EFFECTS OF DDT AND DDE ON IMMUNITY, OXIDATIVE STRESS AND REPRODUCTIVE PARAMETERS IN RODENTS

by

Erica Marie Molina

A dissertation submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Doctor of Philosophy

Auburn, Alabama
May 1, 2021

Keywords: ecotoxicology, DDT, DDE, cellular stress, hormone expression, endocrine disruption

Approved by

Mary T. Mendonça, Chair, Professor, Biological Sciences
Benson T. Akingbemi, Professor, Department of Anatomy, Physiology and Pharmacology,
Andreas N. Kavazis, Professor, School of Kinesiology
Elizabeth H. Schwartz, Associate Professor, Biological Sciences

Abstract

Lingering endocrine disrupting chemicals (i.e. DDT) are a significant threat to wildlife and humans. The effects of DDT have long been documented to cause adverse effects on reproduction, immunity and induce physiological changes. However, this type of ecotoxicological research predominantly focuses on the physiological changes caused by administering acute high dose ranges of these contaminants. There is a significant lack of research on the consequences of chronic exposure to environmentally relevant doses, although this exposure is the most experienced by and impactful to humans and wildlife in our current Anthropocene age. I tested 3 hypotheses in rodent species: (1) *Peromyscus maniculatus* and *Sigmodon hispidus* from the historically contaminated site would exhibit increased tissue contaminant load, decreased immune capability and a higher expression of oxidative stress biomarkers increasing probability of disease and decreased reproductive success; (2) early onset exposure of DDE will cause a greater inhibition of steroidogenesis and induce higher levels of oxidative stress in a dose-dependent manner during laboratory exposure; (3) that rats exposed chronically to environmentally relevant levels of DDE would exhibit decreased levels of testosterone and steroidogenic enzyme capacity (i.e. 3β HSD) in Leydig cells. We found that contaminant load was higher in *Peromyscus maniculatus,* the omnivorous species, than in *Sigmodon hispidus,* the predominantly herbivorous species, with each species showing an increase in a marker for oxidative stress. Additionally, both species exhibited a marked leukocytopenia which may indicate immune dysfunction. However, a traditional marker of exposure to stress (i.e., higher N/L ratio) did not differ significantly in either species when individuals from contaminated and reference areas were compared. Experimental DDE dosing in Long-Evans rats demonstrated a significant reduction in testosterone, with later onset rats being

most affected contrary to our predictions. Moreover, we found an upregulation in a oxidative stress in a dose-dependent manner. Chronically dosed Long-Evans rats also showed a significant reduction of testosterone in Leydig cells which exhibited increased apoptosis and significant differences within the steroidogenic pathway. As a whole, this dissertation provides evidence that low levels of contaminants pose a threat in the environment with implications on immune responsiveness, cellular stress and especially steroidogenesis.

Acknowledgments

The advancement of science is brought about by collaboration and without that I could not have successfully finished my dissertation. I am extremely grateful for the opportunity to so many who supported me and encouraged during the pursuit of my dissertation. Especially for my master's advisor, Hardin Rahe that made the connection for me to attend Auburn University and provided me support during my time there. I would also like to thank my advisors, Mary Mendonça, Benson Akingbemi, Andreas Kavazis and Elizabeth Schwartz who served on my committee, supported me along the way and provided insight during the process of completing and writing up my research. I am very thankful for Redstone Arsenal allowing me to conduct field research on their base, as well as Michael Barbour, Jonathan Armbruster, and Christina Easterwood for facilitating the opportunity. Furthermore, I am grateful to Samantha Bradley, Bamidele Jeminiwa, Rachel Knight, Rita Akinkuotu, Steven Gardner, Meghan Kelly and Hailey Perry for providing a listening ear, helpful hands and constructive input. My deepest gratitude is extended my friends and family that supported me mentally and emotionally throughout everything. For my parents, siblings and Anastacio Perez for countless encouragement and support, and Krischell Webster for providing me a second family and a feeling of being home away from home.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

BMI	Body Mass Index
cPARP	Cleaved Poly-(ADP-ribose) Polymerase Protein
DDD	Dichlorodiphenyldichloroethane
DDE	Dichlorodiphenyldichloroethylene
DDT	Dichlorodiphenyltrichloroethane
DDTs	DDE, DDD and DDT combined
E2	Estradiol
USEPA	United States Environmental Protection Agency
FSHβ	Follicle Stimulating Hormone
HPG	Hypothalamus Pituitary Gonadotropin
HSP 70	Heat Shock Protein 70
HSP 90	Heat Shock Protein 90
LHβ	Luteinizing Hormone
LH-R	Luteinizing Hormone Receptor
PARP	Poly-(ADP-ribose) Polymerase Protein
PND	Post Natal Day
ROS	Reactive Oxygen Species
StAR	Steroidogenic Acute Regulatory Protein
T	Testosterone
WBC	White Blood Cell Count
4-HNE	Lipid peroxidation

17β HSD 17β-Hydroxysteroid dehydrogenases

3β HSD 3β-Hydroxysteroid dehydrogenases

Chapter 1: General Introduction

INTRODUCTION

Many potentially harmful pollutants are introduced into our environment and adversely affect development and physiology by acting as endocrine disruptors (Zala and Penn, 2004). A commonly known pesticide, Dichlorodiphenyltrichloroethane (DDT) and its metabolites Dichlorodiphenyldichloroethylene (DDE) and Dichlorodiphenyldichloroethane (DDD) can affect many hormones, especially sex steroid hormones (i.e. estrogens, progestins, and androgens) inducing physiological changes (Migliaccio et al., 2019; Scippo et al., 2004; Guillette and Gunderson, 2001; Patisaul and Adewale, 2009). Animals are the most sensitive to sublethal levels of endocrine disruptors, such as DDE, during the earliest stages of life and can cause permanent alterations of vertebrae reproductive function (Clotfelter et al., 2004; Wolf et al., 1999; Crews et al., 2000). DDT and its metabolite DDE have estrogenic effects in males by blocking androgen receptors (Sikka and Wang, 2008). Testosterone production and secretion are modulated by Luteinizing Hormone (LH; Dohle et al., 2003) with testosterone having many downstream effects on sexual differentiation, masculinization, and immune function. If environmental changes dictate, the stress axis is pliable and subject to modification and permanent changes (Reeder and Kramer, 2005; Boonstra, 2005). The gonadal steroids regulate many processes including reproduction and thus are a prime target for endocrine disrupting chemicals, particularly by environmental estrogenic compounds (Clotfelter et al., 2004).

Organochlorine pesticides, such as DDT, are relatively resistant to degradation and can be bioaccumulated in the environment, as well as and biomagnified in higher trophic levels (Guillette and Gunderson, 2001). Persistent organic pollutants (POP's), such as DDT and its derivatives, accumulate in mammals within the environment through various pathways, such

as crops, dirt ingestion during foraging and through bioaccumulation in trophic levels (Arroyo-Salgado et al., 2016). Although DDT has been banned, its ubiquitous nature and persistence to degradation in the environment allows it to persist within the environment at sublethal levels for decades, with elevated levels of DDE being found in humans and wildlife (Makita et al., 2003).

Field research plays an essential role in strengthening data collected within a laboratory setting. The majority of experiments involving vertebrates are conducted within a laboratory environment allowing for the control of environmental variables, leading to more targeted testing of specific variables. The question is does exposure to a targeted contaminant (i.e. DDE) administered under a more limited exposure timeline, to highly selected lines of lab rat give us insight to the impact of this contaminant that has been decades in the environment resulting in lifetime (if not generational) exposure of rodents.

Whenever an animal is taken out of its natural environment it generates a different endocrine responses to exogenous stimuli (Calisi and Bentley, 2009). The endocrine system is an important part of a multicellular network that coordinates physiological processes with each other and the environment (Purifoy, 1981). The discrepancy in environmental factors and animal interactions from wild to captive, can yield a difference within an individual's stress response (Calisi and Bentley, 2009). Therefore, analyzing the effects of DDT and/or DDE in both the field and lab has the ability to strengthen interpretation of findings from different studies.

In Alabama, DDT was used for several years at Redstone Arsenal, a classified EPA Superfund site, prior to it being banned. Despite the efforts to cleanup the soil, DDT levels in soil and water still pose a potential risk to the wildlife inhabiting the area. Wildlife species can serve as biomonitors of environmental health and can be used to assess ecotoxicological

consequences when contaminants are introduced into the environment (Phelps, 2009; Espinosa-Reyes et al., 2010). Rodents are commonly used as biomonitors due to their small home range size, abundance, and exposer to contaminated soils through ingestion and inhalation while foraging for their food source (Phelps, 2009). Rodent diets are predominantly omnivorous allowing for biomagnification of pesticides through topic levels (i.e. arthropod ingestion) (Figure 1).

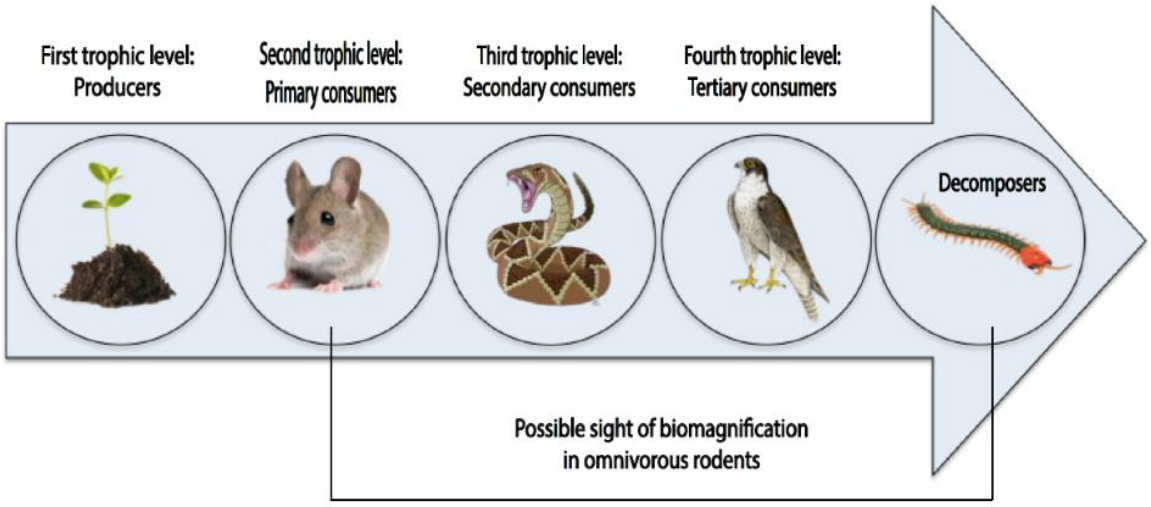

Figure 1 Schematic of how contaminants can be biomagnified through trophic levels. Consumption of arthropods (decomposers) makes rodents particularly vulnerable to biomagnification.

Analyzing the physiological mechanisms and changes that occur when animals colonize toxic soils will aid in the development of better conservation protocols for wildlife. The use of biomarkers (e.g. oxidative stress) has allowed expansion beyond fecundity and growth and given us the ability to evaluate exposure to sub-lethal levels at the cellular level. Other common measures used in POP studies include 4-Hydroxynonena (HNE) to measure lipid peroxidation

and protein carbonyls to measure protein oxidation. Pesticides' lipophilic nature disrupts the lipid rich biomembrane in an organism causing an imbalance between prooxidants and antioxidants in the body causing membrane damage through lipid peroxidation, in turn causing an imbalance in homeostasis (Dwivedi and Flora, 2011; Abd Allah et al., 2018). The physiological responses to short-term exposures of pollutants are relatively well studied, but the understanding of how of long-term pollutants affect an organism is lacking (Isaksson, 2010; Phelps, 2009; Sikka and Wang, 2008).

RESEARCH APPROACH AND HYPOTHESES

The goal of this dissertation was to understand the direct links caused from exposure to lingering DDTs, in the field and in lab, on rodent populations and its implications for human exposure. The objectives of this study were to:(1) assess the effects of chronic low dose exposure to DDT in a Superfund site at Redstone Arsenal in two species of small mammal (an herbivore and an omnivore) and (2) determine if observed pattern differences in animals from impacted and unimpacted sites are repeatable when DDE (a byproduct of DDT degradation) is administered in a modified chronic exposure to laboratory rats in a controlled environment.

Field study

Worldwide surveys have documented the impact of environmental contaminants on soils, terrestrial and aquatic organisms, and their toxic effects on human as well as non-human biota (Carvalho, 2017). In 1947, Redstone Arsenal leased a portion of their land to Calbama Chemical Company to manufacture DDT products. The DDT manufacturing plant was active for 15 years at this location. Since the closing of the DDT manufacturing plant, initiatives have been taken to strip the area of contaminants. However, according to a Resource Conservation and Recovery

Act investigation in 2016 levels of DDT in RSA-117 (former DDT manufacturing area) still posed a potential threat (e.g. >1ppm) to site-specific food chain populations (Table 1).

Parameter	Surface Soil (mg/kg)	Subsurface Soil (mg/kg)
4,4'-DDT	0.0045-7870	0.00308-34000
4,4'-DDE	0.0028-655	0.00137-5700
4,4'-DDD	0.00129- 312	0.00143-1600

Table 1 Levels of DDT contaminant measured at RSA-117 (former DDT manufacturing area).

This study involved consecutively trapping two sites (reference and contaminated area) and analyzing captured rodents for markers of oxidative stress, immune dysfunction and contaminate load. Prior to beginning trapping for the experiment a pre-survey was conducted to determine the two most prominent rodent species in the area. The most trapped species were the deer mouse, *Peromyscus maniculatus,* and the cotton rat, *Sigmodon hispidus.* We predicted that animals exposed to DDT and its metabolites within the field will show an increase in ROS inflicted cellular damage (i.e. 4-HNE, oxyblot, HSP 70/90), increased levels of stress (N/L ratios) and an increase in contaminant load indicating a decreased efficiency in maintaining cellular homeostasis in a chronically contaminated area.

