) | Plateau in response (μg/kg) | Fold-change |
|---|---|---|---|---|
| Glandular epithelium | 1 | 6.75 | 5–50 | 58.2 |
| Myometrium | 0.5 | 3.3 | 5–50 | 2.5 |
| Lamina propria | 1 | 3.45 | 10–50 | 13.3 |
| Luminal epithelium | 2.5 | 3.6 | 5–50 | 22.8 |

both in the luminal and the glandular epithelium (Fig. 2). The LOEL for lactoferrin expression in both compartments was 5 μg/kg per day. Therefore, these cellular parameters are the least sensitive measures of estrogenicity in the uterus.

### 3.3. Estradiol responses in the mammary gland

Based on graphs of each morphometric parameter, the mammary gland appears to exhibit a non-monotonic

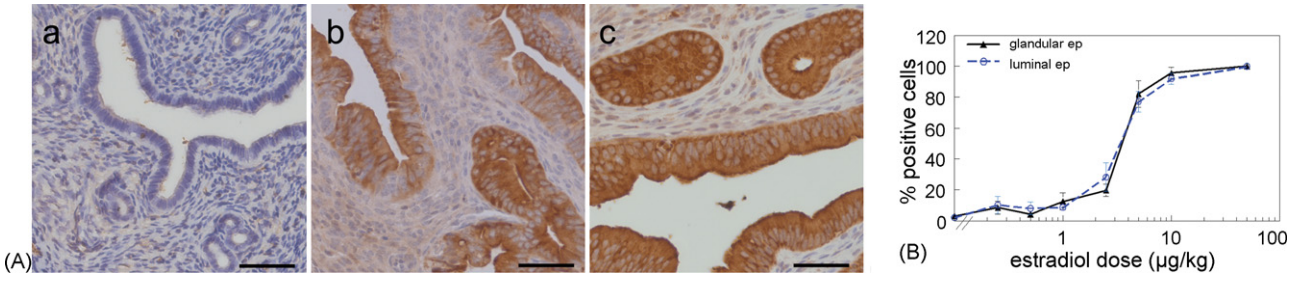


Fig. 2. Expression of lactoferrin in the uterus: (A) immunohistochemistry to detect lactoferrin in the luminal and glandular epithelium of the uterus following exposure to (a) 0 μg/kg per day, (b) 5 μg/kg per day and (c) 50 μg/kg per day estradiol. Scale bar, 200 μm; (B) quantification of lactoferrin expression in the glandular (closed triangle, solid line) and luminal (open circle, dashed line) epithelial compartments of the uterus.

Table 3
Direct and relative morphometric measures in the mammary gland

| Parameter | LOEL (μg/kg) | Half-maximal dose (μg/kg) | Dose of maximal effect (μg/kg) | Fold-change |
|---|---|---|---|---|
| Ductal extension | 0.5 | 0.42 | 2.5 | 3.2 |
| #TEBs | 2.5 | 1.8 | 5 | 9.8 |
| TEB area | 2.5 | 1.6 | 5 | 11.6 |
| Ductal area | 0.5 | 0.4 | 2.5 | 1.4 |
| #TEBs/ductal area | 2.5 | 2 | 5 | 7.0 |
| TEB area/ductal area | 2.5 | 1.6 | 5 | 8.6 |

dose–response to estradiol, with maximal responses for each parameter achieved at one of the intermediate doses of estradiol administered (Table 3) and lesser responses at low and high doses of estradiol (Fig. 3A, a–d). We assessed whether the maximal response (at an intermediate dose) could be statistically distinguished from the response at the highest dose of estradiol (50 μg/kg per day). Based on this criterion, ductal extension (Fig. 3B), number of TEBs (Fig. 3C) and total TEB area (Fig. 3C) were determined to have non-monotonic dose–response curves. The only parameter that failed to meet this criterion was ductal area (Fig. 3B), because the maximal response at 2.5 μg/kg per day cannot be statistically distinguished from responses at higher doses of estrogen. However, the LOEL for ductal area was 0.5 μg/kg per day, the most sensitive parameter in the mammary gland, indicating that this parameter is still a highly sensitive measure of estrogen exposure. The density of epithelial structures was also examined. The number of TEBs/mm$^2$ of ductal area as well as the area of all TEBs/mm$^2$ of ductal area followed non-monotonic dose–response curves (Fig. 3D), with maximal response occurring at 5 μg E2/kg per day (Table 3).

