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The acute effect of propanil on reproductive hormones, ovarian gene expression and splenic cells in C57Bl/6 female mice

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The acute effect of propanil on reproductive hormones, ovarian gene expression and splenic cells in C57Bl/6 female mice

Malia Danielle Berg

Thesis submitted to the Davis College of Agriculture, Natural Resources and Design at West Virginia University in partial fulfillment of the requirements for the degree of

Master of Science in Reproductive Physiology

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Keywords: Propanil, heat-killed *Streptococcus pneumoniae*, reproductive hormones, splenocytes, and steroidogenic gene expression

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Abstract

The acute effect of propanil on reproductive hormones, ovarian gene expression and splenic cells in C57Bl/6 female mice

Malia Danielle Berg

Earlier evidence indicates exposure to propanil, a post-emergent herbicide used on rice and wheat fields, increases the number of antibody-secreting cells (ASC) in the spleen of mice after immunization with heat-killed *Streptococcus pneumoniae* (HKSP, 2×10^8 CFU). Interestingly, an intact hypothalamic-pituitary-gonadal (HPG) axis is required for this immune response assessed on day 7-10 after immunization in female mice. Hence, an overarching premise is that propanil arbitrates the connection between the reproductive and immune system by altering the concentration of hormones involved in the HPG axis, which may then affect the populations of splenic cells. Hypotheses were tested with C57Bl/6 female mice vaccinated with HKSP and assigned among four treatment groups (6 mice each) in a 2x2 factorial arrangement. The first factor represented propanil treatment (propanil, control), and the second factor represented two periods (24h, 72h) of collection for blood, spleen, ovaries, and oviducts after immunization. At 24h, propanil decreased the number of splenocytes contrary to increased cell populations at day 7-10 observed in earlier experiments. Also, propanil increased serum concentrations of prolactin and progesterone but decreased estradiol, corroborated by corresponding changes in mRNA for steroid synthesis in ovaries. To determine if these effects depend on gonadotropin-releasing hormone (GnRH) receptor binding, we utilized the GnRH antagonist (antide; 60 μ g s.q.), given to C57Bl/6 female mice before the same immunization with HKSP. In ovaries, antide increased gene expression of inflammatory and anti-inflammatory cytokines and markers (*Csf1*, *Cxcl1*, *Cxcl10*, *Il-10*, *Irf-1*, *Tlr2*), interacted with propanil effect in HKSP-challenged mice on the expression of serum amyloid A by reducing propanil's effects. In the spleen, antide decreased the number of T-cells, and in the presence of HKSP decreased the number of B-cells and the percentage of monocytes and macrophages. Thus, GnRH receptors may be necessary for the acute responses (24 hours) to propanil and support a hypothesis that immune and reproductive systems interact in female mice challenged with the bacterial vaccine.

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List of Abbreviations

µg	Microgram
µM	Micrometer
Adamts1	A Disintegrin And Metalloproteinase with Thrombospondin Motifs 1
Actb	B-Actin
ALP	Alkaline Phosphatase
AP-1	Activating Protein-1
ASC	Antibody-secreting cell
ASM	Accelerator Mass Spectrometry
AST	Aspartate Aminotransferase
BFU-E	Erythroid Burst-forming Unit
bp	Base Pairs
BSA	Bovine Serum Albumin
C	Celsius
Car3	Carbonic Anhydrase 3
Ccl2	C-C Motif Chemokine Ligand 2
CCL7	C-C Motif Chemokine Ligand 7
CFB	Complement Factor B
CFD	Complement Factor D
CFU-E	Erythroid Colony-forming Unit
CFU-G	Granulocyte Progenitor Cell
CFU-M	Macrophage Progenitor Cell
CFU-S	Myeloid Stem Cell
CHI3L1	Chitinase 3 like 1
CL	Corpus Luteum
cm	Centimeter
CNS	Central Nervous System
ConA	Concanavalin A
CSF	Colony Stimulating Factor
Csf1	Colony Stimulating Factor 1
Cxcl1	C-X-C Motif Chemokine Ligand 1
Cxcl10	C-X-C Motif Chemokine Ligand 10
Cyp11b1	Cytochrome P450 11B1
Cyp17a1	Cytochrome P450 17A1
Cyp19a1	Cytochrome P450 19A1
DCA	3,4-Dichloroaniline
DCPA	3,4-dichloropropionanilide
Dhcr7	7-Dehydrocholesterol reductase
DHT	Dihydrotestosterone

E ₂	Estradiol
EDN2	Endothelin 2
ER	Estrogen Receptor
FSH	Follicle Stimulating Hormone
GM	Granulocyte-macrophage-progenitor cell
GUCA2B	Guanylate Cyclase Activator 2B
GnRH	Gonadotropin Releasing Hormone
GnRHR	Gonadotropin Releasing Hormone Receptor
HCL	Hydrochloric Acid
H&E	Hematoxylin and Eosin
HKSP	Heat-killed <i>Streptococcus pneumoniae</i>
Hprt1	Hypoxanthine-guanine phosphoribosyltransferase
HSD	Honestly Significant Difference
Hsd17b2	Hydroxysteroid 17-Beta Dehydrogenase 2
Hsd17b7	Hydroxysteroid 17-Beta Dehydrogenase 7
HPG	Hypothalamic-pituitary-gonadal
IACUC	Institutional Animal Care and Use Committee
ICAM1	Intercellular Adhesion Molecule 1
IL-2	Interleukin 2
IL-3	Interleukin 3
IL-6	Interleukin 6
IL-10	Interleukin 10
IL-12	Interleukin 12
INF	Interferon
Io	Ionophore A23187
i.p.	Intraperitoneal
Irf1	Interferon regulatory factor 1
kg	Kilogram
L	Liter
Lcn2	Lipocalin 2
LDH	Lactate Dehydrogenase
LPS	Lipopolysaccharide
LS	Least Square
m	Meter
MDA	Malondialdehyde
mg	Milligram
MLR	Mixed Lymphocyte Reactions
mL	Milliliter
mmol	Millimolar
MRAP	Melanocortin 2 Receptor Accessory Protein

NFAT	Nuclear Factor of Activated T-cell
NF κ B	Nuclear Factor kappa B
NK	Natural Killer Cell
ng	Nanogram
NRQ	Normalized Relative Quantity
P4	Progesterone
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PC	Polysaccharide Phosphorylcholine
PGE2	Prostaglandin E2
PGF	Prostaglandin F2 alpha
PMN	Polymorphonuclear Neutrophils
ppm	Part per million
PspA	Pneumococcal surface protein A
RBC	Red Blood Cell
rCSF	recombinant Colony-stimulating Factor
RQ	Relative Quantity
Saa3	Serum Amyloid A 3
SEM	Standard Error of the Means
SERPINA3	Serpin Family A Member 3
Socs3	Suppressor of cytokine signaling 3
S100a8	S100 calcium-binding protein A8
S100a9	S100 calcium-binding protein A9
TD	T-dependent
TI-2	T-independent
Tlr2	Toll-like receptor 2
TNF	Tumor Necrosis Factor
Tnfaip3	Tumor Necrosis Factor, Alpha-induced Protein 3
VCAM1	Vascular Cell Adhesion Molecule 1
XPNPEP2	X-prolyl Aminopeptidase
$\Delta\Delta C_q$	delta-delta Quantification Cycle

Chapter 1: Literature Review

Introduction

Propanil (3,4-dichloropropionanilide, DCPA, **Figure 1**) is a selective herbicide from the acetanilide chemical family (Zhao *et al.*, 1998) used in the United States mainly to regulate broadleaf weeds on rice fields (Bartha and Pramer, 1970; U.S. Environmental Protection Agency, 2003). The particular properties of propanil depend on the ability of plants to metabolize it, thus resisting the propanil-induced inhibition of photosynthesis (Prado *et al.*, 1992), oxidative damage, and cell death. Rice plants contain sufficient enzymatic apparatus to metabolize propanil; for example, rice leaves contain about 60-times higher amounts of the aryl acylamidase enzyme than barnyard grass weeds (Frear and Still, 1968). Propanil exposure in humans via ingested rice seed is minimal. Exposure is limited to agricultural workers, applicators, propanil-manufacturing workers, as well as residents near treated farms, and via direct accidental or intentional ingestion. Past evidence from clinical case reports, laboratory animal studies, and *ex-vivo* and *in-vitro* models indicate that propanil has adverse effects on mammalian physiology. The current studies were designed to expand the understanding of the effect of propanil on the immune system and its interaction with the reproductive system in female mice. Individually, we examined the acute effects of propanil and hypothalamic hormone-binding on pituitary and ovarian hormones, ovarian gene expression, and the cell populations in the spleen.

Figure 1. Chemical structure and hepatic metabolism of propanil, modified (McMillan *et al.*, 1990)

General effects of propanil

Workers involved with manufacturing and applicators of propanil in agriculture are at the highest risk of exposure, while there have been clinical reports of intentional or accidental ingestion of propanil (Eddleston *et al.*, 2002; Roberts *et al.*, 2009) and other non-application exposure (M. Richards, G. Y. H. McClure, T. L, 2001; Roberts *et al.*, 2009).

Upon propanil's entry into the mammalian system, the first step of the metabolism takes place in microsomes of the liver (Williams and Jacobson, 1966; McMillan *et al.*, 1990). The process is enzymatic hydrolysis catalyzed by acylamidase (Williams and Jacobson, 1966; Singleton and Murphy, 1973; McMillan *et al.*, 1990; Lewis *et al.*, 2013) cleaving the propionic side chain and subsequent formation of 3,4-

Dichloroaniline (DCA, **Figure 1**) and propionate. Some authors refer to the enzyme leading to DCA formation as esterase or use esterase and acylamidase interchangeably (McMillan *et al.*, 1990; Roberts *et al.*, 2009) due to two factors: esterase inhibitors block DCA formation (Matsunaka, 1968, DuBois *et al.*, 1968; Singleton and Murphy, 1973; McMillan *et al.*, 1990), and some enzymes have both (esterase and acylamidase) activities (Rajesh *et al.*, 2003).

While the first metabolic step (conversion of DCPA to DCA and propionate) protects propanil-resistant plants like rice, tomatoes, lettuce, sugar beets, okra, peppers and occurs in soil as well (Bartha and Pramer, 1967; Hoagland *et al.*, 1974, 2004), the generation of metabolites in mammals has undesirable physiological consequences. Central nervous system (CNS) suppression, including loss of the righting reflex in mice, and methemoglobinemia and hemolytic anemia have been documented in humans (Morse *et al.*, 1979; Eddleston *et al.*, 2002) and mice (Singleton and Murphy, 1973; Chow and Murphy, 1975) and occur within 30 minutes to 4-8 hours of oral or intraperitoneal (1.3 mmol/kg or 200-800 mg/kg) exposure to propanil. Methemoglobinemia is higher than usual (~1% of hemoglobin) concentration of methemoglobin in the systemic circulation (Curry, 1982). Methemoglobin is an abnormal form of unoxygenated hemoglobin, in which the iron is in the ferric (Fe^{3+}) rather than the ferrous (Fe^{2+}) state and cannot carry oxygen or carbon dioxide (Curry, 1982). This condition can lead to blue-brown discoloration of the skin and mucous membranes, cyanosis (above 1.5%), hypoxia (60%), and, in extreme cases (higher than 70-80%) is lethal (Curry, 1982). Clinical accounts from a Sri Lanka medical center reported 16 human cases of severe propanil poisoning from ingestion with clinical features such as

confusion, reduced consciousness, cyanosis, and development of respiratory depression and death. Laboratory tests demonstrated increased methemoglobin (Hb) levels, alanine, and aspartate transaminases, which were more than twice the upper limit and indicative of severe liver damage (Eddleston *et al.*, 2002). *In vivo* (Weisburger and Weisburger, 1973) and *ex vivo* oxidation of hemoglobin by propanil and its metabolites, such as DCA, N-Hydroxy-3,4-dichloroaniline, and 6-Hydroxy-3,4-dichloroaniline (**Figure 1**), were demonstrated in rat liver microsomes and isolated rat erythrocytes (McMillan *et al.*, 1990). Hemolytic anemia is an abnormal decrease in the number of red blood cells (RBC) (Uthman and Uthman, 1998). The survival of ⁵¹Cr-labelled and transfused RBC in rats decreased in a dose-dependent pattern by intraperitoneal (i.p.) administration of propanil or DCA (McMillan *et al.*, 1991; Eddleston *et al.*, 2002). Also, erythrocyte survival was markedly reduced by i.p. administration or *in vitro* exposure to N-hydroxy-3,4-dichloroaniline. When rats were subjected to three months of various concentrations of propanil mixed into their diet at 3,300 and 10,000 parts per million (ppm), hematologic findings showed an increase in polychromatophilia, more stained cells with specific dyes as compared to controls (Ambrose *et al.*, 1972). This indicated the presence of immature forms of red blood cells not typically found in peripheral blood. Collectively, evidence from clinical cases and research indicates that metabolites of propanil are necessary for the induction of both methemoglobinemia and hemolytic anemia in mammals. The lack of oxygen delivery, depending on dose and length of exposure, may lead to other organ system pathologies.

Additional effects of propanil detectable in circulation are related to the liver, where the majority of propanil metabolism occurs. For instance, increased serum

concentrations of alkaline phosphatase (ALP) and aspartate aminotransferase (AST) were measured in male Sprague-Dawley rats when given 50 mg (i.p.) of propanil daily for 10 days (Santillo *et al.*, 1995). The enzyme ALP mediates the break-down of proteins AST, a key enzyme of amino acid metabolism, is concentrated in heart and liver tissues. Both enzymes are used to assess liver function clinically, and increased levels indicate cellular damage of the liver. Microsomes from the liver, brain, and kidneys of propanil-treated rats (50 mg i.p. daily for 10 days) were examined for lipid peroxidation by measuring the malondialdehyde (MDA), a conventional marker of oxidative stress. Peroxidation in liver microsomes was increased by 95%, and in brain microsomes by 36%, as compared to rats injected with only corn oil (Santillo *et al.*, 1995). No difference was observed in kidney microsomes. Also, a decrease in cholesterol and triglycerides in the serum of propanil-treated rats was seen, which could be due to the hepatic damage or direct oxidative effect of propanil on cholesterol and possibly on triglycerides (Santillo *et al.*, 1995). Another study with F344 rats established that the propanil metabolite, DCA with the addition of hydrochloric acid (HCl) moiety (DCA HCl salt), increased ALT activity at 0.8 and 1.0 mmol/kg doses within 24 hours (Valentovic *et al.*, 1997), indicating liver damage. Histological examination of liver demonstrated loss of nuclei from hepatocytes (0.8 mmol/kg) and necrosis (1.0 mmol/kg).

Decreased urine output and increased kidney weight were observed, and blood urea nitrogen increased in a dose-dependent manner in DCA HCl-salt-treated rats. High infiltration of neutrophils and RBCs were detected using the hematoxylin and eosin (H&E) staining in the bladder, indicative of nephrotoxicity (Valentovic *et al.*, 1997). The

same group demonstrated that the addition of hydrochloric acid (HCl) moiety to DCA did not modify DCA toxicity. Albino Swiss mice fed doses 75, 150, or 300 mg/kg of propanil had a dose-related histopathological alteration in the liver and kidney (Cakici and Akat, 2013). Biometric analyses, such as measurement of the number of central filtering units, glomeruli/renal corpuscle, and the number of nuclei per hepatic cell, were determined. The congestion in both the cortex and medulla and mononuclear cell infiltration into the kidney were gradual from the low to the high dose, and glomerular loss and degeneration were evident in propanil-treated mice. The changes to the liver included swelling, intracellular vacuoles, nucleus loss, an increase in the number of Kupffer cells, and liver congestion. An increase in liver weight and swelling was observed in a subchronic oral toxicity study, when albino rats had propanil mixed into their diet, ranging from 0 to 10,000 ppm (Ambrose *et al.*, 1972); the increase in spleen, liver and heart size was observed at 10,000 ppm. The same group established the median lethal dose of propanil in rats (the $LD_{50} \pm SD$) to be $1,384 \pm 99$ mg/kg and $1,217 \pm 8$ mg/kg for dogs, within three days following gastric intubation (Ambrose *et al.*, 1972). The evidence from rat and mice studies lead to the conclusion that propanil caused liver damage and nephrotoxicity.