Experimental studies

Experimental studies focused on exposing a comparable model system (lab rat in a controlled environment) exposed chronically (i.e. not life time but to a greater time frame

(weeks) than the more traditional acute exposure times (several days) used in traditional toxicological studies) to DDE, a relevant endocrine disruptor to determine if this design will result in a similar suite of physiological changes as seen in the field. All measurements from our field study (HSP70/90, Oxyblot, 4-HNE, and WBC count) were repeated to explore whether the oxidative stress response patterns differ or are similar in a controlled laboratory environment when compared to our field species. Despite animals being taxonomically similar, they can still vary in their response to the same exposure of an endocrine disruptor (Clotfelter et al., 2004). The first experimental study had two aims to test: (1) the dose-response of chronically administered DDE and its subsequent effect on oxidative stress and endocrine function measures and (2) influence of age at onset of DDE exposure on oxidative stress and endocrine function measures. This study involved experimentally dosing DDE under a controlled environment and analyzing tissue, after the completion of the study, to compare oxidative and endocrinological parameters in control animals compared to treated animals. The hypothesis for this study is that early onset of exposure would cause a greater inhibition of steroidogenesis and induce higher levels of oxidative stress in DDE exposed animals. This hypothesis was not supported by finding that later onset of treatment showed a greater change in steroidogenesis than those with an early onset exposure.

The second experimental study further investigated the underlying effects of environmentally relevant levels of DDE on hormonal expression, specifically in Leydig cells. To do this Long-Evan male rats were treated for 4-weeks with DDE in a controlled environment. Leydig cells were then isolated and analyzed to analyze key enzymes and proteins in the steroidogenic pathway and to determine apoptosis in Leydig cells. We hypothesized that rats dosed with chronic low-dose levels of DDE would exhibit a disruption in the steroidogenic

pathway in Leydig cells and decreased levels of testosterone production with implications for reproductive capacity.

WORK CITED

Abd Allah, A. A., E. A Ibrahim, E. S Ibrahim, N. F Mahmoud, and M. M Afify. 2018. Thiocyclam-Induced Reproductive Toxicity, Oxidative Stress and Genomic DNA Damage in Testicular Tissues of Rats: Protective Effects of Green Tea Extract. Journal of Environmental & Analytical Toxicology 08.

Arroyo-Salgado, B., J. Olivero-Verbel, A. Guerrero-Castilla, B. Arroyo-Salgado, J. Olivero-Verbel, and A. Guerrero-Castilla. 2016. Direct effect of p,p'- DDT on mice liver. Brazilian Journal of Pharmaceutical Sciences 52:287–298.

Boonstra, R. 2005. Equipped for Life: the Adaptive Role of the Stress Axis in Male Mammals. J Mammal 86:236–247.

Calisi, R. M., and G. E. Bentley. 2009. Lab and field experiments: are they the same animal? Hormones and Behavior 56:1–10.

Carvalho, F. P. 2017. Pesticides, environment, and food safety. Food and Energy Security 6:48–60.

Clotfelter, E. D., A. M. Bell, and K. R. Levering. 2004. The role of animal behaviour in the study of endocrine-disrupting chemicals. Animal Behaviour 68:665–676.

Crews, D., E. Willingham, and J. K. Skipper. 2000. Endocrine disruptors: present issues, future directions. The Quarterly Review of Biology 75:243–260.

Dohle, G. R., M. Smit, and R. F. A. Weber. 2003. Androgens and male fertility. World J Urol 21:341–345.

Dwivedi, N., and S. J. S. Flora. 2011. Concomitant exposure to arsenic and organophosphates on tissue oxidative stress in rats. Food and Chemical Toxicology 49:1152–1159.

Espinosa-Reyes, G., A. Torres-Dosal, C. Ilizaliturri, D. González-Mille, F. Díaz-Barriga, and J. Mejía-Saavedra. 2010. Wild rodents (Dipodomys merriami) used as biomonitors in contaminated mining sites. Journal of Environmental Science and Health, Part A 45:82–89.

Guillette, L. J., and M. P. Gunderson. 2001. Alterations in development of reproductive and endocrine systems of wildlife populations exposed to endocrine-disrupting contaminants. Reproduction 122:857–864.

Isaksson, C. 2010. Pollution and Its Impact on Wild Animals: A Meta-Analysis on Oxidative Stress. EcoHealth 7:342–350.

Makita, Y., T. Matsuura, R. Ogata, Y. Romero, M. Omura, A. Tanaka, M. Hirata, and N. Inoue. 2003. Systemic effects of orally administered p, p'-DDE on immature male Wistar rats during pubertal period. J Occup Health 45:223–227.

Migliaccio, V., R. Sica, R. Scudiero, P. Simoniello, R. Putti, and L. Lionetti. 2019. Physiological Adaptation to Simultaneous Chronic Exposure to High-Fat Diet and Dichlorodipheniletylhene (DDE) in Wistar Rat Testis. Cells 8:443.

Patisaul, H. B., A. C. Gore, and D. Crews. 2017. Environmental Endocrine Disruption of Brain and Behavior.Pages 63–88 in Hormones, Brain and Behavior. Elsevier.

Phelps, Kendra. 2009. Ecological Characteristics of Small Mammal Communities at a Superfund Site. The American Midland Naturalist. 161. 57-68. 10.1674/0003-0031-161.1.57.

Purifoy, F. E. 1981. Endocrine-Environment Interaction in Human Variability. Annual Review of Anthropology 10:141–162.

Reeder, D. M., and K. M. Kramer. 2005. Stress in free-ranging mammals: integrating physiology, ecology, and natural history. Journal of Mammalogy 86:225–235.
Ryan, J. A., and L. E. Hightower. 1996. Stress proteins as molecular biomarkers for environmental toxicology. Pages 411–424 in Stress-Inducible Cellular Responses. Feige, U., Yahara, I., Morimoto, R.I., Polla, B.S., eds. EXS. Birkhäuser Basel, Basel.

Sikka, S. C., and R. Wang. 2008. Endocrine disruptors and estrogenic effects on male reproductive axis. Asian J Andrology 10:134–145.

Scippo, M.-L., C. Argiris, C. Van De Weerdt, M. Muller, P. Willemsen, J. Martial, and G. Maghuin-Rogister. 2004. Recombinant human estrogen, androgen and progesterone receptors for detection of potential endocrine disruptors. Anal Bioanal Chem 378:664– 669.

Wolf, C., C. Lambright, P. Mann, M. Price, R. L. Cooper, J. Ostby, and L. E. Gray Jr. 1999. Administration of potentially antiandrogenic pesticides (procymidone, linuron, iprodione, chlozolinate, p, p′-DDE, and ketoconazole) and toxic substances (dibutyl- and diethylhexyl phthalate, PCB 169, and ethane dimethane sulphonate) during sexual differentiation produces diverse profiles of reproductive malformations in the male rat. Toxicology and industrial health 15:94–118.

Zala, S. M., and D. J. Penn. 2004. Abnormal behaviours induced by chemical pollution: a review of the evidence and new challenges. Animal Behaviour 68:649–664.

Chapter 2: Chronic exposure to environmental DDT/DDE in two species of small rodents: measures of contaminant load, immune dysfunction and oxidative stress

FORMAT FOR ENVIRONMENTAL TOXICOLOGY AND CHEMISTRY JOURNAL

Chronic exposure to environmental DDT/DDE in two species of small rodents: measures of contaminant load, immune dysfunction and oxidative stress

Erica M Molina[a], Andreas N Kavazis[b], Mary T Mendonça[a]

[a]Department of Biological Sciences, Auburn University, 331 Funchess Hall, 350 South College St, Auburn, AL 36849, USA. Email: emm0044@tigermail.auburn.edu (Corresponding author)

[b]School of Kinesiology, Auburn University, 287 Kinesiology Building, 301 Wire Road, Auburn, AL 36849, USA.
Email: ank0012@auburn.edu

ABSTRACT

Persistent contaminants are ubiquitous in our water and soil; thus, chronic exposure to environmentally relevant levels of these contaminants may pose a risk to humans and wildlife. Two species of small mammals (deer mouse, *Peromyscus maniculatus* and cotton rat, *Sigmodon hispidus*) were collected from historically DDT contaminated and reference areas at the Redstone Arsenal, an EPA designated Superfund site. Soil samples taken concurrently with mammal collection from the DDT abatement site exhibited DDT and DDE levels above 1 ppm [EPA Total Threshold Limit Concentration for DDT and DDE (<1ppm)], while those from the reference area had undetectable levels. Liver samples from individuals of both species collected

in the contaminated areas also had significantly elevated DDT/DDE levels; *P. maniculatus* averaging a ~20x greater load than that documented for *S. hispidus*. Both rodent species collected from the contaminated site had significantly lower total leukocyte counts and total differential blood cell counts (p=0.01; p=0.04) than those collected from the nearby (~7000 m) reference site. Cellular stress indices also trended higher in both species suggesting a potential for chronic exposure to DDT to act as a mediator of oxidative damage. The present study provides support that environmental exposure to low levels of contaminants can cause physiological consequences that may influence immune responsiveness and initiate cellular stress in resident mammals.

KEYWORDS: DDT, Superfund site, cellular stress, immune function

INTRODUCTION

The use of pesticides around the world induces sediment runoff that contaminates our soil and water sources with a wide range of persistent organic pollutants (POPs). Environmental contamination of POPs is a global problem that has increased susceptibility to disease, abnormal growths, dysfunctional immune and reproductive systems, and endocrine disruption (Banerjee 1999; Eskenazi et al. 2009; Köhler and Triebskorn 2013). Many POPs, such as dichlorodiphenyltrichloroethane (DDT), have been banned due to their long half-life in the environment and documented effects on humans and wildlife; DDT can also be further transformed into its endocrine disruptor counterparts dichlorodiphenyldichloroethylene (DDE) and dichlorodiphenyldichloroethane (DDD; Aislabie et al. 1997).

Through a photochemical reaction with sunlight or by dehydrochlorination in bacteria and animals, DDT is degraded into DDE, whereas DDD is formed by reductive dechlorination through microbial mediation or a chemical reaction (Aislabie et al. 1997). The ubiquitous nature

of DDT allows it to persist within the environment at sublethal levels for decades; over time, it bioaccumulates and biomagnifies in higher trophic levels (Guillette and Gunderson 2001; Arroyo-Salgado et al. 2016), potentially increasing its physiological impacts, especially if exposure occurs during the sensitive developmental stages of life, which can lead to long-term dysfunction (Wolfe et al. 1999; Crews et al. 2000; Clotfelter et al. 2004; Patisaul and Adewale 2009; Schug et al. 2011). Historically, DDT caused a decline in American robin *(Turdus migratorius)* populations, eggshell thinning in bald eagles (*Haliaeetus leucocephalus*; Hoffman et al. 2003), reproductive abnormalities in alligators (*Alligator mississippiensis*; Guillette et al. 1994), and a decline in semen quality in humans (Cheek and McLachlan 1998). As ecotoxicology became a predominant research field, the great majority of studies examined the effects of short-term exposures to contaminants, and a complete understanding of how long-term pollutants affect an organism is lacking (Guillette and Gunderson 2001; Sikka and Wang 2008; Phelps 2009; Isaksson 2010).

Many areas within the United States are still heavily contaminated and are classified as Superfund sites by the US Environmental Protection Agency (USEPA). In Alabama, DDT was manufactured at Redstone Arsenal (Huntsville, AL, USA), a USEPA-classified Superfund site, until 1972, when manufacturing ceased as a result of the ban on DDT use in the United States. Despite remediation efforts, DDT, DDD, and DDE levels in soil and water still pose a potential risk to the wildlife inhabiting the area. For example, a study found DDE levels in surface soil samples ranging from 0.0028 to 655 mg/kg and in subsurface soil samples ranging from 0.0014 to 5700 mg/kg, with some of these levels being higher than USEPA-designated safe levels (health reference levels are 1.4 mg/kg in soil and 0.2 μg/L in drinking water) and causing a risk of water contamination (US Environmental Protection Agency 2008). Although DDE in drinking

water has been decreasing, the majority of US residents have detectable levels of DDE in their serum, indicating that exposure to DDE is common among the general population (Centers for Disease Control and Prevention 2019) through the breakdown of historic DDT use.

Wildlife species are commonly used as biomonitors of environmental health to assess ecotoxicological consequences when contaminants are introduced into the environment (Phelps 2009; Espinosa-Reyes et al. 2010). Rodents in particular are highly prone to contaminant exposure due to their small home range size, high population abundance, and chronic exposure to contaminated soils through ingestion and inhalation while foraging for their food source (Phelps 2009). Many rodent species' omnivorous diet would allow for biomagnification of pesticides through trophic levels (i.e., arthropod ingestion). Analyzing the physiological mechanisms and changes that occur in animals inhabiting toxic soils will aid in the development of better conservation protocols for wildlife.

Biomarkers of lipid and protein damage give researchers the ability to measure cellular damage due to damaging reactive oxygen species (ROS; Dalle-Donne et al. 2003; Gaikwad et al. 2015; Finger et al. 2017). The lipophilic nature of pesticides allows the disruption of lipid-rich biomembranes in an organism, causing an imbalance between ROS and antioxidants in the body. This can lead to membrane damage through lipid peroxidation and cause an imbalance in homeostasis (Dwivedi and Flora 2011; Abd Allah et al. 2018). Many pesticides and chemicals are also known to show impairment of the immune response, known as pesticide-induced immune toxicity (Banerjee 1999). Organochlorides have been shown to cause lymphocytopenia and neutrophilia in rats and broiler chicks dosed with 2 ppm monocrotophos (Garg et al. 2004). In addition, marked leucocytopenia was seen after exposure to both monocrotophos and

endosulfan (chlorinated hydrocarbon), indicating potential immune effects after exposure to different contaminants (Garg et al. 2004).