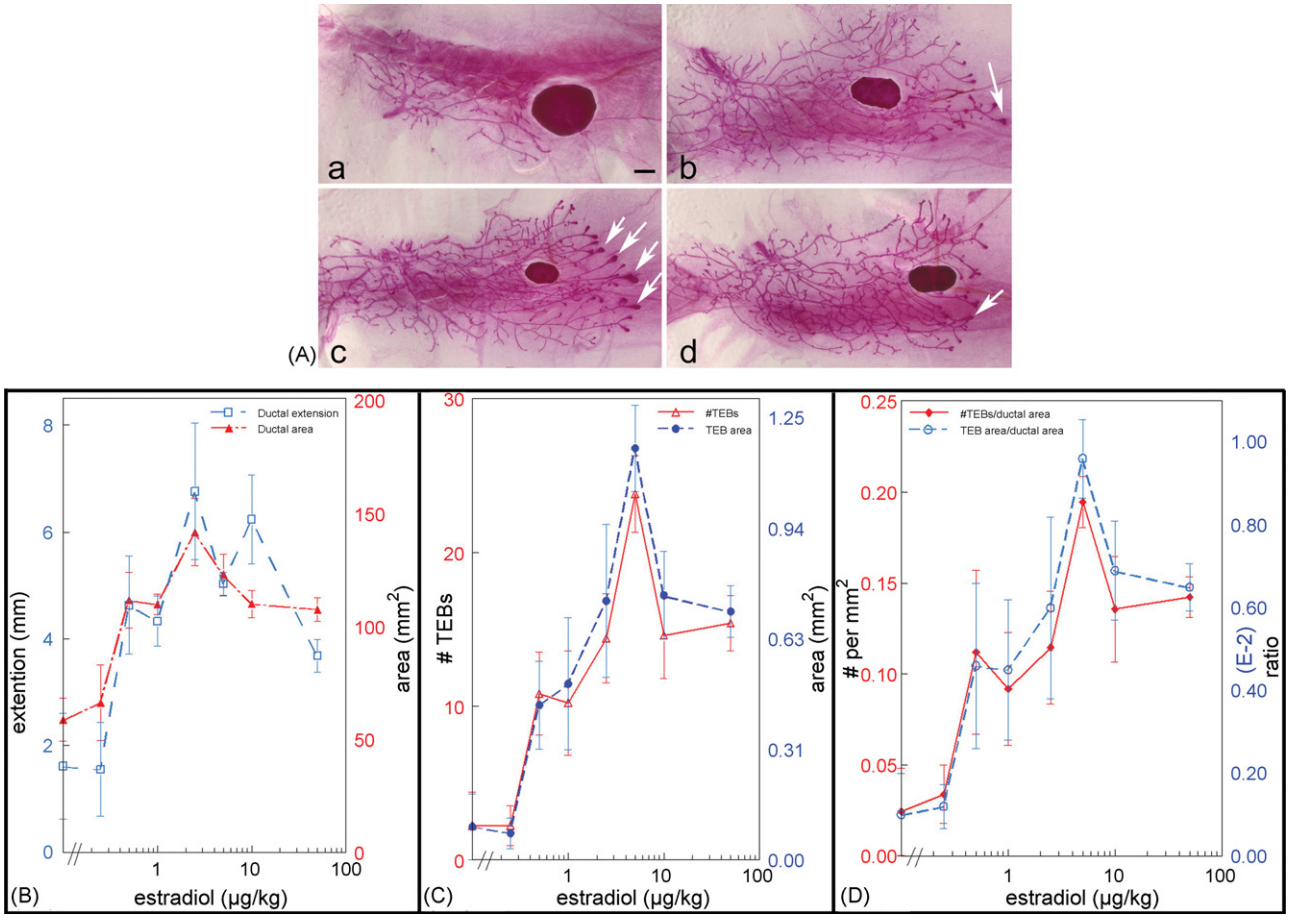


Fig. 3. Morphological responses of the mammary gland to estrogen: (A) whole-mount mammary glands from animals exposed to (a) 0 μg/kg per day, (b) 0.5 μg/kg per day, (c) 5 μg/kg per day and (d) 50 μg/kg per day estradiol. Arrows indicate TEBs. Bar, 1 mm; (B) ductal area (closed triangle) and ductal extension (open square), parameters of the mammary gland that are indicative of overall epithelial growth; (C) number (open triangle) and area (closed circle) of TEBs, parameters of the mammary gland that are indicative of the developmental state of the organ; (D) the density of TEB structures, measured by calculating the number (closed diamond) and area (open circle) of TEBs per area of the epithelial ductal tree.

