The Effect of Pyrethroid Compounds on the Expression of Estrogen Receptors in Mouse Sertoli Cells and Implications for Male Infertility

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Abstract

Male fertility is largely controlled by the hypothalamic-pituitary axis, a careful balance between stimulating and suppressing gene expression and the secretion of hormones. The critical factors for male fertility have in the past been thought to be limited to testosterone and the gonadotropins. Estrogen has only recently been demonstrated to be both a crucial requirement for fertility and a cause of infertility. Reports in the early 1990s demonstrated a decrease in mean sperm counts over the last 50 years. A hypothesis for this observation is the increase of xenoestrogens in the environment that are able to mimic and potential disrupt the natural estrogens involvement in fertility. Although the mechanisms of estrogens involvement are not yet defined, the Sertoli cells are a potential sites of action as they possess receptors for the hormone and are able to locally produce it. Sertoli cells both act to protect and provide for the male germ cells and the developing spermatozoa. Pyrethroids are common synthetic insecticides of which some have previously shown estrogenic activity. Therefore this investigation examined the effects of pyrethoids, whose estrogenicity was confirmed via the yeast assay, on the estrogen receptor expression in mouse Sertoli cells as a model for general effects of estrogenic chemicals on male fertility. The results first confirmed the estrogenicity of some pyrethroids and these pyrethroids when exposed to mouse Sertoli cells effected estrogen receptor mRNA expression however in a different way to the natural ligand 17β-estradiol.

1 Introduction

Understanding the involvement of hormones in male fertility is crucial to the development of treatments for the growing number of sub-fertile men. The interaction of testosterone and the gonadotropins has been well documented but recently there has been a resurgence of interest in estrogens' role as an important contributor to male fertility. Although generally associated with female fertility, estrogen is now recognised as both a requirement and a potential culprit in male fertility. Comprehensive research into estrogens' involvement has been fuelled by the discovery of a second estrogen receptor (ERβ), and enabled by the development of knock-out mice models with targeted disruption of the ERα and ERβ genes. Interest has also been motivated by the suggestion that reduction in sperm counts and an increase in the incidence of testicular tumours in men in some industrialised areas may be due to estrogenic substances in the environment (Whorton *et al.*, 1977, Stone *et al.*, 1991, Swan *et al.*, 2003, Bian *et al.*, 2004, Xia *et al.*, 2004).

1.1 Link between sperm decline and increase presence of xenoestrogens in the environment

Male causes account for up to 50% of infertility in couples and the rate of male infertility in developed countries increases by 0.5-1% every year (Swerdloff, 1985). Initial reports of a decline in semen quantity and quality by Carlsen et al., (1992) were met by fierce criticism due to the methodology employed. The meta-analysis undertaken by Carlsen et al., (1992) combined data sets from 61 studies that included over 14000 men with normal fertility with data dating back to 1930. Analysis showed a significant decrease in mean sperm count from 114 million/ml in 1940 to 66 million/ml in 1990 as well as a decrease inseminal volume. Criticism focused on the limitations of the meta-analysis and possible technical errors in the regression analysis. However re-evaluation of the data by a later study that in addition, included data up to 1996 confirmed the conclusions of the original study (Swan et al., 2000.

An "estrogen" hypothesis was proposed by Sharpe and Skakkebaek (1993) for the observed decline in sperm number over the last 50 years. This hypothesis suggested that the increasing incidence of reproductive abnormalities in the human male may be related to increased oestrogen exposure *in utero* due to the increased concentration of estrogenic

chemicals in the environment. Hence subsequent efforts have been made to elucidate the direct involvement of estrogen in male infertility and whether other sources of estrogen such as xenoestrogens in the environment disrupt estrogens involvement.

The term xenoestrogen refers to a group of endocrine disrupting chemicals (EDCs) with estrogenic activity. Xenoestrogens can induce estrogenic effects in multiple ways: they may act directly through estrogen receptors, or may disturb estrogen metabolism, thus increasing the levels of the endogenously produced ligand, 17β-estradiol. Numerous environmental chemicals, such as many organochlorine pesticides, PCBs, alkylphenol polyethoxylates, phthalates, and phytoestrogens are known to have estrogenic effects in vertebrates or in assays *in vitro* (reviewed in Topparri *et al*., 1996). Yet only a few of the tens of thousands of man-made chemicals have been tested for estrogenic or other endocrine activity. Pyrethroids are synthetic insecticides that are commonly used both in the home and in industry. The estrogenicity of a limited number of pyrethroids has been investigated by other authors however it has been noted that each pyrethroid compound is unique in its ability to influence several cellular pathways and based on evidence thus far, pyrethroids should be considered to be hormone disruptors although further investigations are required (Go *et al.,* 1999).

1.1.2 Localisation of Estrogen Receptors in the Male Reproductive Tissues

The action of a hormone, such as estrogen, in a given tissue is substantiated by the identification of its specific receptor(s) in that tissue. Two estrogen receptor subtypes, ERα (the classical ER) and ERβ (a recently discovered subtype), have been identified (Green *et al.,* 1986, Kuiper *et al*., 1996). Recent investigations have demonstrated the differential expression of these subtypes in testes as well as the presence of additional transcript variants that differ between humans and rodents. ERα has been reported to be strongly expressed in the efferent ductules however it has not been localized in testicular germ line or somatic cells of human or mouse testis. In contrast, ERβ immunostaining has been observed in spermatogonia, spermatocytes, and spermatids, as well as somatic cells (Couse *et al.,* 1997, Denger *et al*., 2001).

1.1.3 Role of Estrogen and Estrogen Receptors in Male Infertility using Knockout-Mice Models

The requirement of estrogen for normal spermatogenesis has been shown experimentally by targeted disruption of the aromatase gene in male mice models. ArKO mice lack an endogenous source of estrogen as the inactivation of the aromatase gene blocks estrogen biosynthesis by preventing the enzyme mediated conversion of testosterone to estradiol. ArKO mice exhibit late onset infertility with defects in round spermatids and increased apoptosis (Fisher *et al*., 1998, Robertson *et al*., 1999). Likewise the requirement for ER has been shown by knock-out mice models. ERαKO mice are also infertile however the phenotype is different to the ArKO mice (Eddy *et al.*, 1996). Testicular function has been shown to decline in male ERαKO mice but as a secondary effect due to impaired function of the efferent ductules. ERβKO mice on the other hand exhibit no obvious disturbances in testicular or epididymal function suggesting that ERβ is not essential to normal spermatogenesis (Ogawa *et al*., 1999). Not surprisingly then, the phenotype of mice with targeted disruption of both ER subtypes resembles ERαKO mice (Krege *et al*., 1998).

1.1.4 Evidence of Estrogens Regulating Germ Cell Development Mediated by ERβ

The finding that ERβ is not a requirement for fertility in adult male mice according to the knockout models is unexpected considering that ERβ is the dominantly expressed ER subtype in both human and rodent testicular cells. There is considerable evidence in other tissues that a main role of ERβ is to moderate estrogen induce proliferation. The majority of evidence regarding the ERα/β interaction relates to the decrease or loss of ERβ expression in estrogen dependent tumour progression (reviewed in Bardin *et al*., 2004). However recent evidence suggests a role of ERβ in moderating neonatal germ cell development in spermatogenesis (Delbes *et al*., 2004). This lends support to a universal role of ERβ in growth regulation of estrogen induced pathways.

Inhibition of germ cell growth through an ERβ mediated process was shown experimentally where ERβ-/- mice showed 50% more germ cells compared to wild type mice, whereas no change in germ cell number was shown in ERα-/- mice. The growth

inhibition by ERβ when activated occurred at endogenous concentrations of estrogen. In addition, approximately the same amount of inhibition was observed in mice heterozygous for ERβ-/+ who only express half as much ERβ protein (Delbes *et al*., 2004). These two factors illustrate that the inhibitory response of ERβ is highly sensitive to estrogen and does not require a large receptor occupancy level for the effect to be observed.

The increase in germ cell numbers in the ERβ inactivated mice was essentially due to a lower rate of apoptosis and, to a lesser extent, to a higher rate of mitosis. Therefore the limiting of germ cell production via estrogen binding to ERβ may indicate ERβ's involvement in activating apoptotic signalling pathways.

1.1.5 Sertoli cells as Models for the Effects of Xenoestrogens on Male Infertility

Among the cells that contain estrogen receptors and could mediate direct effects of estrogens within the testis are the Sertoli cells. Sertoli cells are the somatic cells of the testes that are essential for testis formation and spermatogenesis. Their location within the seminiferous tubules enables them to act as structural support to the spermatogonia as well as a physiological barrier which is thought to mediate hormone signals between the body and the testes (Setchell, 2004). Survival of germ cells has been shown to be proportional to that of the Sertoli cells. Experimental evidence of this relationship derives from three observations. Firstly, no situation exists in which germ cells have been observed in the absence of Sertoli cells (Griswold, 1998). Secondly, manipulating the number of Sertoli cells, by inhibiting their proliferation during testicular development, results in changes in testes size and spermatogenic output (Orth *et al.,* (1988). Finally, the Sertoli cells express the receptors for gonadal steroids and exclusively possess the receptor for follicle stimulating hormone (FSH) involved in regulation of spermatogenesis (Zirkin *et al*., 1994). Two major mechanisms are thought to contribute to the relationship between the germ cells and Sertoli cells. Firstly, the tight junctions between Sertoli cells and germ cells and secondly the Sertoli cell involvement in the Fas mediated apoptotic signalling pathway.

The tight junctions between the Sertoli cells and the germ cells are enabled by N-cadherin (N-cad). N-cad is a calcium dependent cell adhesion molecule that mediates cellular interactions. Both FSH and estradiol increase the mRNA expression of N-cad indicating an

estrogenic involvement in maintaining the junctions (MacCalman *et al*., 1997). Administration of an estrogen antagonist (tamoxifen) compromises structural integrity of the seminiferous epithelium and blocks spermatogenesis (Gill-Sharma *et al*., 1993). Likewise antibodies directed against N-cad inhibit Sertoli cell-germ cell interactions *in vitro* (Newton *et al*., 1993).

Cell death in the testes occurs primarily, if not exclusively, via programmed cell death. Testicular apoptosis involves the Fas system. Sertoli cells produce Fas ligand (FasL), a proapoptotic factor that binds to Fas, a transmembrane receptor protein on germ cells. Thus the balance of prosurvival and proapoptotic signals originating from the Sertoli cells and associated changes in Fas protein in the germ cells are thought to control germ cell survival (Boekelheide *et al*., 2000). Estradiol treatment has been shown to inhibit apoptosis of germ cell *in vitro*. This effect was abolished by treatment with ER antagonist, ICI 182,780 (Pentikainen *et al*., 2000) thus suggesting estradiols' involvement of the Fas mediated apoptotic pathway possibly at the Sertoli cell level.

1.1.6 Signal transduction mediated by estrogen receptors

Estrogen receptor signalling is far from understood yet the complexity that has so far been resolved is extensive. Not only are the receptors able to form either homo or heterodimers if co-expressed but their transactivation ability is dependent on both the ligand bound, the recruitment of co-factor proteins, and even the chromatin structure of the DNA. The activation or inhibition of gene transcription induced by the estrogen receptors is also dependent on whether the ligand bound receptor binds to estrogen response elements (EREs) or activates through other protein recognition sites such as AP-1 and SP-1 (reviewed in Mathews and Gustafsson, 2003).

1.2 Pyrethroids

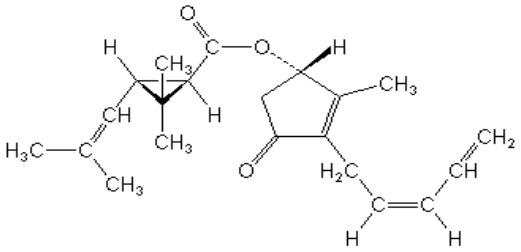
In 1992 pyrethroid based insecticides contributed one fifth of the total insecticide application world wide. Since then use has continued to rise with pyrethroids employed in house hold fly sprays, carpet treatment and in a multitude of both commercial and domestic garden treatments.

Pyrethroids are synthetic derivatives of naturally occurring pyrethrins which are found in pyrethrum, the oleoresin extract of dried chrysanthemum flowers. As a consequence of being derived from a natural insecticide, insecticides containing pyrethroids have been referred to as "safe as chrysanthemum flowers." However pyrethroids were developed to be more toxic with longer persistence times than the natural insecticide (reviewed by Khambay, 2002) and are often combined with other chemicals that increase their absorbance and compromise the human bodies' ability to detoxify them. All of the pyrethroids investigated in this study are licensed for use in New Zealand (NZFSA, 2004) and are available in insecticide preparations for use in domestic gardening.

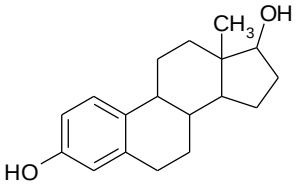
The pyrethroid group is subdivided into two classes based on the different toxicological effects and symptoms, these subtypes also reflect a specific structural difference. (see Figure 1). Type I pyrethroids tend to produce milder poisoning symptoms whereas Type II Pyrethroids, which are identified by the presence of α-cyano group, lead to more severe poisoning symptoms (Ray, 2000, Raymond-Delpech et al., 2005). As insecticides pyrethrins and pyrethroids act as contact poisons; they rapidly penetrate via their highly lipophilic nature to disrupt the nervous system of insects. The primary targets of the insecticides are the sodium channels (reviewed in Raymond-Delpech *et al*., 2005). In addition Type II pyrethroids have been shown to also interfere with chlorine ion channels which increases their toxicity (Bradberry et al., 2005). Selectivity for insects, thus low mammalian toxicity, is a result of ion channel and metabolic enzyme differences as well as an approximate 10°C body temperature difference that negatively affects the pyrethroids potency in mammals.

The two main metabolic mechanisms that degrade pyrethroids in biological systems are enzyme mediated oxidation and ester hydrolysis (Wollen *et al*., 1992, Leng *et al*., 1997, Choi *et al*., 2002, McCarthy *et al*., 2006). Understanding the metabolism of pyrethroids has enabled estimation of the physiological pyrethroid exposure levels based on metabolite levels in urine samples. By analysing the specific metabolites and their isomeric ratio present, both the type of pyrethroid and the route of absorption of the exposure can be determined (Wollen *et al*., 1992). Urine samples are preferable to blood or plasma samples as pyrethroids continue to degrade due to the presence of non-specific esterases in blood and plasma (Leng *et al*., 1997, Ramesh *et al*., 2004).

Pyrethrin I

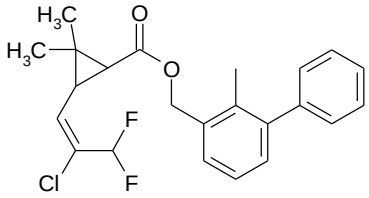


17β-Estradiol

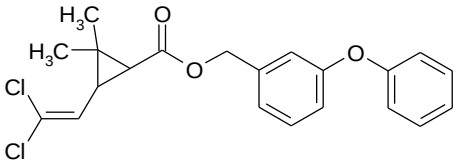


Type I Pyrethroids

Bifenthrin

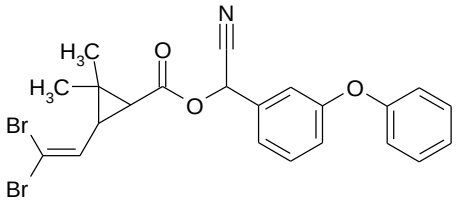


Permethrin

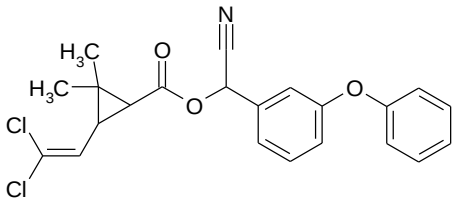


Type II Pyrethroids

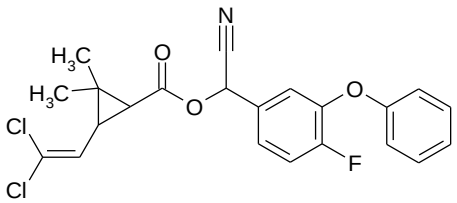
Deltamethrin



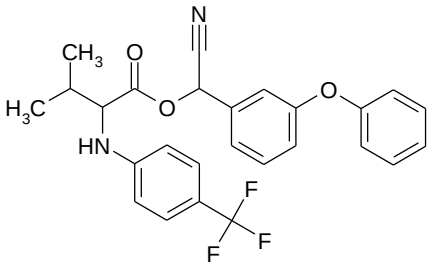
Cypermethrin



Cyfluthrin



Taufluvalinate



Permethrin metabolite
(3-(4-hydroxyphenoxy)benzyl alcohol)

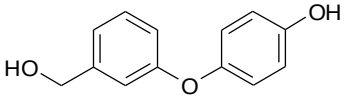


Figure 1 Chemical Structures of pyrethroids investigated in this work as well as pyrethrin I and 17β-estradiol. Pyrethoids are positioned according to their class determined by their physiological symptoms. The structure of the Permethrin metabolite included in the investigation is also given.

Exposure to pyrethroids occurs through ingestion of the residual insecticide left on produce and through inhalation or dermal exposure during treatment. Absorbance is greatest through the gut (bioavailability 36%) and pulmonary membrane with absorbance through the skin less effective (1%) (Wollen *et al*., 1992). The physiological levels of pyrethroid exposure for both the general population and pesticide workers are detailed in the discussion (see section 4.2). Of significance is the consistent detection of pyrethroid metabolites in urine samples of the general population thus indicating widespread low level exposure.

1.2.1 Pyrethroids as Xenoestrogens

Structural similarities between pyrethroids and the natural estrogen 17β-estradiol (refer to Figure 1) are not immediately apparent other than the presence of an aromatic ring. However, examination of both the end products and intermediate products of pyrethroid metabolism reveals structures which are suggested to have higher binding potential compared to their parent compounds based on essential binding characteristics of the estrogen receptor active site (McCarthy et al., 2006). These characteristics have been identified by structure-activity relationship analysis, enabled through competitive binding assays. Such investigations have been carried out for the last 40 years however advances in computer modelling technology has allowed more detailed analysis of a far more diverse set of structures. One such study (Fang *et al*., 2001) determined 5 clearly defined features that indicate a chemicals estrogenic potential. Firstly all chemicals so far found to be estrogenic contain a ring structure. As described in Fang *et al.,* (2001) other structural features can contribute to this basic requirement namely; H-bond ability, precise O-O distance, rigid structure, steric moieties mimic 7α and 11β-position and satisfactory hydrophobicity. Comparison of the permethrin metabolite investigated in this study with 17β-estradiol, illustrates three similarities that may contribute to the permethrin metabolites' ability to bind to the estrogen receptors. The presence of a phenolic ring, electronegative hydroxyl groups on either end of the molecule for H-bonding and similar O-O distance in 2D (see Figure 2).

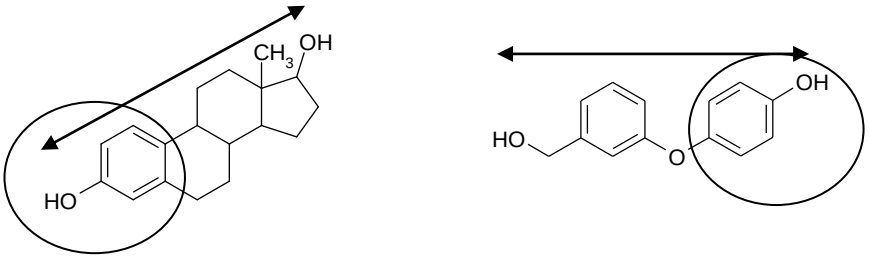


Figure 2 2D structural comparison of 17β-estradiol and the permethrin metabolite (3-(4-hydroxyphenoxy)benzyl alcohol) investigated in this study. Presence of phenolic ring in both structures is circled, arrows suggest similar O-O distance also present is the same electronegative groups on either end of the molecules.

Metabolites are often supposed to be less toxic than their parent compounds as they result from what is primarily a detoxification process. However the possibility remains that the body may inadvertently transform already toxic or bio-active chemicals into even more potent metabolites. An example of metabolic activation in the body is the conversion of diadzein, found in soy products, to one of its major metabolites equol (7-hydroxyisoflavan) (Rowland *et al.*, 2000). Equol has been found to be more estrogenic than its parent compound and thus the metabolism resulted in a more bio-active chemical (Rowland *et al.*, 1999, Markiewicz *et al.*, 2000). The permethrin metabolite included in this work to investigate potential bio-activation of pyrethroids is an intermediate species in the permethrin degradation pathway (McCarthy *et al.*, 2006).

1.2.2 Are Xenoestrogens a Physiological Risk?

An argument against the physiological effects of some xenoestrogens is that due to their low potency compared to 17β-estradiol the dose required to elicit an effect is well outside the physiological exposure range of these chemicals. This theory is heavily modelled on toxicology studies where the assumption is made that extrapolating from the high exposures levels tested is a valid risk assessment for predicting low doses effects in the physiological range. Evidence to the contrary has been presented for the xenoestrogens, bisphenol-A and nonylphenol and the phytoestrogen genistein (phytoestrogen refers to a naturally occurring xenoestrogen). The results were found to be consistent with a U-shaped dose response curve, thus effects were observed at low doses - even with doses below the established no effect level (Sheehan, 2000, Gupta, 2000, Kaiser, 2000). This highlights that effects at near toxic concentrations can be very dissimilar to effects at a physiological concentration and that the relationship is far from linear.