The objectives of the present study were to: 1) determine the abundance of DDTs in soil and liver samples in small mammals from reference versus historically contaminated sites (Superfund site: Redstone Arsenal), and 2) assess the effects of chronic exposure to environmental DDTs on oxidative stress and baseline immunity parameters in 2 species of small mammals inhabiting these areas. Generally, we hypothesized that animals chronically exposed to DDTs would experience cellular alterations due to environmental contamination. We predicted that deer mouse, *Peromyscus maniculatus*, and cotton rat, *Sigmodon hispidus*, individuals from the historically contaminated site would exhibit decreased immune capability and a higher expression of oxidative stress biomarkers, which, in turn, can increase the probability of disease and decreased reproductive success.

MATERIALS AND METHODS

Animals collected

All animal trapping was approved by the Auburn University Institutional Animal Care and Use Committee (Protocol Review No. 2017-3100), as were all protocols and animal handling procedures. Animals were trapped concurrently in both the containment area (~119.4 m; DDT abatement site; Figure 2) and 3 areas within the reference site (107, 93.3, and 108 m; Bobcat Cave; Figure 2) at Redstone Arsenal from February to August 2018. Other areas within the DDT abatement area have undergone heavy remediation to avoid future leaching of DDT from the soil and did not report any high contamination during the same investigative report. The reference site and the contaminated site were approximately 7000 m apart mapped from point to point on ArcGIS Survey 123 (online topographic map; Esri 2020). Sherman traps

were set out in 2 to 4 transects at each site, ranging from 61 to 96 m in length. Along each transect, both a large and small Sherman trap was placed at each point (Figure 2). If an area was too heavily wooded to maintain a straight transect, traps were put at the nearest clear segment and then continued along the original transect.

We trapped a focal area in the contaminated site that was categorized as highly impacted in a 2016 investigative report (CB&I Federal Services 2016; Figure 2). Traps were set in the evening, baited with rolled oats and peanut butter, and then checked for 3 consecutive days, twice/month in the morning and afternoons of each day and reset or rebaited if necessary. The sampling trap effort consisted of approximately 42 nights and days from February to August, with both reference and the contaminated sites being trapped simultaneously. There were 52 traps set in the contaminated area and up to 90 in the reference area, resulting in approximately 2184 and 3780 trap days, respectively. More traps were added in the reference area as the study continued, in an attempt to increase captures in reference areas to equal those from the historically impacted area.

Blood was collected by cardiac puncture immediately after cervical dislocation and used for hematological analysis (white blood cell count). Mass and length were measured prior to cervical dislocation. Mass (g) was taken using a PESOLA Light-Line metric hand-held spring scale (to 0.1 g). Body mass index (BMI; g/mm^2) was calculated by dividing mass by body length-squared (measured from the head to the base of the tail to the nearest 0.1 cm; Novelli et al. 2007). To avoid bias in BMI estimation, females showing reproductive traits (lactating or pregnancy) were omitted from the database. There was no significant difference in body mass in either species when comparing sex from our reference or contaminated areas ($p > 0.05$), nor did body mass by species differ between seasons (spring, summer, and winter, $p > 0.05$). Animals

were transported on ice to a designated dissection area at Redstone Arsenal. Tissues were dissected and snap-frozen in liquid nitrogen. Approximately 65 mg of liver tissue from animals from our reference and contaminated sites (*P. maniculatus*, reference; contaminated area [*n* = 11; 16]; *S. hispidus*, reference; contaminated area [*n* = 6; 25]) were homogenized and used to compare the levels of 4 markers of a cellular stress response. The remaining liver was sent to B&B Laboratories (College Station, TX, USA) for analysis of DDT and its metabolites.

Soil and tissue DDT levels

Several soil samples were taken from multiple subsites in both the contaminated and reference areas along trapping transects (reference site, *n* = 11; DDT abatement area, *n* = 11). Areas with the highest occurrence of animals trapped were selected for soil sampling. Because animals are most likely to be in contact with surface soil while foraging, soil samples were taken from the top 5 to 10 inches of soil. A shovel was used and wiped with an individual clean cloth to ensure no debris remained, to avoid cross-contamination. A different shovel was used to sample soil in the reference area, to avoid cross-contamination. Because DDT is a volatile organic compound, clumped soil was immediately packed inside a precleaned glass mason jar and kept cold to be sent for analysis. Soil samples were sent to the Waters Agricultural Laboratories in Camilla, Georgia (USA) for a pesticide panel analysis.

Liver samples were snap-frozen and stored at −80 °C to use for western blot analysis and pesticide analysis. From the remaining liver, after homogenization for western blot analysis, liver collected within the DDT abatement and our reference sites from *S. hispidus* (reference area, *n* = 6; contaminated area, *n* = 12) and *P. maniculatus* (reference area, *n* = 10; contaminated area, *n* = 12) were sent to B&B Laboratories for analysis of DDT and its metabolites by capillary gas chromatography–mass spectrometry using selected ion monitoring mode following methods

detailed in Sericano et al. (2014). Briefly, calibration solutions were prepared at 6 concentrations ranging from 5 to 500 ng/mL by diluting reference standard solutions containing the compounds of interest. For each analyte of interest, a relative response factor was determined for each calibration level. The 6 response factors were then averaged to produce a mean relative response factor for each analyte. Each analyte is reported as ng/dry g of liver tissue.

Blood cell counts

Once the blood was collected, a blood film was immediately made by streaking approximately 3 μL of whole blood across a glass microscope slide using another microscope slide held at an angle of approximately 45° to create the blood smear. Blood samples were allowed to completely air dry and were fixed within 24 h in Hema 3® fixative (Fisher Scientific). Slides were later stained with a Hema 3® stain set according to the manufacturer's instructions.

We performed 2 different counts to determine the number of each leukocyte type (i.e., neutrophils, lymphocytes, eosinophils, basophils, and monocytes). First, we categorized each leukocyte type out of 100 total leukocytes, further termed leukocyte differentials. If a blood smear did not have 100 total leukocytes, when the entire smear was counted, we recorded the number of leukocytes that were present. Neutrophil to lymphocyte ratios were calculated from the leukocyte differential counts. Because some blood smears did not contain 100 total leukocytes, to further validate and standardize our findings, we also identified leukocytes out of 3000 total blood cells (leukocytes and erythrocytes), further termed total blood cell differential counts. Cells were examined using a light microscope at 1000× magnification with oil immersion and were classified by visual inspection according to cell-specific morphological characters from Restell et al. (2014). Average “total” leukocyte counts, for each species/site were calculated by adding up all the leukocyte differential counts for each species in the contaminated

area versus the reference area to obtain total counts

(neutrophils + lymphocytes + eosinophils + basophils + monocytes = total).

Cellular stress response

We measured the amount of protein oxidation via reactive carbonyl derivatives (OxyBlot assay) and lipid peroxidation via aldehyde–protein conjugates (4-hydroxynonenal [4-HNE]) as indicators of the magnitude of lipid modification. We also measured heat shock protein (HSP) 90 and HSP 70 levels. The relative expressions of proteins were quantified by a single western blot analysis. The liver was homogenized 1:10 (wt/vol) in 5 mM Tris HCl (pH 7.5), 5 mM ethylenediaminetetraacetic acid (pH 8.0), and protease inhibitor cocktail (14224–396; VWR) and centrifuged at 1500 g for 10 min at 4 °C. Protein content of the supernatant was quantified using a standard Bradford assay (Millipore Sigma). For 4-HNE, HSP 70, and HSP 90, equal amounts of proteins were loaded and separated by polyacrylamide gel electrophoresis via 12% polyacrylamide gels (Bio-Rad). For protein carbonyls, homogenates were first prepared as outlined in the manufacturer's instructions for a commercially available kit (S7150; 1:150 dilution, OxyBlot protein oxidation detection kit; Intergen) and then loaded and separated by polyacrylamide gel electrophoresis via 12% polyacrylamide gels.

After electrophoresis, the proteins were transferred to microporous polyvinylidene difluoride membranes. The resulting membranes were stained with Ponceau S to analyze equal loading and transfer. Nonspecific sites were then blocked in phosphate-buffered saline (PBS) solution containing 0.1% Tween 20 and 5% milk. Membranes were then incubated for 1 h with primary antibody directed against 4-HNE (ab46545; 1:1000 dilution; Abcam), primary antibody provided in the OxyBlot kit (S7150; Millipore Sigma), HSP 70 (ab79852; 1:1000 dilution; Abcam), and HSP 90 (ab13495; 1:1000 dilution; Abcam). Following incubation with primary

antibodies, membranes were washed with PBS/Tween (5 min, 3×) and then incubated with the appropriate secondary antibody for 1 h at room temperature. After washing (5 min, 3×), a chemiluminescent system was used to detect labeled proteins (GE Healthcare Life Sciences). Images were taken and analyzed with the ChemiDoc-It Imaging System (UVP). For multiple band analysis, full lane band densitometry was performed using UVP Imager (Parry et al. 2018).

STATISTICAL ANALYSIS

Data comparing different variables by site (reference [R] vs historically contaminated [C]) were analyzed using an unpaired *t* test and checked for normality using a Shapiro–Wilk test with GraphPad Prism software, with error bars representing the standard error of mean (SEM). A 2-way analysis of variance multiple comparison was used to assess interaction between sex and between sites. The differences were considered significant at a $p \leq 0.05$. Data are represented as bar plots with SEM. Soil and liver DDT, DDE, and DDD (collectively referred to as DDTs) concentrations are expressed as mean ± SEM. Because average body mass did not significantly differ between sex ($p > 0.05$), we grouped both sexes to calculate an overall BMI for each species.

RESULTS

Animal collected

Trapping at the sites indicated that the most abundant small mammal species captured were *S. hispidus* and *P. maniculatus*, so they were the species selected for further toxicological and physiological analysis. We captured and euthanized 31 *S. hispidus* (reference: 5 males, 1 female; historically contaminated: 20 males, 5 females) and 30 *P. maniculatus* (reference: 10 males, 9 females; historically contaminated: 8 males, 3 females). In both species, there was no significant difference in body mass between sexes within a site nor was there a significant difference in

body mass between the 2 sites by sex (*P. maniculatus* site: R vs C; sex M vs F, respectively; R 17 ± 1.3 vs 15 ± 0.7; C 19 ± 3.1 vs 21 ± 6.0; *S. hispidus* R 82 ± 18 vs 105 ± 0.0; C 99 ± 8.1 vs 93 ± 6.3; interaction effect: *S. hispidus* $F_{(1,34)} = 0.49$, *P. maniculatus* $F_{(1,30)} = 0.38$; $p > 0.05$ for both species).

The BMI was calculated as an indicator of body condition based on length (*P. maniculatus* site: R 84 ± 1.4 vs C 81 ± 3.4; *S. hispidus* R 179 ± 5.9 vs C 150 ± 3.3) and mass. There were no significant differences in BMI in *S. hispidus* from the reference versus contaminated sites (R vs C 0.0045 ± 0.0006 vs 0.0044 ± 0.0002; $t_{(df=37)} = 0.09$, $p = 0.93$; Figure 3), but *P. maniculatus* captured at the contaminated site had significantly lower BMI values versus those from the reference (R vs C: 0.0025 ± 0.0001 vs 0.0017 ± 0.0002, $t_{(df=35)} = 3.09$, $p = 0.004$; Figure 3).

DDTs levels in soil and liver samples

All soil samples analyzed collected from the historically contaminated site had measurable amounts of DDT or one of its metabolites (DDD or DDE), but soil from the reference site did not have any detectable levels of DDT, DDD, or DDE. Of the 3 DDTs measured in the soil samples, DDT concentrations were highest (10.5 ± 10.4 ppm; Figure 4A), whereas DDE and DDD had lower but similar concentrations (1.5 ± 2 and 1.1 ± 0.9 ppm). Liver tissue from *S. hispidus* captured at the historically contaminated site showed a different pattern, with DDE being the most abundant metabolite at 51 ± 55.7 ng/dry g, followed by DDD at 22.8 ± 19.5 ng/dry g, whereas DDT was the lowest (4.6 ± 7.1 ng/dry g; Figure 4C). Liver tissue from *P. maniculatus* exhibited a similar pattern but at much higher levels (in ng/dry g) than what was observed in *S. hispidus* (DDE: 755.1 ± 1142; DDD: 1100 ± 1532; DDT: 570.2 ± 1271 ng/dry g; Figure 4E). Liver samples from animals trapped in the reference subsites

did not exhibit measurable levels of DDT or its metabolites. Total DDTs for soil and liver tissue concentrations for each species sampled were calculated by summing up DDTs/animal or soil and then using the average values of each substance measured in the historically contaminated area's soil samples to obtain total DDTs (112.6 ± 3.8 ppm; Figure 4B) and liver tissue samples by species (*S. hispidus* 91.4 ± 26.5 ng/dry g [Figure 4D]; *P. maniculatus* 2433 ± 866.6 ng/dry g [Figure 4F]). Total DDTs in *S. hispidus* liver samples ranged from 13 to 221 ng/dry g and from 50 to 9153 ng/dry g in *P. maniculatus* liver samples.

Total leukocyte and total blood cell differential counts

Total leukocyte counts (white blood cell counts) were found to be significantly lower in both *P. maniculatus* ($t_{(df=28)} = 2.63$, $p = 0.01$) *and S. hispidus* ($t_{(df=25)} = 2.67$, $p = 0.01$) collected from our historically contaminated sites compared with animals from the reference site (Figure 5A). Total blood cell differential counts (white blood cells + red blood cells) exhibited the same pattern: individuals from the contaminated sites had significantly lower average total blood cell differentials (i.e., lower numbers of white blood cell/3000 total blood cells counted) compared with their reference counterparts (*P. maniculatus:* $t_{(df=29)} = 2.18$, $p = 0.04$; *S. hispidus* $t_{(df=25)} = 2.14$, $p = 0.04$; Figure 5E and F). There was also a significant difference in the average white blood cell to red blood cell ratio in *P. maniculatus* ($p = 0.02$) and *S. hispidus* ($p = 0.03$) in the historically contaminated site versus animals from the reference site.