Table 4
Curve-fitting models for morphometric parameters

| Parameter | *R*-value sigmoidal model | *R*-value polynomial model | Best fit model/response |
|---|---|---|---|
| # TEBs | 0.76 | 0.89 | Polynomial/non-monotonic |
| TEB area | 0.76 | 0.90 | Polynomial/non-monotonic |
| Ductal area | 0.60 | 0.91 | Polynomial/non-monotonic |
| Ductal extension | 0.56 | 0.91 | Polynomial/non-monotonic |
| # TEBs/ductal area | 0.79 | 0.89 | Polynomial/non-monotonic |
| TEB area/ductal area | 0.79 | 0.91 | Polynomial/non-monotonic |
| Uterine weight | 0.99 | 0.98 | Sigmoidal/monotonic |
| Uterine weight/body weight | 0.99 | 0.97 | Sigmoidal/monotonic |

Finally, we assessed whether the data points from each parameter best fit a sigmoidal/monotonic curve or a polynomial/non-monotonic curve. The curve fits (*R* values) for polynomial curves were higher than the curve fits for sigmoidal curves for all mammary gland morphometric parameters (Table 4) including ductal area.

To determine if all responses to estrogen in the mammary gland were non-monotonic, we next investigated gene expression in the mammary glands of estradiol-treated females; this would allow us to identify the effects of treatment on the level of cellular organization in this target organ. The expression of Msx2, Wnt4, and PR mRNAs was found to increase monotonically with increasing doses of estradiol (Fig. 4 and Table 5). The expression of Msx2 and Wnt4 reached a plateau between 5 and 50 μg E2/kg per day and the half-maximal doses were 1.6 and 2.1 μg/kg per day, respectively. PR expression did not plateau within the dose range tested, with a dose of maximal response of 50 μg E2/kg per day, and a half-maximal dose of 19 μg E2/kg per day, the highest noted in this study. However, because PR expression did not reach its plateau in response, the actual half-maximal dose would likely be even higher for this organ.

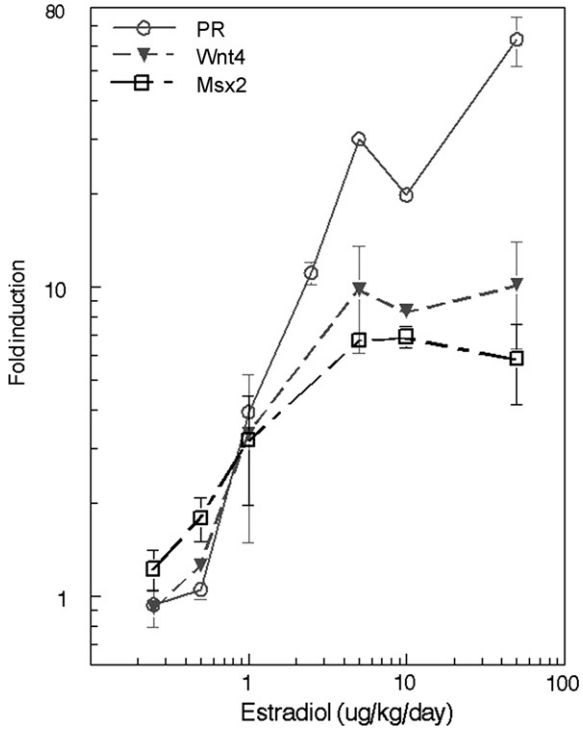


Fig. 4. Gene expression parameters in the mammary gland. Dose–response curves for progesterone receptor (open circle), Wnt4 (closed triangle) and Msx-2 (open square).