Low dose effects of xenoestrogens have been commented on by both the National Institute of Environmental Health/Environmental Protection Agency (NIEHS/EPA) and the World Health Organisation (WHO). Reports issued from these authorities, highlight the need for direct mechanistic evidence and therefore encourage further research into possible xenoestrogen effects especially at different life stages ((NIEHS, 2001, WHO, 2002a). Due to the difficulty of establishing causal associations between between low-level exposure (as found in the general public) and adverse human health effects the exact nature of the effect of xenoestrogens is yet to be determined (Safe, 2005). Thus it appears that direct mechanistic details need to be elucidated before potential health risk is verified by these authorities.

Low potency chemicals may also have the potential to act in an additive or synergistic ways with either other xenoestrogens or in combination with estradiol. Additive effects have been demonstrated where xenoestrogens, combined at below their no-effect-observed-concentrations, have been able to exhibit either an estrogenic response (Payne *et al.,* 2000, Silva *et al*., 2002) or dramatically enhance 17β-estradiols' hormone action (Rajapaksi *et al.,* 2002).

The comparative bioavailability of xeno-estrogens compared to the natural estrogen may also impact on their actual physiological potencies. Specific binding proteins, for example sex hormone binding globulin (SHBG) sequester 95% of circulating 17β-estradiol (Arnold *et al.,* 1996). Circulating xenoestrogens are not sequestered by serum binding proteins to the same degree, due to lowered binding affinity therefore, their free concentration is proportionally higher. In addition lipophilicy of some xenoestrogens, for example nonylphenol, is higher than estradiol which increases the likelihood of bioaccumulation.

Aside from directly perpetuating estrogenic pathways by binding to the receptors, xenoestrogens may also disrupt the normal hormone regulatory mechanisms eventuating in the same end product of endocrine disruption. Estrogens are rapidly metabolised in the testis e.g. by sulphotransferases after which they cannot bind to their receptors. If xenoestrogens, of which many mimic the structure of natural estrogen, are occupying the active sites of metabolic enzymes then this would extend the persistence time of natural estrogen perpetuating its effects (Toparri *et al.,* 1996).

1.3 Aims of this Investigation

The requirement of estrogen in male fertility has been established however, the mechanisms of this involvement are far from understood. There is concern that environmental estrogens may affect endogenous estrogens' signalling pathways yet debate regarding the physiological consequences of this due to insufficient causal associations observed.

The aims of this study were therefore to extend the current understanding of possible estrogenic responses occurring in the testes. To this end, examination of estrogen receptor levels in mouse Sertoli cells in response to treatment with both estradiol and xenoestrogens was investigated. The aspect of estrogen signalling relevant to this investigation is therefore the potential the effect of different ligands on the expression of ERα and ERβ mRNA and possible implications this may have on spermatogenesis.

The xenoestrogens of interest were commonly used pyrethroid compounds which have been detected in the general public. Initial screening of pyrethroids by the Yeast Estrogenic Assay thus aimed to indicate pyrethroids individual estrogenicity to enable selection of the most likely xenoestrogens for the mouse Sertoli cell exposure. Investigating the estrogen receptor levels as a result of the treatments aimed to determine if changes occurred and if so, whether this change was the same for pyrethroids as for the natural ER ligand 17β-estradiol.

2.1 Materials and Methods

2.1.1 Test Chemicals

The commercial insecticides purchased contained the desired pyrethroids as their active ingredient. They are listed as follows, manufacturer, product name and concentration of active ingredient:

M^{c}Gregor's Natural Insecticide, contained 7g/L **Pyrethrins**.

Yates Ant & Spider Spray, contained 2.5g/L **Permethrin**.

Defender Bug-B-Gon, contained 0.03g/L **Bifenthrin**.

BASF Ripcord Plus, contained 15g/L α-**Cypermethrin**.

Kiwicare NO Bugs Super, contained 10g/L **Deltamethrin**.

Yates Mavrik, contained 9.6g/L **Tau-fluvalinate.**

Bayer Rose and Bug Spray, contained 0.2g/L **Cyfluthrin**.

Pure **α-Cypermethrin** was purchased from Sigma-Aldrich.

The **Permethrin metabolite**, 3-(4-hydroxyphenoxy)benzyl alcohol was generously supplied by Anna M^{c}Carthy who synthesised it as part of a PhD at University of Canterbury. The structure was confirmed by both ^{1}H and ^{13}C NMR and the chemical had a stock concentration of 40mg/L.

2.2 Yeast Estrogen Screen

The yeast assay screen was adapted from the method of Routledge and Sumpter (1996). The yeast used in this assay was purchased from the same source - Glaxo group. It had been transfected with the human estrogen receptor (ERα) gene into the main chromosome, and with expression plasmids. The expression plasmids contain estrogen responsive elements (ERE) and the *lac-Z* reporter gene encoding the enzyme β-galactosidase (β-gal). The yeast was incubated in medium containing specific test chemicals and the chromogenic substrate, chlorophenol red-β-D-galactopyranoside (CPRG). If the test chemicals were, or metabolised into, active ligands of ERα, the reporter gene *lac-Z* was expressed and *β-gal* produced and secreted into the medium which caused the CPRG (yellow) to be metabolised into a red product. The colour change measured by absorbance at 540 nm.

2.2.1 Preparation of Yeast Culture

Preparation of the minimal and growth medium for the yeast assay was as per the method described by Routledge and Sumpter (1996). However preparation of the yeast differed from their protocol in that only 0.125 µl of -20°C 10x concentrated yeast stock was used to inoculate the 24 hour culture. Also, from that 24 hour culture only 0.25 ml was added to assay medium compared to the suggested 2 ml. The lowered volumes of yeast used compared to the original paper reflects the faster growth rate of the yeast observed in this lab. The new yeast volumes were based on work by Thomson (2004), and by McCarthy *et al.*, (2006) that established the yeast cultures from Glaxo group, used for this work. The fast growth of the yeast was likewise evident in this work where the yeasts' optical density at 640 nm after 24 hour incubation was on average greater than 2.4, compared to a value of 1.0 for the 24 hour culture reported by Routledge and Sumpter (1996).

2.2.2 Assay Procedure

The test chemicals were diluted with 96% v/v ethanol and 100 µl was aliquot into the first well and diluted across the remaining wells creating a 1:2 dilution gradient. After the serial dilution, 10 µl was transferred to a different row and left until the ethanol evaporated; subsequently 200 µl of assay medium was added. The assay medium consisted of growth medium plus 0.25 ml yeast (from the 24-hour culture OD value generally above 2.4) and 0.5 ml CPRG per 45 mls of minimal media as described in Routledge and Sumpter (1996). A 17β- estradiol standard was prepared in the same way and included on every plate as well as a blank containing only the assay medium. The plates were shaken in a fixed wavelength plate reader (BIO-TEK EL312) for 2 min prior to incubation at 32°C. After 48 hours incubation, the absorbance was measured at both 610 nm and 540 nm in the same plate reader.

2.2.3 Data Analysis

Absorbance at 540 nm was corrected for changes in turbidity caused by yeast growth by adjusting it by the difference between the optical density at 610 nm for wells with and without yeast.

Sample Corrected Absorbance at 540 nm = $OD_{Sample\ 540\ nm}$ - ($OD_{Sample\ 610\ nm}$ – $OD_{Blank\ 610\ nm}$)

The corrected absorbance data was plotted using SigmaPlot 9.0 with the dose response curves fitted by either the Hills four parameter or Sigmoid four parameter equations.
EC_{50} values were calculated by using the equation of the fitted line and the parameters given in Sigmaplot to find the concentration (x) which gave half maximal response, (y = min + ((max-min)/2)).
Relative potency to estradiol was calculated by $EC_{50\ Estradiol}$ /$EC_{50\ Chemical}$
The absolute change in absorbance resulting from each treatment after the correction was assessed for significance using a paired-two-tailed Student's *t*-test.

2.3 TM4 Mouse Sertoli Cell Culture

2.3.1 Cell Culture Protocol

TM4 Mouse Sertoli cells from the American Type Culture Collection (ATCC catalogue number CRL-1715) were cultured in a phenol-red free medium containing a 1:1 mixture of Dulbecco's Modified Eagles Medium and Ham's F12 medium with 2.5 mM L- Glutamine containing 15 mM HEPES, 0.5 mM sodium pyruvate, and 1.2 g/L sodium bicarbonate and supplemented with 5% v/v horse serum and 2.5% v/v fetal bovine serum. Frozen vials stored at -80˚C were resuspended in T75 flasks with approximately 12 mls of media. Incubation was done in a 37°c humidity controlled incubator with 5% v/v CO_2.

Determination of doubling time was carried out by using the SRB assay (protocol below). Identical plates were set up each containing varied initial seeding densities estimated by manual cell counting. Manual cell counting was performed with the aid of trypan blue exclusion stain and a Makler counting chamber. Plates were then tested one per day for five days.

2.3.2 Quantitation of Cellular Protein in Cell Cultures

2.3.2.1 Sulforhodamine B Colorimetric Assay (SRB assay)

Skehan et al., (1990) developed the SRB assay for the *in vitro* measurement of cellular protein content in adherent and suspension cell cultures. The protocol involves fixation and subsequent staining where the SRB dye binds to the basic amino acids. Colorimetric analysis of the stained protein enables the estimation of cell number based on the proportionality of total protein to cell number.

2.3.2.2 SRB General Protocol

In a 96 well flat-bottom microplate, a final volume of 200 µl per well was added consisting of growth medium seeded with an appropriate cell density. After incubation for a minimum of 6 hours to allow attachment, 100 µl of 30% (w/v) trichloroacetic acid (TCA) was added to each well to a final concentration of 10% v/v, and left standing for 20 minutes at 4°C. TCA was removed by blotting on filter paper. The plate was gently washed with tap water and the washing was repeated 5 times. The fixed cells were then stained with 100 µl per well of 0.4% SRB in 1% v/v acetic acid and left standing for 20 minutes at room temperature. After staining the stain was removed and the cells were washed with 1% v/v acetic acid. After over night drying in a fume hood the stain was solubilised with 100 µl of 10 mM Tris-base (Tris(hydroymethyl)methylamine), pH not adjusted. After solubilisation for 20 minutes, the plates were gently shaken for 30 seconds before the absorbance was measured at 540 nm on a fixed wavelength plate reader (BIO-TEK EL312).

2.4 Sertoli Cell Pyrethroid Exposures

Cypermethrin and Ripcord, the commercial product containing cypermethrin, were diluted in 96 % v/v ethanol to two final concentrations each of 36 µM (3.6×10^{-5} M) and 0.36 nM (3.6×10^{-10} M). The permethrin metabolite was diluted to three concentrations, 69 µM (6.9×10^{-5} M), 0.69 nM (6.9×10^{-10} M) and 69 pM (6.9×10^{-11} M). 17β-estradiol was also

diluted to two concentrations of 10 nM (1 x 10^{-8} M) and 0.3 nM (3 x 10^{-10} M). Each of these chemicals, at their specific concentrations, was applied to T25 flasks with three replicates per concentration. The same volume of ethanol was also applied to three other flasks to act as no treatment controls. The ethanol in all flasks was allowed to fully evaporate before the addition of the Sertoli cells. Bulk medium was made to contain 2 x 10^4 cells per ml and 4mls was added per flask giving final concentration of cells per flask at 8 x 10^4. All flasks were incubated for approximately 100 hours before cells were harvested.

2.5 RNA Extraction

RNA was extracted from the exposed cells using an Invitrogen Micro-to-Midi Total RNA Purification System. The manufacturer's protocol was followed but using the following options, where options were given. Harvested cells were frozen in either PBS or trypsin therefore prior to adding lysis solution they were pelleted at 2000 x g (max) for 5 mins straight from the -80 freezer. As advised by the protocol due to the number of cells, 0.6 ml of lysis solution was added to the cell pellet. Homogenisation was achieved by repeated drawing of the cells through a 21 gauge needle using a 5 ml syringe. One volume, 0.6 ml, of 70% v/v ethanol was added to each vial and the precipitate dispersed by pipetting. Spinning and the application of both wash buffer I and II were carried out as per the kit protocol. One aliquot of 30 µl of RNase-free water was used to recover RNA from the spin cartridge due to the low expected yield.

2.5.1 RNA Quantification

Extracted RNA from the exposed cells was quantified using the Quant-iT™ RNA Assay Kit *5-100ng* from Molecular Probes, Invitrogen and using a Stratagene MX3005P Quantitative PCR system to measure fluorescence. The Quant-iT™ reagent bound to RNA has an excitation and emission maxima of 644 and 673 nm, respectively. The CY5 filter in the Stratagene system has excitation and emission wavelengths of 635 and 665 nm respectively. The reagent to buffer dilution was 1:100 and 10 µl of each standard was used. Using 10 µl of each *Ecoli* standard provided in the kit gave a standard curve of 5-100 ng against the corresponding fluorescence values. An R-squared value of >0.985 was suggested in the kit as acceptable and was achieved consistently with standard RNA

amounts up to 80 ng. To calculate the amount of RNA in the unknown sample per µl from the amount given off the standard curve, the volume read, volume diluted and dilution factor were accounted for.
i.e : value off standard curve graph/volume read * dilution factor/volume diluted = ng/ µl

2.2.5 DNase Treatment of Extracted RNA

RNA samples were treated with DNase by adding 1 µl 10X buffer and 10X DNase I (Amplification Grade, Invitrogen) per volume of RNA. This was incubated at 25 ˚C for 15 minutes then the reaction stopped by heating at 75 ˚C for 10 minutes, followed by immediate ice chilling. A no RT-PCR was performed for confirmation that no DNA contamination persisted.

2.6 Two-step Quantitative RT-PCR

2.6.1 First-Strand cDNA Synthesis

First-strand cDNA synthesis from the DNase treated RNA was achieved by using Invitrogen SuperScript™ III Platinum® two step qRT-PCR kit. From the kit, 10 µl of 2x RT reaction mix was combined with 8 µl of the RNA sample to which 2 µl of enzyme mix, also from the kit, was added. Incubation times for the reaction were as per protocol supplied with the kit as well as the addition of RNase H. Fourteen of the RNA samples had relatively small amounts of RNA that were relatively dilute due to the extraction protocol. These samples were lyophilized and resuspended in autoclaved distilled water to enable a higher concentration of RNA in the 8 µl added to the cDNA reaction.

2.6.2 Quantitative PCR

After extensive optimisation, the following reaction conditions were used for the amplification of all cDNA samples. LUX® primer sequences and expected product sizes are given in Table 1.

Each 25 µl reaction contained 2x Platinum® Quantitative PCR SuperMix-UDG, 50 nM of both the β-actin JOE labelled and the unlabelled primer, 50 nM of both the ERα FAM labelled and the unlabelled primer, and 100 nM of both ERβ ALX546 labelled and the unlabelled primer. Reactions also included 50 nM of ROX reference dye and 5 µl cDNA and were made to 25 µl with autoclaved distilled water. The 5 µl of cDNA added per reaction contained the equivalence of 50 ng total RNA. No template controls contained 5 µl of autoclaved distilled water in place of the cDNA. The cycling conditions were one cycle of 50˚C for 2 minutes and 95˚C for 2 minutes, then 45 cycles of 95˚C for 30 seconds and 60˚C for one minute. Fluorescence data was collected by endpoint data collection at the end of each cycle using HEX, CY3, FAM and ROX filters. The filters each detected the fluorescence caused by the Lux® primers extension but only within their specific excitation and emission wavelengths. Hence CY3 filter detected the ALX546 label, HEX filter detected the JOE label and FAM filter detected the FAM label. ROX was measured as a passive reference dye to account for inaccuracies in reaction conditions such as small pipetting errors. The filter gain settings on the Stratagene Mx3005p employed were, x 1 for CY3(ERβ), x 8 FAM(ERα) and x 2 JOE(β-actin).

The same reaction mix as described above for the quantitative PCR was also used for the no RT-PCR using DNase treated RNA in place of cDNA.

Table 1 Lux Primer Details

Gene	Label	Sequence	Expected Fragment Size
β-actin	JOE	CGGTTCTGAACCCTAAGGCCAAC[JOE]G	109
β-actin	-	CCAGAGGCATACAGGGACAGC	
ERα	FAM	CGGCGGAGACTCGCTACTGTGC[FAM]G	144
ERα	-	GGTGCATTGGTTTGTAGCTGGA	
ERβ	ALX546	CGGCCTTCCTCCTATGTAGAGAGC[ALX546]G	89
ERβ	-	GGTGCTGCTGGGAACACTATA	

2.6.3 Post Amplification Data Analysis

The amplification curves produced by the fluorescence data were adjusted by altering the analysis term settings in the Stratagene software. The default setting of adaptive baseline

was changed to non-adaptive baseline and the baseline manually set between 3-15 cycles. Threshold cycle was not altered and hence was calculated by the default set algorithm. The amplification of the β-actin gene was an internal control used to normalise the amplifications of ER alpha and beta against variations in the template amount.

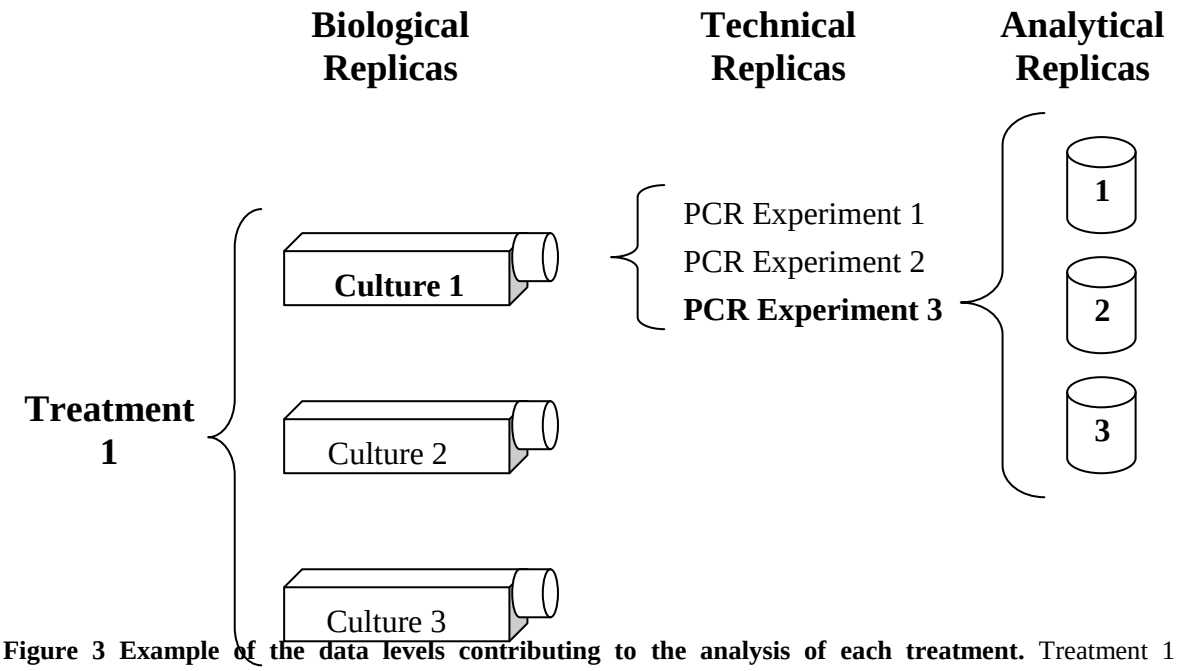


Figure 3 Example of the data levels contributing to the analysis of each treatment. Treatment 1 represents any of the treatments including the non-treated cells. Each treatment included three biological replicate cultures. The cDNA reversed transcribed from the RNA extracted from each culture was assayed in three separate PCR experiments. Within each PCR experiments three analytical replicas per culture sample were included.

The set up of the experiment was such that the raw data required specific averaging of technical, analytical and biological replicas to gather average treatment results. For each treatment three replica cultures were set up (see Figure 3). Each culture contained approximately the same initial cell number. Three separate PCR assays were performed using cDNA reverse transcribed from the RNA of each culture. Within each technical replicate experiment, triplicate analytical replicates were assayed. The data from each culture i.e. the individual culture results, constitutes the average of three separate PCR reactions; this average is plotted on Figure 15. The data from each treatment, Figure 3, compiles of the average of the cultures for that treatment and is graphed on Figure 16.

The relative expression fold-change of ERα and ERβ was calculated based on the mathematical model presented by Pfaffl (2001). The equation encompasses the correction for changes in the control samples, accounting for reaction efficiencies and normalisation with the β-actin expression. Gene of interest, (GOI) refers to either ERα or ERβ and the

normaliser gene (NORM) to β-actin. Individual efficiency of the primer sets in the multiplex was calculated by construction of a standard curve of template concentrations. The resultant fold-changes were compared to the changes in the non-treated control and tested for significance by a paired-two-tailed Student's *t*-test.

$$\text{Fold Change} = \text{Eff}_{(GOI)}^{(\Delta Ct\ GOI)} / \text{Eff}_{(NORM)}^{(\Delta Ct\ NORM)}$$

$$\Delta Ct\ GOI = Ct_{(GOI)}\ \text{control} - Ct_{(GOI)}\ \text{treated}$$

$$\Delta Ct\ NORM = Ct_{(NORM)}\ \text{control} - Ct_{(NORM)}\ \text{treated}$$

The ratio of ERα to ERβ expression was calculated by dividing the fold-change of ERα by the fold-change of ERβ. Whether this ratio increased or decreased significantly from that of the non treated control was tested by a paired-two-tailed Student's *t*-test.