Similar markers of liver damage and metabolic stress were detected in European eels (*Anguilla anguilla*) exposed to sublethal propanil in the aquaria water (3.16 mg/L, 1/10 of 96-hr LC_{50}) for 72 hours. Increases in cortisol, lactate dehydrogenase (LDH) from 24 hours to 48 hours, ALP from 24 hours through hour 168, and AST from hour 2 through hour 168 with a spike at hour 72 were detected in plasma when compared to controls (Sancho *et al.*, 2017). The LDH enzyme is involved in the conversion of sugar

into energy and resides typically inside the cells. Upon cellular damage, it is released into the plasma. Besides liver damage, protein, cholesterol, and energy metabolism were negatively affected, indicative of overall, multiple organ toxicity. The dose of propanil, 3.16 mg/L, was about a quarter of the concentration following agricultural application of 12 mg/L in rice paddies in Spain. However, it was much higher (30-60 times) than propanil concentrations of 50-100 µg/L measured in drainage waters, where the fish may be exposed. Thus, the evidence from the eels may be more pharmacological than physiological (Sancho *et al.*, 2017).

In summary, exposure to propanil via oral (rats, dogs, and humans) intraperitoneal (rats and mice) and aquatic environment (eels), depending on the dose, has adverse effects on oxygen-carrying capacity of RBCs via methemoglobinemia, decrease of RBCs, decrease in liver function, kidney structure, energy metabolism and cholesterol concentration. Marked changes in the amount of cholesterol and triglycerides produced by the liver or metabolized in the blood may have other functional consequences; for example, it could lead to an increase in inflammatory markers or a decrease in steroid hormone production.

Propanil dose-response and mode of exposure

The propanil doses experienced by non-applicators or humans not involved with propanil manufacturing are not well defined (Malerba, 2002). Earlier studies reported a propanil reference risk dose in humans of 0.005 mg/kg/day in the rice-producing paddies area, 0.1-0.228 µg/l in river samples, and 0.0003 mg/kg of propanil in leafy vegetables (Johnson *et al.*, 1984; Gartrell *et al.*, 1986). As propanil is used extensively

in rice production in the Mississippi River delta region, the airborne drift during aerial application may pose a health concern to farming families (McClure *et al.*, 2001). Exposure of families living at rice farms in Arkansas was measured inside and near the house, at an intermediate distance (30m), and the edge of the rice fields after the aerial propanil application (M. Richards, G. Y. H. McClure, T. L., 2001). The amount of propanil increased with the decreasing distance to the field. Inside the houses, when detectable, there were 3 -7 μg of propanil/400 cm^2 surface per 90 minutes, near the house the concentration ranged from 3 - 615 μg /400 cm^2 , in the intermediate distance from the house it varied from 3 to 952 μg /400 cm^2 . The amount was the highest at the edge of the rice fields, ranging from 9 to 12,391 μg /400 cm^2 . Wind velocity and direction contributed to the large variability in the measurements. Farmers in central Italy were tested for occupational exposure to anilide pesticides, including propanil. Most (81.5%) urine samples were positive for the propanil metabolite, DCA, which is now considered a marker of occupational exposure (Vitelli *et al.*, 2007). In a survey of occupationally acquired diseases in workers in a pesticide plant, 17 of 28 production workers with exposure to propanil and DCA had chloracne, an acne form of dermatosis; however, a contaminant tetrachlorazobenzene in propanil and DCA was a likely agent causing the chloracne (Morse *et al.*, 1979). Collectively, the data from non-occupational exposure indicate no significant health concern or effect on immune function in contrast to occupational exposure, accidental, or self-poisoning cases.

Concentration, distribution, and pharmacokinetics of propanil metabolism were evaluated using radiolabeled carbon conjugated to propanil in murine blood, liver and spleen to reconcile higher doses used in the majority of experimental studies with lower

doses typical for environmental exposure (Schafer *et al.*, 2018) using high sensitivity of accelerator mass spectrometry (AMS). The low dose, 854 pg/g of [^{14}C]-propanil per body weight was used to estimate non-occupational exposure, while the high-dose, a mixture of 200 $\mu\text{g/g}$ [^{12}C]-propanil and 854 pg/g of [^{14}C]-propanil, mimicked occupational, accidental or self-ingestion scenarios. The low doses appeared to be metabolized within an hour, and almost all disappeared within 12 hours. Concentrations decreased in the liver faster than in the blood and the spleen. The response likely reflects the presence of the liver's catabolizing enzymes, which are absent in the blood and the spleen. In contrast, the high dose demonstrated a later peak and much longer duration of higher concentrations of propanil in all three tissues indicative of saturation, and detoxification could not be accomplished within the 12 hour time point (Schafer *et al.*, 2018). These data gauge exposure and prevailing concentrations of propanil (and possibly its metabolites) in organs relevant to the toxicology of propanil and are a valuable tool for future toxicological studies.

Effect of propanil on innate immunity

The innate immune system ensures the defense of the organism against exogenous pathogens using physical protection in the form of skin and mucosal tissues as well as multiple cell types equipped with phagocytic, chemical, and killing machinery to eliminate invading pathogens. Besides the elimination of pathogens, cells of the innate immune system also capture and introduce the pathogen to cells of the adaptive immune system. The innate immune system includes monocytes, macrophages, polymorphonuclear neutrophils (PMN), and natural killer (NK) cells. For example, when

whole blood is incubated with HKSP, cytokine production (TNF, IL-6, IL-10, IFN, IL-12, and IL-12p70) from the innate immune cells increases in a time-dependent manner from 4 to 24 hours after exposure (Schultz *et al.*, 1998). Those cytokines are pro-inflammatory cytokines and activate the innate and adaptive immune systems to help clear the foreign bacteria from the host. If propanil affects the innate immune system, then both the elimination of pathogen and initiation of the adaptive immune response may be compromised. NK cells have antimicrobial cytotoxic mechanisms and work by secreting cytokines. Propanil (50, 100, or 200 mg/kg) and DCA decreased in a dose-response pattern on NK lytic activity on day 7 in mice (Barnett *et al.*, 1992). Also, an acute suppressive effect on NK cell activity was reported as early as 12 hours after propanil (Pruett *et al.*, 2000).

A case-control study was conducted in Arkansas, in which the blood samples were collected from 53 people living on 12 farms within 100 yards of rice fields, and their measurements were compared to 55 individuals at 15 farms with crops other than rice (McClure *et al.*, 2001). The immune assessment included a white blood cell count and peripheral blood mononuclear cell (PBMCs) functional assay, such as mitogen-induced cell proliferation, mitogen-induced cytokine production, and cytotoxic PBMC activity (McClure *et al.*, 2001) over two years and three seasons (before, 5-7 days after and post-propanil-spraying season). A comparison of immune function between propanil exposed and control families showed no significant differences in most of the variables and functions, except the percent of natural killer cells in youth living at rice farms were significantly lower (McClure *et al.*, 2001).

In spleen cells that were exposed to propanil (200 mg/kg), IL-2, IL-6, IFN- γ , and GM-CSF production decreased after being activated by ConA (a nonspecific mitogen) (Zhao *et al.*, 1998). TNF production in THP-1 cells (human monocytic cell line derived from an acute monocytic leukemia patient) was suppressed at 6, 12, and 24 hours at different doses of propanil (25, 50, and 100 μ M) (Ustyugova *et al.*, 2007).

In macrophages collected from mice treated with propanil (200 mg/kg, i.p.) had a reduction in TNF- α over time from 3 to 72 hours (Xie *et al.*, 1997). Intracellular pathways and mechanisms of propanil action in macrophages have been examined. Many extracellular inflammatory signals, bacterial cell wall components, like lipopolysaccharide (LPS), peptidoglycan polysaccharide, growth factors, and cytokines initiate signal transduction and intracellularly converge in the activation of transcription factor Nuclear Factor Kappa B (NF- κ B). Activated NF- κ B translocates into the nucleus and upon binding to promoter regions, initiates transcription of genes involved in cytokines synthesis. TNF α depends on the NF- κ B activation and translocation into the nucleus. *In-vitro* experiments conducted in macrophage cell line IC-21 stimulated with LPS found reduced NF- κ B binding as a result of propanil treatment, measured by confocal microscopy and Western blot (Frost *et al.*, 2001).

In summary, both *ex-vivo* murine spleen cells within 24 hours in mice and *in-vitro* cell culture, propanil modified the inflammatory response.

Propanil effects on primary and secondary lymphoid organs and cellularity

The bone marrow is where the B-cells develop from the progenitor cells and where they mature. The spleen is a site for the production of antibodies against pathogens by B-cells. The number of murine pre-B-cells in bone marrow decreased to 50 % of control 7 days after 50 mg/kg i.p. propanil (de la Rosa *et al.*, 2003). However, the number of circulating follicular B-cells was not affected after propanil (Salazar *et al.*, 2005). To summarize, the developing T and immature B-cells in bone marrow were affected by propanil, but that did not translate to measurable changes in T and B-cell numbers in circulation.

Researchers have examined bone marrow and thymus, the primary lymphoid organs, and their cellular populations as possible targets of propanil. Female C57Bl/6 mice were treated with 0, 50, 100, or 200 mg of propanil/kg of body weight on day 0 and bone marrow (BM) collected on day 7 (Blyler *et al.*, 1994). The number of leukocytes from the BM was determined, and cells were cultured for 6 days, after which the recombinant colony-stimulating factor (rCSF) induced colony formation was measured. Various stem cell types and stages of hemopoiesis were enumerated using their respective growth factors and cytokines. Formation of a colony of pluripotent stem cells that are induced to differentiate in the presence of interleukin (IL)- 3 (CFU-IL-3) was inhibited by each dose of propanil (50, 100 and 200 mg/kg). The next developmental stage, myeloid stem cell (CFU-S) function, was measured by transferring bone marrow cells from propanil-injected mice (200 mg/kg) to irradiated syngeneic recipient mice. After 12 days, the splenic foci were enumerated. The number of CFU-S (100 ± 15

colonies per million cells) in propanil-treated mice was about a quarter of the number of CFU-S in control mice (390 ± 30 colonies per million cells). CFU-S mature either into the myeloid lineage, starting with granulocyte-macrophage-progenitor cells (GM) or into the erythroid lineage, depending on growth factor and cytokine. Propanil doses of 50 mg/kg and higher reduced the number of CFU-GM colonies to about half of the control mice. However, the more differentiated cells of the myeloid lineage, such as macrophage progenitor cells (CFU-M) and granulocyte progenitor cells (CFU-G), were not affected by propanil.

Similarly, the earlier progenitor of erythroid cell lineage, the erythroid burst-forming unit (BFU-E) colony number was decreased by propanil, while the later developmental stage, the erythroid colony-forming unit (CFU-E) was not affected. Results might represent selective toxicity of propanil, also suggested by a study where natural killer cell function was significantly decreased while the cytotoxic T-cell function was not affected by propanil (Barnett *et al.*, 1992). The effect of propanil and DCA on human and murine erythroid progenitor lineage BFU-E and CFU-E as well as on the granulocyte/macrophage progenitors (CFU-GM) was carried out in an interspecies comparison of *in-vitro* and *ex-vivo* toxicity, respectively (Malerba, 2002). Human mononucleated cord blood cells and BM cells obtained from CD-1 male mice were each cultured for one to two weeks in their required media with addition of propanil or DCA at 10, 20, 50, 100, 200, 500 and 1000 μM for 3 and 10 days (BFU-E and CFU-E cultures) or for 7 days (CFU-GM cultures). The concentration of propanil or DCA that inhibited 50% of the growth of the cells (IC_{50}) was much lower in murine erythroid progenitors ($<70 \mu\text{M}$) than the granulocyte-macrophage progenitors (170 μM), showing the erythroid

progenitors were about 2.5 to 13-times more sensitive than GM progenitors. DCA was a more potent inhibitor of erythrocyte progenitors than the parent compound. Human cord blood cells, however, were not nearly as sensitive to propanil or DCA as murine progenitors, with average IC_{50} between 200 – 450 μ M for propanil and >500 μ M for DCA on erythroid progenitors and >500 μ M of either propanil or DCA on GM progenitors. The human erythroid progenitors were more sensitive to propanil than to DCA. In both species, the erythroid progenitors were more susceptible to propanil or DCA than the granulocyte/macrophage progenitors, which can relate to the susceptibility of red blood cells to propanil and DCA in (anemia) and hemoglobin modifications (methemoglobinemia), described in both species (Singleton and Murphy, 1973; McMillan *et al.*, 1991; Roberts *et al.*, 2009).

The thymus is a lymphoid organ where the development and maturation of T-cells occur. T-cells perform many functions in the support and regulation of the humoral immune response and work closely with B-cells, responsible for the production and secretion of antibodies. $CD4^+$ T-cells are mainly involved in activation and regulation of the humoral immune response and cell-mediated immune responses, while $CD8^+$ T-cells mainly destroy infected or transformed cells as cytotoxic T lymphocytes (Owen *et al.*, 2013). T-regulatory and suppressor cells operate in a variety of ways to control the adaptive immune response. Weight and thymocyte populations were examined upon i.p. propanil injection using 100 - 400 mg/kg (Barnett and Gandy, 1989; Cuff *et al.*, 1996; de la Rosa *et al.*, 2005). Evidence from multiple experiments demonstrated a marked decrease in thymic weight, decreased thymus to body weight, and thymic atrophy characterized by a decreased number of immature, double-positive, $CD4^+CD8^+$ T-cells

on days 2 to 4 after propanil injection. By day 7, the cells and size of thymus begin to recover, preceded by the proliferation of CD4⁺CD8⁺ T-cells, and by day 14, both cellularity and thymus size completely recovered (Cuff *et al.*, 1996). Corticosterone, which was found negatively correlated with the number of double-positive T-cells in a propanil-dose-dependent manner, is the primary mechanism by which propanil induces thymic atrophy is attributed to (Cuff *et al.*, 1996; de la Rosa *et al.*, 2005). Acute i.p. propanil exposure spiked corticosterone concentration in the serum of C57Bl/6 mice almost 10-times control levels, peaked at one hour after injection (de la Rosa *et al.*, 2005), and returned to normal levels by 12 hours. Collectively, data from *in vivo* experiments examining both bone marrow and thymus, demonstrate the sensitivity of these tissues to propanil.

T-cells also reside in the secondary immune organs, such as the spleen and lymph nodes. Various *in vivo* models, mostly involving the intraperitoneal injection of propanil in mice, as well as *in vitro* systems utilizing T-cell lymphoma cell lines (Jurkat, EL-4), were utilized to study if propanil affects T-cell number and function. At the highest propanil dose (400 mg/kg), a decrease in mixed lymphocyte reactions (MLR), characterizing decreased CD4⁺ function in addition to *ex vivo* proliferation of splenocytes were observed following stimulation with the T-cell mitogen concanavalin A (ConA) (Barnett and Gandy, 1989).