Average total lymphocyte count was significantly lower for both species captured in the historically contaminated site versus the reference site (*P. maniculatus:* $t_{(df=27)} = 2.68$, $p = 0.01$; *S. hispidus*: $t_{(df=25)} = 2.63$, $p = 0.01$; Figure 5C). However, we did not find a significant difference in the neutrophil to lymphocyte ratio in either species compared with reference animals (Figure 5B). All other individual WBC types did not differ compared with

reference animals.

Cellular Stress Response

In both *P. maniculatus* and *S. hispidus* from contaminated sites, cellular stress biomarkers such as HSP 70 and lipid peroxidation (4-HNE) did not exhibit higher levels compared with the respective reference group ($p > 0.05$; Figures 6A and D and 7A and D). We did find that HSP 90 in *P. maniculatus* had significantly higher levels ($t_{(25)} = 2.67_{(25)} = 3.30$, $p = 0.003$), compared with our reference groups (Figure 6B), with *S. hispidus* showing a similar trend of increased HSP 90 levels in individuals from the historically contaminated site ($t_{(28)} = 1.78$, $p = 0.08$; Figure 7B). In addition, *S. hispidus* did exhibit significantly higher levels of protein carbonyl derivatives when reference and contaminated site animals were compared ($t_{(27)} = 2.36$, $p = 0.03$; Figure 7C) but this parameter did not differ in *P. maniculatus* collected from the 2 sites ($t_{(25)} = 1.02$, $p = 0.32$; Figure 6C).

DISCUSSION

The present study demonstrates that individuals of both species (*P. maniculatus* and *S. hispidus*) inhabiting a DDT abatement site (at Redstone Arsenal) exhibited significantly fewer circulating leukocytes and also that each species from the contaminated site exhibited a significantly higher level of an oxidative stress biomarker. In addition, despite mitigation efforts in 1977 at the Redstone Arsenal Superfund site (CB&I Federal Services 2016), we found that 4 decades later, soil levels of DDTs were higher than those designated as the maximum permissible level. Individuals of both species inhabiting the historically contaminated area also had significantly elevated levels of DDTs in liver tissue compared with the reference group, with the species that is categorized as omnivorous, *P. maniculatus*, having significantly higher DDTs

(~20×) in their liver tissue compared with that of *S. hispidus*, a predominantly herbivorous species (Myers et al. 2020). This finding suggests biomagnification of the contaminant.

The environmental persistence of DDTs is due to their resistance to degradation (Aislabie et al. 1997; Mansouri et al. 2017) and their ability to be bioaccumulated in the environment and biomagnified in higher trophic levels (Hu et al. 2010; Song et al. 2016; Patisual et al. 2017). Maximum levels of DDT, DDD, and DDE found in sediment and soil along the southeastern coast of the United States in the mid-1990s were reported to be 151, 34, and 35 ng/g, respectively, with median concentrations below the detection limit (Agency for Toxic Substances and Disease Registry 2002). The soil levels collected at the Redstone DDT abatement site were much higher, (i.e., DDT, DDE, and DDE: 10.5, 1.5, and 1.0 ppm; converted: 10 500, 1500, and 1000 ng/g, respectively). Similarly, liver tissue levels were significantly elevated (ng/dry g): DDT (*P. maniculatus*: 570.2 ± 366.8; *S. hispidus*: 4.5 ± 2.0), DDD (*P. maniculatus*: 1100.0 ± 442.3; *S. hispidus*: 22.8 ± 5.6), DDE (*P. maniculatus*: 755.1 ± 329.6; *S. hispidus*: 51.0 ± 14.4), and total DDTs (2433.0 ng/dry g). Because DDT is lipid soluble, it tends to be deposited in fat tissue (i.e., adipose tissue DDT levels were 100× higher than those found in breast milk and serum of the same individuals: Jaga and Dharmani 2003). Thus, by comparison, the highly elevated DDT levels in the liver (an organ that is not normally fatty) from individuals from our contaminated site indicates that inhabitants of this area probably carry a substantial body burden of these contaminants, which can, in turn, pose a threat to higher consumers.

Predators such as raptors consuming contaminated prey exhibited concentrations several thousand times higher than those found in the environment (Sánchez-Bayo 2011). In bald eagles (*Haliaeetus leucocephalus*), the DDT biomagnification factor in birds that ate contaminated fish was 22, leading to eggshell thinning (Bowerman et al. 1995. Woodwell (1967) tracked

biomagnification from the lowest trophic level (plankton; 0.04 ppm DDT) to minnows (1 ppm DDT) to carnivorous ring-billed gulls (*Larus delawarensis*), resulting in 75 ppm in this upper-level predator. The levels we found in our mice and rat liver tissues were equal to or surpassed those found in minnows, and therefore it is probable that biomagnification would be evident in upper-level predators, which might also impact them adversely. The levels we found in the liver tissue were also similar or surpassed average levels (i.e., 12.2 ng/g DDT and 96.5 ng/g DDE) measured in adipose tissue of 555 women from a 1994 to 1996 study who were exposed to environmental DDT (Jaga and Dharmani 2003). However, in contrast, when immature male rats were exposed for 6 wk in the laboratory to a very large dose of DDE (10 mg/kg), it scarcely affected physical development, sexual maturation, or sperm number in dosed males (Makita et al. 2003). Thus, the exposure that most persists within body tissues and leads to apparent physiological dysfunction is one that is chronic and sublethal, the very way most DDTs are found in the environment, constituting a risk to wildlife and human health.

Unlike the laboratory study using a relatively acute exposure to a high dose of DDT, we did observe several signs of physiological dysfunction in the individuals inhabiting the historically contaminated area. Both species exhibited a significant depression in total leukocyte numbers (Figure 4A), total lymphocyte numbers (Figure 5C), and total blood cell differential counts (Figure 4E and F), suggesting that chronic exposure to DDTs could have an effect on immune function. The number of circulating lymphocytes within the blood acts as an index to assess the ability of the lymphoid organs to respond to a stressor, and thus a reduction in total blood cell differential and leukocyte differential counts may indicate lower immunocompetence (Garg et al. 2004). Previous research with 27 human males employed to spray various inorganic pesticides also indicated a significant decrease in white blood cells compared with control males

who were not in contact with DDT (Gaikwad et al. 2015). The immunosuppressive effect of chronic stressors may be evolutionarily adaptive or may be more proximately mediated as an adaptive mechanism to prevent inflammatory mechanisms (i.e., T-cell activation; Dhabhar and McEwen 1997).

Ecologists predominantly measure a stress response using glucocorticoids; however, relative white blood cell count is considered a reliable and a longer lasting alternative because the adrenal and leukocyte stress responses are tightly linked and can be directly related to stress hormone levels (Davis et al. 2008; Goessling et al. 2015). Many pesticides are known to impair or suppress the immune system. Mice dosed with 50 and 100 ppm DDT for 12 wk exhibited attenuation of both the humoral and secondary immune response (Banerjee 1999). At 3 to 8 wk of exposure to 100 ppm DDT, contamination reduced only the secondary immune response (reduction in plaque-forming cells of the liver and decreased lymphocytes) without having any effect on the primary humoral immune response (Banerjee 1999). The effect of DDT on the immune response seems to be attributed predominantly to exposure time, rather than dose (Banerjee 1987), which correlates with our finding of possible immunosuppression in our chronically exposed animals. Generally, the immune response depends on the successful interaction of the antigen with different leukocytes (i.e., lymphocytes, macrophages, and other accessory cells of the lymphoid organs; Banerjee 1999). The inability to mount a proper immune response during a stressor may cause permanent damage or a disease state.

Although we predicted there would be a change in neutrophil to lymphocyte ratios, because it is commonly used as a marker of stress (Davis et al. 2008; Goessling et al. 2015), neutrophil to lymphocyte ratios remained the same in both contaminated and reference animals (Figure 5B). Because there was no significant difference in neutrophil to lymphocyte ratio

between our 2 sites for either species, this absence of a fairly robust “stress” biomarker could suggest selection or physiological adaptations to this chronic exposure to allow animals to successfully survive and reproduce while maintaining very high body loads of organochlorines. Selection has clearly been shown to play a vital role in resistance to endocrine disruption. Mice that were administered DDT produced DDT-tolerant mice after 9 generations of selection (Ozburn and Morrison 1962). Likewise, quail fed a diet containing 200 ppm of DDT during the quails' first 30 d showed evidence of resistance after the third generation of selection, as indicated by lower mortality among the selected lines compared with a control line that was fed DDT (Patisaul et al. 2017). In addition, the physiological adaptation of hormesis could be present. Hormesis is a biphasic response to stressors (the physiological responses to a low “dose” of the challenge being the opposite of the responses to a high “dose”). Hormesis has been shown in several low-dose exposure toxicants, possibly leading to decreased susceptibility to pesticides in adulthood (Shutoh et al. 2009). It could be that chronic, low-level DDT exposure leads to development of resistance mechanisms to compensate for the decreased numbers of total white blood cells.

Cellular stress response

Studies have shown that animals living in polluted environments had an overall increase in oxidative stress (Isaksson 2010), with protein carbonyls increasing, in a dose-dependent manner, after pesticide exposure (Banerjee et al. 2001; Parvez and Raisuddin 2005; Cecarini et al. 2007). Protein carbonyls are relatively stable and represent an irreversible form of protein modification that can cause damage to proteins and surrounding tissue (Almroth et al. 2008; Weber et al. 2015), as well as an imbalance between pro- and anti-oxidant defense mechanisms (Banerjee et al. 2001). An imbalance of ROS can then influence the function of several cellular processes,

such as cell signaling and structure and can cause DNA alterations (Cecarini et al. 2007). In *S. hispidus*, protein carbonyls were significantly higher in the contaminated site, whereas this measure was not significantly different between sites in *P. maniculatus* (Figures 6C and 7C). Within the liver, mitochondria are the main sources of ROS in hepatocytes and are the prominent target of hepatotoxic chemicals such as DDT. Thus DDT and its metabolites may be causing mitochondrial dysfunction resulting in ROS and leading to oxidative stress (Harada et al. 2016). In our study, *S. hispidus* had lower levels of DDTs stored in their liver but expressed signs of oxidative stress, suggesting that liver levels of DDTs do not necessarily indicate amount of exposure. The combination of oxidative stress and high levels of DDTs within the liver could adversely affect reproduction and cause a decrease in survival.

Both HSP 70 and HSP 90 are frequently up-regulated during stress to assist in the refolding of damaged proteins, and they play a critical role in cell growth, survival mechanisms (i.e., immune response) apoptosis, and inflammatory response (Mitra et al. 2018). In mammals, specifically, HSP 90 has been found to associate with cellular proteins, including steroid receptors, and it exhibits a 3- to 5-fold synthesis increase on exposure to a stressor (Sanders 1990). The HSP 90 protein levels in *P. maniculatus* from the contaminated site (which had a much higher DDT burden than *S. hispidus*) exhibited significantly higher levels than the reference group, whereas the other species did not exhibit this pattern. The function of HSP 90 as a chaperone is an essential protective component during stress (Park and Kwak 2014), and appears to play a protective role for rodents inhabiting the DDT Superfund site. No significant differences were found in HSP 70 or 4-HNE in either species. Our finding of a lack of significant difference in either species for these markers could be due to 1) the lack of morphological damage in the liver that is normally noted prior to lipid peroxidation, or 2) an absence of metal ions to decompose

lipid peroxides, therefore providing very little secondary byproducts (i.e., 4-HNE) to be measured (Repetto et al. 2012). Levels of HSPs in rat brain and algae have been documented to increase in a dose-dependent manner after exposure to DDE (Bierkens et al. 1998; Ammon-Treiber et al. 2004). It is possible that our lack of change in HSP 70 levels may simply be due to adaption from long-term exposure to DDT, with long-term exposure, again, possibly inducing a hormetic effect.

CONCLUSIONS

Overall, the present study documented markedly high DDT, DDE, and DDD soil levels in a DDT abatement site, above those reported in a previous survey, potentially indicating that these contaminants continue to leach and spread in the environment. We also found that individuals of both species inhabiting a historically contaminated area exhibited high levels of DDTs in liver tissue (and, by extension, high body burdens of the DDTs), with *P. maniculatus*, an omnivore, experiencing levels an order of magnitude (20× higher) above those found in *S. hispidus*, a predominantly herbivorous species. Despite this difference in the degree of body burden of DDTs between the 2 species, both resident species exhibited a dramatically lower number of circulating total leukocytes, primarily due to a significantly lower number of lymphocytes. In addition, both species showed a significant increase in an oxidative stress measure (protein carbonyl or HSP 90), which also indicates cellular dysfunction.

The low numbers of circulating lymphocytes in both species may indicate higher disease susceptibility, and the presence of biomarkers of oxidative stress in each species would also indicate lower physiological performance. Despite these indicators, these mammals are persisting and reproducing even with these high body burdens, suggesting there has been some selection or adaptation to compensate for the depression of a critical immune defense. This resistance to high

DDT body burdens allows these mammals to be available to enter the food chain and be a source of biomagnification in higher order consumers. Because many DDTs are ubiquitously found in both terrestrial and aquatic environments, the potential for chronic, low-level exposure throughout the daily life of wildlife and humans is substantial and merits further study.

Work Cited

Abd Allah, A. A., E. A Ibrahim, E. S Ibrahim, N. F Mahmoud, and M. M Afify. 2018. Thiocyclam-Induced Reproductive Toxicity, Oxidative Stress and Genomic DNA Damage in Testicular Tissues of Rats: Protective Effects of Green Tea Extract. *J Anal Toxicol*.

Aislabie, J. M., N. K. Richards, and H. L. Boul. 1997. Microbial degradation of DDT and its residues—A review. *New Zealand J Agric Res.* 40:269–282.

Almroth, B. C., J. Sturve, E. Stephensen, T. F. Holth, and L. Förlin. 2008. Protein carbonyls and antioxidant defenses in corkwing wrasse (*Symphodus melops*) from a heavy metal polluted and a PAH polluted site. *Mar Environ Res.* 66:271–277.