Table 5
Gene expression in the mammary gland

| mRNA | LOEL (μg/kg) | Half-maximal dose (μg/kg) | Plateau in response (μg/kg) | Fold-change |
|---|---|---|---|---|
| Msx2 | 0.5 | 1.6 | 5–50 | 5.9 |
| Wnt4 | 1.0 | 2.1 | 5–50 | 9.2 |
| PR | 0.5 | 19 | 50 | 62.0 |

## 4. Discussion

This work was initially undertaken to quantify the estrogen-induced growth of the mammary gland ductal tree at puberty. A second objective was to compare the sensitivity of different end points involved in the growth of the mammary ductal tree with that of the classical end points in the uterus of the same animals. The results of these experiments indicate that both the mammary gland and the uterus respond to increasing levels of estradiol in a monotonic fashion when the end point examined is gene/protein expression. In contrast, examination of effects occurring at higher orders of organization revealed that the uterus displays a monotonic dose–response whereas the mammary gland exhibits a non-monotonic response to increasing levels of estradiol.

The finding that morphometric parameters in the mammary gland respond to estradiol in a non-monotonic fashion while the uterine response is monotonic has theoretical and practical relevance. When a given end point exhibits a monotonic dose–response curve to estradiol, all effective doses will result in a qualitatively similar effect. On the contrary, if the dose–response curve is non-monotonic, dissimilar or even opposite effects will be observed at different doses. From a theoretical point of view, monotonic phenomena can be easily modeled, by assuming that each step in a linear pathway behaves according to the law of mass action [29]. From a practical point of view, a monotonic end point is easily studied because high doses will give predictably high effects. On the contrary, a non-monotonic effect will require

complex experimental design to disentangle its components before it can be modeled. It is believed that non-monotonic dose–response curves are generated by the integration of two or more monotonic dose–response curves that are occurring through different pathways and affecting a common end point with opposing effects [28]. For these end points, one cannot test a single high dose to assess whether or not a given chemical produces a biological effect [30,31].

Also worth noting is that non-monotonic dose–response curves have been observed following exposure to xenoestrogens. For instance, prenatal exposure of rats to low doses of the xenoestrogen bisphenol-A (BPA) led to a significant increase in body weight in adulthood compared to unexposed animals, but a higher dose of this chemical did not have this effect [32]. Additionally, fetal exposure of mice to 0.1 μg/kg per day of diethylstilbestrol (DES) or to 10 μg/kg per day of methoxychlor led to significantly heavier uteri following an estrogen challenge in adulthood. However, a 1000-fold higher dose of DES or a 10,000-fold higher dose of methoxychlor induced significantly lighter uteri which were not significantly different from the controls [31]. Collectively, these experiments have illustrated that non-monotonic dose–response curves are common for endocrine endpoints.

While BPA, like other xenoestrogens, has a lower affinity for ERα and ERβ than estradiol [33–35], prenatal exposure to low levels of this chemical has significant effects in estrogen-target organs such as the uterus [36] and the mammary gland [18–20]. The developing female mouse fetus is affected by doses of BPA several orders of magnitude lower than doses required to evoke a uterotrophic response in the prepubescent female [27]. However, this dose discrepancy between fetal and adult effects has not yet been satisfactorily explained. It is yet unknown by which mechanism xenoestrogens affect the fetus [37,38]. Some researchers believe that the low dose actions occur through ERα and β by an additive effect with estrogen already present in the organism [39], while others postulate mediation by an ER residing in the plasma membrane [40].

### 4.1. The uterus exhibits a monotonic dose–response to estrogen

We have examined the response of the uterus to estradiol at several levels of organization: protein expression (lactoferrin), cellular (epithelial cell height), tissue (volume of each uterine component) and organ level (uterine weight). While some parameters reached a maximal response at an intermediate dose, we noted a plateau in response at higher doses and therefore found that each response behaved in a monotonic fashion.