A lower dose, 200 mg/kg, stimulated a decrease in interleukin-2 (IL-2) and IL-6 production, suggesting the functionality of T-cells was affected by propanil. *In vitro* studies led to similar results. IL-2 cytokine production by EL-4 cells (human T-lymphoma), stimulated with phorbol 12-myristate 13-acetate (PMA), a mitogen,

decreased in propanil (20 mM)-treated cells (Zhao *et al.*, 1999). Stimulation of T-cells via the T-cell receptor leads to the secretion of IL-2 and cell proliferation. Transcription of the IL-2 gene decreased to about 50% of control levels in the presence of propanil, and IL-2 mRNA message stability decreased (Zhao *et al.*, 1999). The human IL-2 gene has a complex promoter region involving multiple transcription factors, including activating protein-1 (AP-1). Utilizing Jurkat cells (human T-lymphoma), stimulated with PMA and ionophore A23187 (Io), IL-2 mRNA decreased due to propanil (Brundage, 2004). The PMA and Io mimic the natural two-signal activation of T-cells, one via the T-cell receptor, second through the co-stimulatory molecule CD28. Also, propanil-treated Jurkat cells had decreased DNA-AP-1 binding activity (Brundage, 2004). Influx in extracellular calcium (Ca^{2+}), following internal Ca^{2+} depletion, is important for optimal T-cell activation. Further work with Jurkat cells, as well as with primary T-cells from mouse spleen- stimulated with anti-CD3 antibodies and propanil demonstrated that intracellular calcium concentrations decreased in a propanil-dose-dependent pattern (Lewis *et al.*, 2008). Also, a transcription factor involved in IL-2 signaling, Nuclear Factor of Activated T-cells (NFAT), decreased to 60% of controls in propanil-treated and CD3- stimulated Jurkat cells (Lewis *et al.*, 2008). A similar decrease of IL-2 and NFAT in T-cells was also observed with propanil metabolites, DCA, 6OH-DCA, and NOH-DCA in a dose-response model (Lewis *et al.*, 2013). The results of the *in-vitro* studies with EL-4 (Zhao *et al.*, 1999) and Jurkat cells (Brundage, 2004; Lewis *et al.*, 2008) suggest the signal transduction pathway and transcription factor important in IL-2 transcription being adversely affected by propanil treatment. Collectively, decreased thymus size, via increased glucocorticoids, decreased percentage of double-positive (CD4^+ , CD8^+)

immature T-cells, and reduction in IL-2 cytokine production are the main adverse effects in the thymus and T-cell function in the spleen.

Numerous studies found a significant increase in spleen weight 7 days after propanil injection, referred to as splenomegaly. The cellular examination of the spleen revealed a substantial increase in the number of leukocytes (leukocytosis). The metabolism of propanil to DCA and its metabolites could result in leukocytosis after propanil or DCA. Alternatively, the propanil could have a direct mitogenic effect, or leukocytosis could be a consequence of methemoglobinemia (Barnett *et al.*, 1992).

Bacterial challenges with common pathogens were utilized to evaluate if propanil suppressed the immune system. Propanil administered to C57Bl/6 mice together with *Listeria monocytogenes* (*L. monocytogenes*) decreased the *ex-vivo* secretion of interferon (INF)- γ but did not change the in vivo concentration of various inflammatory cytokines (Watson *et al.*, 2000). Another model examining antibody response to heat-killed *Streptococcus pneumoniae* (*S. pneumoniae*) injected i.p. to C57Bl/6 mice concomitantly with propanil was utilized (Salazar *et al.*, 2005). In the United States, four million estimated cases of bacterial pneumonia occur every year (Schultz *et al.*, 1998). *S. pneumoniae*, a gram-positive bacterium, is the most commonly identified pathogen in community-acquired pneumonia. Heat-killed *S. pneumoniae* (HKSP), inactivated by heating to 60°C for one hour (Salazar *et al.*, 2005), was used as an immune challenge to study the response of mice to propanil. The cell wall of the *S. pneumoniae* contains antigens that can elicit two kinds of responses, classified as T-independent (TI-2) and T-dependent (TD) (Wu *et al.*, 1999, 2000). Polysaccharide phosphorylcholine (PC) is an *S. pneumoniae* antigen that usually elicits TI-2 response, while pneumococcal surface

protein A (PspA) is a TD kind of antigen. The main finding in studies of HKSP given as an immunogen with the propanil, as the immune modulator, was the spleen of propanil-treated mice had a four-fold increase in PC-specific (but not PspA-specific) antibody-secreting cells (ASC) (B cells) at day 5 after exposure (Salazar *et al.*, 2005). When mice were given only HKSP, there was not a marked increase in ASC as with HKSP and 50 or 150 mg/kg propanil. Propanil further increased the PC-specific ASC in the spleen on day 7, followed by a gradual decrease to normal by day 14 (Salazar *et al.*, 2005). However, this increase in ASC depended on the presence of ovaries in female mice, as evidenced by the loss of the ASC increase in ovariectomized mice (Salazar *et al.*, 2006).

Collectively, antibody response to HKSP was increased by propanil, but the increased response in female mice depends on the intact hypothalamic-pituitary-gonadal axis, further discussed in the next section. The requirement of ovaries or HPG axis in amplifying the humoral immune response and function of corticosterone on immature T-cells and B-cells in the bone marrow and thymus highlight potential interactions of endocrine and immune systems. Before the discussion of the existing evidence of interactions of reproductive and immune function, mostly in mice, a summary of the main murine reproductive functions and hormones is in place.

Reproductive Hormones

Rodents are polyestrous species meaning that they have multiple and regular estrous cycles during the year. While the wild mice are seasonal polyestrous species and breeding season is mostly in spring and summer, the inbred mice, like C57Bl/6J breed year-round. However, A/J mice, which also breed in laboratory conditions, have

more offspring in spring and summer months than in the fall and winter (Schile, Jackson Laboratory). The estrous cycle in mice on average is four days long, with each day being a different phase in the estrous cycle: proestrus, estrus, metestrus, and diestrus. Proestrus and estrus are part of the follicular phase when follicles are growing and maturing and start to secrete estradiol. The estrous stage includes the ovulation, release of an ovum or egg, or multiple eggs in some species, from the follicle and ovary, into the oviduct. Mono-ovulatory species, like humans, normally ovulate one follicle per estrous cycle; polyestrous species, like mice, rats, pigs, and cats, ovulate several follicles in one estrous cycle (European Society of Human Reproduction and Embryology, ESHRE, Capri Workshop Group, 2003). Metestrus and diestrus are part of the luteal phase, covering the time from after ovulation until the corpora lutea (CL) regress, which is known as luteolysis. The primary hormone during the luteal phase is progesterone that is secreted by the CL (Senger, 2012). The CL is a transient endocrine gland that forms by remodeling of the empty, ovum-free, post-ovulatory follicle. The former theca and granulosa cells in the ovulatory follicle get re-purposed into small and large luteal cells. Hormonally, the production of estrogen is turned off at ovulation, and steroidogenic function is switched to progesterone. Overview of steroidogenesis from cholesterol, including the substrates and enzymes, is listed in **Figure 2**.

Figure 2. Steroid Synthesis Pathway (Moller and Backstrom, 2016).

The master hormone, gonadotropin-releasing hormone (GnRH), governing the regular cyclical changes in the ovaries, is secreted from the hypothalamus.

Hypothalamic-pituitary-gonadal (HPG) axis is a conduit of reproductive hormones utilizing circulation and second messenger signaling in target tissues. GnRH is needed to start the follicular phase of an estrous cycle. GnRH is produced in the hypothalamus and transported via neurons and specialized blood portal plexus to bind to GnRH receptors (GnRHR) on gonadotropin cells in the pituitary gland. Once GnRH binds to the receptor, the gonadotropin cells release luteinizing hormone (LH) and follicle-stimulating hormone (FSH). The gonadotropins are transported in blood and bind to their respective receptors on follicles of specific sizes in the ovary. LH binds to receptors on theca interna cells to start producing testosterone, which diffuses across the membrane to granulosa cells where FSH binds to its receptor to mediate the production of aromatase (CYP19A1). Aromatase converts testosterone into estradiol (**Figure 2**), which attaches to transport proteins in capillaries to be delivered to the reproductive tract, pituitary, hypothalamus, and other organs. The process of estrogen production in

the follicle is referred to as The Two-Cell-Two Gonadotropin Model, as it involves the two follicular cell types (theca and granulosa) and two gonadotropins (LH and FSH) (Senger, 2012). Estradiol acts via positive and negative feedback on the hypothalamus, depending on the stage of the cycle (Senger, 2012). During the follicular stage, estradiol (E_2) from growing follicles has positive feedback on the surge center in the hypothalamus, stimulating the preovulatory surge of GnRH. At the same time, E_2 causes inhibition of FSH from the anterior pituitary in a negative feedback fashion. Thus, the ratio of FSH and LH changes and LH increases into the ovulatory surge immediately before ovulation (Senger, 2012). The major effects of estrogen in the reproductive tract are increased blood flow, swelling, change in tissue electrical conductivity, increased number of leukocytes, an increase in smooth muscle motility and initiation of uterine growth (Sanger, 2012).

In addition to E_2 , granulosa cells of follicles in the ovary generate activin and inhibin, the glycoprotein hormones that activate or inhibit, respectively, the FSH secretion by the pituitary. During the luteal phase, progesterone from the CL has many functions. It induces negative feedback on the hypothalamic GnRH to allow for time without ovulation, it induces growth of mammary gland and growth of uterine lining with uterine glandular secretion, while at the same time prevents contractions of smooth muscle cells in the myometrium. Another hormone involved in the estrous cycle is oxytocin, a neuropeptide mostly produced by the hypothalamus and stored in the posterior pituitary. Oxytocin is also synthesized by the CL (Sanger, 2012). The target tissue of oxytocin is uterine myometrium, endometrium, and mammary gland. It is responsible for uterine motility, promotion of uterine prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), and milk

ejection. Another hormone important in rodent reproduction is prolactin, a protein produced in the anterior lobe of the pituitary gland. The known target tissues are mammary cells, CL (in rat and mouse), and the brain (Sanger, 2012). $\text{PGF}_{2\alpha}$, a C-20 fatty acid, is secreted by uterine myometrium and targets the CL for luteolysis at the end of the luteal phase, promotes uterine tone and contractions and is also involved in ovulation. Hormone counteracting $\text{PGF}_{2\alpha}$ actions is prostaglandin E_2 (PGE_2), secreted by ovaries, uterus, and embryonic membranes.

GnRH function is often tested by utilizing a GnRH antagonist. Antide is a GnRH antagonist that binds to the GnRHR to block GnRH from binding. Antide injected every 12 hours for 48 hours into female rats increased ovarian weight and number of preovulatory follicles (Parborell *et al.*, 2005). They also found that antide inhibits cell death in preovulatory follicles by decreasing the protein BAX translocation to the mitochondria (Parborell *et al.*, 2005).

Collectively, hormones that are important in mice reproduction include GnRH, FSH, LH, prolactin, progesterone, testosterone, E_2 , activin, inhibin, oxytocin, $\text{PGF}_{2\alpha}$, and PGE_2 . However, these hormones are also involved in immune function and regulation. For example, prolactin stimulates T-lymphocytes (Bachelot and Binart, 2007). Dopamine from the brain inhibits the synthesis of prolactin (Ben-Jonathan and Hnasko, 2001). Prolactin has been suggested to enhance mature B cell function by increasing IgG and IgM antibody production (Lahat *et al.*, 1993; Yu-Lee, 1997). Prolactin regulates the immune system through the transcription factor IRF-1, which can lead to the proliferation of T cells, differentiation of B cells, differentiation of CD8^+ T cells, antigen presentation, and macrophage activation (Yu-Lee, 1997; Book McAlexander and Yu-

Lee, 2001). Prolactin is required for T helper cells to proliferate when the proliferation is IL-2 driven (Clevenger *et al.*, 1990). An *in vivo* study demonstrated that rodents become immunosuppressed if there was a decrease in circulating prolactin levels (Hartmann *et al.*, 1989). Healthy humans given recombinant human prolactin showed a decrease in pulsatile LH leading to the conclusion that high prolactin levels suppress GnRH secretion (Page-Wilson *et al.*, 2006).

Estrogens play a vital role in immune function. In chronic exposure, E₂ has been observed to increase the number of immunoglobulin-producing cells in the blood, while decreasing the number of splenic B-cells (Verthelyi and Ahmed, 1998). A study confirmed that estrogen receptor signaling regulates innate immune cells such as macrophages and monocytes (Straub, 2007). When mice are ovariectomized (OVXed), they have an increase in leukocytes, mostly neutrophils, and lymphocytes in the blood (Stubelius *et al.*, 2017). They determined that sex steroids and especially estrogen can regulate inflammation, specifically during ovulation.

Progesterone also is a primary immunomodulatory sex hormone. Previous research has shown that progesterone can induce TH2 cytokine production. An *in vitro* study confirmed that when human cloned T cells from patients with multiple sclerosis were cultured with progesterone, the cells produced IL-4 and suppressed inflammatory cytokine production that included IL-10, TGF- β , INF- γ , and TNF- $\alpha\beta$ (Correale *et al.*, 1998).

Reproductive hormones play an important role in maintaining the estrous cycle, fertility as well as helping the immune system. Each hormone acts on the immune

system in specific ways: estrogen is proinflammatory, while progesterone can be anti-inflammatory. Overall, almost all the reproductive hormones discussed also affect immune function, specifically on B-cells and T-cells.

Effects of propanil on the endocrine, including reproductive, system

Propanil was termed an endocrine disruptor (Kojima *et al.*, 2004; Salazar *et al.*, 2006). Propanil at 75, 100, and 150 mg/kg increased corticosterone over several hours in mice and simultaneously caused a dose-responsive decrease in thymocyte numbers (Pruett *et al.*, 2000). Propanil decreased the weight of the thymus of female mice exposed to an injection (i.p.) of propanil at 200 mg/kg, and an about 10-fold increase in corticosterone (ng/ml) was observed at one-hour post-exposure (de la Rosa *et al.*, 2005). Adrenalectomy partially abrogated the decrease in immature T-cells in the thymus after propanil (Cuff *et al.*, 1996), providing the evidence of corticosteroids from the adrenal gland mediating the propanil effect on decreased thymus weight and cellularity. European eels treated with propanil for 72 hours had an increase in cortisol from 24 hours through hour 144 (Sancho *et al.*, 2017). Collectively, the evidence suggests propanil induces the physiological stress, represented by increased corticosteroids, as an endpoint of the hypothalamic-pituitary-adrenal axis. Elevated corticosteroids may then affect the function of immune cells in many different ways. For example, short-term stress may lead to induced immuno-protective functions or immuno-pathology. Chronic or long-term stress may lead to induced immunopathology as well as immuno-suppression (reviewed by Dhabhar, 2014).

The connection between the effect of propanil on the spleen and its increase of *S. pneumoniae* – specific ASC being conditional on the presence of ovaries or intact HPG axis in female mice was examined and discussed (Salazar *et al.*, 2006, 2008). Propanil, given to mice with HKSP, caused a three to six-fold increase in the number of PC– specific antibody-secreting cells (ASC) on day seven post-exposure, compared to only HKSP group (Salazar *et al.*, 2005, 2006). Both male and female mice had increases; however, in females, the increase was revoked by ovariectomy (OVX), but not by orchidectomy in males (Salazar *et al.*, 2006). Because of the known effects of steroids on the immune system, experiments testing specific steroid hormones produced by ovaries were designed to examine the interaction with propanil on the PC-specific, ASC increase (Salazar *et al.*, 2006; Holásková *et al.*, 2015). Utilizing the “remove and replace” model, exogenous estradiol given to OVX mice did not restore the effect of the propanil-mediated enhancement on PC-specific ASC increases. Estrogen receptor alpha antagonist, ICI 182,780, did not prevent the PC-specific, ASC increase (Salazar *et al.*, 2006), demonstrating the HKSP/PC-specific ASC increase may be happening independently of the estrogen receptor. This was supported further by the findings propanil did not bind to either ER- α or ER- β at day 7 in female mice (Kojima *et al.*, 2004, Salazar *et al.*, 2006).