Ammon-Treiber, S., G. Grecksch, R. Stumm, U. Riechert, H. Tischmeyer, A. Reichenauer, and V. Höllt. 2004. Rapid, transient, and dose-dependent expression of Hsp70 messenger RNA in the rat brain after morphine treatment. *Cell Stress Chapter.* 9:182.

Arroyo-Salgado, B., J. Olivero-Verbel, A. Guerrero-Castilla, B. Arroyo-Salgado, J. Olivero-Verbel, and A. Guerrero-Castilla. 2016. Direct effect of p,p'- DDT on mice liver. *Brazilian J Pharm Sci.* 52:287–298.

ATSDR. 2002. ToxGuide for DDT/ DDD/DDE. Available from https://www.atsdr.cdc.gov/toxguides/toxguide-35.pdf

Banerjee, B. D. 1987. Effects of sub-chronic DDT exposure on humoral and cell-mediated immune responses in albino rats. *Bull Environ Contam Toxicol.* 39:827–834.

Banerjee, B. D. 1999. The influence of various factors on immune toxicity assessment of pesticide chemicals. *Toxicol Lett.* 107:21–31.

Banerjee, B. D., V. Seth, and R. S. Ahmed. 2001. Pesticide-Induced Oxidative Stress: Perspectives and Trends. *Rev Environ Health* 16.1:1-40.

Bierkens, J., J. Maes, and F. V. Plaetse. 1998. Dose-dependent induction of heat shock protein 70 synthesis in Raphidocelis subcapitata following exposure to different classes of environmental pollutants. *Environ Pollut.* 101:91–97.

Bowerman WW, Giesy JR, Best DA, Kramer VJ. 1995. A review of factors affecting

productivity of bald eagles in the Great Lakes region: implications for recovery. *Environ Health Perspect.* 103:9.

CB&I Federal Services LLC. 2016. RCRA Facility Investigation Report (Project Number 14685). *Environmental management division.*

CDC. Washington, DC: Centers for Disease Control and Prevention. 2019. Vol 2 Fourth National Report on Human Exposure to Environmental Chemicals. Available at: http://www.cdc.gov/exposurereport/

Cecarini, V., J. Gee, E. Fioretti, M. Amici, M. Angeletti, A. M. Eleuteri, and J. N. Keller. 2007. Protein oxidation and cellular homeostasis: Emphasis on metabolism. *BBA- Mol Cell Res.* 1773:93–104.

Cheek, A.O., & McLachlan, J.A. (1998). Environmental hormones and the male reproductive system. *J Androl* 19 (1):5-10.

Clotfelter, E. D., A. M. Bell, and K. R. Levering. 2004. The role of animal behaviour in the study of endocrine-disrupting chemicals. *Anim Behav.* 68:665–676.

Crews, D., E. Willingham, and J. K. Skipper. 2000. Endocrine disruptors: present issues, future directions. *Q Rev Biol.* 75:243–260.

Dalle-Donne, I., R. Rossi, D. Giustarini, A. Milzani, and R. Colombo. 2003. Protein carbonyl groups as biomarkers of oxidative stress. *Clin Chim Acta.* 329:23–38.

Davis, A. K., D. L. Maney, and J. C. Maerz. 2008. The use of leukocyte profiles to measure stress in vertebrates: a review for ecologists. *Funct Ecol.* 22:760–772.

Dhabhar, Firdaus S., and Bruce S. Mcewen. 1997. Acute Stress Enhances While Chronic Stress Suppresses Cell-Mediated Immunity in Vivo: A Potential Role for Leukocyte Trafficking. *Brain, Behavior, and Immunity* 11(4): 286–306.

Dwivedi, N., and S. J. S. Flora. 2011. Concomitant exposure to arsenic and organophosphates on tissue oxidative stress in rats. *Food Chem Toxicol.* 49:1152–1159.

Eskenazi, B., J. Chevrier, L. G. Rosas, H. A. Anderson, M. S. Bornman, H. Bouwman, A. Chen, B. A. Cohn, C. de Jager, D. S. Henshel, F. Leipzig, J. S. Leipzig, E. C. Lorenz, S. M. Snedeker, and D. Stapleton. 2009. The Pine River Statement: Human Health Consequences of DDT Use. *Environ Health Perspect.* 117:1359–1367.

Espinosa-Reyes, G., A. Torres-Dosal, C. Ilizaliturri, D. González-Mille, F. Díaz-Barriga, and J. Mejía-Saavedra. 2010. Wild rodents (Dipodomys merriami) used as biomonitors in contaminated mining sites. *J Environ Sci and Health, Part A.* 45:82-89.

Finger, J. W., J. Botero, Y. Zhang, S. E. Still, A. J. Hoffman, A. N. Kavazis, D. A. Cristol, and H. Wada. 2017. No Effect of Lifelong Methylmercury Exposure on Oxidative Status in Zebra Finches (Taeniopygia guttata): A Demonstration of Methylmercury-Induced Selection? *Bull Environ Contam Toxicol.* 99:668–672.

Gaikwad, A. S., P. Karunamoorthy, S. J. Kondhalkar, M. Ambikapathy, and R. Beerappa. 2015. Assessment of hematological, biochemical effects and genotoxicity among pesticide sprayers in grape garden. *J Occup Med Toxicol.* 10:11.

Garg, U. K., A. K. Pal, G. J. Jha, and S. B. Jadhao. 2004. Haemato-biochemical and immuno-pathophysiological effects of chronic toxicity with synthetic pyrethroid, organophosphate and chlorinated pesticides in broiler chicks. *Int Immunopharmacology.* 4:1709–1722.

Goessling, J. Kennedy, H. Mendonça, M., Wilson, A. 2015 A meta-analysis of plasma corticosterone and heterophil:lymphocyte ratios – is there conservation of physiological stress responses over time? *Funct Ecol.* 29:1189-1196

Guillette, L. J., T. S. Gross, G. R. Masson, and A. R. Woodwardff. 1994. Developmental abnormalities of the gonad and abnormal sex hormone concentrations in juvenile alligators from contaminated and control lakes in Florida. *Environ Health Perspect.* 102:9.

Guillette, L. J., and M. P. Gunderson. 2001. Alterations in development of reproductive and endocrine systems of wildlife populations exposed to endocrine-disrupting contaminants. *Repro.* 122:857–864.

Harada, T., M. Takeda, S. Kojima, and N. Tomiyama. 2016. Toxicity and Carcinogenicity of Dichlorodiphenyltrichloroethane (DDT). *Toxicol Res.* 32:21–33.

Hu, W., T. Wang, J. S. Khim, W. Luo, W. Jiao, Y. Lu, J. E. Naile, and J. P. Giesy. 2010. Organochlorine pesticides (HCHs and DDTs) in soils along the north coastal areas of the Bohai Sea, China. *Chem Ecol.* 26:339–352.

Hoffman, D. J., B. A. Rattner, G. A. Burton, Jr. C. John, Jr (Ed). 2003. Handbook of ecotoxicology. 2nd ed. Lewis Publishers, Boca Raton.

Isaksson, C. 2010. Pollution and Its Impact on Wild Animals: A Meta-Analysis on Oxidative Stress. *EcoHealth.* 7:342–350.

Jaga, K., and C. Dharmani. 2003. Global Surveillance of DDT and DDE Levels in Human Tissues. *Int J Occup Med Environ Health.* 16(1):7-20.

Köhler, H.-R., and R. Triebskorn. 2013. Wildlife Ecotoxicology of Pesticides: Can We Track Effects to the Population Level and Beyond? *Sci.* 341:759–765.

Makita, Y., T. Matsuura, R. Ogata, Y. Romero, M. Omura, A. Tanaka, M. Hirata, and N. Inoue. 2003. Systemic effects of orally administered p, p'-DDE on immature male Wistar rats during pubertal period. *J Occup Health.* 45:223–227.

Mansouri, A., M. Cregut, C. Abbes, M.-J. Durand, A. Landoulsi, and G. Thouand. 2017. The Environmental Issues of DDT Pollution and Bioremediation: a Multidisciplinary Review. *Appl Biochem Biotechnol.* 181:309–339.

Mitra, T., A. Mahanty, S. Ganguly, G. K. Purohit, S. Mohanty, P. K. Parida, P. R. Behera, R. K. Raman, and B. P. Mohanty. 2018. Expression patterns of heat shock protein genes in Rita rita from natural riverine habitat as biomarker response against environmental pollution. *Chemosphere.* 211:535–546.

Myers, P., R. Espinosa, C. S. Parr, T. Jones, G. S. Hammond, and T. A. Dewey. 2020. The Animal Diversity Web (online). Accessed at https://animaldiversity.org.

Novelli, E. L. B., Y. S. Diniz, C. M. Galhardi, G. M. X. Ebaid, H. G. Rodrigues, F. Mani, A. A. H. Fernandes, A. C. Cicogna, and J. L. V. B. Novelli Filho. 2007. Anthropometrical parameters and markers of obesity in rats. *Lab Anim.* 41:111–119.

Ozburn, G., Morrison, F. 1962. Development of a DDT-Tolerant Strain of Laboratory Mice. *Nature.* 196, 1009–1010.

Parry HA, Kephart WC, Mumford PW, Romero MA, Mobley CB, Zhang Y, Roberts MD, Kavazis AN. 2018. Ketogenic diet increases mitochondria volume in the liver and skeletal muscle without altering oxidative stress markers in rats. *Heliyon*. 4(11): e00975.

Parvez, S., and S. Raisuddin. 2005. Protein carbonyls: novel biomarkers of exposure to oxidative stress-inducing pesticides in freshwater fish Channa punctata (Bloch). *Environ Toxicol Pharm.* 20:112–117.

Park, K., and I.-S. Kwak. 2014. Characterize and Gene Expression of Heat Shock Protein 90 in Marine Crab Charybdis japonica following Bisphenol A and 4-Nonylphenol Exposures. *Environ Health Toxicol.* 29.

Patisaul, H. B., A. C. Gore, and D. Crews. 2017. Environmental Endocrine Disruption of Brain and Behavior. Pages 63–88 in Hormones, Brain and Behavior. Elsevier.

Phelps, Kendra. (2009). Ecological Characteristics of Small Mammal Communities at a Superfund Site. *The Am Midl Nat.* 161.1: 57-68.

Repetto, M., J. Semprine, and A. Boveris. 2012. Lipid Peroxidation: Chemical Mechanism, Biological Implications and Analytical Determination. Lipid Peroxidation. Catala, A., ed. InTech.

Restell, T. I., L. C. Porfirio, A. S. de Souza, I. S. Silva, T. I. Restell, L. C. Porfirio, A. S. de Souza, and I. S. Silva. 2014. Hematology of Swiss mice (*Mus musculus*) of both genders and different ages. *Acta Cirurgica Brasileira.* 29:306–312.

Sanders, B.M. 1990. Stress-proteins: Potential as multitiered biomarkers. In L. Shugart and J. McCarthy _eds. Environmental Biomarkers, pp. 165-191. Boca Raton, Florida: Lewis Publishers.

Sánchez-Bayo F. 2011. Impacts of Agricultural Pesticides on Terrestrial Ecosystems. *Ecological Impacts of Toxic Chemicals.* 63-87.

Schug, T. T., A. Janesick, B. Blumberg, and J. J. Heindel. 2011. Endocrine Disrupting Chemicals and Disease Susceptibility. *J Steroid Biochem Mol Biol.* 127:204–215.

Sericano JL, Wade TL, Sweet ST, Ramirez J, Lauenstein GG. 2014. Temporal trends and spatial distribution of DDT in bivalves from the coastal marine environments of the continental United States, 1986–2009. *Mar Pollut Bull*. 81(2):303–316.

Shutoh, Yasufumi, Makio Takeda, Ryoichi Ohtsuka, Atsuko Haishima, Satoru Yamaguchi, Hideaki Fujie, Yutaka Komatsu, Keizo Maita, and Takanori Harada. 2009. Low dose effects of dichlorodiphenyltrichloroethane (DDT) on gene transcription and DNA methylation in the hypothalamus of young male rats: implication of hormesis-like effects. J. Toxicol. Sci. 34(5): 469-482.

Sikka, S. C., and R. Wang. 2008. Endocrine disruptors and estrogenic effects on male reproductive axis. *AJA* 10:134–145.

Song, Q., P. Zheng, L. Qiu, X. Jiang, H. Zhao, H. Zhou, Q. Han, and X. Diao. 2016. Toxic effects of male Perna viridis gonad exposed to BaP, DDT and their mixture: A metabolomic and proteomic study of the underlying mechanism. *Toxicol Lett.* 240:185–195.

U.S. Environmental Protections Agency. 2008. Health effects support document for 1,1-Dichloro-2,2-bis (p-chlorophenyl)ethylene (DDE) (Report number EPA-822-R-08-003). Retrieved from https://www.epa.gov/sites/production/files/2014-09/documents/health_effects_support_document_for_dde.pdf

Weber, D., M. J. Davies, and T. Grune. 2015. Determination of protein carbonyls in plasma, cell extracts, tissue homogenates, isolated proteins: Focus on sample preparation and derivatization conditions. *Redox Biol*. 5:367–380.

Wolf, C., C. Lambright, P. Mann, M. Price, R. L. Cooper, J. Ostby, and L. E. Gray Jr. 1999. Administration of potentially antiandrogenic pesticides (procymidone, linuron, iprodione, chlozolinate, p, p′-DDE, and ketoconazole) and toxic substances (dibutyl-and diethylhexyl phthalate, PCB 169, and ethane dimethane sulphonate)

during sexual differentiation produces diverse profiles of reproductive malformations in the male rat. *Toxicol Ind Health.* 15:94–118.

Woodwell, George M. 1967. Toxic Substances and Ecological Cycles. *SciAm.* 216(3): 24–31.