2.6.4 Gel Electrophoresis of PCR Products

Amplification products were run on pre-made 4% agarose gels (E-Gel, Invitrogen) that separate 20 – 500bp DNA fragments. Wells were filled with either 20 µl of sample or 20 µl of 100bp DNA ladder (Invitrogen). Running time and voltage settings were as per protocol supplied with the E-Gel.

3 Results

3.1 Estrogenicity using the yeast assay

The estrogenicity of the pyrethroids of interest was investigated using an *in vitro* yeast assay, with a representative set of results for the dosage based effects shown in Figure 4.

The concentrations of the chemicals were serially diluted with the highest concentration on the left of the figure. The specific concentration ranges of each chemical used in the assay are shown in the box at the end of each row. This figure illustrates the yellow background colour of the medium (row K in Figure 4) where there was no estrogenic response and the colour change to red when there was a positive estrogenic response (for example see row A). The presence or absence of a colour change was quantified by measuring the absorbance of the culture medium. The plates were read at 540 nm for the red colour development and 610 nm to account for the turbidity of the yeast growth. Absorbance data, corrected for the yeast growth are shown in Figure 5A-I. Some of the colour changes are visually detectable, but are statistically insignificant when graphed (Figure 4, Figure 5).

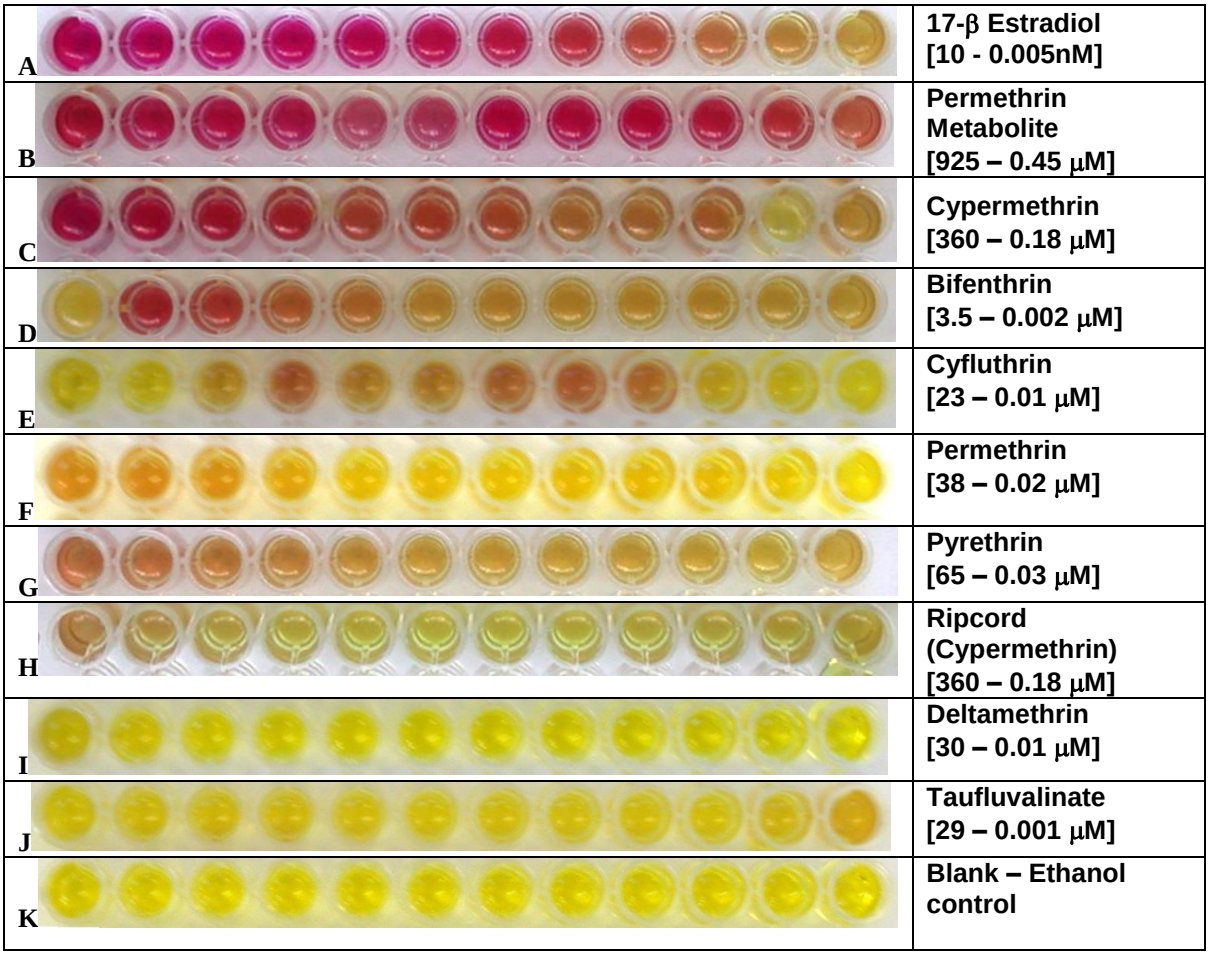


Figure 4 A-K. Representative photos of the colour change response of the yeast growth medium to test compounds. Serial dilutions run left to right. Chemical name and concentration ranges are indicated to the right of each row.

The yeast assay successfully identified 17β-estradiol as estrogenic by its positive colour change and the response also displayed the expected dose based effect with the colour change most extreme at the highest concentration (see Figure 4, Row A). The permethrin metabolite and pure cypermethrin in rows B and C, respectively, show strong positive

responses over a wide concentration range. Bifenthrin also showed a positive colour change for most concentrations. The lack of colour change in the first well of the bifenthrin dilution (Row D) is an example of a false negative. This well appears yellow and quite clear but this was due to the yeast dying rather than a lack of response. The results for cyfluthrin show it was able to elicit an estrogenic response but within a narrow concentration range (see Figure 4, Row E). The lack of colour change seen at the higher concentrations of cyfluthrin in row C was thought to result from toxicity, similar to that seen at the highest concentration of bifenthrin in row D. A weak colour change at the high end of the concentration ranges tested was shown in rows F, G and H whereas no colour change was shown in rows I and J thus mirroring the control in which only ethanol was added (Row K).

As outlined in the methods section, corrected absorbance at 540 nm was used in the analysis of the yeast assay data. Each of the pyrethroid chemicals were graphed according to the concentration ranges in which they were assessed, along with 17β - estradiol (Figure 5, A-I). Estradiol served as both a positive control and bench mark to highlight the differences in the concentration ranges tested. The error bars represent the standard error and illustrate the variation between the replicate assays for each chemical. The curves were plotted using the program Sigmaplot and regression lines calculated by either the Hills 4 parameter test (for the estradiol data) or Sigmoid 4 parameter test (the other chemicals). For both bifenthrin and cyfluthrin, the wells at the high end of their concentration ranges were excluded from the graphs because the lack of a response was the result of toxic effects of the chemicals.

The estradiol data depicted on every graph showed the expected increase in absorbance with increase in concentration for a positive estrogenic chemical. This dose based response can be predicted by the equation of the sigmoid regression line fitted. The R-squared value shown on individual graphs indicates the robustness of the fitted regression line with a value of 1.0 representing a perfect fit. Thus, it specifies whether the equation of the line is a good representation of the data with accurate predictive power.

The permethrin metabolite and the pure cypermethrin (Figures 5A and 5B respectively) induced the strongest response next to that of estradiol. These responses also displayed the best R-squared values for fit with their respective regression lines. This signifies that their

responses followed the non-linear sigmoidal shaped curve well and that the equation of the fitted line could be used to predict either absorbance or concentration with accuracy.

For the bifenthrin graph (Figure 5C), the value for the well with the highest concentration was excluded because the toxic effect of bifenthrin killed all the yeast cells (see Figure 4, Row D). Dead and non-responsive yeast were differentiated by the cloudiness of the culture medium by measuring absorbance at 610 nm as yeast growth increased the turbidity and hence the optical density of the culture medium. The absorbance measurements for bifenthrin at the lower concentrations were very consistent as indicated by the very small error bars. The response appears to saturate at a much lower absorbance level compared to estradiol which may be an indicate the beginnings of toxicity.

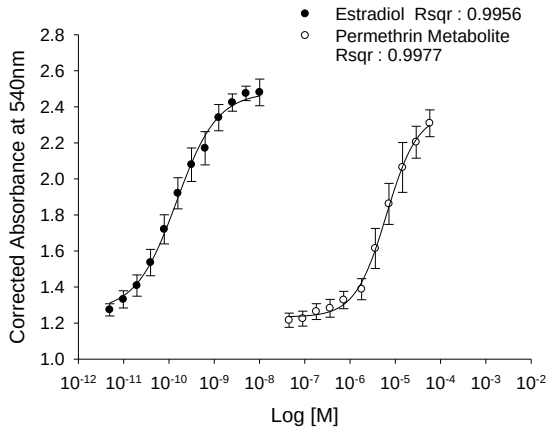
At high concentrations cyfluthrin also caused cell death. Thus the data from concentrations above 0.18 μM where the colour change disappeared were removed and the remaining data from lower concentrations are shown on Figure 5D. The dose response of cyfluthrin appears almost linear, due in part to the very limited number of data points available at concentrations that were not affected by toxicity. This also means that the EC_{50} calculated for cyfluthrin (Table 2) is only tentative as the maximum response could not be achieved in the assay due to toxicity.

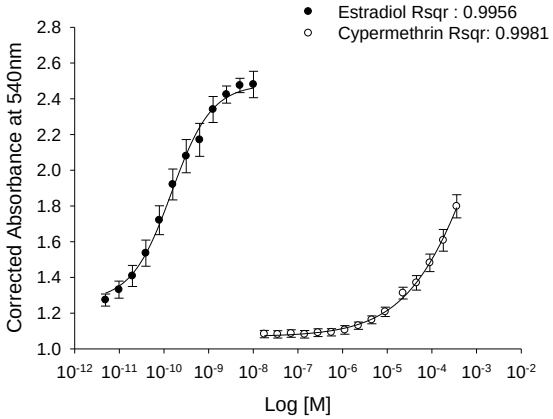
Pyrethrin and Permethrin (Figures 5E and 5F) show relatively good R-squared values despite the sigmoidal curve appearing flat compared to that of estradiol. Colour change can be visually detected, although much weaker than that of estradiol (Figure 4).

The lack of colour change for taufluvalinate and deltamethrin (see Figure 4, rows J and I) resulted in effectively flat lines and hence relatively low R-squared values (see Figure 5G and H respectively).

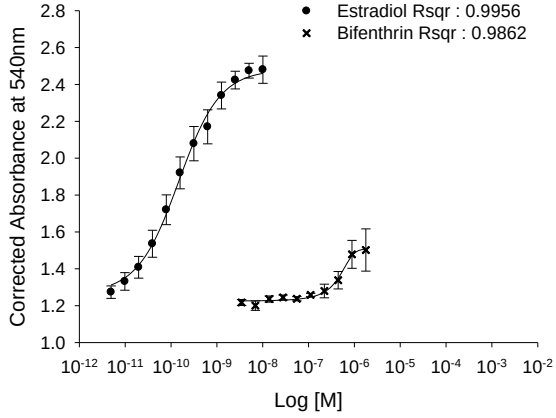
Ripcord, a commercial product containing cypermethrin, showed no obvious change in absorbance over the concentration range tested (Figure 5I). However, the error for the higher concentrations was large, which could be masking the small colour change observed in Figure 4, Row H.

A **B**

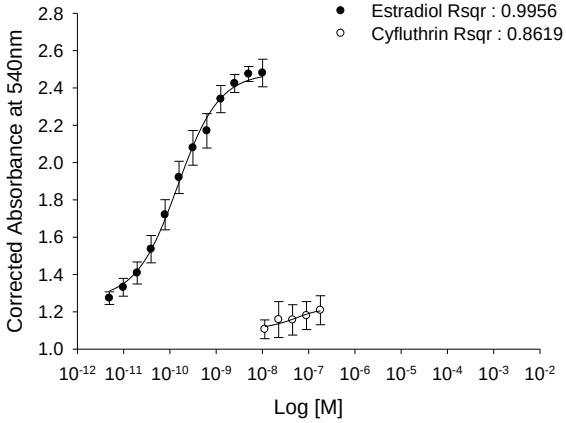

Estradiol Rsqr : 0.9956
Permethrin Metabolite Rsqr : 0.9977
Corrected Absorbance at 540nm
Log [M]


Estradiol Rsqr : 0.9956
Cypermethrin Rsqr: 0.9981
Corrected Absorbance at 540nm
Log [M]

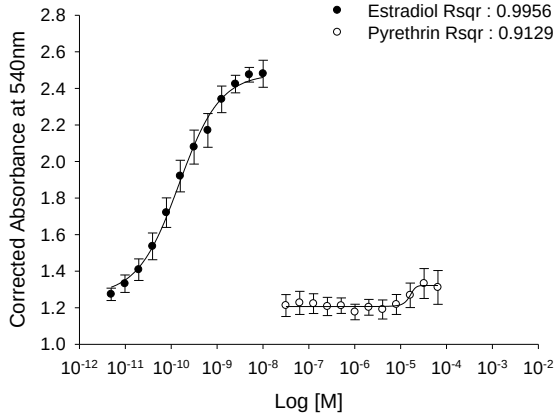
C


Estradiol Rsqr : 0.9956
Bifenthrin Rsqr : 0.9862
Corrected Absorbance at 540nm
Log [M]

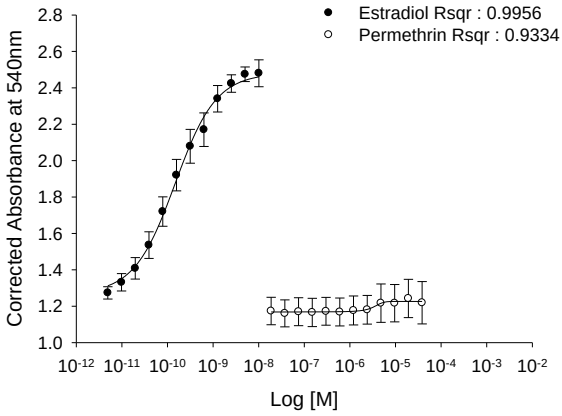
D


Estradiol Rsqr : 0.9956
Cyfluthrin Rsqr : 0.8619
Corrected Absorbance at 540nm
Log [M]

E


Estradiol Rsqr : 0.9956
Pyrethrin Rsqr : 0.9129
Corrected Absorbance at 540nm
Log [M]

F


Estradiol Rsqr : 0.9956
Permethrin Rsqr : 0.9334
Corrected Absorbance at 540nm
Log [M]

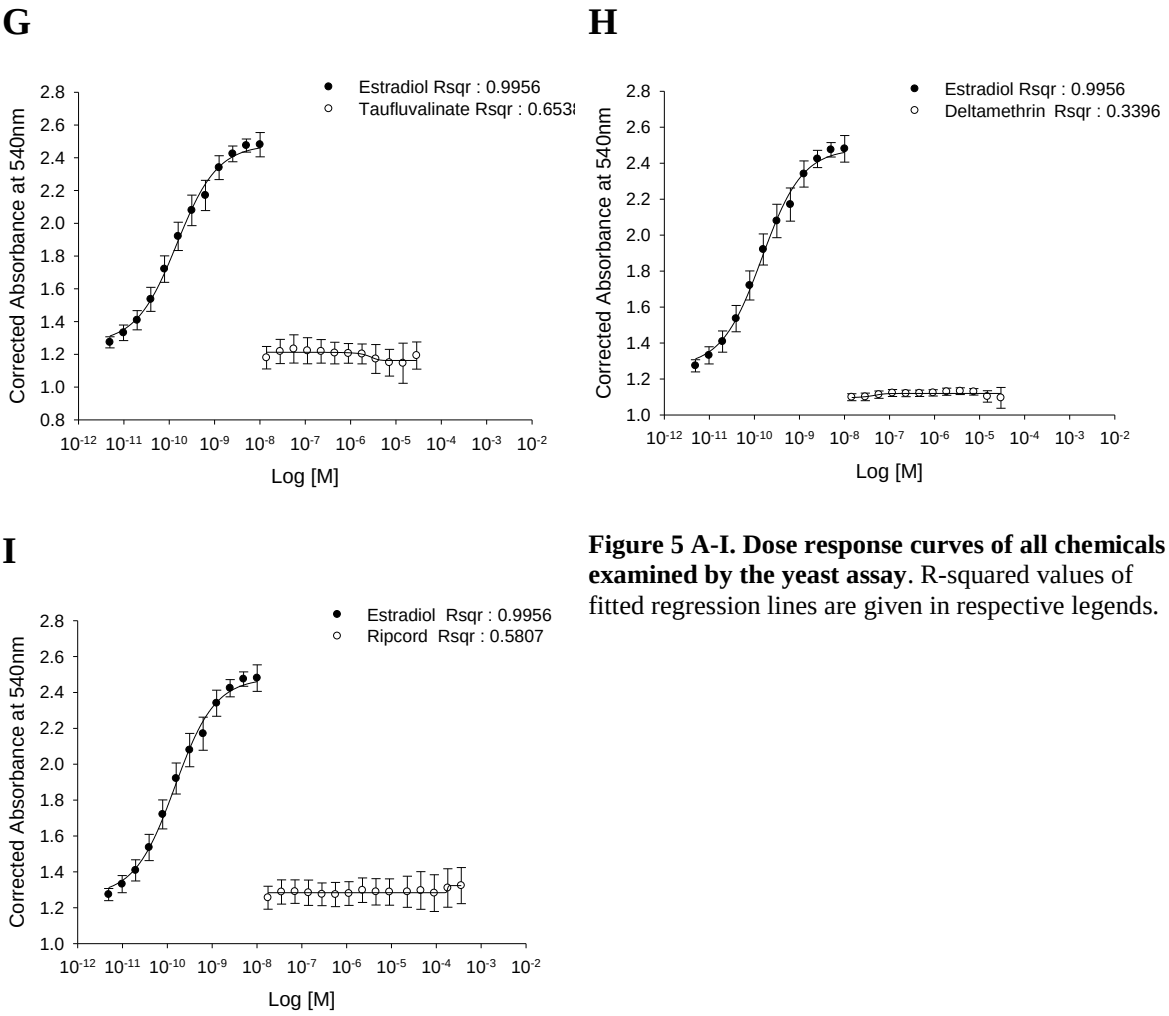


Figure 5 A-I. Dose response curves of all chemicals examined by the yeast assay. R-squared values of fitted regression lines are given in respective legends.

The estrogenic response that was detected visually, EC_{50} values, the relative potency and significance level of each chemical tested are shown in Table 2. The EC_{50} values were determined from the regression lines where the EC_{50} was the concentration at which half the maximum absorbance was observed. The lower the concentration of the EC_{50} value, the more potent the chemical was found to be in that specific assay. Significance was calculated by a paired-two-tailed Student's *t*-test, which compared the absorbance at the highest and at the lowest concentrations tested for each chemical. In the case of bifenthrin and cyfluthrin, where data at some concentrations were removed, the absorbance at the highest and lowest remaining concentrations was compared to test for a significant change.

Table 2 Summary of the responses of the chemicals assessed by the yeast estrogen screen.

Chemical	Response $^{\Psi}$	EC_{50} (M) $^{\lambda}$	Relative Potency to 17β-Estradiol $^{\phi}$	Significance level $^{\delta}$
17β-Estradiol	**+**	**1.5×10^{-10}**	**1**	$P << 0.00005$
Cyfluthrin	+			NS
Taufluvalinate	-	–	–	NS
Permethrin	+	3.6×10^{-6}	4.1×10^{-5}	NS
Permethrin Metabolite	+	5.7×10^{-6}	2.6×10^{-5}	$P < 0.001$
Bifenthrin	+	4.5×10^{-7}	3.3×10^{-4}	$P < 0.06$
Pyrethrin	+	1.5×10^{-5}	9.9×10^{-6}	NS
Deltamethrin	-	–	–	NS
Cypermethrin	+	4.8×10^{-5}	3.1×10^{-6}	$P < 0.05$
Ripcord (cypermethrin)	+	1.6×10^{-5}	9.4×10^{-6}	NS

$^{\Psi}$Response based on colour change. $^{\lambda}EC_{50}$ was calculated from the regression lines of the absorbance data. $^{\phi}$Relative potency was calculated by the ratio between the estradiol EC_{50} and the EC_{50} of each other chemical $^{\delta}$Significance was calculated by a paired-two-tailed Student's *t*-test comparing the change in absorbance. NS indicates non significant p values.

Only deltamethrin and taufluvalinate showed no estrogenic response over their respective concentration ranges assessed. However, although a positive response was visually detectable in the rows containing permethrin, cyfluthrin and pyrethrin assays, the changes were not statistically significant.