Similarly, subcutaneous treatment with progesterone antagonist RU486, given two hours before propanil, 12 hours after propanil and every 24 hours during the duration of the experiment, did not prevent the PC-specific ASC increase (Salazar *et al.*, 2006). This was evidence that blocking endogenous progesterone did not block the PC-specific ASC rise in the spleen. Treatment of mice with testosterone propionate, given

to female pups peri- and postnatally to “masculinize” the spleen response, altered neither the rise in ASC in propanil-treated, HKSP-challenged female nor OVXed mice (Holásková *et al.*, 2015). The masculinization of reproductive function was evident by highly irregular estrous cycles determined by vaginal lavage, but the masculinization of the PC-specific ASC response did not occur. The evidence suggested the ovarian-spleen relationship in HKSP/propanil – treated females occurs independently of testosterone as well. Removal of endogenous testosterone source by orchidectomy did not affect the PC-specific ASC response in the spleen of male mice (Salazar *et al.*, 2006).

A weak antiandrogenic function of propanil was demonstrated using the androgen receptor assay, and propanil was shown to inhibit transcriptional activity by dihydrotestosterone (DHT) in mouse hepatoma Hepa1c1c7 cells (Kojima *et al.*, 2004). DHT is the most active form of testosterone; therefore, they hypothesized propanil inhibits the transcriptional activity of DHT by blocking the androgen receptor, which is a nuclear receptor. Their data provided evidence that propanil may show antiandrogenic activity *in vitro*. Contrasting were the findings with propanil metabolite DCA, using *ex vivo* androgen-sensitive, calf uterus cytosol assay, where DCA misplaced only a minuscule fraction of [³H] dihydrotestosterone (DHT) on the androgen receptor, 6.2×10^{-5} (Bauer *et al.*, 1998). In summary, the experimental evidence eliminated the main ovarian steroids, such as estrogen, progesterone, and testosterone, as possible mediators of the sexually dimorphic PC-specific ASC response.

However, an injection of antide, an antagonist of gonadotropin-releasing hormone (GnRH), into female mice attenuated the increase ASC (Salazar *et al.*, 2006)

at day seven after exposure to propanil, similar to the effect to OVX in mice. Therefore, propanil may interact with the hypothalamus-pituitary-gonadal axis (HPG axis) to increase ASC. Known target tissue of GnRH is the pituitary gland (Senger, 2012); nonetheless, GnRH receptors are present on other tissue, for example, spleen (Jacobson *et al.*, 1998) and ovary (Torrealdy *et al.*, 2013). In thymus and spleen, GnRH receptors (mRNA) are detected in a pattern similar to the pituitary gland, suggesting the estrous cycles and feedback mechanisms of steroid and nonsteroidal hormones might be affecting immune function as well (Jacobson *et al.*, 1998). DBA/2 mice had an increase in GnRHR mRNA in the evening of proestrus when represented with an autoradiograph of competitive RT-PCR analysis of GnRHR in the spleen and thymus while only showed an increase in GnRHR during estrus in the thymus (Jacobson *et al.*, 1998). Mice that were in proestrus or estrus showed to have a significant increase in GnRH in both the thymus and the spleen (Jacobson *et al.*, 1998). GnRH is barely detectable in adult circulation but is very high during fetal development. It was demonstrated that GnRH signaling is involved in thymic development (Melnikova *et al.*, 2019).

Propanil exposure during pregnancy

There are a few reports on the effects of propanil on reproductive functions or hormones during pregnancy in mammals. In a chronic oral toxicity study (Ambrose *et al.*, 1972), three generations of albino rats had 0, 100, 300 or 1,000 ppm of propanil incorporated into their diet before mating and during pregnancy, parturition, lactation, and various gestational and histopathological indexes, including fetal autopsies, were examined. No deleterious effects on any reproduction performance were observed in

any of the diet groups of propanil-treated rats studied. (Ambrose *et al.*, 1972). However, at a higher dose (1,600 ppm) and chronic oral exposure of rats over 2 years, an increase in testes organ-to body weight ratio in males, as well as increase spleen size of male and female rats, were noticed. In the same report, subchronic oral toxicity, feeding 10,000 ppm propanil to rats for three months resulted in increased testes, heart, and spleen weight in males (Ambrose *et al.*, 1972).

To determine pregnancy outcome after being exposed to propanil, researchers injected female rats with three different doses on different days of pregnancy. Rats were orally given propanil at 175 mg/kg per day and 350 mg/kg per day on days one through three of pregnancy had a decrease in the number of implants and the number of pups born and an increase in pre-implantation loss (Ratnasooriya and Perera, 1997). A very similar pattern of embryonic loss occurred when the higher dose (350 mg/kg per day) of propanil was given to rats in the first two days of pregnancy (Ratnasooriya and Perera, 1997). Propanil needed to be administered at least two consecutive days at a dose of 350 mg/kg to see a response in pregnancy variables. Three different mechanisms could explain the embryonic losses: Firstly, propanil could have a luteolytic effect on CL and cause functional regression of CL and the loss of progesterone-production. Decreased size and altered morphology (color) or the rat corpora lutea accompanied pre-implantation and post-implantation losses (Ratnasooriya and Perera, 1997). Altered serum estrogen and progesterone balance can prevent implantation (Bonney and Franks, 1990). The second possible mechanism of propanil could be alteration in the speed of embryonic transport mediated by a decrease of estrogen (Kaplan-Kraicer *et al.*, 1995). Estrogen stimulates the motility of the oviduct and propelling the fertilized egg

towards the uterus. If propanil decreased estradiol, that would reduce the rate of embryonic transport that could lead to an increase in preimplantation loss. However, propanil does not decrease estrogen directly as it does not bind to the estrogen receptor α or β (Salazar *et al.*, 2006), making this mechanism unlikely. The indirect mechanism of the effect of propanil on estrogen by modifying the availability of key steroid-synthesis enzymes, like aromatase (a.k.a. CYP19A1, **Figure 2**) remains an option to be examined. The third possibility could be the interruption in uterine blood supply (Antebi *et al.*, 1991). Lack of oxygen-carrying capacity via methemoglobinemia or decrease in red blood cells due to propanil (McMillan *et al.*, 1990, 1991) could be detrimental to the CLs, developing embryos or post-implantation fetuses. Clinical evidence from deliberate poisoning reported a case of pregnant females losing pregnancies (Eddleston *et al.*, 2002).

Freshwater snails exposed to propanil at a concentration of 10⁻⁵ M had reduced egg-maturation and an increase in dead embryos by the end of the twentieth day and overall delayed development of embryos (Kosanke *et al.*, 1988). Propanil affected length, survival, and fecundity of small planktonic crustacean, *Daphnia magna*, treated in water for 21 days with different doses (Villarroel *et al.*, 2003). They also showed a dose-dependent decrease in days to first brood, the number of young ones per adult, brood size, and the number of broods per adult with the most significant decrease at the highest dose of 0.55 mg/l.

In summary, propanil has adverse effects on pregnancy. The exact mechanism is not defined yet, but steroid synthesis, luteolytic action, or lack of oxygen may be involved.

The connection between the spleen and ovary

When ovulatory gonadotropin stimulation occurs in the ovary, it activates an apparent local acute inflammatory response (Espey, 1980). This inflammatory response during ovulation is similar to the response seen during an infection (Oakley *et al.*, 2011). Leukocytes are involved in three main phases of ovarian function. These functions include loosening of the follicular wall, which allows the follicle to grow and ovulate, tissue repair after the follicle ruptures (ovulation), and lastly, the formation of the CL and its regression after progesterone decreases (Oakley *et al.*, 2011). Leukocytes are recruited from the spleen to the ovary after the release of cytokine from the inflamed tissue around the ovulated follicle (Oakley *et al.*, 2011). Therefore, the authors' suggested the connection between the ovary and spleen during ovulation be called the pituitary-ovarian-splenic axis. They hypothesized that leukocytes carrying specific markers traverse from the spleen and are attracted to chemokines produced by follicles. The leukocytes then move from the capillary into the ovary to increase the number of leukocytes. They suggested that the spleen is a bridge between the immune and reproductive systems by serving as a reservoir for leukocytes needed for ovulation. Collectively, data available on propanil and reproductive function are sparse and inconsistent. Determination of the effect of propanil in the HKSP/propanil murine model on ovaries and ovarian function-related hormones would aid in understanding the interaction of reproductive and immune systems in the face of immune/chemical challenge.

Summary

Overall, propanil affects the circulatory, reproductive, endocrine, nervous, and immune systems. Interactions of these systems are evident but not well understood. When studying the connection between the spleen and the female reproductive system, HKSP can be useful for challenging the murine immune system to enhance the impact propanil has on the immune and reproductive target tissues. Steroid synthesis (hormones and enzymes for progesterone, testosterone, and estrogen), neuropeptides (GnRH and oxytocin), glycoproteins (FSH, LH, activin, and inhibin), protein (prolactin) and prostaglandin (PGF_{2α} and PGE₂) hormones may all be possibly affected by propanil. Subsequently, the affected hormone(s) may then signal immune cells in the spleen and affect the initial decrease and later increase in the spleen size and the number of HKSP/PC-specific ASC. Therefore, the experiments were designed to determine whether ovarian, systemic, and splenic factors are expressed differentially 24 hours after propanil/HKSP immune/chemical challenge and could indicate early changes that can influence the spleen HKSP/PC-specific ASC numbers in spleen seven days later. Individually, we examined the effect of propanil/HKSP on 1) ovarian genes that are activated or suppressed, 2) effects on systemic hormone concentrations, 3) splenic cell populations, and 4) involvement of GnRH antagonist in this model of endocrine disruption/immune challenge.

Chapter 2: Statement of Problem

A preliminary step in detecting compounds that ovaries secrete used a working model of propanil (200 mg/kg/BW i.p.), vaccine HKSP (Salazar *et al.*, 2005, 2006; Holásková *et al.*, 2015) and microarray data analysis (murine Affimetrix chip, Cleveland Clinic, unpublished, Dr. Schafer). Ovarian mRNA expressions of 106 genes were significantly modified at 24 hours due to propanil. Sixty-four were upregulated, and 42 were downregulated. Top genes upregulated included inflammatory markers, cytokines, and chemokine immune regulators, transcription factors, and adhesion molecules, and are listed with their abbreviation and known ovarian function in parentheses (determined using Ingenuity Pathway Analysis, Qiagen, Hilden, Germany): Serum Amyloid A 3 (SAA3, ovulation), S100 calcium binding protein A8 and A9 (S100A8 and S100A9, both involved in ovulation); chemokine (C-C motif) ligand 7 (CCL7), serpin family A member 3 (SERPINA3); chitinase 3 like 1 (CHI3L1), guanylate cyclase activator 2B (GUCA2B), lipocalin 2 (LCN2), complement factor B (CFB), glycosylation dependent cell adhesion molecule 1 (Glycam 1), vascular cell adhesion molecule 1 (VCAM1), C-X-C motif chemokine ligand 2 (CXCL2), suppressor of cytokine signaling 3 (SOCS3, involved in oocyte maturation and luteolysis), C-C motif chemokine ligand 2 (CCL2, involved in luteolysis), interleukin 18 receptor 1 and intercellular adhesion molecule 1 (ICAM1, follicle rupture, ovulation, luteinization and luteolysis).

Top genes decreased due to propanil in 24 hours in murine ovaries included enzymes involved in steroid synthesis, such as cytochrome P450 family 11 subfamily B member 2 (Cyp 11B2, involved in corticosterone synthesis), dehydrocholesterol

reductase (DHCR7), cytochrome P450 family 17 subfamily A member 1 (CYP17A1, involved in hydroxy-progesterone conversion from progesterone), ADAM metalloproteinase with thrombospondin type 1 motif 1 (ADAMTS1, involved in ovarian follicle development, ovulation, follicle rupture and luteinization), carbonic anhydrase 3 (CA3), melanocortin 2 receptor accessory protein (MRAP), X-prolyl aminopeptidase (XPNPEP2), complement factor D (CFD), and endothelin 2 (EDN2).

Based on the literature and preliminary analysis of microarray data, there are two aims of the proposed research. The first aim is in light of evidence that the presence of the ovaries is vital for the increase in the number of PC-specific antibody-secreting cells in the spleen on Day 7 (Salazar *et al.*, 2006). Thus, we first determined the acute effect of propanil on the changes in expression of genes in ovaries and oviducts, systemic concentrations of specific reproductive hormones, and splenic weight and cell populations. We hypothesize that ovarian and oviductal genes involved in ovulation, inflammation, steroid hormone synthesis, and tissue remodeling are modified at 24 and 72 hours after propanil/HKSP injections in female mice. We hypothesize that prolactin, progesterone, and estradiol are affected by propanil. Also, even though adaptive immune response (rise in PC-specific-ASCs) cannot be evident in the spleen in such a short time, we expect that the immune cell populations vary in composition and number due to propanil as early as 24 or 72 hours. The experiments testing these hypotheses comprise Trials 1 and 2.

The second aim is inspired by the fact that an intact HPG axis was necessary for the increase of HKSP/PC-specific antibody-secreting cells response in the spleen on Day 7 (Salazar *et al.*, 2006). We examined the interaction of GnRH (using a GnRH

antagonist, antide) and propanil on the expression of genes in the ovaries and oviducts, systemic concentrations of specific reproductive hormones, and splenic weight and cell populations.

We hypothesize that GnRH is involved in the propanil-mediated effects. Therefore, antide (GnRH antagonist) would interfere with propanil influences on ovarian gene expression, spleen cell populations, and reproductive hormones indicating that an intact HPG axis is necessary for the acute propanil-mediated responses. The experiment testing these hypotheses is Trial 3.

Chapter 3: Materials and Methods

Animals. Eighty-four of 6-9 weeks old C57Bl/6 female mice (trial 1, n=24; trial 2, n=12; trial 3, n=48), weighed 18 to 20 grams (Jackson Laboratory, Bar Harbor, ME), were housed 3-4 per cage at West Virginia University (WVU) vivarium. The mice were on 12 hour-light and dark cycles and provided food and water *ad libitum*. The mice were acclimated to the WVU vivarium for 5 days before the start of an experiment. All procedures were approved by the WVU Institutional Animal and Care Committee (IACUC # 1603001079.8).

HKSP preparation. *S. pneumoniae* strain R36A is an avirulent, non-encapsulated bacterial strain used as an antigen to activate the immune system. The vaccine was prepared as described previously (Wu *et al.*, 1999; Salazar *et al.*, 2005). *S. pneumoniae* strain R36A was grown to mid-log phase in Todd–Hewitt broth supplemented with 2% yeast extract (BD Biosciences) at 37 °C. The culture was grown to OD₆₀₀ of 0.4, and immediately heat-killed at 60°C for 1 h, washed twice in 0.85% saline and resuspended in saline at 2×10^8 CFU/ml. Sterility and the number of CFU were confirmed by culture on blood agar plates before the killing, and after, the heat-killed stock was stored at -80 °C.

HKSP immunization and propanil exposure. The schedule of immunizations and treatments for all three trials are in **Figure 3**. Mice were treated with 200 mg/kg propanil (N-10800-500MG, Chem Service, West Chester, PA) dissolved in peanut oil or peanut oil alone (control) and HKSP (2×10^8 CFU) dissolved in PBS. The injections were given intraperitoneally (i.p.) at hour 0. Mice were monitored throughout the day for signs

of lethargy until hour 8 (Chow and Murphy, 1975). The mice were euthanized with Euthasol (100 mg/kg, i.p.) (Henry Schein, Inc., Melville, NY) at 24 and 72 hours (Trial 1) or only at 24 hours (Trial 2 and 3) after treatment. Immediately after euthanasia, blood, ovaries, and spleen (Trial 1 and 3) or blood, ovaries, oviducts, and spleen (Trial 2) were collected.