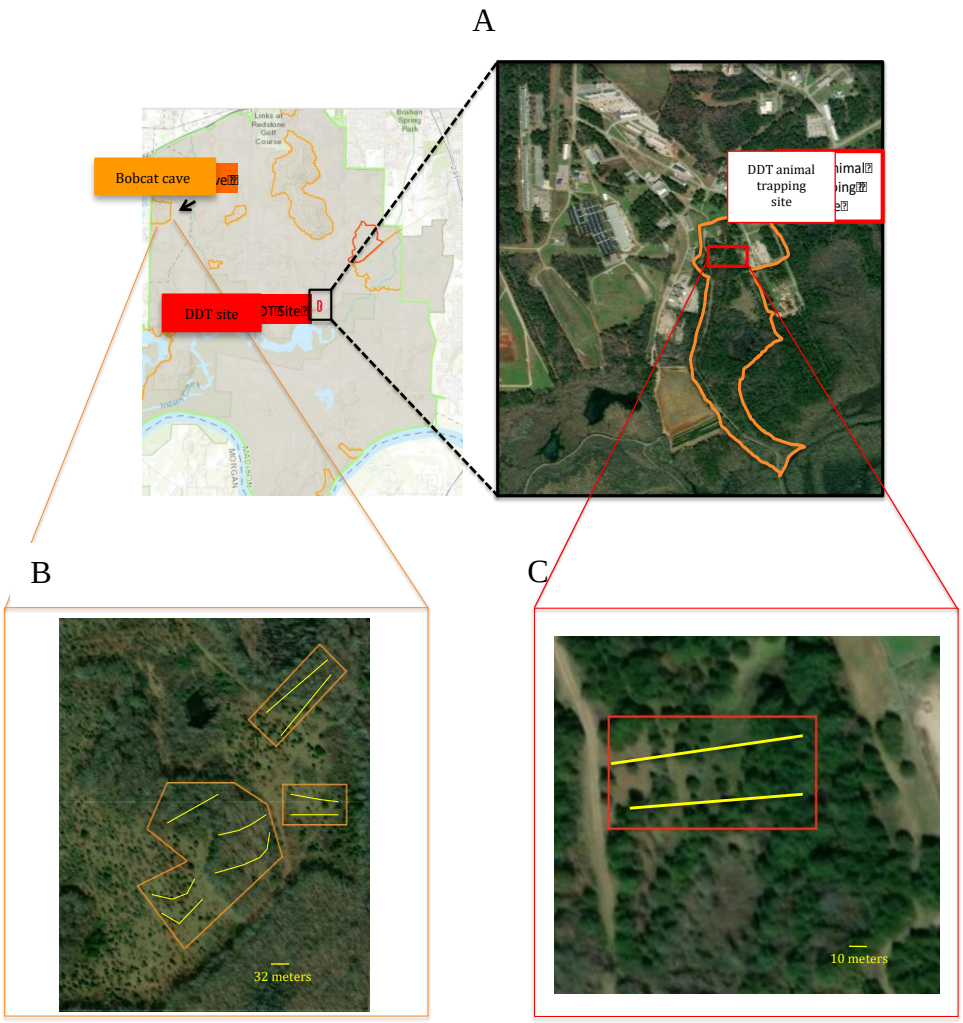

Figure 2. (A & C) DDT abatement area (contaminated site) and (B) Bobcat Cave (reference site) sites at Redstone Arsenal in Huntsville, AL. Yellow lines representing each transect placed at each site.

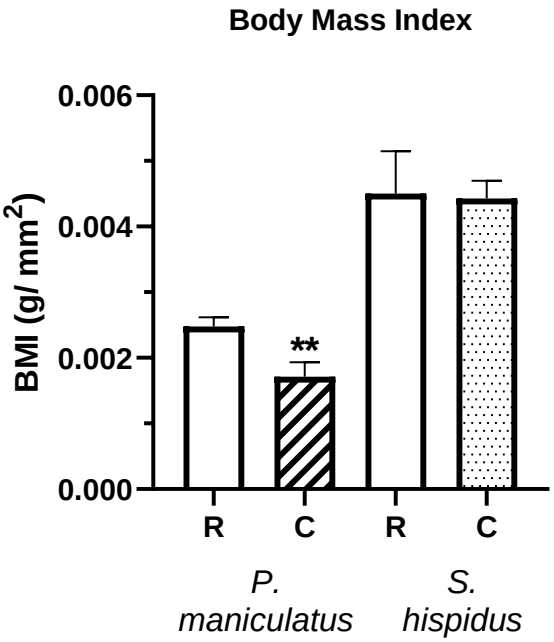

Figure 3. Body mass index for all animals from start of trapping to conclusion, deer mice (*Peromyscus maniculatus*) and cotton rats (*Sigmodon hispidus*) in our reference site (R) versus our contaminated site (C). (*Peromyscus maniculatus*- R: n=23, C: n= 14; *S. hispidus*- R: n=4, C: n=35). *Peromyscus maniculatus* BMI was significantly decreased in contaminated site compared to reference site. (***p= 0.002).*

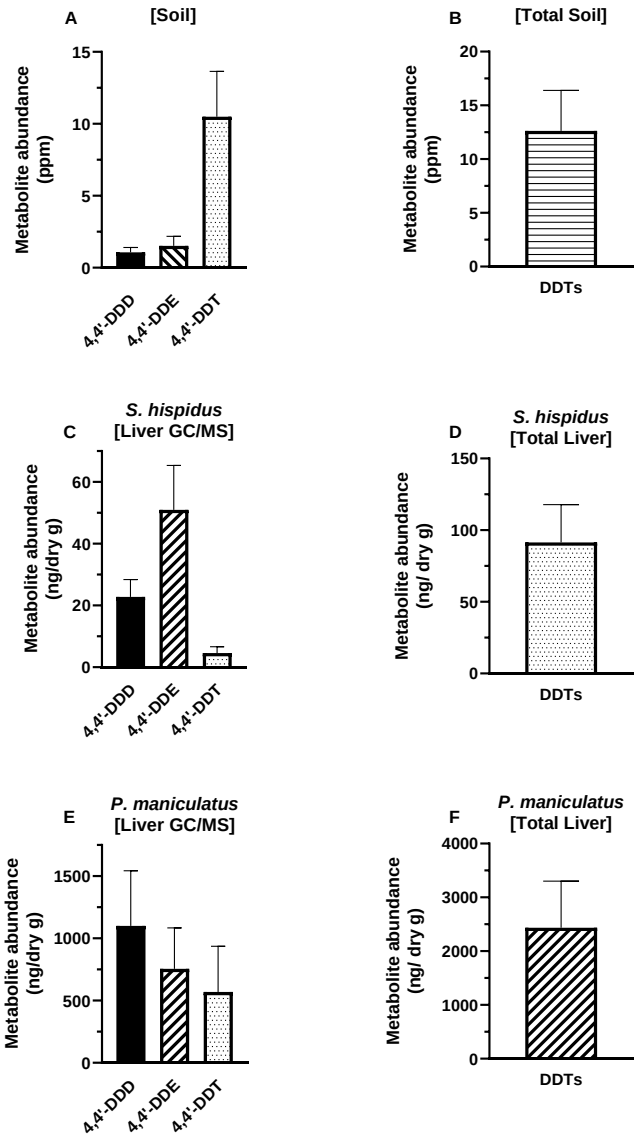

Figure 4. Abundance of DDTs within (A) soil samples (n=11) and (B) total abundance of soil DDTs (combined concentrations of DDT, DDD and DDE) added up from every sample taken. (C) Liver samples from cotton rat (*Sigmodon hispidus*) within the DDT abatement site (n=12) and (D) total abundance of liver DDTs (DDT, DDD and DDE) added up from every sample taken. (E) Liver samples from deer mice (*Peromyscus maniculatus*) within the DDT abatement site (n=12) and (F) total abundance of liver DDTs (DDT, DDD and DDE) added up from every sample taken. Liver and soil from our reference area showed levels below detection for all metabolites (data not shown).

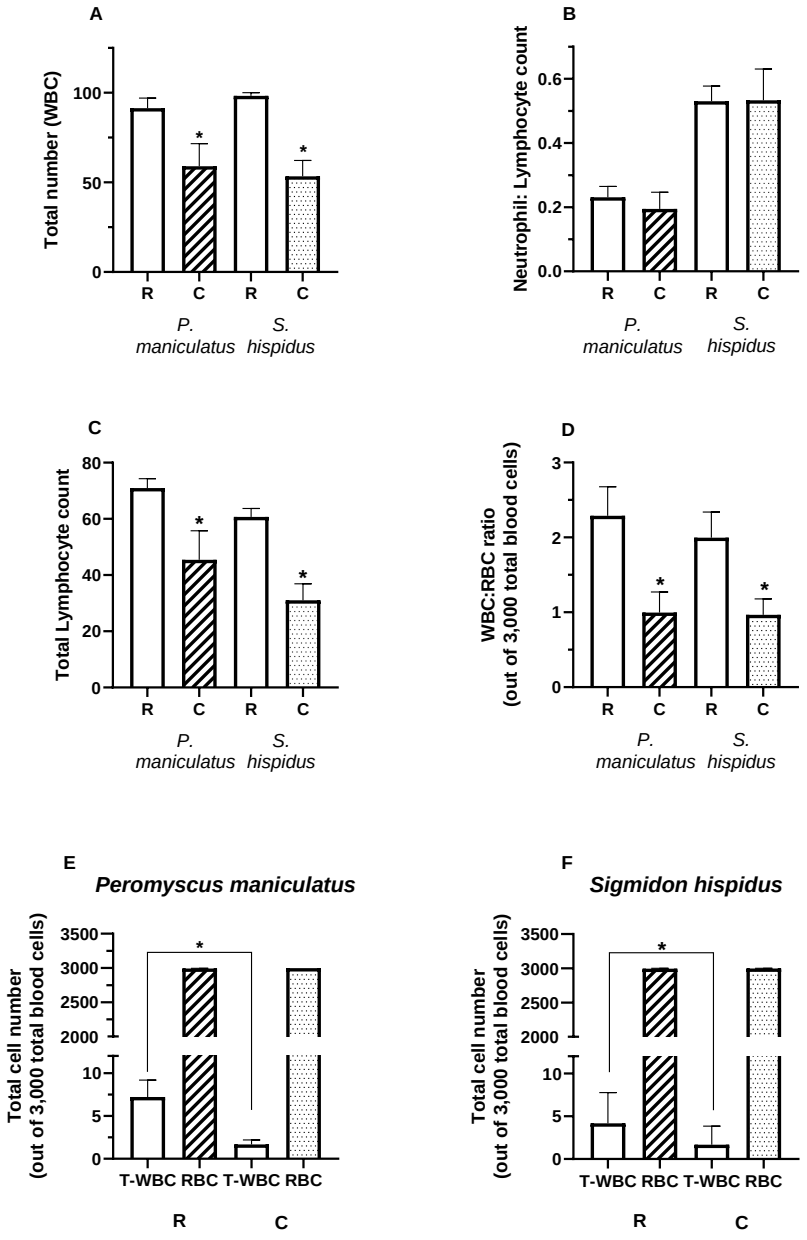

Figure 5. Analysis of (A) total white blood cell count (B) Neutrophil:Lymphocyte ratio, (C) total lymphocyte count, (D) WBC:RBC differential ratio and (E, F) total blood cell differential via microscopic examination of blood smears and (F). Total white blood cell count, total lymphocyte count, leukocyte differential counts and WBC:RBC differential ratio were found to be significantly lower in both deer mice (*Peromyscus maniculatus*) *and* cotton rat (*Sigmodon hispidus*) collected from our contaminated sites when compared to animals from the reference site. (**p=0.05*)

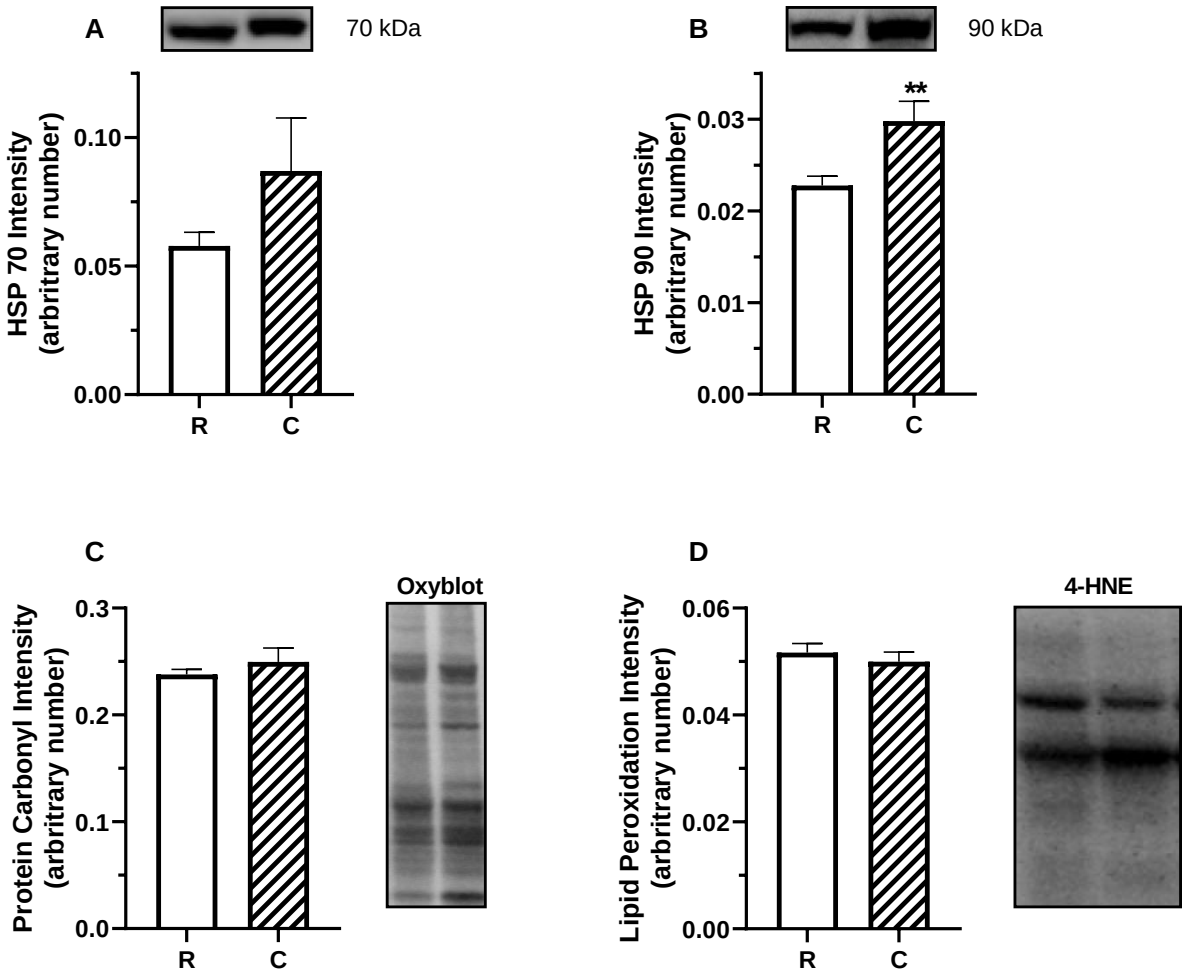

Figure 6. Effects of DDT on oxidative stress markers in the liver of deer mice (*Peromyscus maniculatus*) after environmental exposure at a DDT superfund site. (A) HSP 70 (B) HSP 90 (C) Oxyblot and (D) Lipid peroxidation (4-HNE) protein was analyzed in liver. Protein levels were normalized to total protein using a Ponceau stain on the same membrane prior to antibody incubation. (R: n=13-17; C: n=10-11; ***p= 0.002*)