Of the pyrethroid chemicals that showed a statistically significant estrogenic response, all were much less estrogenic than estradiol. Relative potencies of 10^{-4}, 10^{-5} and 10^{-6} were determined for bifenthrin, permethrin metabolite and cypermethrin respectively.

3.2 Cell Culture of Sertoli Cells

The Sulforhodamine B colorimetric assay was used as a fast and simple estimate of cell numbers through total protein measurement. The SRB dye binds to the basic amino acids which after removal of the unbound dye can be quantified by the absorbance at 540 nm. The SRB assay was established by Skehan *et al*., (1990) and has been validated by its routine use as part of the E-SCREEN assay (Soto *et al*., 1995). A five day growth experiment was used to chart the growth of mouse Sertoli cells from varying initial seeding densities (see Figure 6). The highest initial cell density, approximately 4.8 $x10^5$/well, was estimated by manual cell counting, subsequent cell numbers were estimates based on the dilution of the cells in the first well. The absorbance data at 540 nm was used to plot

growth curves for five of the different seeding densities over the five day incubation period (see Figure 7).

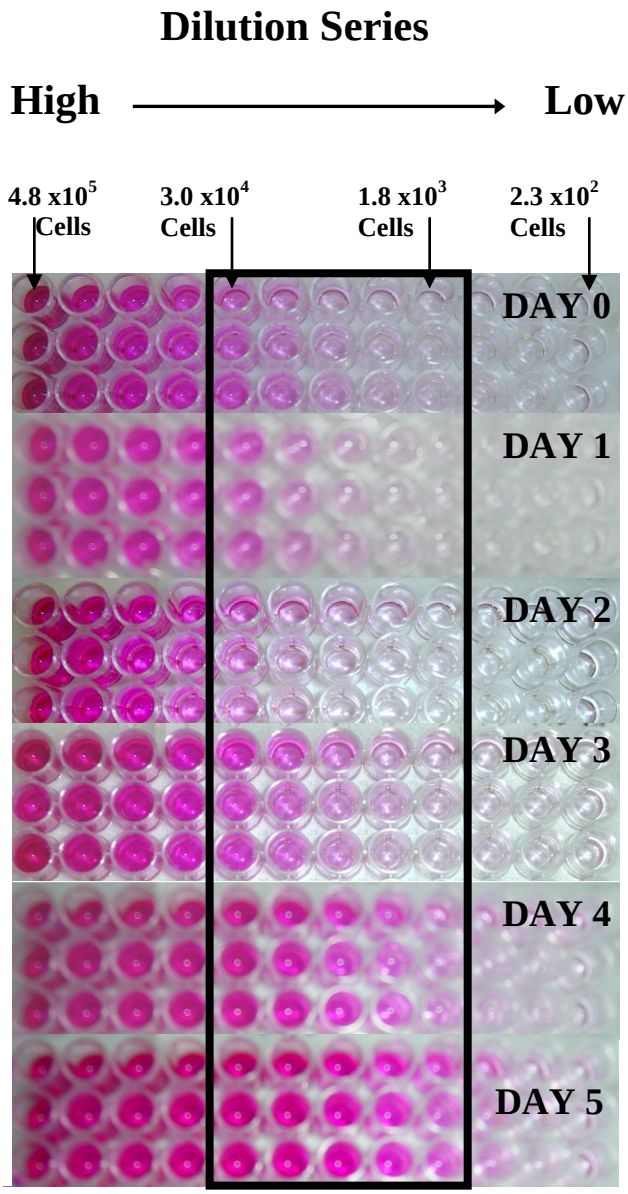


Figure 6 Photographs showing the amount of stained protein according to the number of days incubated. Highest initial cell density per well is on the left (4.8×10^5) with a serial 1:2 dilution towards the right (2.3×10^2). Inside the black box are the dilutions (3.0×10^4 - 1.8×10^3) whose measurements were used to plot Figure 7.

The progressive increase of staining in each well over the 5 days indicates an increase in protein, hence growth of the Sertoli cells. The highest initial cell density on the left of the Figure 6, stained strongly from Day 0 and the colour intensity approached the upper level of spectroscopic discrimination. The wells within the black box had little or no staining initially and progressed to mostly full colour. These wells within the black box are graphed in Figure 7. The detection range of the SRB assay is also demonstrated by the staining patterns on Figure 6. All wells had at least 200 cells initially, however many to the right of the black box did not show any visible staining on Day 0 or until after Day 2.

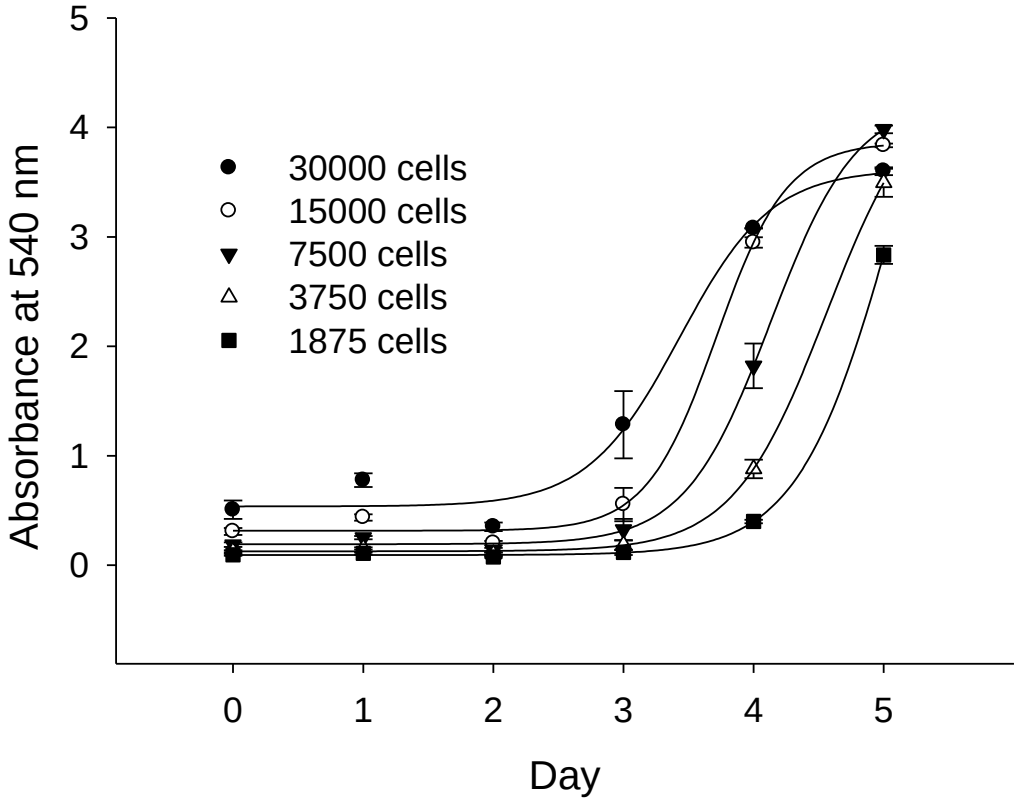


Figure 7 Growth of TM4 mouse Sertoli cells over five days measured by absorbance at 540 nm (± SE, n=3) after fixing and staining using SRB reagent. Legend indicates the initial approximate cell seeding densities calculated from the original cell count and the serial dilution factor.

The TM4 mouse Sertoli cells exhibited normal growth characteristics of a lag phase and log growth phase (see Figure 7). As space and nutrients became a limiting factor, a slight decline or plateau was observed as reflected in the growth curve of the cells seeded at 30000 cells per well. The R-squared value for the curve corresponding to the initial cell density of 30000 was the lowest at 0.987 whereas the other curves scored over 0.997. The measurement of Day 0 was taken after an initial 6 hours attachment time. This was required to prevent the cells from being lost during the fixing and staining process. The lag phase lasted for between two and four days; the exponential growth phase appeared around Day 2 and Day 4, depending on the initial seeding density.

3.3 Exposure of Mouse Sertoli cells to pyrethroid chemicals

The exposure to test chemicals of mouse Sertoli cells involved multiple treatments consisting of four different chemicals at multiple concentrations and a non-treated control. Each exposure experiment was carried out in T25 flasks in triplicates over an

approximately 100 hours of incubation. Photographs of the cell cultures were taken throughout the incubation period to assess changes in gross morphology and to estimate cell death. The increase in cell numbers over the three days for both the non-treated cells and the 36 μM cypermethrin treatment are shown in Figure 8. The approximate confluence of the cells on Day 3 are shown for the other treatments (see Figure 9). Confluence was used as a cell density estimate, referring to the coverage of the flasks' surface by the cells.

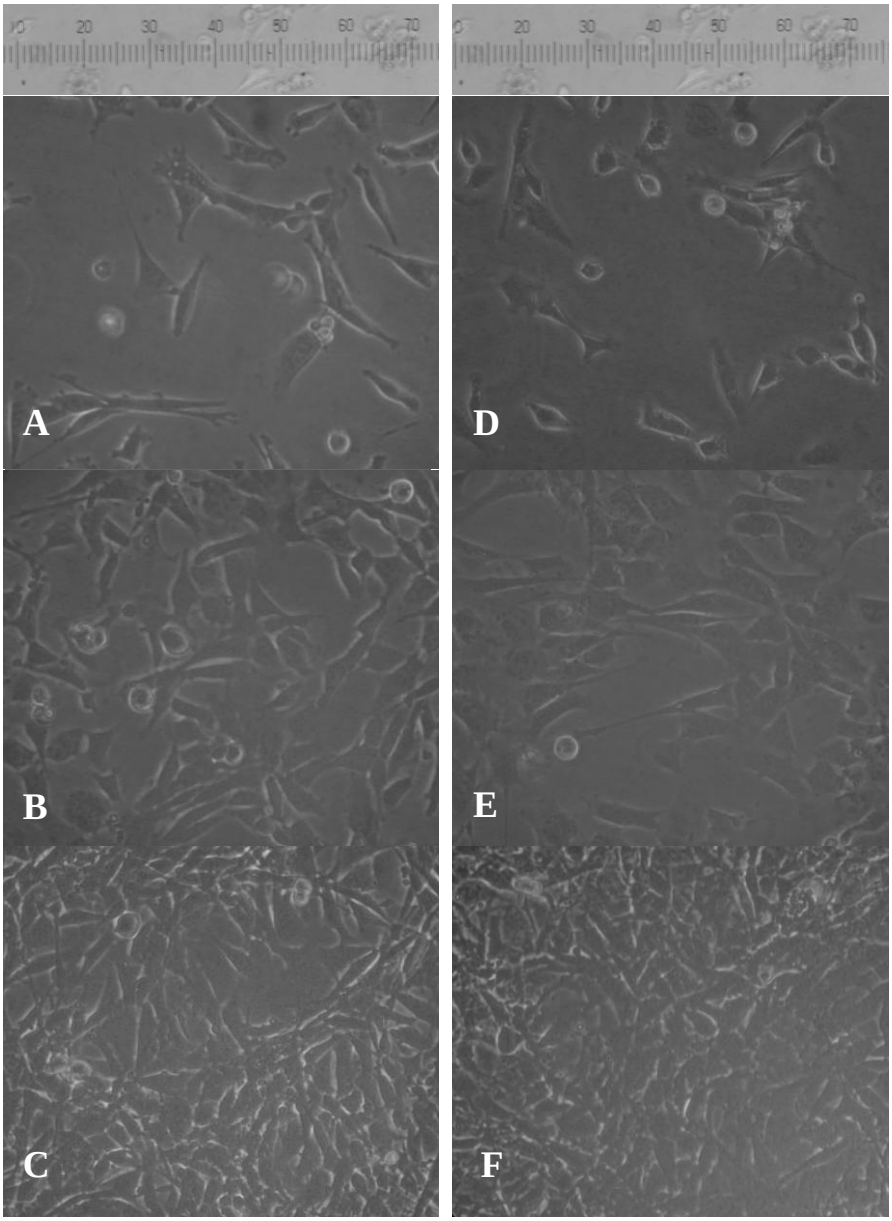


Figure 8 Sertoli cell growth over the exposure period. A,B,and C Non treated cells, Day 1,2 and 3 respectively. D,E and F Cypermethrin treatment at 36 μM, Day 1,2 and 3 respectively**.** Scale (nm).

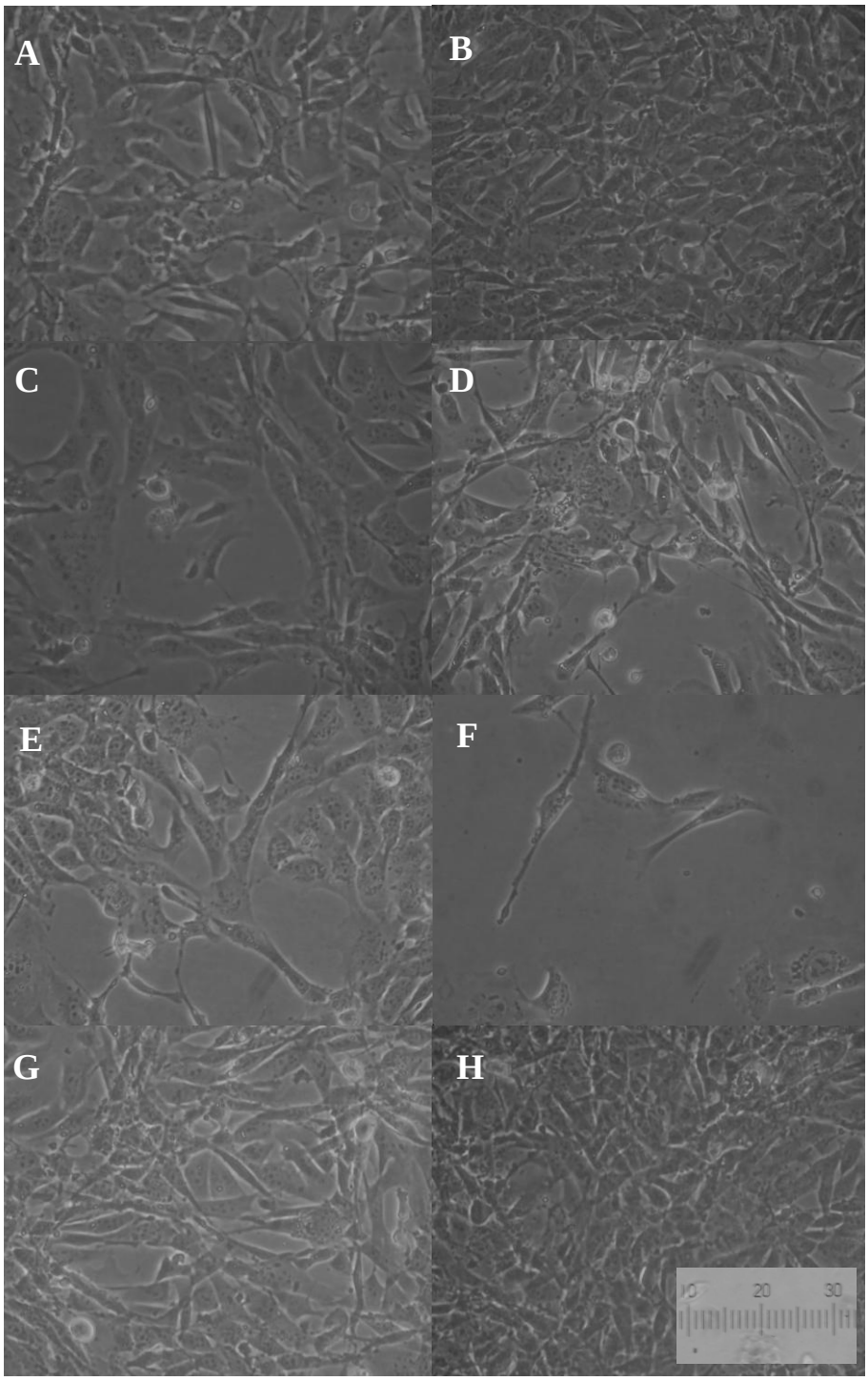


Figure 9 Sertoli cell growth on Day 3 of the exposure period. Treatments A and B, 17β-estradiol at 0.3 nM and 10 nM respectively. C and D Ripcord (cypermethrin) at 0.36 nM and 36 μM respectively. E, and F Permethrin metabolite at 0.69 nM and 69 μM respectively. G and H cypermethrin at 0.36 nM and 36 μM respectively. Scale (nm).

The cells exposed to the cypermethrin treatment at 36 μM showed similar growth progression as the non-treated cells over the three days (see Figure 8). This suggests the cypermethrin at that concentration did not cause increased toxicity relative to the non-treated cells. Other treatments did result in either growth inhibition or increased toxicity and therefore showed a decrease in cell density at day three (see Figure 9). In particular the

culture treated with the metabolite of permethrin at 69 μM, resulted in very few cells that were sparsely distributed (see Figure 9F). The culture treated with the lower concentration of the permethrin metabolite was also less dense than the control (see Figure 9E). Ripcord (cypermethrin) at both concentrations lowered the final confluence of cells (Figure 9C and D), however not to the same degree as the permethrin metabolite treatment. The higher concentrations of both the estradiol treated flasks (Figure 9B) and the cypermethrin treated flasks (Figure 9H) showed more cells compared to the low concentrations (Figures 9A and G) and even relative to the control cultures (Figure 8C). It should be pointed out that the cells were not uniformly distributed over the surface of the flasks, hence, the photos are only used to demonstrate a representative section of a culture in a particular treatment.

3.4 Quantification of Extracted RNA

Quantification of the extracted RNA was required to enable approximately equal amounts of cDNA, reverse transcribed from the RNA, to be added to the each quantitative PCR reaction. Quantification of the extracted RNA from the exposed cells was performed by measuring the absorbance of the Quant-iT ™ reagent, a dye that fluoresces when bound to single-stranded RNA. Dilution of the extracted RNA samples was required to limit the fluorescence to within the linear portion of the standard curve. Due to the instability of the dyes' fluorescence over time and when exposed to light, the standard curves were repeated for each run. The standard curve was consistently linear between runs for up to 80 ng of RNA (Figure 10). After calculation to adjust for the dilution and the amount read, final quantification results for each extraction are graphed on Figure 11. The RNA concentrations of the replicate cultures were not averaged as each sample was used individually in qRT-PCR.

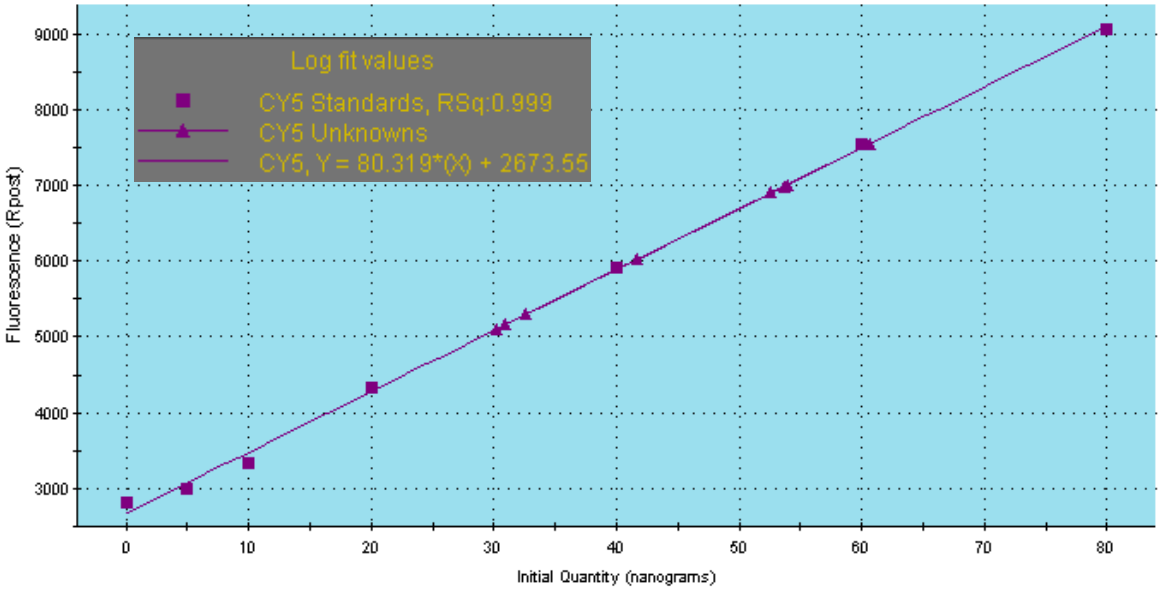


Figure 10 Standard curve of RNA quantities by fluorescence detected using the CY5 filter on Stratagene Mx3005p. Each data point represents the average of triplicate samples. Unknowns were diluted to within a range of up to 80 ng/well. Unknown samples are indicated by triangles which represent the average of duplicate reactions.

Generally the different cultures of the same treatment yielded approximately the equal concentrations of RNA (Figure 11). The RNA concentrations form replicate cultures were not averaged as each sample was independently amplified by real time RT-PCR. The large variation in the different treatments reflects both the amount of cells that were present at the time of harvest and their growth phase, hence, their transcriptional activity.