Antide. In trial 3, mice were treated with 60 mg of antide dissolved in 0.9% propylene glycol or 0.9% propylene glycol alone (control) every other day for a total of six injections. The injection was given subcutaneously between 1300-1400 hours.

Prolactin, Progesterone, and Estradiol ELISA. Blood was collected by cardiac puncture after euthanasia at 24-hours or 72-hours after treatment. Serum prolactin levels were determined using a mouse/rat prolactin ELISA kit (PR063F-100), mouse/rat estradiol ELISA kit (ES180S-100), and progesterone ELISA kit (PG362S, Calbiotech, Spring Valley, CA). Samples were measured at 450 nm using an Epoch Microplate Spectrophotometer (BioTek, Winooski, VT). AssayZap (Biosoft, Great Shelford, Cambridge) was used to determine concentrations.

Figure 3. Timeline for experimental design. Trial 1 and 2 (A) and trial 3 (B).

Splenocyte Isolation and Flow Cytometry. Single-cell suspensions of splenocytes were generated using tissue dissociation through nylon mesh (89000-044, VWR International, Indianapolis, IN) in RPMI-1540 (BioWhiteaker, Walkersville, MD), 10% heat-inactivated fetal bovine serum (FBS, Hyclone Laboratories, Inc, Logan, UT), 10 mM HEPES (Sigma), 1mM L-glutamine (Gibci, Rockville, MD), 5×10^{-5} M 2-

mercaptoethanol (Sigma), 100 U/ml penicillin (Gibco) and 100 mg/ml streptomycin (Gibco). Red blood cells were lysed with Tris-buffered ammonium chloride. Cell viability and quantity were enumerated with trypan blue dye exclusion, and splenocytes from individual mice (1×10^6) were stained with fluorochrome-conjugated antibodies: CD4-FITC (553729, BD Bioscience, San Jose, CA), CD8- PE (561095), B220- Pacific blue (558108) and CD11b- Alexa Fluor 700 (56-0112-80) for phenotypic analysis using flow cytometry at 24 hours. Staining was performed by adding 1×10^6 cells to individual wells on a 96-well plate and centrifuged for 5 minutes at 1500 rpm. The supernatant was removed, and the cells were resuspended in 100 μ l of flow cytometry buffer (PBS with 1% FBS and 0.04% sodium azide). The cells were centrifuged again, and 25 μ l of the specific antibody was added to each well. The plate with cells was then wrapped in foil and left on ice for 20 minutes. The cells were washed twice with flow cytometry buffer to remove the unbound antibodies. The cells were then fixed with 100 μ l of paraformaldehyde, wrapped in foil and stored at 4°C overnight. Data were acquired using a FACS Aria III, employing FACS Diva software version 8.0 (BD Biosciences, USA) and analyzed using FCS Express software version 5 (DeNovo Software, Glendale, CA). A total of 10,000 events were collected and analyzed for each sample, including controls, including individual cell stains and no stain. The percentage of particular cell types detected by flow cytometry multiplied by the total number of cells determined by hemocytometer gave the cell numbers for each mouse.

RNA Extraction and Quantitative Real-time PCR (qRT-PCR). Total RNA was extracted from frozen whole ovaries (Trial 1) or oviductal tissue (Trial 2) using the ENZA Total RNA Kit I (catalog number R6834-01, Omega BIO-TEK, Norcross, GA). After RNA

extraction, High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, catalog number 4368814) was used to transcribe RNA into cDNA for real-time PCR. Primers used from PrimerBank were purchased from Invitrogen (**Table 1**). The PCR reactions consisted of 15 µL PowerUp SYBR green master mix (Applied Biosystems, Foster City, CA, catalog number A25778,) and 5 µL of sample developed during an initial temperature of 50°C for 2 minutes then 95°C for 2 minutes followed by 40 cycles consisting of melting at 95°C for 15 seconds and annealing and extension at 60°C for 1 minute using the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA). CFX Maestro (Bio-Rad, Hercules, CA) software was used to collect the data. Qbase+ (version 3.2, Biogazelle, Zwijnaarde, Belgium) geNorm function was completed to determine the most suitable housekeeping genes from a panel of commonly used housekeeping genes (*Hprt1*, *Gapdh*, *Actb*, *18s*, *B2m*, *Hmbs*, *Ppia*, and *Sdha*). Qbase+ was used to normalize the qRT-PCR data using *Hprt1* and *Actb* as the two housekeeping genes. Qbase+ uses a generalized delta-delta quantification cycle method ($\Delta\Delta C_q$, also known as $\Delta\Delta C_t$), where reads were normalized to both housekeeping genes (Hellemans *et al.*, 2007). The equation to determine the relative quantity (RQ) of each gene was the following $RQ=2^{(35-C_t)}$. The RQ values were divided by the geometric mean of the control genes relative quantity to obtain the normalized relative quantity (NRQ) then multiplied by 100. After the relative expression was determined, the data was log-transformed before statistical analyses.

Ovarian Histology. Ovaries were removed after euthanasia, and the left ovary was frozen in liquid nitrogen for the RNA isolation while the right ovary was placed into 40% formalin for assessment of ovarian histology. Formalin- preserved ovaries were

processed and stained with hematoxylin and eosin (H&E) at the WVU histology core. Namely, the ovaries were embedded in paraffin, sectioned to 5 μ m sections until the entire ovary was used up, keeping the ribbon of the slices intact until mounting. Three serial sections were mounted on each slide, and the first and sixth of the consecutive slides from each mouse were stained for a total of six ovarian H&E sections per mouse. Photographs of the entire ovarian area of six sections were taken using a Primo Star Microscope with AxioCam ERc5s camera (Zeiss, Thornwood, NY). The size of the ovarian area was estimated using ImageJ software (NIH, Bethesda, MD) on all H&E sections of each ovary and average size estimated from six sections for the entire ovary. Similarly, numbers of primary, secondary, and antral follicles were determined at each of the six stained sections and averaged per ovary.

Analysis. All continuous variables were confirmed for normality of distribution by the Shapiro-Wilk W test. Most continuous variables in Trials 1 and 2 (weights, total splenocytes counts, hormonal concentrations, and gene expressions) were analyzed using 2x2 factorial ANOVA testing main effect of treatment (HKSP, HKSP + propanil), time (24 hours, 72 hours) and their interactions. A significant F-test was followed by multiple comparison tests- Tukey's honestly significant difference (HSD). All flow cytometry data (% and counts), were analyzed by two-sided t-tests comparing HKSP and HKSP + propanil at 24 hours. Due to a large number (37) of similar analyses, Benjamini-Hochberg adjustment to multiple analyses was completed using a false discovery rate of 0.1 (Benjamini and Hochberg, 1995). The continuous variables from Trial 2 (weights and gene expressions) were analyzed by a two-sided t-test comparing

HKSP and HKSP + propanil at 24 hours, followed by Benjamini-Hochberg adjustment to multiple analyses using a false discovery rate of 0.1.

All continuous variables from Trial 3 (weights, splenocytes counts, flow cytometry percent and counts, hormonal concentrations, counts of ovarian follicles, ovarian area, and gene expressions) were analyzed using 2x2 factorial ANOVA testing main effect of propanil (peanut oil, propanil), antide (propyl. glycol, antide) and their interactions within the saline-treated and separately within HKSP-treated mice. In case a significant F-test was found, Tukey's Honestly Significant Differences (HSD) was determined. The saline-treated mice (without HKSP) served as an overall reference, as it was expected the HKSP, as an immune challenge, affects responses in a significant way, and statistical comparison of HKSP vs. saline (its control) was not the intention of this research.

All statistical analyses were conducted in JMP (JMP® version Pro 14.0, SAS Institute, Cary NC), and the significance criterion alpha for all tests was 0.05.

Chapter 4: Results

The first aim of these studies was to determine the acute effect of propanil with HKSP at 24 and 72 hours after exposure on splenic cell populations, reproductive hormones, ovarian, and oviductal gene expression. Experiments (trials 1 and 2) were designed to test the hypotheses that propanil not only affects the above tissues but also that this effect may be relatively short-lasting, thus different at hour 24 vs. 72 post-exposure. Therefore, the interaction of treatment (propanil vs. control) and time (24, 72 hours) in HKSP injected mice were tested. The second aim was to estimate if the acute effect of propanil at hour 24 is mediated by Gonadotropin-releasing hormone (GnRH) by utilizing GnRH antagonist, antide, and measuring the same dependent variables as in hypotheses of aim one. The main effect of antide and interaction of propanil and antide on immune and reproductive tissues from C57Bl/6 female mice challenged with or without HKSP, in a similar model as above, where the purpose of trial 3. **Table 2 lists** significant findings and least square (LS) means standard error of the means (SEM) from trials 1 and 2. Statistical analyses (factorial ANOVA) were done, and results (p-values, LS means and SEM) from trial 3 are reported separately for groups without HKSP, listed in **Table 3**, and groups with HKSP, detailed in **Table 4**. Figures from all trials are interwoven together.

Propanil was related to a many-fold increase in spleen size on day 7 (Barnett *et al.*, 1992b; Salazar *et al.*, 2005, 2006). We observed about 20% reduction in spleen weight at 24 hours but not at 72 hours (**Figure 4 A, B**), supported by more than 50% reduction of the total number of splenocytes (**Figure 5 A, B**). The antide effect was

evaluated to test if the GnRH antagonist could modify these results. In mice without HKSP, the reduction of spleen size due to propanil was not significant and not affected by antide. In mice immunized with HKSP regardless (with or without) of antide propanil caused near 20% decrease in spleen weight (**Figure 4 C**). Antide, however, increased spleen weight in mice treated with HKSP and corn oil (without propanil).

Parallel to decrease in spleen weight, propanil exposure decreased the total number of spleen cells, but only in HKSP (**Figure 5 A and B**) at 24 hours. Overall control, reference treatment mice (without HKSP, antide, or propanil) had about $33 \pm 8 \times 10^6$ splenocytes. In HKSP-challenged, without propanil or antide, the counts were similar, although more variable, 37 ± 15 Million splenocytes. However, mice treated with HKSP and antide with or without propanil had double the number of splenocytes (about 65 Million) compared to the two groups treated without antide (about 30 Million) (**Figure 5 B, HKSP side**), which would suggest GnRH involvement in the number of splenocytes. Multiple comparisons detected a significantly highest increase of splenocyte number (89 ± 15 Million splenocytes) in a group with HKSP, with antide and without propanil, over a group with HKSP, without antide, and with propanil.

Adaptive immune response, marked by a detected increase in antigen-specific antibodies takes typically 5-7 days (Owen *et al.*, 2013) In the spleen of propanil/HKSP-treated mice, PC-specific IgG and IgM ASC peaked on day 7 (Wu *et al.*, 1999; Salazar *et al.*, 2005). We hypothesized that propanil might modify the elemental composition of the spleen as early as the second day after HKSP/propanil challenge. Flow cytometry results and LS means (cell percent and counts) and SEM of t-test comparing treatments at 24 hours are listed in **Table 2**. The specific cell populations affected by propanil

include B cells (**Figure 6**), CD4⁺ T cells (**Figure 7**), CD8⁺ T cells (**Figure 8**), and CD11b⁺ cells (**Figure 9**). The percentage composition of the spleen was modified, such as percent of B-cells decreased (**Figure 6A**), while the percent of CD4⁺ T-cells did not change, (**Figure 7A**), percent of CD8⁺ T-cells increased (**Figure 8A**) and percent of granulocyte/monocytes (**Figure 9A**) increased due to propanil in trial 1. Decreased numbers in all four main cell types (**Figures 6-9 B**), such B-cells, CD4⁺ and CD8⁺ T-cells, and granulocyte/monocytes cells, due to propanil, matched the decrease in total cell number. Within the mice vaccinated with HKSP and exposed to antide, there was an increase in the number of B cells, indicated by the significant main effect of antide. On average, antide-treated mice had almost the double number of B-cells than HKSP mice without antide (regardless of propanil). Specific multiple comparisons pinpointed the difference mostly due to the antide without propanil group registering the highest number of B-cells (about 63 ± 12 Million) compared to without antide, propanil-treated, HKSP-challenged mice (about $13 \text{ Million} \pm 12$ B-cells) (**Table 4, Figure 6 D**).

T-cell function in thymus on days 2-4 (Cuff *et al.*, 1996; Rosa *et al.*, 2005), in the spleen on day 7 (Barnett and Gandy, 1989; Zhao *et al.*, 1998) and *in vitro* functional assays with IL-2 and its intracellular transcription factors (24-48 hours) (Brundage *et al.*, 2004; Lewis *et al.*, 2008, 2013) were examined in propanil-treated mice and overall, general, decreased numbers and suppressed T-cell function/activation were observed. In this experiment, mice vaccinated with HKSP had a decrease in the splenic number of CD4⁺ T cells (**Figure 7 B**) 24 hours after exposure to propanil. The possible relationship of GnRH and T-cells stems from the evidence that GnRH receptor and its mRNA in spleen (and in thymus) was observed to fluctuate in a pattern identical to pituitary

changes of GnRH receptor due to the cyclical hormonal changes (Jacobson *et al.*, 1998) and GnRH is involved in T-cell development in thymus (Melnikova *et al.*, 2019). Mice exposed to saline and antide had a decrease in percent CD4⁺ cells (**Figure 7 C**), suggesting that even without immune challenge, GnRH can affect the T-cells. Mice vaccinated with HKSP and exposed to propanil had almost a 50% decrease in the number of CD4⁺ cells (**Figure 7 D**).

The percentages of CD8⁺ T cells (**Figure 8 A**) populations were enhanced by propanil while the number of CD8⁺ T cells (**Figure 8 B**) was decreased at 24 hours. Antide in the main effect decreased the percent of CD8⁺ cells in mice treated with saline (without HKSP) (**Figure 8 C**). However, the statistical significance *versus* the biological significance of the decrease in the percent of CD8⁺ T cells from about 3.5% to 2.5% needs to be addressed. The number of CD8⁺ cells increased in mice treated with HKSP and antide with or without propanil (**Figure 8 D**). Multiple comparisons within the HKSP-challenged group detected a higher number of CD8⁺ T cells in antide group without propanil than without antide with propanil.

CD11b is a marker expressed on the surface of many leukocytes, including monocytes, neutrophils, natural killer cells, granulocytes, and macrophages, and about 8% of spleen cells. Lytic activity of NK cells from spleen was decreased with 50 mg/kg dose of propanil on day 7 (Barnett *et al.*, 1992), but numbers or percent different font of CD11b⁺ cells were not enumerated on in 24 hours after propanil. Results indicated the mice treated with propanil showed an increase in the percent of CD11b⁺ cells (**Figure 9 A**) but a decrease in the number of CD11b⁺ cells (**Figure 9 B**). In a macaque corpus luteum, antide treatment increased the number of CD11b⁺ cells (Bishop *et al.*, 2015).

Within mice vaccinated with HKSP and exposed to antide, there was a main effect of antide demonstrated by a decrease in percent CD11b⁺ cells, more so in the group without propanil (**Figure 9 C**).

We hypothesized that propanil would modify hormones involved in ovarian function, and antide interfere with this effect. Concentrations of prolactin were higher in propanil treated than control in HKSP vaccinated mice at 24 hours (**Figure 10 A, B**); however, antide did not modify the propanil's effect on prolactin. The concentrations of progesterone were statistically enhanced only at 24 hours in propanil treated mice (**Figure 10 C**), demonstrated by a significant interaction of treatment and time. There was no significant difference in progesterone concentrations (**Figure 10 D**) after mice were treated with propanil and antide. There was not a significant difference in estradiol concentrations (**Figure 10 E**) in the propanil treated mice at either time point, while mice exposed to propanil without HKSP demonstrated a decrease in estradiol concentration (**Figure 10 F**) within the serum.