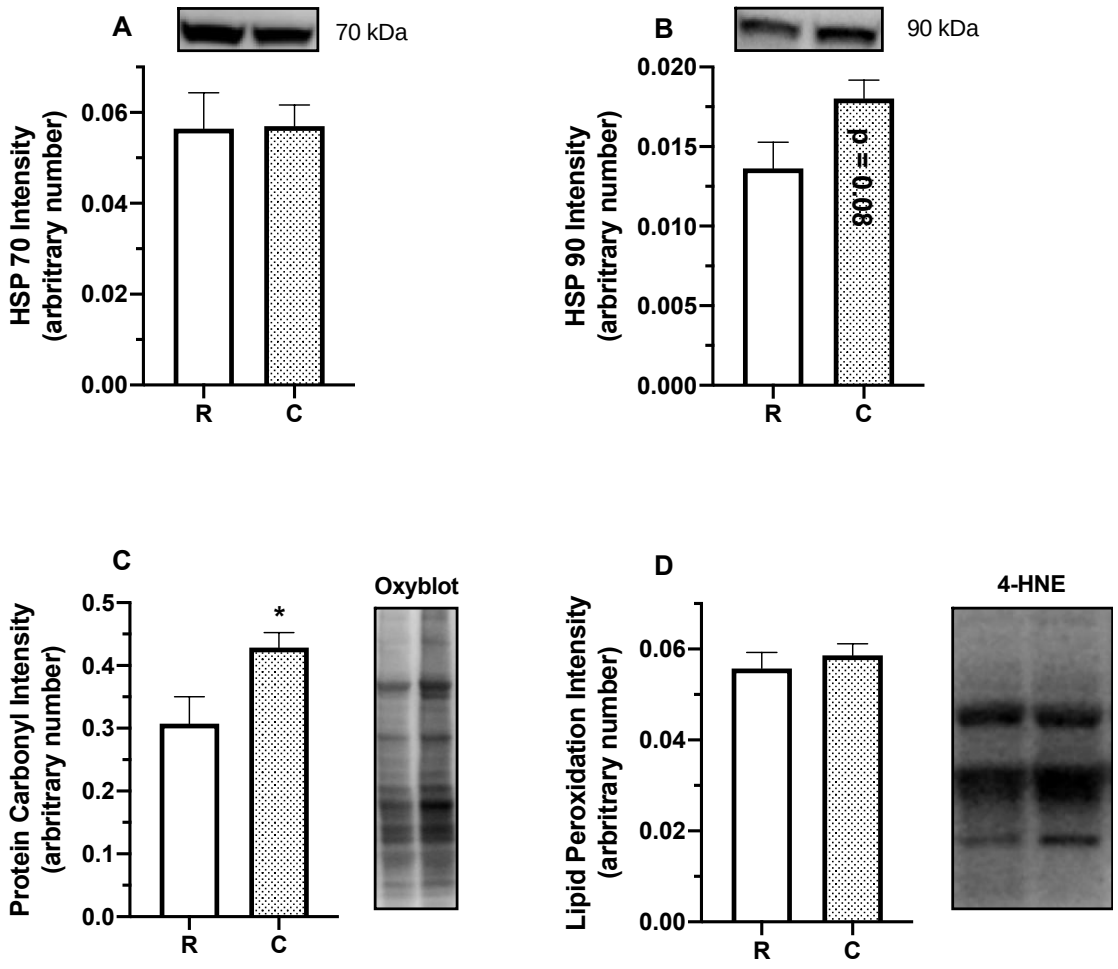

Figure 7 Effects of DDT on oxidative stress markers in the liver of cotton rats (*Sigmodon hispidus*) after environmental exposure at a DDT superfund site. (A) HSP 70 (B) HSP 90 (C) Oxyblot and (D) Lipid peroxidation (4-HNE) protein was analyzed in liver. Protein levels were normalized to total protein using a Ponceau stain on the same membrane prior to antibody incubation. (R: n=6; C: n=17-24; *$p= 0.05$)

Chapter 3: Effects of different DDE exposure paradigms on testicular steroid hormone secretion and hepatic oxidative stress in male Long-Evans rats

FORMAT FOR GENERAL AND COMPARATIVE ENDOCRINOLOGY

Effects of different DDE exposure paradigms on testicular steroid hormone secretion and hepatic oxidative stress in male Long-Evans rats

Erica M Molina[a], Andreas N Kavazis[b], Mary T Mendonça[a], Benson T Akingbemi[c]

[a]Department of Biological Sciences, Auburn University, 331 Funchess Hall, 350 South College St, Auburn, AL 36849, USA. Email: emm0044@tigermail.auburn.edu (Corresponding author)

[b]School of Kinesiology, Auburn University, 287 Kinesiology Building, 301 Wire Road, Auburn, AL 36849, USA.
Email: ank0012@auburn.edu

[c]Department of Anatomy, Physiology and Pharmacology, Auburn University, 1130 Wire Road, Auburn, AL 36849, USA.
Email: akingbt@auburn.edu

HIGHLIGHTS

1) Relatively chronic exposure to very low DDE resulted in exposed male rats having significantly decreased serum T levels and disrupted the normal T: E2 ratio

2) Contrary to our initial prediction, the above effect was more pronounced in pubertal rather than pre-pubertal male rats indicating DDE exposure affects male steroidogenesis and potentially sexual maturation

3) Testicular explants of DDE exposed pubertal rats when challenged with LH had significantly different T:E2 ratios, again most pronounced in pubertal individuals

4) Same DDT exposure level also produced an significant increase in an oxidative stress biomarker in liver tissue (HSP 90), but not in other expected biomarkers

ABSTRACT

Chronic exposure to low doses of anthropogenic chemicals in the environment continues to be a major health issue. Due to concerns about the effects in humans and wildlife, use of persistent organic pollutants, such as dichlorodiphenyltrichloroethane (DDT), is prohibited. However, their ubiquitous nature and persistence allows them to remain in the environment at low levels for decades. Dichlorodiphenyldichloroethylene (DDE) is the most persistent metabolite of DDT and has been shown to cause hepatotoxicity, nephrotoxicity, hormonal disorders, and induce oxidative stress in many organisms. Although the effects of acute exposure to DDT and its metabolite DDE have been extensively studied, the chronic effects of sub-lethal DDE exposure at levels comparable to those found in the environment have not been well documented. Long-Evans male rats were used to determine the effect of relatively chronic and short term DDE (doses ranged from 0.001-100 μg/L) exposure on endocrine function and oxidative stress at different developmental time points. We found that circulating serum testosterone (T) levels were significantly decreased and T secretion in testicular explants were significantly influenced in a dose dependent manner in both pre-pubertal and pubertal male rats after DDE exposure, with pubertal rats being the most affected contrary to our original prediction. Additionally, exposure to DDE increased expression of protein oxidation indicating a possible increase in cellular damage caused by oxidative stress. These results suggest that chronic exposures to environmentally relevant levels of DDE affected testicular function and decreased T secretion with implications for reproductive capacity.

Keywords: DDE; oxidative stress; chronic exposure; hormone expression

1.1 INTRODUCTION

A variety of anthropogenic pollutants, many of which disrupt natural endocrine function and adversely affect development and physiology (Zala and Penn, 2004), have been and continue to be introduced into the environment. Dichlorodiphenyltrichloroethane (DDT) and its metabolite dichlorodiphenyldichloroethylene (DDE) are chemically stable compounds that can remain in the environment for decades. Because of their lipohilic nature, DDT and DDE accumulate within food chains and be found in exposed human blood samples, hair, milk and fat (Alhama et al., 2018; Koureas et al., 2019). In humans, greater than 80% of the ingested pesticide is consumed through food sources, with studies showing higher than allowable DDE and DDT levels in daily food intakes (Muralidharan et al., 2009). DDT can be found in parts per *trillion* in water but has been documented in parts per *million* in animals, especially top carnivores (Khan et al., 2008).

The most persistent metabolite of DDT is DDE and it has been shown to cause hepatotoxicity, nephrotoxicity, hormonal disorders and induce oxidative stress in many organisms (Migliaccio et al., 2019). Studies have indicated that individuals are most sensitive to sub-lethal doses of endocrine disruptors, such as DDE, during their early life stages (Clotfelter et al., 2004; Wolf et al., 1999; Crews et al., 2000). For example, offspring of dams that were gavaged with DDE (100 mg/kg) for 14-18 days during pregnancy had a high incidence of retained nipples and shortened anogenital distance at birth, indicating an antiandrogenic effect (Kelce et al., 1995). Krause (1997) found that exposing male rats to 100 mg/kg of DDT daily for two weeks resulted in a significant decrease in testicular and serum testosterone (T) concentrations.

Male sexual differentiation is critically dependent on the actions of androgens and an imbalance in the ratio of estrogens to androgens can disturb it, which could result in a decrease in sperm production and fertility (Rochira et al., 2016). Over the last 50 years, a decline in semen quality has occurred worldwide (Cheek and McLachlan, 1998). The introduction of estrogenic compounds such as DDT into the environment was associated with a decrease in adult sperm count and increasing incidents in testicular anomalies (De Jager, 2006; Kelce et al., 1965; Jeng, 2014). The process of spermatogenesis is especially vulnerable to toxicants due to the large number of cell divisions that occur at this time (Bonde, 2010). Oxidative stress is one of the many mediators of male infertility that has been identified as a cause of sperm dysfunction (Agarwal et al., 2014). Since DDT binds to estrogen receptors and its metabolite DDE has been documented to have anti-androgenic effects, both have been linked to reproductive tract anomalies although direct evidence is still lacking (De Jager, 2006).

DDT and DDE can affect circulating sex hormones (i.e. estrogens, progestins, and androgens) and adversely affect reproductive physiology (Migliaccio et al., 2019; Scippo et al., 2004; Guillette and Gunderson, 2001; Patisaul and Adewale, 2009). For example, DDE exposures at 20 mg/kg induced apoptotic cell death in the testis of rats (Shi et al., 2009). Alligators maturing in Lake Apopka Florida located next to an Environmental Protection Agency (EPA) Superfund site with documented DDT contamination had an array of reproductive abnormalities, including altered testicular steroidogenesis, decreased T concentrations, and high estradiol (E2) concentrations (Guillette et al., 1994). Furthermore, changes within the endocrine system (i.e. hypothalamus-pituitary-

gonadal axis (HPG axis)) in rodents have been shown to affect the rate of meiotic segregation errors (McAuliffe et al., 2012).

The liver appears to be one of the main targets of toxicity due to DDT and its metabolites (U.S. Department of Health and Human Services, 2019). The liver is hematopoietic and responds to injury by producing extensive cell proliferation (Li et al., 2017). In mice it was shown that stem cells within the transplanted liver can produce leukocytes to rescue immunodeficient mice (Seki et al., 2000), possibly allowing it to rescue cells from DDE toxicity. DDE exposure has also been implicated in inducing physiological and cellular oxidative stress. Therefore, measures of cellular dysfunction such as oxidative stress measures (i.e. presence of reactive carbonyl derivatives, lipid peroxidation (4-HNE) and heat shock proteins) in the liver can aid in assessing cellular damage linked to chemical exposure.

Acute *in vitro* and *in vivo* organochloride exposure has been documented to cause immunosuppression and cellular apoptosis, potentially due to the formation of reactive oxygen species (ROS; Dowling et al., 2006). During exposure to stressors, most organisms can produce proteins that facilitate defense against cellular damage that is linked to ROS (Lewis et al., 1999). If normal defense mechanisms are overwhelmed, excess cellular damage and increased production of ROS results in irreversible introduction of aldehyde and ketone (carbonyl) groups to amino acid side chain, resulting in protein inactivation and degradation.

Standard biomarkers of oxidative stress include the presence of reactive carbonylated proteins (to assess protein damage) and 4 hydroxy 2 nonenal (4-HNE), the most reactive aldehyde, to assess lipid peroxidation (Dowling et al., 2006; Braconi et al.,

2011; Shulaev and Oliver, 2006; Breitzig et al., 2016). Other measures used to assess an organism's attempt to mitigate ROS or other environmental stressors are heat shock proteins (HSPs). HSPs are highly conserved in all organisms and play a pivotal role in cellular protective responses to environmental stressors and pollutants (Lewis et al., 1999; Sanders, 1990; Kim et al., 2014; Mitra et al., 2018; Lewis et al., 1999; Mukhopadhyay et al., 2003).In particular, HSP 70 and HSP 90 are found to be upregulated during stress to assist in the refolding of damaged proteins (Mitra et al., 2018). This cellular response can lead to a measurable upregulation of HSP 70 in animals during a stressor.