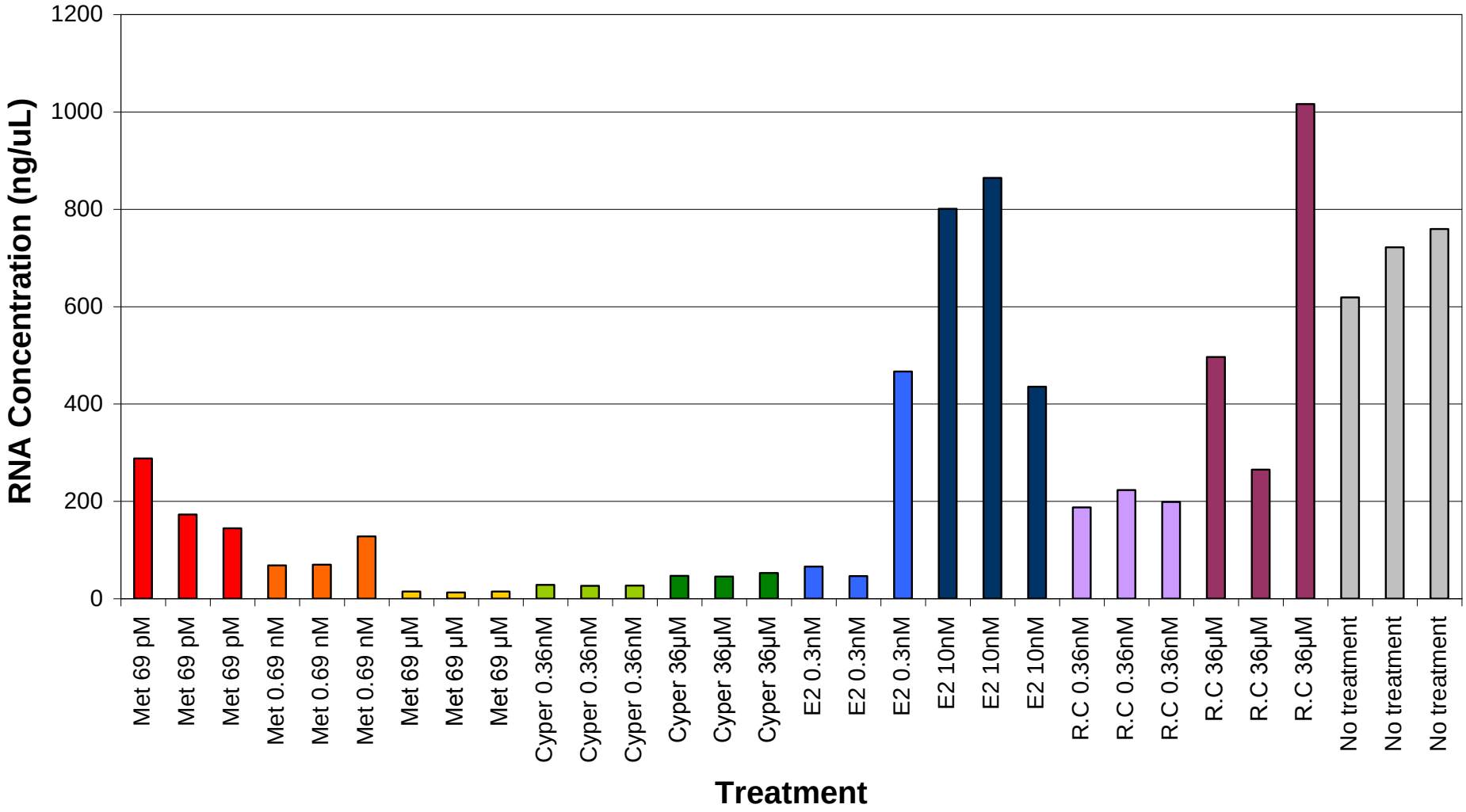


Figure 11 Concentration of RNA in ng/µl obtained from Sertoli cell cultures exposed to various treatments. Each bar represents a separate culture with all treatments having three replicate cultures. Abbreviations; Met – Permethrin Metabolite, Cyper – Cypermethrin, E2 – 17β-Estradiol, R.C – Ripcord.

3.51 Real-time multiplex RT-PCR of the Estrogen receptors

Real-time multiplex reverse transcriptase polymerase chain reaction was used to amplify three target sequences, ERα, ERβ and β-actin. The estrogen receptors were the target genes with β-actin acting as a normalising gene. LUX® primers with different fluorescent labels on one of each of the primer pairs enabled the amplification to be detected and graphed in real time by the Stratagene Mx3005p software. The cycle thresholds (Ct values) were used to calculate the relative expression of each target gene. Extensive optimisation was carried out to allow the fluorescence of all primers to be detected below the filter saturation levels and to minimise background noise.

The optimisation of the multiplex reaction included alteration of LUX® primer concentrations, template concentration, and adjustments of the filter gain settings on the Stratagene MX3005P. Increasing specific reaction mix components such as magnesium and dNTP concentrations was investigated but did not improve reaction efficiency for all primer pairs.

The reaction efficiency when less than 100%, increases the number of amplification cycles required to reach the cycle threshold. Hence, the efficiency of each primer set in the multiplex reaction was calculated by construction of a standard curve using a dilution series of cDNA template quantities (Figure 12) and adjusted for in the equation employed to calculate the relative expression levels. Dissociation curves and agarose gels were also performed to investigate the presence of non-specific products which can adversely affect reaction efficiency and distort the actual gene expression levels detected (see Figures 13 and 14).

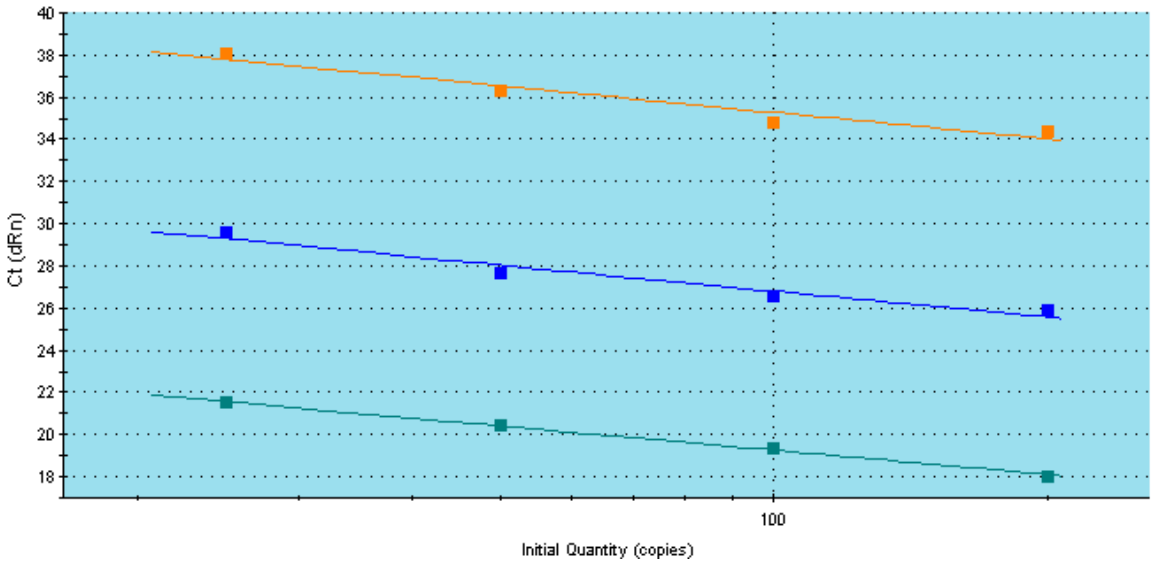


Figure 12 Efficiency of each primer set in the multiplex real time PCR. Construction of the standard curve was enabled by a dilution series of cDNA template and their respective Ct values. Each point on the standard curves is the average of triplicate reactions. Legend shows individual efficiencies, the equation of each line and their respective R-Squared values.

The efficiency of the amplification of ERα and ERβ genes under the multiplex reaction conditions, outlined in the methods section, were found to be similar, 75.4% and 74.1% respectively (Figure 12). The efficiency of the β-actin PCR is slightly higher but as this gene acts to normalize the other Ct values, both values will have the same adjustment thus not altering relative amounts.

The standard curves of all three primer pairs shown in Figure 12 also illustrates that even over multiple dilutions the expression of the three genes do not overlap. The range of raw Ct values for β-actin illustrate that β-actin had the highest expression levels compared to the other target genes as indicated by the lowest range of Ct values approximately 18-22. ERα gave Ct values between 26 and 30 over the dilution range and ERβ showed the lowest expression with raw Ct values between 34 and 38.

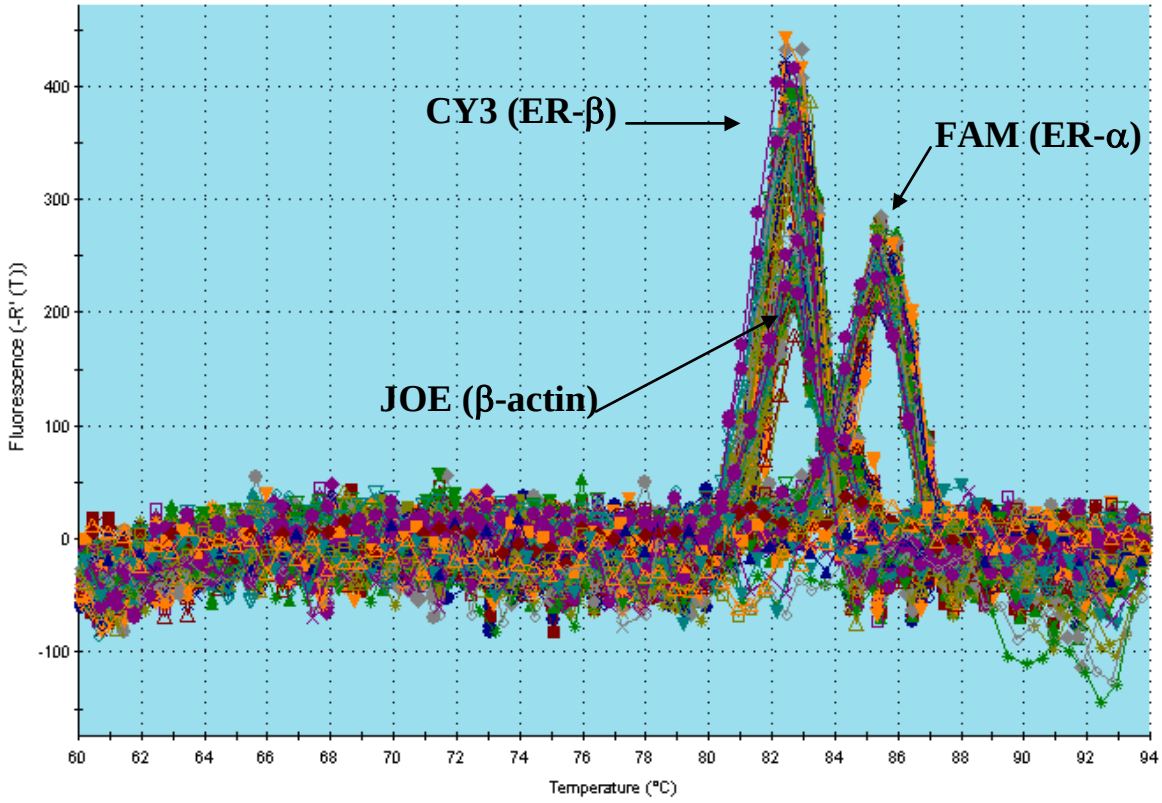


Figure 13 Disassociation curve for one run of the multiplex reactions of all samples and no-template controls. Each peak represents the specific disassociation of the products of one primer set indicated by labels.

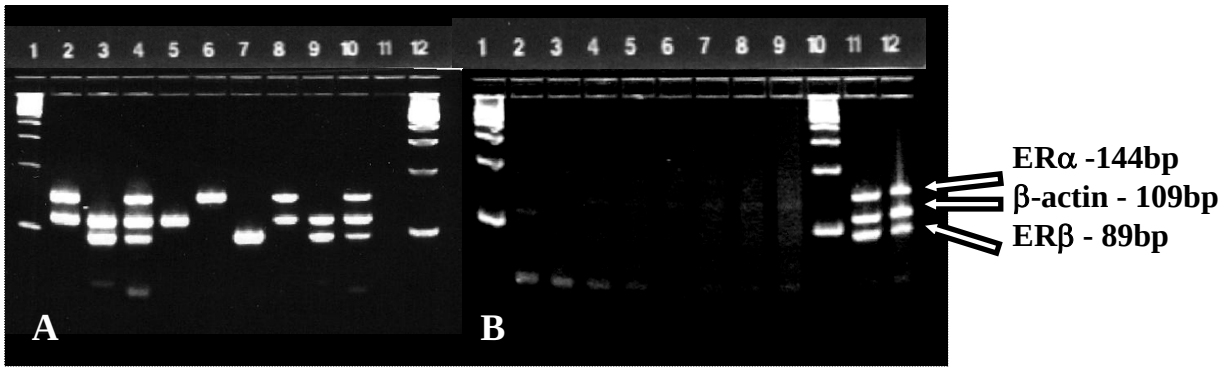


Figure 14 4% agarose gel of RT-PCR products from Singleplex,Duplex and Triplex assays as well as No Template Controls. A Lane 1 10 µl 100bp ladder. Lane 2 20 µl ERα and β-actin duplex, Lane 3 20 µl β-actin and ERβ duplex. Lane 4 20 µl ERα, ERβ, β-actin multiplex. Lane 5 20 µl β-actin singleplex. Lane 6 20 µl ERα singleplex. Lane 7 20 µl ERβ singleplex. Lane 8 10 µl of ERα and β-actin duplex, Lane 9 10 µl of β-actin and ERβ duplex. Lane 10 10 µl of ERα, ERβ, β-actin multiplex. Lane 11 empty. Lane 12 20 µl 100bp ladder. B Lane 1 10 µl 100bp ladder, Lane 2-9 No template control replicates. Lane 10 20 µl 100bp ladder. Lane 11 and 12 20 µl ERα, ERβ, β-actin multiplex.

Both the disassociation curves and the agarose gel analysis show that the multiplex reaction was successful in amplifying the three target sequences without non-specific products. The products visualised on the agarose gel are of the expected relative sizes with

ER β migrating the furthest followed closely by β-actin and the ER α band after a slightly larger gap (see Figure 14). Only three peaks are present on the disassociation curve indicating no non-specific product formation (Figure 13). The height of the peaks is related to the fluorescence of the primer label and not to the amount of product formed.

3.6 Analysis of ERα and ERβ expression

Expression fold-change shows the relative expression of both ERα and ERβ as a result of the specific treatments compared to their expression in the non-treated cells. Thus it is the amount the expression increases or decreases compared to the non-treated cultures arbitrarily given the expression value of one for both genes.

The three biological replicates of two treatments, 69 μM permethrin metabolite and 0.36 nM cypermethrin, were unable to be consistently amplified due to low RNA yield. These treatments are therefore not included in the real time RT-PCR results.

3.6.1 Individual Culture Results

The fold-change data was averaged between separate PCR experiments for each individual culture (see Figure 15). Each PCR experiment contained triplicate samples from each individual culture. Thus the error bars represent the standard error of the mean between the PCR experiments, where the mean represents the average of the triplicates within each PCR experiment.

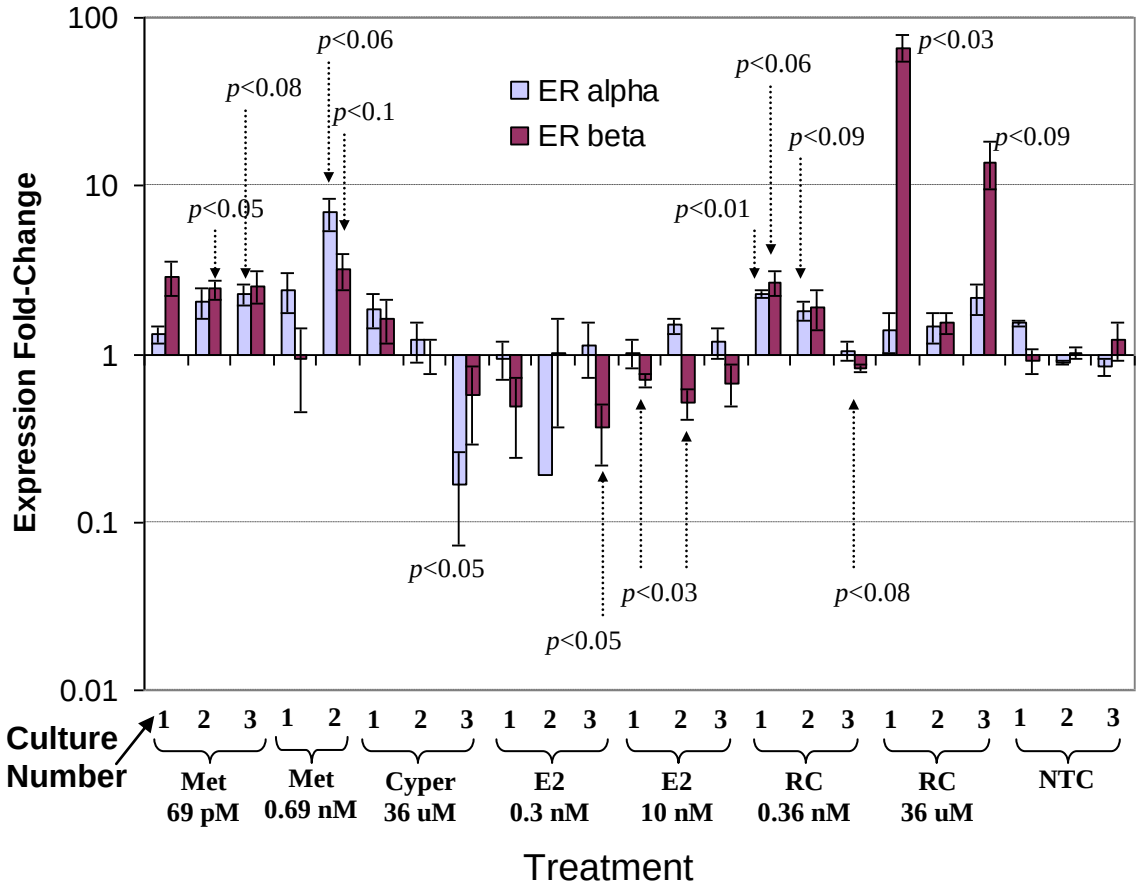


Figure 15 Average fold-change of ER alpha and ER beta mRNA expression over three PCR runs per replicate culture (± SEM, n=3). The cDNA from each culture was assayed in three separate PCR experiments with triplicate culture samples. Abbreviations used in data labels: Met – Permethrin metabolite, Cyper – Cypermethrin, E2 - 17β-estradiol, RC- Ripcord and NTC refers to No Treatment Control. Significance compared to NTC tested by paired-two-tailed Student's *t*-test, *p* values ≤ 0.1 are indicated on the Figure.

Generally, the exposure of mouse Sertoli cells to cypermethrin and the permethrin metabolite caused an increase in expression of both ER alpha and beta mRNA whereas exposure to estradiol resulted in a decrease in expression. Varying degrees of expression change resulted from the different concentrations of treatments however, most followed the same trend. The variation between what should be similar biological replicates is notable particularly for the replicates of Ripcord 36 μM treatment (see Figure 15). Two of these replicates show an over 10-fold increase in ERβ mRNA expression however the third shows no relative change. The 36 μM cypermethrin treatment resulted in a significant decrease in ERα but in only one replicate and the other replicates failed to follow the same trend. A significant increase in the relative expression of both ERα and ERβ for the permethrin metabolite at 0.69 nM is evident, however, both increases occurred in one replica only. Ripcord 0.36 nM treatment similarly resulted in a significant increase in both ERα and ERβ for two of the replicates however the third replicate

exhibited a significant, though very minor, decrease. The results for the permethrin metabolite at 69 pM illustrate that the variability between the three PCR experiments, represented by the error bars, contributes to whether a change was statistically significant. For those replicas, the increased expression for ERβ in particular was very similar however only one change was significant. This reflects the fact that the larger the variability the less likely a *t*-test will be able to distinguish significant change from the variation in the control. Both concentrations of the estradiol treatments resulted in a decrease in expression of ERβ with the three individual cultures showing significant change. The change in ERα expression was not as consistent at both concentrations though no changes were significant.

3.6.2 Treatment Results

The average fold-change data for individual cultures over three PCR experiments was averaged with other cultures of that specific treatment to show the overall treatment trend (see Figure 16). Hence the error bars represent the variation between separate biological replicas of each treatment. They are standard errors of the mean as the average over all PCR experiments for each culture was used in calculating the treatment average.