Efficacy of antide was verified by decreased ovarian weight and area and uterine weight shown both with or without HKSP (**Figure 11 A, B**, and **Figure 12, Figure 13**). This decrease likely happens indirectly via the HPG axis through the decreased output of luteinizing (LH) and follicle-stimulating hormone (FSH) from pituitary after lack of GnRH-stimulation with antide. However, GnRH receptors are also present in ovaries. The propanil effect on ovaries at 24 hours was tested as a first step in answering why are ovaries required to induce PC-specific ASC number in the spleen observed in earlier studies (Salazar *et al.*, 2006). Propanil caused a decrease in ovarian weight in

mice treated with saline (**Figure 11 A**). Hypotheses were tested about propanil affecting specifically chosen gene expression. Genes in the ovaries involved in steroid synthesis, ovulation, extracellular and remodeling molecules, as well as inflammatory markers, were studied, and the interaction of antide with propanil on the gene expressions was also examined.

Propanil caused a significant increase in *Dhcr7* (7-dehydrocholesterol reductase) mRNA abundance at 24 hours but not at 72 hours (**Figure 14 A**), established by a significant interaction of treatment and time. Antide decreased the mRNA expression of *Dhcr7* in mice treated in saline and HKSP (**Figure 14 B**). *Cyp17a1* (17 α -hydroxylase) mRNA expression decreased in the propanil treated mice at 24 hours (**Figure 14 C**), demonstrated by a significant interaction of treatment and time. A significant interaction of propanil and antide was observed in mice vaccinated with HKSP; a decrease in *Cyp17a1* mRNA that was observed in propanil treated mice was eliminated by antide injection (**Figure 14 D**). Mice treated with propanil demonstrated a decrease in gene expression of *Cyp19a1* (aromatase) mRNA expression at 24 hours (**Figure 14 E**). Mice vaccinated with HKSP and exposed to propanil decreased *Cyp19a1* (**Figure 14 F**) mRNA expression. Antide decreased the expression of *Cyp19a1* in mice vaccinated with HKSP. *Hsd17b2* mRNA abundance showed a significant effect of time between 24 and 72 hours (**Figure 14 G**). HKSP vaccinated mice had a decreased in *Hsd17b2* expression after being treated with antide (**Figure 14 H**).

Antide decreased the mRNA expression of *Cyp11b1* in mice vaccinated with HKSP, and the saline-treated mice (**Figure 14 I**). Mice treated with saline and antide or vaccinated with HKSP and antide showed a decrease in mRNA expression of *Hsd17b7*

when compared to mice treated without antide (**Figure 14 J**). Increase in expression of *Gnrhr* mRNA in mice treated with antide with or without HKSP (**Figure 1J K**).

The expression of *Irf1* increased in both saline-treated and HKSP-vaccinated mice treated with antide (**Figure 15 A**). Propanil increased expression of *Irf1* in mice treated with saline (**Figure 15 A**). Propanil increased expression of *Socs3* in mice vaccinated with HKSP while antide also increased expression in mice treated with saline (**Figure 15 B**).

Propanil increased the expression of *Lcn2* (**Figure 16**) at 24 hours but not at 72 hours after exposure. Antide increased expression of *Adamts1* (**Figure 17 A**), *Csf1* (**Figure 17 B**), and *Cxcl10* (**Figure 17 D**) in mice treated with saline or vaccinated with HKSP. In saline-treated mice, there was an increase in *Cxcl1* expression (**Figure 17 C**). In mice vaccinated with HSKP or treated with saline, antide increased the expression in mRNA of *Il10* (**Figure 17 E**), and *Tlr2* (**Figure 17 F**). A significant interaction of propanil and antide on *Saa3* mRNA expression was observed in mice vaccinated with HKSP; without antide no changes were observed due to propanil, but with the addition of antide, in peanut oil group had an increase in *Saa3* mRNA expression in ovaries, giving an impression the propanil decrease the *Saa3* in antide-treated mice (**Figure 17 G**).

Propanil had no effect on gene expression on any of the 22 genes analyzed (**Table 1**) from the oviductal tissue of female mice exposed to propanil and HKSP.

Gene Name	Gene Symbol	Forward Primer (5'→ 3')	Reverse Primer (5'→ 3')	Length	Efficiency (%)
Interferon regulatory factor 1	<i>Irf1</i>	ATGCCAATCACTCGAATGCG	CCTGCTTTGTATCGGCCTGT	203	94
Suppressor of cytokine signaling 3	<i>Socs3</i>	ATGGTCACCCACAGCAAGTTT	TCCAGTAGAATCCGCTCTCCT	145	101
Cytochrome P450 17A1	<i>Cyp17a1</i>	GCCCCAAGTCAAAGACACCTAAT	GTACCCAGGCGAAGAGAATAGA	159	96
Cytochrome P450 11B1	<i>Cyp11b1</i>	CAGATTGTGTTTGTGACGTTGC	CGGTTGAAGTACCATTCTGGC	164	108
Cytochrome P450 19A1	<i>Cyp19a1</i>	ATGTTCTTGGAATGCTGAACCC	AGGACCTGGTATTGAAGACGAG	150	99
Hydroxysteroid 17-Beta Dehydrogenase 2	<i>Hsd17b2</i>	ATGAGCCCCGTTTGCCTCTG	CCACAGGTAACAAGTCTTGGTC	244	93
Hydroxysteroid 17-Beta Dehydrogenase 7	<i>Hsd17b7</i>	CCACCTGTGTTTGGCGTGTA	GAGGTTGAATTGTGGATTAGGCA	217	93
7-Dehydrocholesterol reductase	<i>Dhcr7</i>	AGGCTGGATCTCAAGGACAAT	GCCAGACTAGCATGGCCTG	184	94
S100 calcium-binding protein A1	<i>S100a8</i>	AAATCACCATGCCCTCTACAAG	CCCCTTTTATCACCATCGCAA	165	93
C-X-C Motif Chemokine Ligand 1	<i>Cxcl1</i>	CTGGGATTACCTCAAGAACATC	CAGGGTCAAGGCAAGCCTC	117	98
C-X-C Motif Chemokine Ligand 10	<i>Cxcl10</i>	CCAAGTGCTGCCGTCAATTTTC	GGCTCGCAGGGATGATTTCAA	157	94
C-C Motif Chemokine Ligand 2	<i>Ccl2</i>	TTAAAAACCTGGATCGGAACCAA	GCATTAGCTTCAGATTTACGGGT	121	90
Colony Stimulating Factor 1	<i>Csf1</i>	GTGTCAGAACACTGTAGCCAC	TCAAAGGCAATCTGGCATGAAG	101	96
Lipocalin 2	<i>Lcn2</i>	TGGCCCTGAGTGTCTGTG	CTCTTGTAGCTCATAGATGGTGC	239	94
Serum Amyloid A 3	<i>Saa3</i>	TGCCATCATTCTTTGCATCTTGA	CCGTGAACCTTCTGAACAGCCT	248	90
Toll-like receptor 2	<i>Tlr2</i>	GCAAACGCTGTTCTGCTCAG	AGGCGTCTCCCTCTATTGTATT	231	100
A Disintegrin And Metalloproteinase with Thrombospondin Motifs 1	<i>Adamts1</i>	TACTGACTTCCTGGATAGTGGG	TGGCTCAAACCTGTAGCTTGTG	105	98
Carbonic Anhydrase 3	<i>Car3</i>	TGACAGGTCTATGCTGAGGGG	CAGCGTATTTTACTCCGTCCAC	125	93
Gonadotropin Releasing Hormone Receptor	<i>Gnrhr</i>	TGCTCGGCCATCAACAACA	GGCAGTAGAGAGTAGGAAAAGGA	114	110
Tumor Necrosis Factor, Alpha-induced Protein 3	<i>Tnfaip3</i>	CTGGATGTCAATCAACAATGGGA	ACTAGGGTGTGAGTGTTTTCTGT	116	102
Interleukin 6	<i>Il6</i>	AGCCAGAGTCCTTCAGAGAGA	TGGTCTTGGTCCTTAGCCAC	155	93
Interleukin 10	<i>Il10</i>	GCTCTTACTGACTGGCATGAG	CGCAGCTCTAGGAGCATGTG	105	106
B-Actin	<i>Actb</i>	GGCTGTATTCCCCTCCATCG	CCAGTTGGTAACAATGCCATGT	154	94
Hypoxanthine-guanine phosphoribosyltransferase	<i>Hprt1</i>	CAGTCCCAGCGTCGTGATTA	GGCCTCCCATCTCCTTCATG	167	93

Table 1. Primer sequences for genes analyzed by qRT-PCR. The names of genes analyzed, the sequences for the primers, length, and efficiency for all genes in all trials are listed in their respective columns.

Organ	Variable	24 Hours		72 Hours	
		HKSP	HKSP + Propanil	HKSP	HKSP + Propanil
Spleen	Spleen Weight (mg)	71.67±2.288 ^a	55.00±2.288 ^b	78.33±2.288 ^a	72.00±2.506 ^a
	Splenocyte Count	81x10 ⁶ ±10x10 ⁶ ^a	29x10 ⁶ ±10x10 ⁶ ^b	105x10 ⁶ ±10x10 ⁶ ^a	98x10 ⁶ ±12x10 ⁶ ^a
	Percent B220 ⁺ Cells	60.64±1.57 ^a	48.74±1.57 ^b	N/A	N/A
	Number B220 ⁺ Cells	49x10 ⁶ ±5x10 ⁶ ^a	14x10 ⁶ ±5x10 ⁶ ^b	N/A	N/A
	Percent CD4 ⁺ Cells	13.20±0.79 ^a	14.13±0.79 ^a	N/A	N/A
	Number CD4 ⁺ Cells	10x10 ⁶ ±8x10 ⁵ ^a	4x10 ⁶ ±8x10 ⁵ ^b	N/A	N/A
	Percent CD8 ⁺ Cells	3.95±0.22 ^a	5.79±0.22 ^b	N/A	N/A
	Number CD8 ⁺ Cells	3x10 ⁶ ±3x10 ⁵ ^a	2x10 ⁶ ±3x10 ⁵ ^b	N/A	N/A
	Percent CD11b ⁺ Cells	5.57±0.68 ^a	9.43±0.67 ^b	N/A	N/A
	Number CD11b ⁺ Cells	5x10 ⁶ ±5x10 ⁵ ^a	3x10 ⁶ ±5x10 ⁵ ^b	N/A	N/A
Blood	Prolactin (ng/ml)	99.06±17.19 ^a	172.36±17.19 ^b	94.58±17.19 ^a	102.78±18.84 ^{ab}
	Progesterone (ng/ml)	4.10±0.59 ^a	7.40±0.59 ^b	3.91±0.59 ^a	3.30±0.65 ^a
	Estradiol (pg/ml)	3.198±0.349 ^a	3.718±0.349 ^a	3.378±0.349 ^a	4.423±0.383 ^a
Ovaries	<i>Dhcr7</i> Expression	0.003±0.05 ^a	0.35±0.05 ^b	-0.17±0.05 ^{ac}	-0.20±0.05 ^c
	<i>Cyp17a1</i> Expression	-0.30±0.17 ^a	-1.08±0.17 ^b	0.63±0.17 ^c	0.75±0.19 ^c
	<i>Cyp19a1</i> Expression	-0.07±0.08 ^a	-0.50±0.08 ^b	0.37±0.08 ^c	0.10±0.09 ^{ac}
	<i>Hsd17b2</i> Expression	-0.04±0.06 ^{ab}	-0.21±0.06 ^b	0.11±0.06 ^a	0.17±0.07 ^a

Table 2. Effect of propanil and time after HKSP/propanil on spleen weight, spleen cell populations, systemic hormones, and ovarian gene expression for trial 1. Female C57Bl/6 mice were immunized with HKSP (2x10⁸ CFU/ml) and injected (i.p., 200 mg/kg) with propanil 24 or 72 hours before euthanasia. Heart-puncture-blood, spleen, ovaries, and oviducts were immediately collected (trials 1 and 2). Total spleen cell count was determined by hemocytometer and trypan blue exclusion. Hormones were measured in serum utilizing specific ELISAs. Total RNA from both ovaries was extracted, reversely transcribed to cDNA before the mRNA expression was measured by qRT-PCR for genes involved in

steroid synthesis, inflammation, or ovarian remodeling (listed in Table 1). A significant F-test in a two-way ANOVA was followed by a multiple comparison test -Tukey's honestly significant difference (HSD). Least Square means $\pm$ SEM for each treatment group are listed for only variables that were significantly affected by treatment or by the interaction of treatment and time. Superscripts correspond to HSD within each row, such that any pair with differing superscript letters are different from each other. For example, in prolactin measured in blood, group with superscript **a** (HKSP in 24 hours, 99.06 ng/ml) differs statistically from group with superscript **b** (HKSP+Propanil in 24 hours, 172.36), but the first group (HKSP in 24 hours, 99.06)^a is not different from the third group (HKSP in 72 hours, 94.58 ng/ml)^a that is why the third group has the same superscript, **a**. The fourth group (HKSP+Propanil in 72 hours, 102.78 ng/ml) has superscript **ab**, and since **a** in **ab** is the shared letter with group one (99.06)^a and three (94.58 ng/ml)^a, it indicates it is not different from either of those, and it is also not different from the second group, as they share the superscript **b**.