The classical uterotrophic assay measures an increase in the wet weight of the uterus; this response results from the integration of a large number of effects occurring at diverse levels of organization. Early events observable upon estradiol administration are water imbibition of the lamina propria and inhibition of cell death in the luminal epithelium. Following these events, cell number increases (hyperplasia) in the luminal and glandular epithelia of the endometrium, and the thickness of the lamina propria increases as well. The alterations observed in the lamina propria vary depending on the age of the animal; in ovariectomized adult rodents, alterations are due mostly to an accumulation of extracellular matrix and fluid, while in immature animals estradiol induces hyperplasia of cells in the stroma [41]. At the cellular level, the luminal epithelial cells become enlarged and their shape is changed from a cuboidal to a columnar type; estradiol induces cellular hypertrophy as well [41,42]. At the biochemical level, a number of gene products are increased upon estrogen exposure, among them lactoferrin and complement C3 [43]. In addition to wet weight, several of the effects mentioned above have been proposed to complement and complete the standard uterotrophic assay to decrease the probability of reporting false negatives when screening compounds for their potential estrogenicity [44,45]. Nonetheless, we found that in the mouse, the weight of the uterus, the highest level of complexity among the end points tested, was the most sensitive, while the cellular parameters were less sensitive.

### 4.2. Morphometric parameters in the mammary gland exhibit a non-monotonic dose–response to estrogen

The role of estrogen in the growth of the mammary gland has been explored using ovariectomized rodents and genetic tools such as ER knockout mice. Here, we have shown that parameters in the mammary gland that are thought to be controlled mainly by estrogen, including ductal growth and TEB development, do in fact change following the administration of estradiol to ovariectomized animals. In addition, the present comprehensive study showed that the response of each of these parameters is not dependent on progesterone, however, it does not exclude the possibility that both hormones would likely contribute to these responses in the intact animal.

By quantifying changes in ductal extension, ductal area, number of TEBs, TEB area, and the density of TEBs in the mammary gland, we found that each morphometric parameter in the mammary gland responds in a non-monotonic manner to increasing doses of estradiol with the exception of ductal area, which also showed an inverted U-shape but did not satisfy the stringent criteria used to determine non-monotonicity. Notably, data reported previously demonstrated a non-monotonic dose–response to estrogen in the percentage of the mammary gland fat pad filled with epithelial structures [46], an estimate of ductal area, although the estrogen doses used were significantly higher than the ones in our data set.

*In vitro* studies with human breast epithelial cells as well as other estrogen-target cell lines showed non-monotonic dose–response curves in response to increasing doses of estrogen [47,48]. This type of dose–response curve suggests that estrogens can evoke different effects (i.e. induction of cell proliferation, inhibition of cell death [49,50], inhibition

of cell proliferation, induction of cell death [51,52]) depending on the different doses at which these effects were tested. The combined effect of these variable responses is reflected in the overall cell number [52]. Similarly, in the mammary gland, estrogens induce proliferation, manifested as ductal growth [2], while concurrently inducing apoptosis, manifested as lumen formation [20,53]. These non-monotonic patterns are not exclusive for estradiol. Low doses of androgens can mediate a proliferative response in androgen-target cells while at a higher dose, they inhibit cell proliferation [54,55]. When the end point is cell number, the resulting curve has the shape of an inverted U. Studies have shown that these two pathways are induced independently of each other because they can be separated, generating two differently behaving cell types, one that shows a monotonic proliferative response (i.e. the cell number increases as the androgen dose increases) and another that shows a monotonic inhibitory response (i.e. the cell number decreases as the hormone concentration increases) [28]. Understandably, the biochemical events underlying these effects are distinct [56]. The data presented herein are compatible with the interpretation that the integration of two monotonic events underlie the non-monotonic response to estradiol in the mammary gland.

Several studies have also shown that the length of hormone administration may produce different effects, including non-monotonic responses. When the length of estrogen treatment is short, the rate of cell proliferation in the uterus and pituitary is high. However, when longer treatments of estrogen are administered, cell proliferation in both organs is inhibited [57,58]. Similar results are seen in the prostate of castrated animals treated with androgen [59]. In our studies, we observed a plateau in response between 10 and 50 μg E2/kg per day in uterine weight; in the standard uterotrophic assay (3 days of estradiol treatment), this plateau would not likely be present [44,45]. Therefore, the effects observed in our studies may indicate a potential inhibition of cell proliferation and/or downregulation of ER in some compartments of the uterus following treatment with high estradiol levels in this organ [60].