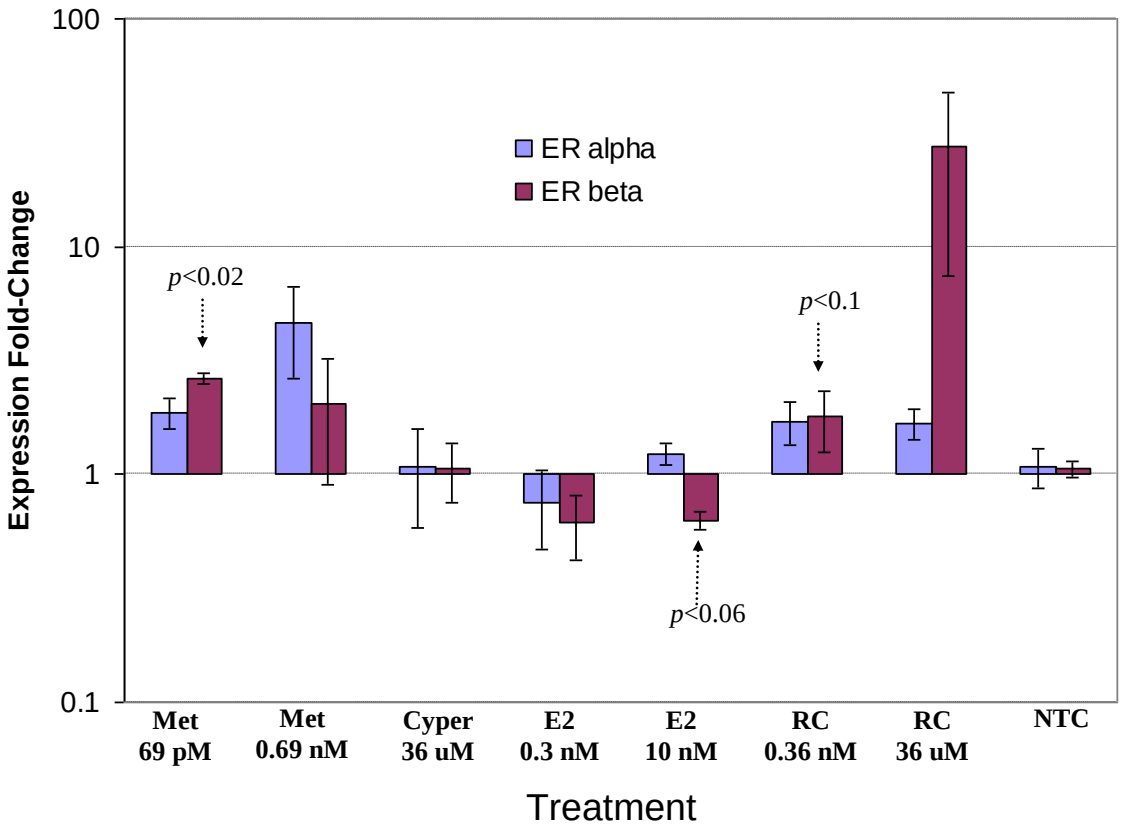


Figure 16 Average fold-change of ER alpha and ER beta by treatment (± SEM). Each treatment represents the average of the three replicate cultures, with the exception of the permethrin metabolite at 0.69 nM which only had two cultures available. The cDNA made from each culture was run in three separate PCR experiments with triplicate culture samples in each. Abbreviations used in data labels: Met – Permethrin metabolite, Cyper – Cypermethrin, E2 - 17β-estradiol, RC- Ripcord and NTC refers to No Treatment Control. Significance compared to NTC tested by paired-two-tailed Student's *t*-test, *p* values ≤ 0.1 are indicated on the Figure.

Due to the averaging of quite disparate biological replicas the retention of significance for the average fold-change per treatment against any change in the control average is in itself notable. For ERβ expression two treatments resulted in a significant change (see Figure 16). The permethrin metabolite at 69 pM caused an increase in ERβ mRNA expression whereas 17β-estradiol at 10 nM caused a decrease relative to the non-treated control. ERα expression only changed to a significant extent after treatment with Ripcord at 0.36 nM which caused an increase in fold-expression. Only the estradiol treatment at 10 nM resulted in receptor levels changing in different directions, all other treatments showed the same effect to varying degrees for both receptors. Some treatments that appear visually significant such as the fold-change of the ERβ expression after Ripcord treatment at 36 μM, are not statistically significant because of the large standard deviation. The large variability in this case was due to the fold-change not occurring in one of the biological replicas that were included in the averaging.

3.6.3 Ratio of Expression Fold-Change

The ratio of ERα expression fold-change to ERβ expression fold-change was calculated and plotted on Figure 17. This ratio indicates whether the relative expression level of either gene to the other has changed. A positive ratio change represents an increase in the difference between the net amounts of ERα to ERβ whereas a negative ratio change indicates that the difference between the net amounts of ERα to ERβ has decreased. Therefore if the ratio remains the same it can either indicate that there was no change in expression for either of the genes or that both changed but by the same degree.

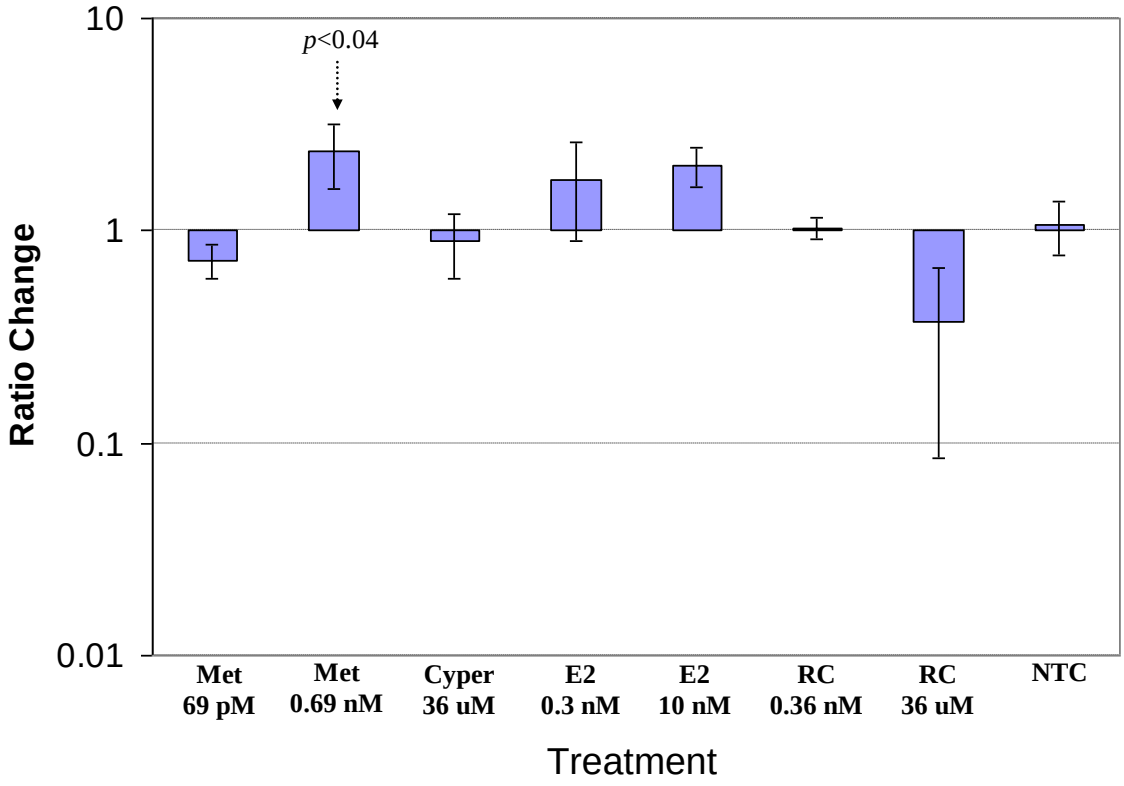


Figure 17 Ratio of the fold change of ER alpha/ER beta expression (± SEM, n=3). Abbreviations used in data labels: Met – Permethrin metabolite, Cyper – Cypermethrin, E2 - 17β-estradiol, RC- Ripcord and NTC refers to No Treatment Control. Significance compared to NTC tested by paired-two-tailed Student's *t*-test, *p* values ≤ 0.1 are indicated on the Figure.

After treatment with permethrin metabolite at 0.69 nM the mRNA expression levels of the estrogen receptors were altered resulting in a greater ratio between them (see Figure 17). As the ERα receptor was consistently more highly expressed in all samples the increase in ratio indicates that either ERα expression increased further or ERβ decreased thus making the overall balance further in the ERα receptors favour. Comparison with Figure 16 reveals that although the relative expression of ERα and ERβ increased, the increase in

ERα expression was significantly greater than the change in ERβ expression. While the other ratio changes were not statistically significant; the trend for both the estradiol concentrations was an increase in ratio, whereas, all other pyrethroid treatments, except, the permethrin metabolite at 0.69 nM, either showed a decrease or no change in ratio.

4 Discussion

4.1 Estrogenicity of Pesticides as Determined by the Yeast Assay

The yeast assay was used to test for potential estrogenic activities of pyrethroid chemicals that are licensed for use in New Zealand. Changes observed in this assay system may not represent the total response of cells because the model is limited to binding of chemicals to the estrogen receptor alpha (ERα). Normal cells are known to have a second estrogen receptor, beta (ERβ) as well as an array of co-activators and repressors. The yeast assay is a good system for preliminary screening for potential estrogenic activities of compounds.

The current study found that, among the various pyrethroids tested in the yeast system, cypermethrin, bifenthrin and the permethrin metabolite were significantly estrogenic (Table 2). Of the remaining pyrethroids, permethrin, pyrethrin, Ripcord (cypermethrin) and cyfluthrin were found to be weakly estrogenic; however, these activities were not statistically significant. Taufluvalinate and deltamethrin were found to be non-estrogenic.

4.1.1 Comparison of Results with Published Literature

The majority of the literature related to the estrogenicity of the pyrethroids tested in this study, concentrates on the more commonly used pesticides such as cypermethrin and permethrin. Very few reports are available for cyfluthrin and deltamethrin and no reports on the estrogenicity of taufluvalinate and bifenthrin were found. In addition to these reports which are summarised in Table 5, the estrogenicity of pyrethrum of which pyrethrin is a component has been reported as well as the estrogenicity of the specific permethrin metabolite used in this study.

Cypermethrin in this assay displayed both a strong visual colour change and statistical significance. However, there are contradictory results in the literature regarding its estrogenicity. Kojima *et al.* (2004) found that cypermethrin was estrogenic through the ERα receptor but not ERβ. Chen *et al*. (2002) also reported cypermethrin to be estrogenic (Table 5). Both reports support the current findings that cypermethrin was estrogenic.

Conversely, other authors have reported cypermethrin to be non-estrogenic (Saito *et al*., 2000, Nishihara *et al*., 2000, Kim *et al*., 2004 and also by Tyler *et al*., 2000).

The current study found that permethrin had a weak but positive estrogenic response. This supports the findings of Tyler *et al*., 2000 who concluded permethrin to be a weak estrogen agonist. Conversely permethrin has been found to be non-estrogenic by Garey and Wolff 1998, Go *et al*., 1999, and Saito *et al*., 2000.

In contrast to the parent compound, the permethrin metabolite that was used in this work, 3-(4-hydroxyphenoxy)benzyl alcohol, was found to be significantly estrogenic displaying a very strong colour change. This confirms the findings of McCarthy *et al*., (2006) who also obtained a similar EC_{50} value for this specific metabolite (see Table 4).

Bifenthrin was found to be one of the pyrethroids that induced a statistically significant colour response, however, the physiological significance of this is likely to be more heavily influenced by the fact that toxicity occurred within a fold higher concentration than that which was required to exhibit the estrogenic response. This suggests that any cell that is exposed to bifenthrin at what was found to be an estrogenic concentration maybe more likely to be affected by the beginnings of toxicity rather than any estrogenic influence. However, the physiological relevance of both the estrogenicity and the toxicity are increased because the bifenthrin used in this work was the active ingredient in a commercial product. Hence, compared to a purified laboratory chemical, there is a higher likelihood of exposure as the commercial product is sold over the counter for recreational garden use. Exposure to humans is less likely to cause toxicity due to our mass and physical barriers while the estrogenic potency of bifenthrin was the greatest amongst the pyrethroids at only 10,000-fold below that of estradiol. Other xeno-estrogens such as o,p' - DDT and BPA are 30,000-fold and 17,000-fold less potent than estradiol in the yeast assay screen (Rajapakse *et al.* ,2001) highlighting the significance of bifenthrins potency.

Cyfluthrin, in this work, exhibited a positive response (colour change) over a narrow concentration range, thought to be limited due to the chemicals toxicity. Kojima *et al*., (2004) have previously reported that cyfluthrin acted as an estrogen agonist through ERα, which is consistent with the current finding. Deltamethrin showed no estrogenic response

in the yeast assay which is supported by Andrade *et al*., 2002; Kojima *et al*., 2004, where neither suggested any agonist activity.

Taufluvalinate displayed no colour change although solubility issues may have contributed to this result. In this study, when ethanol was used as a solvent for the commercial product that contained taufluvalinate a white viscous body solidified in the solution, though most of this viscous body eventually dispersed. It is, nevertheless possible that the intended quantity of taufluvalinate was not accurately transferred to the assay wells. Thus, an alternate solvent may be more appropriate for taufluvalinate to confirm its lack of estrogenicity.

Ripcord (cypermethrin) and pyrethrin showed a positive estrogenic activity as judged by the colour change, although these activities were not statistically significant. As there was no visually detectable colour change in the control, this suggests that these compounds are likely to be mildly estrogenic. While the chromogenic substrate used in the assay medium, CPRG (or its β-galactosidase degradation product chlorophenol red) has been shown to act as a weak estrogen (Vanderperren *et al.,* 2001). This potential contamination was minimized by the short incubation time (48 hours) and monitored by the inclusion of a blank control in each experiment.

The weak estrogenic activities observed in some of the compounds tested may be due to solubility, which would lower the bioavailability of these test chemicals to the yeast cell. There are suggestions that adsorption of chemicals into the plastic plates may occur or that the chemicals may be actively transported out of the cells by means of efflux pumps known to be active in the yeast cells (De Boever *et al.,* 2001). The highly lipophilic nature of pyrethroids aids in their absorption through the cell membranes of mammalian cells however the presence of the yeast cell wall almost certainly alters their typical uptake and transportation (Rajapakse *et al.,* 2001). Beresford *et al.* (2000), however, suggested that, where a submaximal response is observed in the yeast assay, it is most likely related to the binding affinity of the chemical to the estrogen receptor rather than any solubility or adsorption problems. Problems associated with solubility in the yeast assay and some specific chemicals such as DDT, and organochlorine have previously been encountered (Rajapaksi *et al*. 2001) as well as in this study as mentioned for taufluvalinate.

4.1.2 EC_{50} Comparison

The EC_{50} value for 17β-estradiol calculated from the data in this work was of the same order of magnitude as values reported in the literature (Table 3). The EC_{50} value of the permethrin metabolite determined in this study was consistent with that reported by McCarthy *et al*. (2006) who generously supplied the chemical for the present study (see Table 4). These comparisons confirm the accuracy of the assay in this work.

Table 3 Comparison of the EC_{50} value of 17β-Estradiol calculated in this work and values given in literature.

EC_{50} 17β-Estradiol [M]	Reference
1.5×10^{-10}	**This work (2006)**
$3.49 \pm 0.16 \times 10^{-10}$	McCarthy *et al*., 2006
$2.7 \pm 0.93 \times 10^{-10}$	Thomson, 2004
1.3×10^{-10}	Rajapakse *et al*., 2001
$1.44 \times 10^{-10} - 10.78 \times 10^{-10}$ Ψ	De Boever *et al*., 2001
$2.1 \pm 0.35 \times 10^{-10}$	Tyler *et al*., 2000
$2.2 \pm 0.22 \times 10^{-10}$	Beresford *et al*., 2000
2.0×10^{-10}	Arnold *et al*., 1996

Ψ95% confidence intervals. EC_{50} values are unique to the organism, all values in the table are from similar yeast assays.

Table 4 Comparison of Permethrin Metabolite EC_{50} with literature value

EC_{50} Permethrin Metabolite (M) (3-(4-hydroxy-phenoxy)benyl alcohol)	Reference
5.7×10^{-6}	This work
$6.75 \pm 2.28 \times 10^{-6}$	McCarthy *et al*., 2006

Investigations have found that increasing the number of yeast cells or extending the incubation time has no effect on relative potency of chemicals compared to estradiol however it does effect the position of the dose response curves, shifting them to the left. Thus it has an effect on the EC_{50} values (Beresford *et al*., 2000). This effect could have contributed to the EC_{50} value of estradiol calculated in this work which was found to be slightly more potent compared to those reported in the literature, (see Table 3). The yeast batch from the Glaxo group used in this work had an increased growth rate as mentioned in the methods section. Thus even with the decreased inoculation volume, the final yeast concentration at the end of the incubation would have been expected to have been greater than that for slower batches used in other studies.

4.1.3 Influence of the Yeasts' Metabolic Capabilities on the Pyrethroid Induced Responses

The permethrin metabolite that was used in this work, 3-(4-hydroxyphenoxy)benzyl alcohol, as previously mentioned, was found to be significantly estrogenic confirming the findings of McCarthy *et al*., (2006). Previously another metabolite of permethrin, 3-phenoxybenyl alcohol has also been found to be estrogenic by both McCarthy *et al*., (2006) and Tyler *et al*., (2000). As some of the metabolites of permethrin are proving to be more estrogenic than their parent compounds and this may to some extent explain the disparity of the parent compounds results.

The weak response of permethrin observed in this work could reflect the yeasts ability to metabolise the compound to estrogenic metabolites. Hence the permethrin itself may not be estrogenic and the weak response could be accounted for by the accumulation of its metabolites. The submaximal response compared to its metabolite is consistent with the required metabolic conversion of permethrin which would delay the reporter genes response hence, limit the maximal absorbance over the short incubation period. Alternatively, permethrin may be weakly estrogenic in its own right and the degradation products could simply contribute to the colour development.

Likewise, some metabolites of cypermethrin have also shown estrogenicity (McCarthy *et al*., 2006) however, the very strong colour change and good dose response curve seen for cypermethrin suggests that either the metabolic conversion was rapid or that the parent compound was estrogenic with potential additional estrogenic contribution from its metabolites. The metabolic capabilities of the different assays in Table 5 most likely contributed to the varied results for permethrin and cypermethrin.

The disparity between the pure cypermethrin and the commercial cypermethrin that were both tested at the same concentrations may also be explained by the metabolic processes occurring. The lack of response from Ripcord cypermethrin could indicate that the yeast were unable to metabolise this compound. Unlike the pure compound the commercial product could contain a synergist chemical that are often used in combination with pyrethroids to enhance their performance as pesticides. Synergists such as piperonyl butoxide, which is licensed for use in New Zealand, act by inhibiting the activity of metabolic enzymes preventing the degradation of pyrethroids and hence, extending their

persistence (NZFSA, 2004, Tomlin, 1994). As cypermethrin potentially requires metabolic conversion prior to exerting an estrogenic response a synergist may significantly block this process.

4.1.4 Considerations Relevant to the Yeast Assay Model

Phytoestrogens such as genistein and coumestrol have been shown to have higher affinity, approximately 20-fold and 7-fold respectively, for ERβ over ERα through competitive binding assays (Kuiper and Gustafsson 1997, Kuiper *et al.*, 1998, Mueller *et al*., 2003, Gruber *et al.*, 2002). Thus there is potential for this yeast assay to underestimate the relative potencies of some chemicals as they might have a higher affinity for ERβ similar to the phytoestrogens. However, cypermethrin, permethrin, cyfluthrin and deltamethrin have all previously been shown to interact with ERα but not ERβ (Kojima *et al*., 2004). The presence of only the alpha receptor in the yeast assay model is less likely to underestimate the pyrethroids estrogenicity compared to assaying phytoestrogens by the same method.

The yeast assay identifies chemicals that bind to the estrogen alpha receptor however it does not directly indicate whether they act as agonists, antagonists or anti-estrogens. The yeast assay can identify some anti-estrogens by the co-treatment of estradiol with the test chemical (Routledge and Sumpter, 1997, Sohoni and Sumpter, 1998). A decrease in response significantly different from estradiols' individual dose response thus shows potential anti-estrogenic activity of the test chemical. Amongst the disparate literature reports, anti-estrogenic activity has previously been found for cypermethrin, permethrin and deltamethrin (Table 5). Thus further experiments to investigate the pyrethroids effects on estradiols' estrogenic activity would be of interest.

The yeast assay can not identify all anti-estrogens, even when employing co-treatment with estradiol. For example the pure anti-estrogen, ICI 182,780, referred to as a type II anti-estrogen, is known to produce a purely agonistic response in the yeast screen with no inhibition of the estradiol-induced response. This lead Gaido *et al.,* (1997) to suppose that the yeast may not contain the full complement of appropriate repressor proteins necessary for antagonism in this case. Considering the estrogen receptor was transfected into yeast cells which do not usually contain the estrogen receptor, the lack of necessary co-

repressors and co-activators is likely. This lack of co-factors may also limit the response induced by some chemicals as they could require binding of the co-factors, to the ligand bound receptor, to further alter the receptors' conformation and improve its binding to the estrogen response elements. Thus the yeast screen is not a comprehensive model however

	General Assay Type

as long as its limitations are recognised it contributes valuable information to the complex process of risk assessment.