Organ	Variable	Significant Effect	Treatment			
			Saline (without HKSP)			
			Propyl. Glycol		Antide	
			Peanut Oil	Propanil	Peanut Oil	Propanil
Spleen	Spleen Weight (mg)	Propanil (0.0143)	71.233 ± 4.883	58.533 ± 4.883	79.5 ± 4.883	66 ± 4.883
	Splenocyte Count (1x10 ⁶)	NS	33.434 ± 8.419	22.304 ± 8.419	33.7 ± 8.419	28.552 ± 8.419
	Percent B220 ⁺ Cells	NS	54.308 ± 3.353	53.158 ± 3.353	62.192 ± 3.353	56.96 ± 3.353
	Number B220 ⁺ Cells (1x10 ⁶)	NS	18.975 ± 5.263	12.915 ± 5.263	20.816 ± 5.263	15.515 ± 5.263
	Percent CD4 ⁺ Cells	Antide (0.0087)	21.48 ± 1.337	23.72 ± 1.337	17.87 ± 1.337	19.555 ± 1.337
	Number CD4 ⁺ Cells (1x10 ⁶)	NS	6.449 ± 1.381	4.97 ± 1.381	5.95 ± 1.381	5.309 ± 1.381
	Percent CD8 ⁺ Cells	Antide (0.0286)	2.05 ± 0.272	2.8 ± 0.272	1.607 ± 0.272	1.793 ± 0.272
	Number CD8 ⁺ Cells (1x10 ⁶)	NS	0.988 ± 0.184	0.491 ± 0.184	0.665 ± 0.184	0.626 ± 0.184
	Percent CD11b ⁺ Cells	NS	11.61 ± 1.734	11.452 ± 1.734	8.257 ± 1.734	8.473 ± 1.734
	Number CD11b ⁺ Cells (1x10 ⁶)	NS	2.794 ± 0.464	2.22 ± 0.464	2.623 ± 0.464	2.123 ± 0.464
Blood	Prolactin (ng/ml)	NS	22.598 ± 5.25	35.571 ± 5.25	28.687 ± 5.25	31.325 ± 5.25
	Progesterone (pg/ml)	NS	5.859 ± 0.99	6.066 ± 0.99	6.198 ± 0.99	6.42 ± 0.99
	Estradiol (pg/ml)	Propanil (0.0232)	5.284 ± 0.524	4.031 ± 0.524	5.132 ± 0.524	3.809 ± 0.524
Uterus	Uterine Weight (mg)	Antide (0.0394)	71.4 ± 7.346	61.167 ± 6.706	51.16 ± 7.346	50.167 ± 6.706
Ovary	Ovarian Weight (mg)	Propanil (0.017) Antide (<0.0001)	8.667 ± 0.544	6.433 ± 0.544	5 ± 0.596	4.317 ± 0.544
	Ovarian Area (mm ²)	Antide (0.0243)	192.782 ± 30.895	193.317 ± 28.203	133.522 ± 28.203	107.099 ± 30.895
	<i>Adams1</i> Expression	Antide (0.0025)	-0.16 ± 0.053	-0.061 ± 0.053	0.131 ± 0.053	0.105 ± 0.053
	<i>Csf1</i> Expression	Antide (0.0055)	-0.126 ± 0.039	-0.05 ± 0.039	0.036 ± 0.039	0.079 ± 0.039
	<i>Cxcl1</i> Expression	Antide (0.0005)	-0.228 ± 0.051	-0.125 ± 0.051	0.104 ± 0.051	0.111 ± 0.051
	<i>Cxcl10</i> Expression	Antide (0.0095)	-0.161 ± 0.068	-0.078 ± 0.068	0.087 ± 0.068	0.136 ± 0.068
	<i>Cyp11b1</i> Expression	Antide (0.0371)	1.669 ± 0.536	0.562 ± 0.536	-0.323 ± 0.536	-0.125 ± 0.536
	<i>Cyp17a1</i> Expression	NS	0.194 ± 0.316	-0.584 ± 0.316	0.39 ± 0.316	0.242 ± 0.316
	<i>Cyp19a1</i> Expression	NS	-0.088 ± 0.119	-0.125 ± 0.119	0.154 ± 0.119	0.022 ± 0.119
	<i>Dhcr7</i> Expression	Antide (0.0048)	0.276 ± 0.081	0.137 ± 0.081	-0.21 ± 0.081	-0.003 ± 0.081
	<i>Gnrhr</i> Expression	Antide (0.0003)	-0.284 ± 0.072	-0.179 ± 0.072	0.193 ± 0.072	0.224 ± 0.072
	<i>Hsd17b2</i> Expression	NS	-0.022 ± 0.059	-0.07 ± 0.059	0.065 ± 0.059	0.065 ± 0.059
	<i>Hsd17b7</i> Expression	Antide (0.0074)	0.57 ± 0.255	0.537 ± 0.255	-0.404 ± 0.255	-0.305 ± 0.255
	<i>Il10</i> Expression	Antide (0.0005)	-0.275 ± 0.072	-0.148 ± 0.072	0.186 ± 0.072	0.193 ± 0.072
	<i>Irf1</i> Expression	Propanil (0.0053) Antide (<0.0001)	-0.134 ± 0.02	-0.08 ± 0.02	0.012 ± 0.02	0.112 ± 0.02
	<i>Saa3</i> Expression	NS	0.368 ± 0.344	-0.501 ± 0.344	0.197 ± 0.344	-0.286 ± 0.344
	<i>Socs3</i> Expression	Antide (0.0037)	-0.087 ± 0.044	-0.152 ± 0.044	0.03 ± 0.044	0.089 ± 0.044
	<i>Tlr2</i> Expression	Antide (0.002)	-0.152 ± 0.041	-0.047 ± 0.041	0.091 ± 0.041	0.079 ± 0.041

Table 3. Effect of antide after saline/propanil on spleen weight, spleen cell populations, systemic hormones, and ovarian gene expression for mice without the immune challenge. Female C57Bl/6 mice were repeatedly injected (s.q.) with antide (or propylene glycol as control), immunized with HKSP (2×10^8 CFU/ml) or saline and injected (i.p., 200 mg/kg) with propanil or peanut oil 24 hours before euthanasia. Heart-puncture-blood, spleen, ovaries, and uterus were immediately collected (trial 3). Total spleen cell count was determined by hemocytometer and trypan blue exclusion. Hormones were measured in serum utilizing specific ELISAs as described in methods. Total RNA from both ovaries was extracted, reversely transcribed to cDNA before the mRNA expression was measured by qRT-PCR for genes involved in steroid synthesis, inflammation, or ovarian remodeling (listed in Table 1). A significant F-test in a two-way ANOVA was followed by multiple comparison tests -Tukey's honestly significant difference (HSD). Least Square means $\pm$ SEM for each treatment group are listed for only variables that were significantly affected by treatment or by the interaction of treatment and time.

Organ	Variable	Significant effect	Treatment HKSP			
			Propyl. Glycol		Antide	
			Peanut Oil	Propanil	Peanut Oil	Propanil
Spleen	Spleen Weight (mg)	Propanil (<0.0001) Antide (0.0147)	73.667 ± 3.682	50.333 ± 3.682	88.167 ± 3.682	55.5 ± 3.682
	Splenocyte Count (1x10 ⁶)	Propanil (0.0437) Antide (0.0364)	37.013 ± 15.415	22.165 ± 15.415	89.919 ± 15.415	38.39 ± 15.415
	Percent B220 ⁺ Cells	NS	57.993 ± 3.434	55.953 ± 3.434	67.223 ± 3.434	60.343 ± 3.434
	Number B220 ⁺ Cells (1x10 ⁶)	Antide (0.0424)	22.701 ± 11.9	12.971 ± 11.9	63.03 ± 11.9	24.241 ± 11.9
	Percent CD4 ⁺ Cells	NS	19.622 ± 1.53	19.912 ± 1.53	15.482 ± 1.53	20.037 ± 1.53
	Number CD4 ⁺ Cells (1x10 ⁶)	Propanil (0.0473)	7.109 ± 2.295	4.152 ± 2.295	13.333 ± 2.295	6.592 ± 2.295
	Percent CD8 ⁺ Cells	NS	1.7 ± 0.205	1.753 ± 0.205	1.617 ± 0.205	1.907 ± 0.205
	Number CD8 ⁺ Cells (1x10 ⁶)	Antide (0.0151)	0.449 ± 0.206	0.296 ± 0.206	1.229 ± 0.206	0.785 ± 0.206
	Percent CD11b ⁺ Cells	Antide (0.0216)	9.58 ± 0.967	10.332 ± 0.967	6.428 ± 0.967	8.662 ± 0.967
	Number CD11b ⁺ Cells (1x10 ⁶)	NS	3.512 ± 1.242	2.431 ± 1.242	5.922 ± 1.242	2.901 ± 1.242
Blood	Prolactin (ng/ml)	Propanil (0.0173)	31.986 ± 8.519	56.48 ± 8.519	32.604 ± 8.519	52.311 ± 8.519
	Progesterone (pg/ml)	NS	6.386 ± 1.335	8.789 ± 1.335	6.23 ± 1.335	6.78 ± 1.335
	Estradiol (pg/ml)	Propanil (<0.0001)	6.225 ± 0.418	4.522 ± 0.418	5.931 ± 0.418	3.582 ± 0.418
Uterus	Uterine Weight (mg)	NS	81 ± 12.823	68.5 ± 12.823	46.017 ± 12.823	68.95 ± 12.823
Ovary	Ovarian Weight (mg)	Antide (<0.0001)	6.75 ± 0.476	6.233 ± 0.476	4.067 ± 0.476	4 ± 0.476
	Ovarian Area (mm ²)	Antide (0.008)	181.537 ± 21.175	202.436 ± 21.175	99.35 ± 21.175	159.816 ± 21.175
	<i>Admts1</i> Expression	Antide (0.0361)	-0.128 ± 0.049	-0.003 ± 0.049	0.068 ± 0.049	0.048 ± 0.049
	<i>Csf1</i> Expression	Antide (0.0319)	-0.059 ± 0.032	0.008 ± 0.032	0.04 ± 0.032	0.073 ± 0.032
	<i>Cxcl1</i> Expression	NS	-0.036 ± 0.069	-0.007 ± 0.069	0.021 ± 0.069	0.159 ± 0.069
	<i>Cxcl10</i> Expression	Antide (0.0183)	-0.02 ± 0.061	-0.153 ± 0.061	0.084 ± 0.061	0.105 ± 0.061
	<i>Cyp11b1</i> Expression	Antide (0.0123)	0.217 ± 0.276	-0.221 ± 0.276	-1.082 ± 0.276	-0.697 ± 0.276
	<i>Cyp17a1</i> Expression	Propanil (0.0002) Antide*Propanil (0.0088)	0.659 ± 0.149	-0.815 ± 0.149	0.179 ± 0.149	-0.266 ± 0.149
	<i>Cyp19a1</i> Expression	Propanil (0.013) Antide (0.0353)	0.444 ± 0.126	-0.105 ± 0.126	-0.023 ± 0.126	-0.277 ± 0.126
	<i>Dhcr7</i> Expression	Antide (0.0291)	-0.002 ± 0.12	0.221 ± 0.12	-0.131 ± 0.12	-0.288 ± 0.12
	<i>Gnrhr</i> Expression	Antide (0.0052)	-0.17 ± 0.077	-0.1 ± 0.077	0.199 ± 0.077	0.117 ± 0.077
	<i>Hsd17b2</i> Expression	Antide (0.049)	0.01 ± 0.078	-0.21 ± 0.078	0.108 ± 0.078	0.053 ± 0.078
	<i>Hsd17b7</i> Expression	Antide (0.0262)	0.022 ± 0.176	0.258 ± 0.176	-0.349 ± 0.176	-0.329 ± 0.176
	<i>Il10</i> Expression	Antide (0.0103)	-0.184 ± 0.067	-0.018 ± 0.067	0.147 ± 0.067	0.099 ± 0.067
	<i>Irf1</i> Expression	Antide (0.0002)	-0.054 ± 0.018	-0.017 ± 0.018	0.066 ± 0.018	0.096 ± 0.018
	<i>Saa3</i> Expression	Antide*Propanil (0.0215)	-0.213 ± 0.174	-0.031 ± 0.174	0.637 ± 0.174	-0.171 ± 0.174
	<i>Socs3</i> Expression	Propanil (0.0261)	-0.134 ± 0.054	0.098 ± 0.054	0.048 ± 0.054	0.108 ± 0.054
	<i>Tlr2</i> Expression	Antide (0.006)	-0.017 ± 0.031	-0.083 ± 0.031	0.079 ± 0.031	0.051 ± 0.031

Table 4. Effect of antide after HKSP/propanil on spleen weight, spleen cell populations, systemic hormones, and ovarian gene expression for mice treated with HKSP. Female C57Bl/6 mice were repeatedly injected (s.q.) with antide (or propylene glycol as control), immunized with HKSP (2×10^8 CFU/ml) or saline and injected (i.p., 200 mg/kg) with propanil or peanut oil 24 hours before euthanasia. Heart-puncture-blood, spleen, ovaries, and uterus were immediately collected (trial 3). Total spleen cell count was determined by hemocytometer and trypan blue exclusion. Hormones were measured in serum utilizing specific ELISAs. Total RNA from both ovaries was extracted, reversely transcribed to cDNA before the mRNA expression was measured by qRT-PCR for genes involved in steroid synthesis, inflammation, or ovarian remodeling (listed in Table 1). A significant F-test in a two-way ANOVA was followed by multiple comparison test -Tukey's honestly significant difference (HSD). Least Square means $\pm$ SEM for each treatment group are listed for only variables that were significantly affected by treatment or by the interaction of treatment and time.

Figure 4. Spleen weights post-HKSP immunization +/- propanil and antide

exposure. Whole spleens were weighed in grams then converted into milligrams. Trial 1 (A) included 24 and 72 hours, while trial 2 (B) only included 24 hours, and trial 3 (C) included 24 hours, and the mice were treated with +/- antide. Data were analyzed by two-way ANOVA followed by Tukey-HSD for (A) and (C, within Saline or HKSP group) and by t-test (B). Interaction of treatment and time (A) was detected. Statistical differences are denoted by different letters for trial 1 (A) and * represents $p \leq 0.05$ for trial 2 (B). Statistical differences in C are also denoted by different letters (saline treatment a-c and HKSP treatment d-f). Group means (bars) that do not share the same letter within the group (saline or HKSP) differ from each other. For example, within saline (left ½ of the graph C) group **b** differs from the group with **a**, but neither **ab** is different from **a**, nor it is different from **b**. Within HKSP (right ½ of the graph C), **d** is

different from either **e**, **d** is different from **f**, **e** is different from **f**, but the two **e** groups are not statistically different from each other.

Figure 5. The total number of splenocytes post-HKSP immunization +/- propanil exposure. Trial 1 (A) included 24 and 72 hours, and trial 3 (B) only included 24 hours, and the mice were treated with +/- antide. The spleen was removed from mice and processed into a single cell suspension. After lysis of RBC, leukocytes were counted on a hemocytometer using trypan blue exclusion. Data were analyzed by two-way ANOVA, followed by Tukey-HSD. Statistical differences are denoted by different letters trial 1 (a-b) and trial 3 (saline treatment a-c and HKSP treatment d-f). Group means (bars) that do not share the same letter within the group (saline or HKSP) differ from each other.

Figure 6. Percentage and the total number of B cells in the spleen 24 hours post-HKSP immunization +/- propanil exposure. The B cell populations from trial 1 (A and B) and trial 3 (C and D). Isolated splenocytes were analyzed by flow cytometry (A, C).

The total number of cells was multiplied by the percent of cells determined by flow cytometry (B, D). For trial 1 the data were analyzed by one-way ANOVA (* $p \leq 0.05$) and trial 3; the data were analyzed by two-way ANOVA followed by Tukey-HSD. Statistical differences are denoted by different letters (saline treatment a-c and HKSP treatment d-f). Group means (bars) that do not share the same letter within the group (saline or HKSP) differ from each other.

Figure 7. Percentage and the total number of CD4⁺ T cells in the spleen 24 hours post-HKSP immunization +/- propanil exposure. The CD4⁺ T cell populations from trial 1 (A and B) and trial 3 (C and D). Isolated splenocytes were analyzed by flow cytometry (A, C). The total number of cells was multiplied by the percent of cells determined by flow cytometry (B, D). For trial 1 the data were analyzed by one-way ANOVA (* $p \leq 0.05$) and trial 3; the data were analyzed by two-way ANOVA followed by Tukey-HSD. Statistical differences are denoted by different letters (saline treatment a-c and HKSP treatment d-f). Group means (bars) that do not share the same letter within the group (saline or HKSP) differ from each other.

Figure 8. Percentage and the total number of CD8⁺ T cells in the spleen 24 hours post-HKSP immunization +/- propanil exposure. The CD8⁺ T cell populations from trial 1 (A and B) and trial 3 (C and D). Isolated splenocytes were analyzed by flow cytometry (A, C). The total number of cells was multiplied by the percent of cells determined by flow cytometry (B, D). For trial 1 the data were analyzed by one-way ANOVA (* $p \leq 0.05$) and trial 3; the data were analyzed by two-way ANOVA followed by Tukey-HSD. Statistical differences are denoted by different letters (saline treatment a-c and HKSP treatment d-f). Group means (bars) that do not share the same letter within the group (saline or HKSP) differ from each other.

Figure 9. Percentage and the total number of CD11b⁺ T cells in the spleen 24 hours post-HKSP immunization +/- propanil exposure. The CD11b⁺ T cell populations from trial 1 (A and B) and trial 3 (C and D). Isolated splenocytes were analyzed by flow cytometry (A, C). The total number of cells was multiplied by the percent of cells determined by flow cytometry (B, D). Data were analyzed by two-way ANOVA, followed by Tukey-HSD. Statistical differences are denoted by different letters trial 1 (a-d) and trial 3 (saline treatment a-c and HKSP treatment d-f). Group means (bars) that do not share the same letter within the group (saline or HKSP) differ from each other.