Collectively, the results previously observed using *in vitro* and *in vivo* models indicate that the same hormone can affect a given end point differently depending on the length of exposure (acute versus chronic) and the dose (low versus high) [52]. In the present study, all animals were treated with estrogen for 10 days, hence, only the effects of chronic estradiol treatment were assessed. The long-term effects of estrogen on the growth of the mammary ductal tree clearly show a non-monotonic dose–response curve. Therefore, we infer that estrogen action on the proliferation of epithelial ductal cells in the mammary gland may reflect the response seen *in vitro* using estrogen-target mammary epithelial cells: low and moderate doses increase epithelial proliferation, while high doses inhibit it.

A less simplistic but perhaps more realistic interpretation would take into consideration the role of stromal-epithelial interactions in mammary gland morphogenesis. For instance, *in vitro* cultures of mammary epithelial and stromal tissue have shown that the presence of stroma can enhance the response of epithelium to estrogen [61,62], which supports the idea that some of the effects of estrogen on mammary epithelium may be partially mediated through stromal receptors.

The non-monotonic dose–response curves observed in the mouse mammary gland may be explained by the complex interactions that govern mammary gland development throughout the lifespan of an individual. In the fetal mouse mammary gland, expression of ERα is predominant in the mammary mesenchyma while low levels of ERβ are expressed in the same region [63]. However, in the pubescent and adult rodent mammary gland, ERα is expressed in the epithelium whereas both epithelial and stromal cells express ERβ [15]. In the uterus, most of the effects of estrogens are mediated by ERα; hence, the initial events of estradiol action are likely to be channeled through the ERα pathway. In contrast, in the mammary gland it seems that ERβ also participates in the proliferative response to estradiol. For example, Cheng et al. [64] found that ligands selective for ERβ induce proliferation in the epithelial compartment of the rodent mammary gland. Furthermore, treatment with estrogen down-regulates the expression of ERα and up-regulates the expression of ERβ. Thus, it is likely that the effects we have observed in the mammary gland are mediated by both receptors acting in both tissue compartments.

### 4.3. Gene expression in the mammary gland

The mRNA levels of genes that can be induced by estrogen, namely, Msx2 [65], Wnt4 [66] and PR [12], all showed monotonic dose–response curves in the mammary glands from the same animals in which non-monotonic responses in morphometric parameters were observed. It is worth noting that Wnt4, a known estrogen-responsive gene in the uterus [67], which acts downstream of progesterone during lateral branching of the adult mammary gland [68], was also induced by estrogen in our experiments.

At the cellular level, RNA expression can be altered within a few hours of estrogen treatment [69]. Therefore, we expect that following 10 days of treatment with estradiol, the gene expression changes we observe do not necessarily resemble the alterations in gene expression that would occur soon after the initiation of estrogen treatment. Furthermore, because we expect that modifications in gene expression are responsible for altered mammary gland morphology following estrogen exposure, gene expression changes we measured *following* this reorganization are not necessarily the same as those required to *induce* such morphological differences.

## 5. Conclusions

Estrogen plays a key role in the development of the uterus and the mammary gland. Examination of effects occurring at

the tissue level of organization reveals that the uterus displays a monotonic dose–response whereas the mammary gland exhibits a non-monotonic response. However, all responses in the mammary gland were not non-monotonic, as expression of several estrogen-induced genes demonstrated monotonic responses to estrogen. Additionally, the results we have reported here indicate that the mammary gland is highly sensitive to estrogen.

Studies in ovariectomized and ovariectomized, hypophysectomized and adrenalectomized animals have contributed greatly to our knowledge of how different hormones affect the growth and development of the mammary gland. However, the observations presented herein offer quantitative data to delineate the degree to which different levels of estrogen can affect mammary gland morphogenesis. While much is known about how the loss of estrogen signaling can stunt the growth of the mammary gland ductal system, our results illustrate the relationship between estradiol dose and the magnitude of the mammary gland response regarding number and size of terminal end buds, ductal elongation, and area subtended by the epithelial tree.

Finally, the increasing prevalence of non-monotonic dose–response curves in biological phenomena underlies the importance of understanding how these complex biological phenomena are regulated [29]. Non-monotonic dose–response curves have been obtained *in vitro* and *in vivo*, and are not unusual for endocrine end points. These patterns again highlight the unreliability of assuming that the effect of exposure to low doses of a natural or environmental hormone or other toxicant can be extrapolated from the response to high doses of the compound [29–31,70].

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