		Reporter Gene	E-Screen (MCF-7 proliferation)	Hershberger and Uterotrophic	Other assays
Permethrin	**Estrogenic**	Tyler *et al*., 2000 Kojima *et al*., 2004	Go *et al*., 1999 Chen *et al*., 2002	Kim *et al*., 2005	
	Non-estrogenic	Nishihara *et al*., 2000 Saito *et al*., 2000	Soto *et al*., 1995	Kunimatsu *et al*., 2002	Garey and Wolff 1998
	Anti-estrogenic		Kim *et al*., 2004		
Cypermethrin	**Estrogenic**	Kojima *et al*., 2004	Chen *et al*., 2002		
	Non-estrogenic	Nishihara *et al*., 2000 Saito *et al*., 2000	Kim *et al*., 2004		
	Anti-estrogenic	Tyler *et al*., 2000			
Cyfluthrin	**Estrogenic**	Kojima *et al*., 2004			
	Non-estrogenic	Nishihara *et al*., 2000			
Deltamethrin	**Anti- estrogenic**	Kojima *et al*., 2004			

Table 5 Summary of Literature Reporting Estrogenicity of some of the Pyrethroids used in this work

4.2 Selection of Chemicals for Exposure of Mouse Sertoli Cells

As a result of the yeast assay investigations, three chemicals plus estradiol and the ethanol control were chosen as treatments for the exposure of Sertoli cells. The chemicals that were selected were both the pure cypermethrin and the commercial compound containing cypermethrin - Ripcord - and the permethrin metabolite. There were various considerations as to why these chemicals were selected and at what concentrations were they to be used. Firstly not only did the permethrin metabolite and the cypermethrin induce near maximal estrogen receptor mediated β-galactosidase expression, thus a clear estrogenic response, but they were also the two pure chemicals and therefore lacked the complication of extensive contamination. On the other hand Ripcord cypermethrin, the garden solution, was diluted to give the same concentration of cypermethrin as the pure sample yet it did not induce a significant response. Thus the comparison between a laboratory chemical and a commercial gardening product, which is the more likely source of physiological exposure, was of interest. Although comparison between the permethrin metabolite and its parent compound was considered, the only permethrin available was from a garden preparation and thus comparison with the chemically synthesised metabolite would already include purity differences not related to the parent-metabolite relationship.

The concentrations of the test chemicals were selected to examine both a high, non-physiological dose effects and low dose effects designed to be within the reported physiological range of the chemicals. Physiological levels of estradiol are known to be higher in the male reproductive tract than in the general circulation. Estrogen levels in peripheral blood range from 0.002-0.18 μg/L depending upon the species, whereas in one report, the estrogen concentration in the rete testis fluid of the rat was as high as 0.25 μg/L (Reviewed in Hess 2003). The estradiol concentrations selected for this study were 300 mg/L as a high dose and 3 μg/L as a low dose. Although the low dose concentration is still ten-fold higher than the reported values, these levels fit well with other cell exposure studies where the concentrations varied from the milli-gram to the nano-gram range (Saceda *et al.*, 1998, Robertson *et al.*, 2002, Diel *et al*., 2002, Wozniak *et al*., 2005). The lower exposure concentration of 3 μg/L estradiol is consistent with the K_d values for binding to both ERα and ERβ by estradiol (Kuiper *et al.*, 1997).

The consistent detection of pyrethroid metabolites in the urine from non-specifically exposed individuals indicates that physiological exposure to pyrethroids exists for the general population. These background levels of pyrethroid metabolites in urine have been investigated for various populations (Butte *et al*., 1998, Hardt *et al*., 1999, Heudorf and Angerer, 2001, Whyatt *et al*., 2003, Leng *et al.,* 2003, Leng *et al.,* 2006). For example, the extensive study by Heudorf and Angerer, 2001 who examined the metabolites in urine from over one thousand volunteers who were not occupationally exposed, nor had recent indoor exposure to pyrethroids, found metabolite levels corresponding with 95% values of 0.3 to 1.5 μg/L depending on the metabolite.

It has been suggested that the pyrethroid metabolites detected in the general population are the result of dietary exposure thus, discrepancies between the detected levels of individual metabolites in different populations would be expected due to the variations in diet (Hardt *et al*., 1999, Heudorf and Angerer, 2001, Leng *et al*., 2003). This supports the findings of Heudorf and Angerers' earlier work that the metabolite levels did not correlate with the pyrethroid levels as measured from the household dust. Dietary exposure was also corroborated by subsequent analysis of the same data set by Schettgen et al., 2002 who demonstrated that the ratio of metabolite isomers found in the urine was 2:1 in favor of the trans- isomer. This ratio is indicative of oral exposure whereas dermal exposure has been shown to result in an approximately equal ratio of isomers (Woolen et al., 1992).

The exposure levels for pest control operators have also been measured by detection of metabolites in urine. The reported levels vary due to the specific chemical concentrations, different exposure periods and the times at which the samples were obtained. Leng *et al*., (1997) reported levels up to 277 μg/L with a median level of 30 μg/L however in their later studies (Leng *et al*., 2003, 2005) they found lower mean concentrations between 0.2-13.4 μg/L most likely due to safer application techniques.

The concentrations chosen for the pyrethroid exposures in this study were 300 mg/L and 3 μg/L for all chemicals with an extra dilution of the permethrin metabolite to 0.3 μg/L in this study. The 3 μg/L concentration is clearly in the physiological range for the general population as the recovery of the metabolites (up to 1.5μg/L) represents only a percentage of the actual pyrethroid exposure, (recovery is reported to be highly varied between 40 –

80% (Leng *et al.,* 2006)). The high dose level 300 mg/L, is 100,000-fold higher than the physiological dose, and is well above the metabolite levels reported even for the pest control workers (highest reported as 277 μg/L Leng et al., 1997). However the possible high dose effects are of interest as they reflect the fact that the potency of the pyrethroids to that of estradiol are up to a million fold lower. Thus the high dose concentration is closer to a level at which the pyrethroids may be capable of exerting an estrogenic response as predicted by the yeast assay results. Similarly, other researchers have examined chemicals at concentrations reflective of their relative potencies rather than their physiological levels. For example Diel *et al.*, (2002) when examining the effects of raloxifene, coumestrol, DDT, and BPA on MCF-7 cells and likewise Kakko *et al*., (2004) when examining the affects of permethrin, cypermethrin and pyrethrin on SH-SY5Y neuroblastoma cells.

The concentrations chosen for the chemical exposures to the Sertoli cells were not all evaluated using the yeast assay as summarised in Table 6. Thus none of the pyrethroids were tested at concentrations as low as those used in the Sertoli cell exposures. Extrapolation from the responses at the higher concentrations suggests that the concentrations would be too low to exhibit any detectable estrogenic affect via the yeast assay. Therefore, if estrogenicity in the yeast assay is predictive of cellular responses to estrogens; the exposure of mouse Sertoli cells to the high doses were expected to affect the estrogen receptors expression where as the low doses were expected to be effectively similar to the non treated cells. Despite the yeast assay no-effect prediction, the inclusion of the low dose exposures are key investigations because although the low concentrations of pyrethroids would be unlikely to show any response in the yeast assay model, the mouse Sertoli cells offer a higher degree of biological complexity that may affect the potency of the chemicals.

Differences between the yeast model and the mouse Sertoli cells have the potential to affect the potencies of the pyrethroids due to differences in the expression level of ERα as well as the presence of the β-subtype, the availability of specific co-factors and different metabolic capabilities. Support for observing chemicals at concentrations below the predicted no dose effect was reviewed by Welshons *et al*., (2003) who highlighted the error in extrapolating from the high toxicological doses commonly tested in risk assessment. In particular they presented evidence on inverted-U dose-response

relationships observed for some chemicals where the effects induced are different at high and low concentrations. For example the MCF-7 cell line exhibits estrogen-dependent stimulation of cell proliferation by natural and xenobiotic estrogens however the same chemicals that stimulate the growth at low concentrations can slow MCF-7 growth at higher concentrations (Welshons *et al*., 2003 and references there in).

Table 6 Summary of the chemicals and concentrations used in the Sertoli cell exposures relative to their yeast assay dose responses

Chemical	Concentration used in cell exposures	Response in yeast assay at the corresponding concentrations[ψ]
Estradiol	300 mg/L	100 %
	3 μg/L	40 %
Cypermethrin	300 mg/L	1 %
	3 μg/L [ρ]	Not Tested
Ripcord (cypermethrin)	300 mg/L	No Response
	3 μg/L	Not Tested
Permethrin Metabolite	300 mg/L [ρ]	50 %
	3 μg/L	Not Tested
	0.3 μg/L	Not Tested

[ψ] **The percentages given indicate the approximate amount of receptor activation at that concentration determined by the increase in absorbance in the yeast assay.**

[ρ] **Gene expression of the cells from of these treatments could not be quantified due to low yield.**

4.3 Pyrethroids effect on mouse Sertoli cells

4.3.1 Variations between observed cell densities and extracted RNA yields

Comparison between the approximate cell density of the cultures prior to harvest and the yield of RNA reveals some inconsistencies. The cultures exposed to the permethrin metabolite treatment that exhibited either toxicity or growth inhibition showed the expected reduced RNA yield that was predicted due to the lower number of cells. Likewise, the Ripcord cypermethrin treatment had a slight effect on the cell growth at both concentrations which was also reflected in the RNA yields. However the pure cypermethrin treatments resulted in surprisingly low RNA yields despite the high cell

numbers observed for both concentrations of the treatment. For the pure cypermethrin treated cultures, the difference in RNA yield compared to the control cells, of approximately equal confluence, was greater than could be attributed to a decrease transcriptional activity, even if the cells had been in a stationary phase. Thus the extremely low yield suggested a problem with the extraction of RNA from these samples.

The extraction of the RNA samples was performed in four batches. One of these batches included all of the pure cypermethrin treatments but only two of the samples for the estradiol treatment at 0.3nM. Thus it is likely that a handling problem specific to when this batch was extracted, affected the yields of RNA for these samples. This is supported by the much higher RNA concentration of the third estradiol sample at 0.3nM that was extracted on a separate occasion.

Although extraction problems are suspected for the pure cypermethrin treatments and two of the estradiol samples, the low yield from other samples such as from the permethrin metabolite treatment at 69 μM, was thought to be purely related to the very few cells present at harvesting. Despite taking steps to concentrate the low RNA yield from these samples, neither the permethrin metabolite at 69 μM treatment samples nor the pure cypermethrin at 0.36 nM treatment samples were able to be amplified.

The RNA quantification was shown to be relatively accurate based on the low variation within the raw Ct values of the β-actin product gained from the subsequent RT-PCR amplification of the samples. Although indirect, the correct dilution of cDNA - as shown by the similar β-actin raw Ct values - supports the initial quantification data.

Interestingly, the slight increase in growth, relative to the control cells, induced by the high concentrations estradiol as seen by the cell photographs was supported by increased RNA yield from two of the estradiol cultures compared to the control. Increase in growth was also observed for Ripcord cypermethrin at high concentrations however, the RNA yields suggest this effect was only substantial in one of the cultures thus a more variable response.

4.3.2 Potential Mechanisms for the Pyrethroid treatments effects on mouse Sertoli Cell growth

Despite the inconsistencies that have been accounted for, the growth of mouse Sertoli cells was clearly affected by their exposure to some of the pyrethroid treatments. The greatest effect, as seen by the cell photographs, resulted from the permethrin metabolite treatment followed by Ripcord, whereas no obvious cytotoxicity was observed as a result of the pure cypermethrin or estradiol treatments relative to the control cultures. The toxicity could have resulted from the pyrethroids pesticide properties rather than any estrogenic affects however mediation of apoptosis via the estrogen receptor has previously been shown for both xeno-estrogens and endogenous estrogens.

4.3.2.1 Possible contributions of insecticidal properties of pyrethroids to toxicity

At the cellular level the important target sites of pyrethroids in mammals are the cell membrane and specifically the sodium channels (reviewed in Raymond-Delpech *et al*., 2005). Cells differ in their sensitivity to pyrethoids due to the multiple sodium channel isoforms that vary in their biophysical and pharmacological properties within and between species (Kakko *et al.*, 2004). Significant toxicity was not observed in the yeast assay from either of the cypermethrin compounds however, the presence of the yeast cell wall most likely afforded the yeast a higher level of protection than the mammalian cell membrane which readily absorbs the highly lipophilic pyrethroids.

The toxicity of Ripcord cypermethrin to mammalian cells has previously been investigated by Kakko *et al*., 2004 using a neural cell line SH-SY5Y. Concentrations of Ripcord ranging from 10 μM up to 100 μM showed significant morphological changes eventually resulting in the rounding-up of the neural cells indicating extensive toxicity. In comparison, the current study observed a decrease in cell density compared to the control cells, for cultures treated with 0.36 nM of Ripcord. This decrease in cell density may indicate toxicity however the gross morphology of the cells did not change. At the higher exposure concentration, 36 μM, no toxicity or decrease in density was observed in the Sertoli cells whereas toxicity increased with dose in the neural cell line. Hence it appears that the Sertoli cells were less sensitive to Ripcord cypermethrin than the neural cell line.

In addition, pyrethroid metabolism involves the formation of reactive oxygen species which increases oxidative stress and can trigger or contribute to apoptosis (Kale et al., 1999). Thus apart from the actions at the cell membrane, cypermethrin could further affect the cells due to its metabolism.

4.3.2.2 Involvement of Estrogenic mechanisms in toxicity of pyrethroids

The permethrin metabolite showed the greatest toxicity compared to both the Ripcord and pure cypermethrin. The permethrin metabolite lacks key structural features required for pyrethroid like function yet it has been shown to possess the ability to bind and activate the human estrogen receptor in the yeast assay. Therefore, in regards to this chemical, the toxicity observed could be linked with the binding to the estrogen receptors rather than classic pyrethroid toxicity. Direct interaction with the estrogen receptors has previously been implicated in the apoptotic signally pathways in the testes and thus may explain the notable cell death seen in this study (Pentikainen *et al.*, 2000).

4.4 ERα and ERβ expression in Sertoli Cells

Generally speaking the exposure of mouse Sertoli cells to cypermethrin and the permethrin metabolite caused an increase in expression of both ERα and β whereas exposure to estradiol resulted in a decrease in expression. The different concentrations produced varying degrees of changes however overall it appears that the effect of the xenoestrogens was not the same as the effect of estradiol. This may reflect different transactivation and subsequent autoregulation properties of the ER when bound to these different ligands. The lack of relationship between the expression changes and toxicity observed also suggests that the expression changes were independent of the toxicity and vice versa.

The real-time RT-PCR indicated that ERα mRNA is predominately expressed over the ERβ mRNA in TM4 mouse Sertoli cells under all treatments. This finding contradicts the publish literature where only ERβ has been found to be localised in adult mouse Sertoli cells (Makinen *et al*., 2001). The presence of both receptors may be an artefact of the TM4

cells being a permanent cell line which, in its establishment, could have altered the estrogen responsiveness. Suppression of both ERα (Lau et al., 2000) and ERβ (Skliris 2003, Zhao 2003) expression dependent on the cell type has been shown to occur via gene silencing by methylation of the promoter sequences (Sasaki et al., 2002). This is a reversible process and exposing cells to de-methylating agents restores expression of both mRNA and protein.

The different expression of the estrogen receptors compared to previous reports is an important factor in determining the physiological significance of these results. The presence of both receptors increases the possible signally pathways that the pyrethroids or estradiol could be involved in. Estrogen receptors when co-localised within the same cell have been shown to form heterodimers, and moderating effects of ERβ on ERα become possible (Matthews and Gustafsson, 2003).

The implications of changes in estrogen receptor expression for male infertility are at this stage speculative yet comparison with the more thoroughly investigated changes in ER expression in cancer may provide some insights. A comprehensive review of changes in ER expression in tissues such as breast, ovaries, prostate and colon, that are proposed to be subject to estrogen mediated tumour progression demonstrated that the overall trend was an increase in the ratio of ERα to ERβ expression(Bardin *et al*., 2004). This was almost always a result of decreases in ERβ and or complete loss of expression. Increases in ERα occurred in both the ovary and breast tissue but not change occurred in the prostate or colon. This most likely reflects that ERα is not highly expressed in normal cells in these tissues compared to breast and ovary expression (Bardin *et al*., 2004).

Although spermatogenesis is a very different process to cancer growth both involve cell proliferation. Spermatogenesis is a tightly controlled process of cell growth and differentiation much like normal tissues. Cancer on the other hand is a situation of uncontrolled cell growth. It could then be suggested that infertility is a state of "over-controlled" cell growth.

It has been found that infertile men have higher serum concentrations of estrogen. Whether the increase in estradiol observed is a result of the infertility originating from other factors, or a cause of the infertility is unknown. If the increased estrogen

concentration is found to be a large contributor to the infertility in these men rather than a subsequent side effect, the mechanism of estrogens action could possibly be through "over-control" of spermatogenesis. This could occur due to increased ERβ expression as a result of the increased estrogen concentrations by estrogen activating the additional transcription of the receptor.

In the current study the estrogenic exposure of mouse Sertoli cells resulted in a significant ratio change of ER mRNA expression for the permethrin metabolite. The increase in ratio was a result of increased ER alpha and beta expression however, the relative increase in ERα was greater. According to the cancer studies ERα is the growth promoter whereas ERβ is the inhibitor or controller. Thus if comparison with the expression in other cell types and cancer progression is in any way indicative of testicular growth control then the ratio change would suggest a pro-growth environment. Thus, in this context the ratio change would be beneficial to spermatogenesis however arriving at that conclusion encompassed a large cohort of assumptions and speculations.

Although the ratio change for the estradiol treatments were not significant the relative expression level of ERβ decreased. In relation to male infertility, estradiol has been shown to act as a survival factor decreasing programmed cell death. This process could be enabled by decreasing the growth control of ERβ through suppressing mRNA expression.

Conclusions as to implications for male infertility are severely limited by the complexity of the estrogen signalling pathways and the vast amount of information still to be obtained. Despite this, the results of this study suggest that xenoestrogens do not effect estrogen receptor expression in the same way as the endogenous estrogen 17β-estradiol in mouse Sertoli cells.

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6 Appendices

6.1 Yeast Assay Raw Data

Chemical	Concentration	Raw Data Replicates					
Estradiol	4.92E-12	1.300	1.418	1.292	1.175	1.187	1.390
	9.84E-12	1.551	1.582	1.573	1.170	1.205	1.685
	1.97E-11	1.568	1.711	1.683	1.222	1.293	1.929
	3.94E-11	1.719	1.993	2.099	1.273	1.418	1.859
	7.87E-11	2.205	2.087	2.250	1.452	1.653	2.009
	1.57E-10	2.247	2.328	2.466	1.674	1.906	2.311
	3.15E-10	2.539	2.603	2.465	1.444	2.225	2.530
	6.3E-10	2.520	2.706	2.556	2.096	2.330	2.424
	1.26E-09	2.561	2.642	2.524	2.421	2.465	2.640
	2.52E-09	2.543	2.673	2.570	2.402	2.480	2.591
	5.04E-09	2.465	2.682	2.502	2.493	2.506	2.610
	1.01E-08	3.096	2.593	2.395	2.507	2.479	2.137
	4.92E-12	1.157	1.171	1.093	1.415	1.171	1.391
	9.84E-12	1.204	1.220	1.083	1.076	1.109	1.488
	1.97E-11	1.226	1.319	1.096	1.065	1.144	1.632
	3.94E-11	1.354	1.438	1.131	1.112	1.154	1.855
	7.87E-11	1.593	1.618	1.201	1.160	1.304	2.034
	1.57E-10	1.756	1.769	1.311	1.257	1.461	2.276
	3.15E-10	1.996	2.074	1.448	1.385	1.654	2.390
	6.3E-10	2.078	2.139	1.618	1.526	1.907	1.350
	1.26E-09	2.229	2.169	1.814	1.623	2.015	2.583
	2.52E-09	2.245	2.214	2.141	2.023	2.104	2.606
	5.04E-09	2.362	2.193	2.077	2.452	2.231	2.643
	1.01E-08	2.463	2.243	1.830	2.563	1.853	2.722
	4.92E-12	1.362	0.907	1.292	1.441	1.392	1.366
	9.84E-12	1.465	1.114	1.207	1.275	1.489	1.468
	1.97E-11	1.530	1.180	1.216	1.368	1.634	1.533
	3.94E-11	1.671	1.294	1.320	1.413	1.861	1.678
	7.87E-11	1.864	1.439	1.437	1.747	2.042	1.870
	1.57E-10	2.060	1.677	1.672	2.045	2.279	2.069
	3.15E-10	2.053	1.922	1.897	2.316	2.392	2.086
	6.3E-10	2.487	2.079	2.090	2.478	1.352	2.504
	1.26E-09	2.574	2.155	2.024	2.524	2.576	2.577
	2.52E-09	2.620	2.363	2.361	2.447	2.602	2.639
	5.04E-09	2.646	2.454	2.416	2.523	2.639	2.650
	1.01E-08	2.725	2.411	2.498	2.682	2.692	2.749
Taufluvalinate	1.4E-08	1.026	1.303	1.136	1.375	1.056	
	2.8E-08	1.128	1.330	1.138	1.447	1.044	
	5.59E-08	1.091	1.340	1.143	1.519	1.071	
	1.12E-07	1.079	1.343	1.145	1.472	1.070	
	2.24E-07	1.094	1.345	1.156	1.430	1.066	
	4.47E-07	1.080	1.335	1.155	1.393	1.075	
	8.95E-07	1.092	1.347	1.146	1.354	1.090	
	1.79E-06	1.062	1.340	1.167	1.349	1.094	
	3.58E-06	0.900	1.336	1.152	1.387	1.084	
	7.16E-06	0.875	1.331	1.157	1.295	1.087	
	1.43E-05	0.705	1.358	1.174	1.388	1.104	
	2.86E-05	1.076	1.453	1.020	1.320	1.093	