Figure 10. Serum hormone concentrations post-HKSP immunization +/- propanil exposure. Trial 1 represents graphs A, C, E, and trial 3 represents graphs B, D, F. the tree hormone measured included prolactin (A, B), progesterone (C, D), and estradiol (E, F) at twenty-four hours after exposure. Data were analyzed by two-way ANOVA, followed by Tukey-HSD. Statistical differences are denoted by different letters trial 1 (a-d) and trial 3 (saline treatment a-c and HKSP treatment d-f). Group means (bars) that do not share the same letter within the group (saline or HKSP) differ from each other.

Figure 11. Effect of propanil and antide exposure in +/- HKSP immunization on ovarian weights and ovarian area. The whole ovaries (A) were weighed. Ovarian area (B) was measured with ImageJ from sections stained with H&E. Data were analyzed by two-way ANOVA, followed by Tukey-HSD. Statistical differences are denoted by different letters (saline treatment a-c and HKSP treatment d-f). Group means (bars) that do not share the same letter within the group (saline or HKSP) differ from each other.

Figure 12. Ovarian histology after mice received six doses of antide. Antide effect on ovarian area represented by histology mice treated with propylene glycol, peanut oil, and HKSP (A), mice treated with propylene glycol, propanil, and HKSP (B), mice treated with antide, peanut oil, and HKSP (C), and mice treated with antide, propanil, and HKSP (D) twenty-four hours after exposure to propanil.

Figure 13. Propanil and antide exposures on uterine weights +/- HKSP

immunization. The whole uterus was weighed. Data were analyzed by two-way ANOVA, followed by Tukey-HSD. Statistical differences are denoted by different letters (saline treatment a-c and HKSP treatment d-f). No statistical differences were detected in multiple comparisons in uterine weight.

Figure 14. Expression of steroid synthesis enzymes and *Gnhrh* post-HKSP

immunization +/- propanil exposure. The expression of genes was log-transformed after being normalized to the two housekeeping genes (*Hprt1* and *Actb*). Significantly different expression was observed in *Dhcr7* (A, B), *Cyp17a1* (C, D), *Cyp19a1* (E, F) and *Hsd17b2* (G, H), *Cyp11b1* (I), *Hsd17b7* (J) and *Gnhrh* (K), a gene involved in steroid hormone synthesis except for *Gnhrh*. Trial 1 (A, C, E, G) and Trial 3 (B, D, F, H, I, J, and K). Data were analyzed by two-way ANOVA, followed by Tukey-HSD. Statistical differences are denoted by different letters trial 1 (a-d) and trial 3 (saline treatment a-c and HKSP treatment d-f). Group means (bars) that do not share the same letter within the group (saline or HKSP) differ from each other.

Figure 15. Expression at 24 hours post-exposure of genes involved in prolactin synthesis. The gene was normalized to two housekeeping genes (*Hprt1* and *Actb*). The *Irf1* (A) and *Socs3* (B) are involved in the Prolactin synthesis pathway. Data were analyzed by two-way ANOVA, followed by Tukey-HSD. Statistical differences are denoted by different letters (saline treatment a-c and HKSP treatment d-f). Group means (bars) that do not share the same letter within the group (saline or HKSP) differ from each other.

Figure 16. Expression at 24 hours post-exposure of *Lcn2*. The gene was normalized to two housekeeping genes (*Hprt1* and *Actb*). Data were analyzed by two-way ANOVA, followed by Tukey-HSD. Statistical differences are denoted by different letters. Group means (bars) that do not share the same letter differ from each other.

Figure 17. Expression at 24 hours post-exposure of genes involved in extracellular matrix remodeling and immune regulation. The gene was normalized to two housekeeping genes (*Hprt1* and *Actb*). The *Adamts1* (A), *Csfl* (B), *Cxcl1* (C),

Cxcl10 (D), *Il10* (E), *Tlr2* (F) and *Saa3* (G) are involved in the immune system regulation. Data were analyzed by two-way ANOVA, followed by Tukey-HSD. Statistical differences are denoted by different letters (saline treatment a-c and HKSP treatment d-f). Group means (bars) that do not share the same letter within the group (saline or HKSP) differ from each other.

Figure 18. Proposed model for acute propanil-mediated effects on the HPG axis leading to immunomodulation. The red arrows with a black outline are representing the antide effect. Green arrows with black outlines represent the propanil effect. The dotted arrows are showing where antide (red) and propanil (green) may be acting on the HPG axis. The black arrows represent the HPG axis and the proper pathway of the HPG axis.

Chapter 5: Discussion

These studies aimed to determine the acute effect of propanil in female mice immunized with HKSP at 24 and 72 hours after exposure on splenic cell populations, reproductive hormones, and ovarian and oviductal gene expression. Propanil had an acute effect, mostly in 24 hours, on spleen weight, splenocytes, number and percent of splenic cell populations, reproductive hormones, and steroid synthesis genes. Another goal was to examine if GnRH antagonist (antide) interfered with propanil's acute response since GnRH receptors have been found on the spleen in the rat (Jacobson *et al.*, 1998) and ovaries in mice (Torrealdy *et al.*, 2013). Also, antide abrogated the PC-specific, ASC response on day 7 in propanil-treated, HKSP-challenged mice (Salazar *et al.*, 2006). Antide bound to the GnRH receptors, as evidenced by the decrease in ovarian weight, ovarian area, and uterine weight. GnRH may induce signals not only through the pituitary gland via the HPG axis but bind directly in the ovary.

The spleen weight and the number of splenic cells decreased at 24 hours after propanil. The spleen weight and total splenocyte counts in our study were back to normal at 72 hours. In contrast, a dramatic increase in spleen size, up to 17 times larger spleen, occurred seven days after propanil and HKSP exposure (Barnett and Gandy, 1989; Barnett *et al.*, 1992; Salazar *et al.*, 2005). The enlargement on day 7 may be partially explained by induced clonal expansion in antibody production by B cells due to HKSP, which is not occurring as early as 24 or 72 hours, and partially by propanil. These conflicting results may indicate propanil involvement in two different processes: innate immune system inflammation/anti-inflammatory process in 24 hours versus

adaptive immune response on day 7. Alternatively, extramedullary hematopoiesis in the spleen could occur (Barnett *et al.*, 1992). Another possibility is the migration of cells to the spleen or a direct mitogenic effect of propanil on splenocytes (Barnett *et al.*, 1992), or it could be due to hemoglobinemia or hemolytic anemia. The decrease in spleen weight and splenocyte count is not fully understood or why this occurs at 24 hours then increases to the same level as the control at 72 hours.

Why the percent of specific cell populations increased (CD8⁺ and CD11b⁺) while the percent and numbers of B cells decreased are not known. The decrease in total splenic cell number after propanil reflected decreases in the specific cell populations at 24 hours. The decrease in the B-cells due to propanil, however, was still observed in antide-HKSP-propanil treated mice, but antide increased the number of B-cells to double in peanut oil controls (40×10^6 vs. 20×10^6 , Figure 6D). Thus, either B-cells have GnRH receptors and are directly affected by antide or affected indirectly via another mediator. Decreased immune cell population induced by propanil exposure may result in leukocytes being released from the spleen and going to the ovary or other specific organs to assist in the propanil mediated response. Possible candidate organs may be the liver and kidneys as these organs were enlarged in acute response to propanil (Ambrose *et al.*, 1972; Valentovic *et al.*, 1997; Cakici and Akat, 2013).

Antide blocked propanil response on the number of ASC on day 7 using a similar (HKSP + propanil) model (Salazar *et al.*, 2006). Thus, it indicated the importance of the HPG axis in this system. In the spleen at 24 hours, we observed the effect of antide on percent and the number of CD8⁺ cells. Specifically, antide without HKSP decreased the percent of CD8⁺ cells, while in HKSP challenged animals, antide doubled the number of

CD8⁺ cells (from a 0.5 to 1 x 10⁶ per spleen). It is uncertain what is the connection between antide and CD8⁺ cells in the spleen without knowing if CD8⁺ cells contain the GnRH receptors. Human healthy and leukemic T-cells produce two isoforms of GnRH: I and II (Chen *et al.*, 2002). Those authors suggested that the GnRH-I and II act in an autocrine or paracrine fashion to trigger laminin receptor gene transcription, adhesion, and chemotaxis of T-cells. Our results with CD8⁺ T-cells support the finding that antide disrupts the effect of propanil exposure in mice. We can conclude that at both 24 hours and 7 days post-exposure to propanil, antide modifies propanil response (albeit different response) in the spleen.

In 2006 Salazar showed that if the ovaries were removed, the increase in ASC did not occur; therefore, the propanil must affect the ovaries to mediate an increase in ASC and spleen weight (Salazar *et al.*, 2006). Our results indicate that propanil interferes with steroid synthesis due to the increase in progesterone in the serum. Modified gene expression of enzymes involved in steroid synthesis in ovaries also supports this as we observed decreased changes in 7-dehydrocholesterol reductase (*Dhcr7*), cytochrome P450 family 11 subfamily B member 1 (*Cyp11b1*), cytochrome P450 family 19 subfamily A member 1 (*Cyp19a1*), 17-beta 2-hydroxysteroid dehydrogenase (*Hsd17b2*), 17-beta 7-hydroxysteroid dehydrogenase (*Hsd17b7*), and cytochrome P450 family 17 subfamily A member 1 (*Cyp17a1*). These genes are responsible for producing cholesterol, cortisol, estradiol, testosterone, and 17-hydroxy pregnenolone.

Because DHCR7 converts 7-dehydrocholesterol into cholesterol, an increase in this enzyme would allow more cholesterol for steroid synthesis. *CYP11B1* converts

deoxycorticosterone into corticosterone and 11-deoxycortisol into cortisol; a decrease in this enzyme would prevent the synthesis of corticosterone and cortisol. *Cyp11b1* expression decreased in the ovaries due to antide regardless of propanil exposure. Therefore, GnRH receptors may not mediate the effect of propanil at 24 hours. The GnRH receptor may ensure compartmentalization of steroid synthesis in the ovary to focus on testosterone, estrogens, and progestins.

The enzyme CYP17A1 converts pregnenolone into 17-hydroxy pregnenolone then to dehydroepiandrosterone. If the gene expression is reduced, the synthesis of those steroids are lower, and pregnenolone is available as a substrate for progesterone. Thus, progesterone production should increase as observed. In parallel, CYP17A1 converts progesterone to 17-hydroxyprogesterone and further to androstenedione, which is a substrate for estrone and testosterone. A consequence of decreased *Cyp17a1* may be reduced substrate for testosterone and estradiol and accumulation of progesterone, further supporting the increased progesterone results. Increased synthesis of progesterone can lead not only to effects on reproductive or immune tissues but also could serve as a reservoir for corticosterone, a substrate for cortisol, or to the major mineralocorticoid – aldosterone, involved in stress and renal function, respectively. Data in mice and eels also demonstrated an increase in corticosterone and cortisol respectively after treated with propanil (Cuff *et al.*, 1996; Pruett *et al.*, 2000; de la Rosa *et al.*, 2005; Sancho *et al.*, 2017).

The enzyme *CYP19A1* (aromatase) is responsible for converting androstenedione into estrone and testosterone into estradiol by adding a hydrogen ion to oxygen for a hydroxy group. The decrease of *Cyp19a1* observed due to propanil in

24 hours in our study should lead to a decrease in estradiol, which occurred in trial 2, but not in trials 1 and 3. In trial 1, the estradiol levels did not change, and that could be due to the sensitivity of the assay.

HSD17B7 converts estrone into estradiol. The reduction in *Hsd17b7*, as well as *Dhcr7*, supports the decrease observed in estradiol concentrations in serum. *HSD17B2* is an enzyme that converts estradiol into estrone by removing a hydrogen ion. The decrease in *Hsd17b2* should prevent the conversion of estradiol to estrone; therefore, an accumulation of estradiol, which could inhibit GnRH via negative feedback.

In the ovaries, antide alone increased the amount of *Gnrhr* mRNA in saline and HKSP treated mice. Therefore, we can conclude that antide blocked GnRH binding the GnRH receptor. Thus, GnRH may act directly on the ovary. GnRH receptor has been detected in mouse ovaries, specifically in granulosa cells by immunohistochemistry (Singh *et al.*, 2011). Even though we observed the strong stimulatory effect on *Gnrhr* expression in the ovaries, this effect happened regardless of propanil or HKSP exposure; therefore, it does not mediate the effect of propanil at 24 hours.

Because propanil did not affect any studied gene expression on the oviduct, propanil may be acting on the ovary only and not the oviduct in the female mice. Propanil is acting as an endocrine disruptor to decrease enzymes that are responsible for producing steroid hormones important to reproduction.

Antide induced pro-inflammatory cytokines and other immune molecules, independently of an immune challenge with HKSP. Hence, the increase in inflammatory cytokines could be mimicking an infection, inflammation, or ovulation in the ovary when

antagonist blocked GnRH receptors was suggested that GnRH exerts direct actions at the level of the immune cells (Jacobson *et al.*, 1998). It was reported earlier that GnRH resembles a cytokine with immunostimulatory action on IL-2 receptor in *ex vivo* splenocytes and thymocytes from female rats (Batticane *et al.*, 1991). With the increase in the immune molecules, antide is an immune activator and may be acting on more than one organ, including the spleen and ovary. A disintegrin and metalloproteinase with thrombospondin motif 1 (*Adamts1*), colony-stimulating factor 1 (*Csf1*), C-X-C motif chemokine ligand 1 (*Cxcl1*), and C-X-C motif chemokine ligand 10 (*Cxcl10*) and toll-like receptor 2 (*Tlr2*) expression increased in the ovaries after antide regardless of propanil exposure, therefore, GnRH may not mediate the effect of propanil at 24 hours. While there was a multiple-fold increase in ovarian *Saa3* in 24 hours after propanil/HKSP compared to HKSP only detected by microarray analysis (unpublished observations), we did not detect an increase in ovarian *Saa3* after propanil with qRT-PCR. The significant interaction between antide and propanil on serum amyloid A3 (*Saa3*) expression as observed in HKSP-challenged mice, such that there was an upregulation in *Saa3* in antide treated group without propanil (peanut oil), compared to propanil group but not in propylene glycol group. The increase in the peanut oil and antide-treated group could be a result of the increase in other pro-inflammatory molecules. More research is needed to understand how these molecules fit into the propanil model.

Figure 18 shows a proposed model for acute propanil-mediated effects on the HPG axis leading to immunomodulation. The different color arrows describe how propanil might act in the female mouse. We do not know if propanil is working directly on the ovaries, but propanil decreased steroid production and influenced splenocyte

production at 24 hours in intact female mice. The proposed model demonstrates that antide and propanil have different responses within the spleen; therefore, we can suggest that antide is blocking the effect of propanil within the spleen. Antide does not seem to affect the changes made by propanil in the ovary as the trends in steroid synthesis are the same if the animal is exposed to antide and propanil. Therefore, we can conclude that propanil is acting directly on the spleen, but antide is not acting directly on the spleen.

In conclusion, propanil causes an acute effect on spleen weight, splenic cell populations, reproductive hormones, steroid synthesis pathway in mice ovaries. Antagonizing GnRH with antide affected immune and reproductive variables and modify propanil's acute effect in female mice. Therefore, GnRH may be necessary for the acute phase (24 hours) of propanil/HKSP challenge but may still involve other potential components of the HPG axis. More research is needed to determine where propanil is acting within the female mouse.

Chapter 6: Literature Cited

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