Cyfluthrin	1.12E-08	1.098	1.198	1.024
	2.25E-08	1.121	1.341	1.014
	4.5E-08	1.129	1.310	1.032
	8.99E-08	1.282	1.224	1.034
	1.8E-07	1.319	1.248	1.059
	3.6E-07	1.276	1.207	1.057
	7.2E-07	1.214	1.041	1.063
	1.44E-06	1.211	1.016	1.103
	2.88E-06	1.323	1.008	1.097
	5.76E-06	1.174	1.014	1.043
	1.15E-05	1.096	1.030	1.025
	2.3E-05	1.091	1.026	1.026
Permethrin	1.87E-08	1.253	1.023	1.246
	3.74E-08	1.257	1.015	1.211
	7.49E-08	1.263	1.018	1.230
	1.5E-07	1.259	1.012	1.228
	2.99E-07	1.259	1.020	1.238
	5.99E-07	1.254	1.016	1.236
	1.2E-06	1.270	1.019	1.242
	2.4E-06	1.281	1.026	1.236
	4.79E-06	1.380	1.020	1.252
	9.58E-06	1.364	1.019	1.268
	1.92E-05	1.360	1.033	1.336
	3.83E-05	1.443	1.052	1.163

Pure Cypermethrin	1.76E-08	1.150	1.142	1.151	0.912	1.060
	3.52E-08	1.135	1.147	1.144	0.905	1.075
	7.04E-08	1.151	1.162	1.132	0.907	1.078
	1.41E-07	1.144	1.175	1.113	0.904	1.073
	2.81E-07	1.165	1.188	1.113	0.911	1.077
	5.63E-07	1.174	1.177	1.103	0.927	1.082
	1.13E-06	1.197	1.210	1.128	0.910	1.085
	2.25E-06	1.202	1.219	1.153	0.966	1.109
	4.5E-06	1.263	1.263	1.160	0.993	1.134
	9.01E-06	1.335	1.352	1.144	1.049	1.157
	2.25E-05	1.083	1.465	1.228	1.315	1.471
	4.5E-05	1.082	1.556	1.261	1.395	1.555
	9.01E-05	1.130	1.689	1.340	1.552	1.698
	0.00018	1.186	1.900	1.456	1.602	1.895
	0.00036	1.339	2.141	1.652	1.801	2.058
Ripcord Cypermethrin	1.76E-08	1.106	1.361	1.364	1.192	
	3.52E-08	1.101	1.383	1.391	1.276	
	7.04E-08	1.118	1.367	1.413	1.260	
	1.41E-07	1.104	1.389	1.404	1.238	
	2.81E-07	1.109	1.375	1.372	1.243	
	5.63E-07	1.093	1.371	1.383	1.246	
	1.13E-06	1.111	1.380	1.390	1.235	
	2.25E-06	1.115	1.391	1.412	1.272	
	4.5E-06	1.121	1.387	1.434	1.212	
	9.01E-06	1.128	1.413	1.412	1.197	
	2.25E-05	1.084	1.388	1.412	1.272	
	4.5E-05	1.080	1.459	1.434	1.212	
	9.01E-05	1.073	1.445	1.412	1.197	
	0.00018	1.094	1.475	1.454	1.218	
	0.00036	1.088	1.457	1.453	1.296	
Permethrin Metabolite	4.52E-08	1.116	1.327	1.247	1.211	1.177
	9.03E-08	1.129	1.349	1.238	1.233	1.173
	1.81E-07	1.147	1.379	1.311	1.257	1.224
	3.61E-07	1.160	1.384	1.357	1.195	1.314
	7.23E-07	1.186	1.404	1.397	1.268	1.384
	1.81E-06	1.239	1.362	1.555	1.105	1.396
	3.61E-06	1.317	1.784	1.851	1.127	1.503
	7.23E-06	1.584	2.088	2.066	1.191	1.707
	1.45E-05	1.720	2.425	2.190	1.256	1.919
	2.89E-05	1.997	2.462	2.235	1.356	2.120
	5.78E-05	2.121	2.526	2.304	1.434	2.284

Pyrethrin	3.17E-08	1.174	1.300	1.060	1.315
	6.33E-08	1.172	1.349	1.075	1.311
	1.27E-07	1.185	1.319	1.087	1.299
	2.53E-07	1.162	1.295	1.088	1.283
	5.06E-07	1.190	1.298	1.103	1.254
	1.01E-06	1.144	1.266	1.075	1.222
	2.03E-06	1.181	1.295	1.094	1.241
	4.05E-06	1.155	1.292	1.061	1.254
	8.1E-06	1.147	1.349	1.111	1.265
	1.62E-05	1.154	1.399	1.152	1.368
	3.24E-05	1.187	1.527	1.208	1.409
	6.48E-05	1.243	1.583	1.171	1.248
Bifenthrin	1.73E-09	1.179	1.253	1.267	1.452
	3.46E-09	1.176	1.210	1.252	1.231
	6.93E-09	1.187	1.138	1.261	1.216
	1.39E-08	1.193	1.271	1.251	1.229
	2.77E-08	1.212	1.268	1.265	1.233
	5.54E-08	1.215	1.233	1.270	1.233
	1.11E-07	1.233	1.272	1.269	1.259
	2.22E-07	1.270	1.185	1.366	1.298
	4.43E-07	1.294	1.322	1.475	1.262
	8.87E-07	1.451	1.503	1.664	1.296
	1.77E-06	1.238	1.391	1.743	1.636
	3.55E-06	1.154	1.263	1.295	1.225
Deltamethrin	1.45E-08	1.117	1.104	1.044	1.131
	2.9E-08	1.096	1.132	1.043	1.132
	5.8E-08	1.103	1.155	1.062	1.132
	1.16E-07	1.128	1.158	1.068	1.135
	2.32E-07	1.107	1.154	1.075	1.143
	4.64E-07	1.105	1.153	1.078	1.147
	9.28E-07	1.111	1.160	1.081	1.140
	1.86E-06	1.104	1.156	1.088	1.169
	3.71E-06	1.120	1.160	1.085	1.164
	7.42E-06	1.108	1.160	1.089	1.158
	1.48E-05	1.123	1.174	1.093	1.022
	2.97E-05	1.190	1.184	1.060	0.947

6.2 Raw Data for SRB Growth curve assay

Initial Cell Number		475000	237500	118750	59375	29688	14844
Day O	Rep 1	2.999	2.576	1.407	0.866	0.387	0.263
	Rep 2	3.027	2.846	1.660	1.009	0.465	0.292
	Rep 3	3.031	2.822	2.099	1.205	0.668	0.369
Day 1	Rep 1	2.979	2.914	2.59	1.618	0.881	0.487
	Rep 2	2.819	2.911	2.475	1.455	0.666	0.385
	Rep 3	3.05	2.81	2.511	1.376	0.783	0.435
Day 2	Rep 1	2.761	1.944	1.229	0.509	0.294	0.171
	Rep 2	2.822	2.183	1.279	0.769	0.425	0.239
	Rep 3	2.827	2.054	0.0975	0.619	0.334	0.193
Day 3	Rep 1	2.927	2.938	2.763	2.37	1.595	0.471
	Rep 2	2.94	2.935	2.763	2.447	1.585	0.849
	Rep 3	2.22	2.668	1.949	1.177	0.669	0.342
Day 4	Rep 1	3.141	3.127	3.065	3.108	3.075	2.92
	Rep 2	3.207	3.174	3.22	3.108	3.084	3.041
	Rep 3	3.117	3.02	3.091	3.099	3.075	2.883
Day 5	Rep 1	3.135	3.225	3.108	3.224	3.135	3.109
	Rep 2	2.971	3.076	3.051	3.262	3.071	3.086
	Rep 3	3.066	3.085	3.142	3.111	3.193	3.143

Initial Cell Number		7422	3711	1855	928	464	232
Day O	Rep 1	0.119	0.086	0.084	0.062	0.065	0.263
	Rep 2	0.118	0.087	0.076	0.063	0.068	0.292
	Rep 3	0.146	0.111	0.092	0.074	0.066	0.369
Day 1	Rep 1	0.161	0.127	0.084	0.078	0.068	0.487
	Rep 2	0.166	0.109	0.085	0.075	0.073	0.385
	Rep 3	0.134	0.09	0.091	0.076	0.073	0.435
Day 2	Rep 1	0.08	0.061	0.064	0.063	0.057	0.171
	Rep 2	0.128	0.088	0.08	0.064	0.074	0.239
	Rep 3	0.094	0.078	0.068	0.063	0.063	0.193
Day 3	Rep 1	0.146	0.1	0.096	0.087	0.069	0.471
	Rep 2	0.274	0.161	0.12	0.129	0.103	0.849
	Rep 3	0.137	0.088	0.079	0.072	0.076	0.342
Day 4	Rep 1	0.98	0.43	0.226	0.147	0.11	2.92
	Rep 2	0.949	0.377	0.305	0.174	0.155	3.041
	Rep 3	0.711	0.39	0.16	0.135	0.096	2.883
Day 5	Rep 1	1.995	0.617	0.301	0.161	0.128	3.109
	Rep 2	1.767	0.885	0.349	0.144	0.132	3.086
	Rep 3	2.213	0.836	0.366	0.129	0.153	3.143

6.3 PCR Raw Ct Values

6.3.1 PCR Experiment 1

No.	Sample	β-Actin	ERα	ERβ	No.	Sample	β-Actin	ERα	ERβ
1	Met 69 pM	21.05	28.93	35.79	21	E2 10 nM	19.46	27.6	35.77
1	Met 69 pM	22.01	29.3	36.27	21	E2 10 nM	19.9	27.58	38.52
1	Met 69 pM	21.47	28.63	35.6	21	E2 10 nM	20.72	28.01	38.49
2	Met 69 pM	21.27	28.48	35.57	22	RC 0.36 nM	20.94	27.51	34.58
2	Met 69 pM	21.69	28.19	35.88	22	RC 0.36 nM	20.71	26.75	34.69
2	Met 69 pM	21.72	28.31	36.58	22	RC 0.36 nM	20.76	27.88	34.92
3	Met 69 pM	20.7	26.65	35.25	23	RC 0.36 nM	21.3	27.84	36.05
3	Met 69 pM	20.89	26.94	35.69	23	RC 0.36 nM	20.51	27.91	35.74
3	Met 69 pM	21.14	27.31	36.17	23	RC 0.36 nM	20.3	27.46	34.13
4	Met 0.69 nM	22.55	28.24	38.82	24	RC 0.36 nM	19.43	26.78	35.36
4	Met 0.69 nM	22.18	27.93	39.57	24	RC 0.36 nM	19.29	26.37	34.72
4	Met 0.69 nM	22.47	28.57	39.21	24	RC 0.36 nM	19.24	27.11	34.7
6	Met 0.69 nM	23.31	27.38	37.05	25	RC 36 uM	21.78	30.56	30.34
6	Met 0.69 nM	23.56	27.78	37.69	25	RC 36 uM	22.35	30.28	30.6
6	Met 0.69 nM	23.82	27.96	38.4	25	RC 36 uM	22.36	31.46	30.63
13	Cyper 36uM	23.66	30.34	38.4	26	RC 36 uM	20.28	27.3	34.83
13	Cyper 36uM	23.73	30.54	38.87	26	RC 36 uM	19.98	26.31	34.32
13	Cyper 36uM	23.62	29.78	38.55	26	RC 36 uM	20.66	27.56	34.42
14	Cyper 36uM	21	27.42	36.38	27	RC 36 uM	21.9	29.54	34
14	Cyper 36uM	19.91	27.17	36.14	27	RC 36 uM	21.77	29.69	34.13
14	Cyper 36uM	20.57	27.54	36.44	27	RC 36 uM	21.9	28.91	34.94
15	Cyper 36uM	21.28	30.84	38.68	28	NTC	21.3	28.94	36.65
15	Cyper 36uM	21.97	32.37	40.01	28	NTC	21.68	28.53	37.18
15	Cyper 36uM	21.69	31.34	39.06	28	NTC	22.24	29.95	38.25
16	E2 0.3 nM	21.65	28.92	38.71	29	NTC	20.63	28.66	36.12
16	E2 0.3 nM	20.76	28.12	37.98	29	NTC	21	29.06	36.54
16	E2 0.3 nM	21.07	28.55	39.03	30	NTC	20.93	29.09	35.67
17	E2 0.3 nM	21.67	31.78	39.62	30	NTC	20.61	29.32	36.21
17	E2 0.3 nM	22.36	34.97	39.61	21	NTC	20.83	28.88	35.94
17	E2 0.3 nM	22.71		39.95	21	NTC	21.22	29.82	36.81
18	E2 0.3 nM	21.89	28.05	38.74					
18	E2 0.3 nM	20.93	28.38	39.72					
18	E2 0.3 nM	21.08	28.28	38.9					
19	E2 10 nM	20.24	28.41	36.64					
19	E2 10 nM	20.82	28.14	37.2					
19	E2 10 nM	20.91	27.8	37.24					
20	E2 10 nM	18.8	26.41	35.94					
20	E2 10 nM	19.28	25.46	36.65					
20	E2 10 nM	19.47	26.64	37.14					

6.3.2 PCR Experiment 2

No.	Sample	β-Actin	ERα	ERβ	No.	Sample	β-Actin	ERα	ERβ
1	Met 69 pM	22.44	28.24	35.16	21	E2 10 nM	21.95	27.75	38.24
1	Met 69 pM	22.3	27.78	35.32	21	E2 10 nM	21.5	28.06	37.13
1	Met 69 pM	22.08	28.53	35.67	21	E2 10 nM	21.82	28.37	36.42
2	Met 69 pM	21.82	27.24	34.75	22	RC 0.36 nM	21.5	26.84	34.87
2	Met 69 pM	21.76	27.79	35.21	22	RC 0.36 nM	21.85	26.43	35.14
2	Met 69 pM	21.78	26.55	34.43	22	RC 0.36 nM	21.84	25.8	35.42
3	Met 69 pM	21.86	26.73	34.74	23	RC 0.36 nM	22.64	27.49	36.54
3	Met 69 pM	22.34	26.96	34.93	23	RC 0.36 nM	21.7	27.68	37.04
3	Met 69 pM	21.92	26.74	34.71	23	RC 0.36 nM	22.15	27.22	36.96
4	Met 0.69 nM	22.71	27.68	36.41	24	RC 0.36 nM	20.39	26.17	35.32
4	Met 0.69 nM	22.93	26.84	36.58	24	RC 0.36 nM	20.14	25.91	34.85
4	Met 0.69 nM	23.16	27.69	37.02	24	RC 0.36 nM	19.92	25.54	34.79
6	Met 0.69 nM	23.7	27.62	37.11	25	RC 36 uM	22.42	28.12	31.19
6	Met 0.69 nM	23.98	27.82	37.65	25	RC 36 uM	22.96	28.18	30.93
6	Met 0.69 nM	25.73	28.27	38.98	25	RC 36 uM	23.88	29.14	30.74
13	Cyper 36uM	23.5	28.98	37.09	26	RC 36 uM	20.68	25.32	34.9
13	Cyper 36uM	24.57	30.38	37.33	26	RC 36 uM	19.97	25.44	34.09
13	Cyper 36uM	22.54	29.05	36.41	26	RC 36 uM	19.91	25.14	33.27
14	Cyper 36uM	20.98	27.34	35.75	27	RC 36 uM	23.48	27.85	32.89
14	Cyper 36uM	20.77	26.99	34.56	27	RC 36 uM	23.68	28.1	33.4
14	Cyper 36uM	20.91	26.9	34.71	27	RC 36 uM	24.2	29.06	34.2
15	Cyper 36uM	22.02	31.95	36.64	28	NTC	21.57	26.24	35.95
15	Cyper 36uM	21.63	32.9	35.82	28	NTC	20.98	25.71	35.76
15	Cyper 36uM	22.58	34.29	37.64	28	NTC	20.91	26.28	35.09
16	E2 0.3 nM	22.25	28.09	36.5	29	NTC	20.52	26.63	34.72
16	E2 0.3 nM	20.59	27.64	35.61	29	NTC	20.87	26.84	35.45
16	E2 0.3 nM	20.75	27.9	36.21	30	NTC	20.88	26.92	35.87
17	E2 0.3 nM	22.9		36.13	30	NTC	20.81	27.1	36.26
17	E2 0.3 nM	22.14	43.59	36.4	21	NTC	20.64	26.95	36.14
17	E2 0.3 nM	23.7		36.74	21	NTC	20.64	27.11	34.94
18	E2 0.3 nM	21.25	27.71	36.31					
18	E2 0.3 nM	21.5	27.94	36.96					
18	E2 0.3 nM	21.02	26.92	37.43					
19	E2 10 nM	21.24	27.05	36.17					
19	E2 10 nM	21.45	27.54	37.13					
19	E2 10 nM	21.73	27.27	36.75					
20	E2 10 nM	21.1	26.45	36.77					
20	E2 10 nM	21.86	26.32	37.01					
20	E2 10 nM	21.24	26.89	37.01					

6.3.3 PCR Experiment 3

No.	Sample	β-Actin	ERα	ERβ	No.	Sample	β-Actin	ERα	ERβ
1	Met 69 pM	23	26.79	36.61	21	E2 10 nM	21.59	26.81	38.13
1	Met 69 pM	22.99	27.36	36.54	21	E2 10 nM	21.81	26.38	38.53
1	Met 69 pM	22.79	26.78	37.31	21	E2 10 nM	21.57	24.41	37.67
2	Met 69 pM	22.85	25	37.45	22	RC 0.36 nM	21.29	25.45	34.85
2	Met 69 pM	21.99	25.15	36.36	22	RC 0.36 nM	21.64	24.14	35.43
2	Met 69 pM	21.62	25.98	35.77	22	RC 0.36 nM	21.88	24.96	35.43
3	Met 69 pM	22.02	25.12	36.85	23	RC 0.36 nM	21.75	24.2	36.67
3	Met 69 pM	22.4	26.18	37.65	23	RC 0.36 nM	20.97	24.92	36.46
3	Met 69 pM	22.36	26.26	36.66	23	RC 0.36 nM	21.72	25.73	36.96
4	Met 0.69 nM	22.05	26.46	38.26	24	RC 0.36 nM	18.96	23.98	33.92
4	Met 0.69 nM	22.33	26.29	36.9	24	RC 0.36 nM	19.34	24.31	33.83
4	Met 0.69 nM	21.78	26.43	37.95	24	RC 0.36 nM	19.26	23.9	33.94
6	Met 0.69 nM	25.3	27.07	40.37	25	RC 36 uM	23.2	27.01	31.13
6	Met 0.69 nM	23.46	25.55	38.09	25	RC 36 uM	23.23	26.99	31.25
6	Met 0.69 nM	24.59	25.92	37.42	25	RC 36 uM	22.89	26.1	30.67
13	Cyper 36uM	22.81	26.03	36.75	26	RC 36 uM	20.03	25.05	35.51
13	Cyper 36uM	22.06	27.45	36.43	26	RC 36 uM	19.15	24.13	34.84
13	Cyper 36uM	23.31	26.33	37.26	26	RC 36 uM	19.43	23.75	34.45
14	Cyper 36uM	20.55	25.19	35.97	27	RC 36 uM	23.49	26.68	33.63
14	Cyper 36uM	20.43	24.56	35.48	27	RC 36 uM	24.52	27.25	34.14
14	Cyper 36uM	20.33	25.79	35.16	27	RC 36 uM	22.91	26.11	32.95
15	Cyper 36uM	21.54	32.07	39.46	28	NTC	20.78	25.5	36.03
15	Cyper 36uM	21.46	29.74	38.29	28	NTC	20.84	25.67	36.4
15	Cyper 36uM	21.08	30.09	37.66	28	NTC	21.87	25.08	37.08
16	E2 0.3 nM	19.86	25.67	35.72	29	NTC	20.77	25.57	36.03
16	E2 0.3 nM	19.79	24.45	35.76	29	NTC	20.72	25.77	36.59
16	E2 0.3 nM	20.21	25.3	36.19	30	NTC	21.59	26.53	38.26
17	E2 0.3 nM	22.24	No Ct	37.29	30	NTC	21.65	26.07	38.51
17	E2 0.3 nM	21.79	No Ct	37.16	21	NTC	21.59	26.81	38.13
17	E2 0.3 nM	23.26	44.31	38.49	21	NTC	21.81	26.38	38.53
18	E2 0.3 nM	20.16	26.45	36.16					
18	E2 0.3 nM	20.69	25.24	37.97					
18	E2 0.3 nM	20.37	26.08	37.1					
19	E2 10 nM	20.82	27	37.56					
19	E2 10 nM	21.44	26	38.81					
19	E2 10 nM	21.08	26.96	38.36					
20	E2 10 nM	20.25	24.34	37.63					
20	E2 10 nM	20.31	24.87	37.54					
20	E2 10 nM	19.92	24.32	38.66					