The physiological responses to short-term exposures of pollutants are relatively well studied, but the understanding of how long-term exposure (i.e. life- long exposure to sub-lethal, environmentally relevant levels) affects an organism is lacking (Molina et al., 2021). Characterizing the ability of chronic low exposures to impact reproductive function and induce oxidative stress will give a better insight into mechanisms of toxicity due to lifetime exposure to endocrine disruptors. Therefore, the objective of this work is to assess the influence of DDE exposures at environmentally relevant to sub-lethal doses on 1) reproductive endocrine function (i.e. changes in circulating serum T and E2 levels, pituitary gonadotropin levels, and testicular steroidogenesis) 2) oxidative stress (lipid peroxidation; reactive carbonyl derivatives) and cellular stress (i.e. HSP 70; HSP 90), and 3) age (pre-pubertal vs pubertal) of onset of DDE exposure. We hypothesize that early onset exposure of DDE will cause a greater inhibition of steroidogenesis and induce higher levels of oxidative stress in pre-pubertal male rats than in pubertal male rats in a dose-dependent manner.

1.2 MATERIALS AND METHODS

1.2.1.Experiment 1- Chronic (one month) DDE exposure

We investigated the dose-response of chronic exposure (4-weeks) in pre-pubertal Long-Evans male rats to DDE. Pre-pubertal male rats at postnatal day (PND) 21 (n= 6/dose) were exposed daily for 28 days to a range of DDE doses (0, 20, 40, 50 and 100 μg/L in drinking water) and then assessed for changes in markers of oxidative stress and gonadal sex steroid hormone secretion. Male rats were obtained from Harlan-Teklad (Indianapolis, IN) and allowed to acclimate for three days at the College of Veterinary Medicine Division of Laboratory Animal Health Housing. Animals were separated into groups of three per standard plastic cage (length, 0.47m; width 0.25m; height 0.22m) lined with wood chip bedding. DDE (Sigma-Aldrich, 35487) was provided, *ad libitum* in glass water bottles, according to assigned treatment dosage groups from 21 to 49 days of age. Doses were extrapolated from previous research that showed feminization, increased liver weights and an increase in testosterone in animals exposed to DDT and DDE (ASTDR, Leavens et al.,2002; Mathur and D'Cruz, 2011). Animals were maintained under standard light:dark conditions (12L:12D) and room temperature (20-23^{o}C), with *ad libitum* access to soy- and alfalfa-free pelleted food (X2020, Harlan-Teklad, Indianapolis IN). Animals were euthanized within 24 h of terminating chemical treatment to obtain blood and tissues for analysis (i.e. blood, testes, liver, and pituitary glands). All liver, pituitary and testis were flash frozen in liquid nitrogen and stored at -80^{o}C until the time of assay. Blood was refrigerated and then centrifuged to obtain serum after clotting occurred. Serum was then stored at -20^{o}C until the time of assay. All experimental and euthanasia procedures were performed in accordance with a protocol

approved by Auburn University Institutional Animal Care and Use Committee (PRN 2018-3399) and in compliance with the recommendations of the panel on Euthanasia of the American Veterinary Medical Association.

1.2.2 Experiment 2- Influence of age at onset of DDE exposure

We compared the influence of age on DDE exposure on oxidative stress and endocrine function measures. Two sets of male Long-Evans rats were obtained from Harlan-Teklad, one set at PND 21 (n=5/dose; designated pre-pubertal) and the second set at PND 35 (n=5/dose; designated as pubertal). Rats were allowed to acclimate for three days at the College of Veterinary Medicine Division of Laboratory Animal Health Housing. Animals were separated into groups of three per standard plastic cage (length, 0.47m; width 0.25m; height 0.22m) lined with wood chip bedding. Water was provided, *ad lib* in glass bottles, according to treatment from Pre-pubertal PND 21 to 35 and Pubertal PND 35- 49 (n=5/group). Animals were maintained under constant 12L:12D and temperature (20-23^{o}C), with ad lib access to pelleted soy- and alfalfa-free diet. Both groups of male rats were simultaneously exposed daily for two weeks to a lower range of DDE than in experiment 1 (0, 0.001, 0.01, 0.1, 1 and 10 μg/L of DDE in drinking water). All animals were euthanized within 24 h of terminating chemical treatment to obtain tissues for analysis, including blood, testis, liver, and pituitary glands. Tissues and blood were processed as stated in section 1.2.1.

1.3 Steroid hormone secretion

Serum was collected from trunk blood at the end of each experiment after animals were sacrificed and frozen at – 20 C until the time of assay. We measured basal serum testosterone (T) and estradiol (E2) concentration in males of all treatment groups using a

tritium-based radioimmunoassay protocol (Cochran et al., 1981), with an interassay variation of 7%–8%.

1.3.1 Follicle stimulating hormone and luteinizing hormone protein levels

We also measured levels of luteinizing hormone (LHβ) and follicle stimulating hormone (FSHβ) in pituitary tissues from DDE-exposed animals using primary antibodies for FSHβ is a mouse monoclonal raised against human amino acids 48-129 mapping at the C-terminus of FSHβ (sc374452; 1:1000) and LHβ, a mouse monoclonal specific for an epitope mapping between amino acids 109-135 near the C-terminus of Lutropin of rat origin (sc373941; 1:1000) Santa Cruz Biotechnology, Inc were used following the western blot procedure.

1.3.2 Testicular steroid hormone secretion

Immediately after euthanasia, testicular explants were obtained from males in both Experiments 1 and 2. Testicular explant aliquots were assigned as basal (incubated without LH) or LH-stimulated (incubated with LH added) to measure testicular T and E2 production. Explants were incubated in 1 ml DMEM/Ham's F-12 culture medium in triplicate without (basal) or containing 100 ηg/ml ovine LH (NIDDK, NIH) and put into a shaking water bath at 34° C for three hours. Explants were then centrifuged at 3000 RPM for 15 min and the supernatant was transferred into fresh tubes and stored at -20° C until aliquots of spent media were analyzed for E2 and T concentrations using a tritium-based RIA (Cochran et al., 1981). In testicular explants, hormone production was normalized to nanograms per testicular mass (milligrams).

1.3.3 Cellular stress response

We quantified the degree of protein oxidation in liver tissues from animals

exposed to the different doses of DDE using two methods: (1) measurement of protein oxidation via reactive carbonyl derivatives (oxyblot assay) and (2) measurement of lipid peroxidation via aldehyde-protein conjugates (4-HNE) as indicators of the magnitude of protein modification. In addition, we measured HSP 90 and HSP 70 levels in separate liver tissue samples. The relative concentration of proteins was quantified by Western blot analysis. The liver was homogenized 1:10 (wt/vol) in 5 mM Tris HCl (pH 7.5) and 5 mM EDTA (pH 8.0), and protease inhibitor cocktail (14224–396, VWR, Radnor, PA, USA) and centrifuged at 1500 g for 10 min at 4 °C. Protein content of the supernatant was quantified using a standard Bradford assay (Sigma). Equal amounts of proteins were loaded and separated by polyacrylamide gel electrophoresis via 12% polyacrylamide gels (BioRad, Hercules, CA, USA). After electrophoresis, the proteins were transferred to PVDF membranes via electrophoresis. The resulting membranes were stained with Ponceau S to analyze equal loading and transfer. Nonspecific sites were then blocked in phosphate-buffered saline (PBS) solution containing 0.1% Tween 20.

Membranes were incubated for 1 hour with primary antibody directed against lipid peroxidation (4-HNE), a rabbit polyclonal to 4-HNE, (Abcam: ab46545; 1:1000 dilution, Cambridge, MA, USA). Protein oxidation was measured by comparing the relative expression of protein carbonyls using a commercially available kit (S7150; 1:150 dilution, Oxy-Blot protein oxidation detection kit; Intergen, Purchase, NY, USA). For heat shock protein analysis, membranes were incubated for 1 hr with primary antibodies for HSP 70 a polyclonal (Ab79852; 1:1000) and HSP 90 (Ab13495; 1:1000)

Following incubation with primary antibodies, membranes were washed with PBS-Tween (5 min, 3x) and then incubated with appropriate secondary antibodies for 1 h

at room temperature. After washing (5 min, 3x), a chemiluminescent system was used to detect labeled proteins (GE Healthcare Life Sciences, Pittsburgh, PA). Images were taken and analyzed with the ChemiDoc-It Imaging System (UVP, Upland, CA). For multiple band analysis, full lane band densitometry was performed using UVP Imager.

1.4 STATISTICAL ANALYSIS

Data were analyzed by one-way analysis of variance (ANOVA) followed by the Dunnett test for post-hoc multiple group comparisons (GraphPad Prism software, San Diego, CA). Bar graphs are presented using SEM. The differences were considered significant when a $p \leq 0.05$ was obtained.

1.5 RESULTS

1.5.1 Steroid hormone secretion

Serum from groups of pre-pubertal male rats (PND 21-49: Experiment 1) was analyzed for T levels (Fig 8A) and E2 concentrations (Fig 8D). Male rats in the lowest DDE dose group (20 μg/L) exhibited significantly lower serum T concentration when compared to the other dose groups (p=0.008). However, the serum T in males exposed to higher doses did not differ significantly from the control group (doses – 40 μg/L; p=0.50, 50 μg/L; p=0.29 and 100 μg/L; p = 0.82; Fig 8A). On the other hand, males exposed to the highest doses (50 μg/L and 100 μg/L) had decreased serum E2 concentrations in a dose-dependent manner (p = 0.0001 and p=0.0001, respectively, Fig 8D), compared to control or lower DDE dose groups (doses – 20 μg/L; p=0.50 and 40 μg/L; p=0.29; Fig 8D). Also, T:E ratios were higher in males exposed to the highest DDE doses (50 μg/L and 100 μg/L; Fig 9A) and significantly so at the 100 μg/L dose (p=0.001). In contrast, lower dose exposures (i.e. 20 μg/L and 40 μg/L; Fig 9A) showed lower T:E2 ratios but

this did not differ significantly from that of the control group.

1.5.2 Experiment 2

Pubertal male rats which were exposed to DDE at different ages for two weeks, at doses much lower than Experiment 1, again exhibited significantly lower serum T concentrations in 0.1 μg/L, 1 μg /L and 10 μg /L dose groups (p=0.03, p=0.03 and p=0.03; Fig 10D) compared to control. Males exposed to the very lowest doses (i.e. 0.001 and .01 ug/L) did not exhibit this effect and had serum T similar to control (p=0.99, and p=0.46, respectively; Fig 10D). In contrast to observations for our pubertal males, pre-pubertal males did not exhibit the dose-response patterns in serum T observed in pubertal animals nor the patterns observed at the higher dose, longer exposures (Fig 10A).

Unlike pubertal males, pre-pubertal males exhibited significantly higher serum E2 at the 0.001 μg /L dose (p=0.03; Fig 11A). Pre-pubertal males exposed to 0.1 μg/L also had higher serum E2 concentrations compared to control (p =0.002 Fig 11A), causing a decreased T:E2 ratio at 0.1 μg/L exposure dose (p=0.03). Pubertal males did exhibit significantly higher serum E2 at the higher 10 μg /L dose compared to control (p=0.05, Fig 12A), but these higher levels did not result in a significanlt difference in their T:E2 ratios (p=>0.05, Fig 12D).

1.5.3 Testicular steroid hormone secretion

We challenged testicular explants from animals exposed to DDE for 28 days with LH in experiment 1. When testicular explants from pre-pubertal (PND 21-49) rats (Experiment 1) were incubated with LH, they did not differ significantly in steroid testicular T production at any doses when compared to control (Fig 8B). E2 secretion by the testes exposed to different DDE doses was similar in all groups, in contrast to the

pattern of circulating serum E2 concentration (Fig 8 E, F). Basal testicular T: E2 secretion did not differ significantly compared to controls. E2 secretion in LH stimulated testicular explant was higher, but not significantly so, in the 20 μg/L dose group than in controls (Fig 8) causing a parallel decrease in T:E ratio (p=0.04; Fig 9). All other E2 testicular explant levels did not differ significantly by dose compared to the control group.

In experiment two, we exposed pre-pubertal (PND 21-35) and pubertal (PND 35-49) male rats to a much lower DDE dose range for two weeks. DDE exposed pubertal male rats exhibited significantly lower levels of serum T than pre-pubertal males (even though they were exposed to the same range of DDE doses, $p<0.005$; Fig 10D). Additionally, testicular explants from pubertal males also exhibited, over a range of lower DDE dose exposures, significantly decreased T secretion both in the control (basal; 0.01 μg/L, 0.1 μg/L and 10 μg/L ($p = 0.02$, $p= 0.02$, and $p=0.04$; Fig 10E),) and LH-stimulated testicular T explants (0.01 μg/L, 0.1 μg/L, and 1 μg/L doses ($p=0.003$, $p = 0.001$, and $p= 0.01$; Fig 10F). In sharp contrast, only testicular explants from pre-pubertal males exposed to 0.001 μg/L of DDE showed a significantly lower T secretion compared to control ($p=0.03$; Fig 10B).

LH- stimulated testicular T:E2 ratios mirrored a similar pattern: pubertal males had significantly lower T:E2 ratios at 0.001 μg/L, 0.01 μg/L, 0.1 μg/L, 1 μg/L and 10 μg /L doses (respectively; $p=0.005$, $p=0.05$, $p=0.008$, $p=<0.0001$, $p=0.001$; Fig 5F). Furthermore, we saw significantly lower basal testicular T:E ratio at the 1 μg/L and 10 μg/L doses compared to control animals ($p=0.05$ and $p=0.03$; Fig 12E). All other T:E2 ratios and testicular E2 production did not differ significantly compared to control

animals (p> 0.05).

1.5.4 Follicular stimulating hormone and luteinizing hormone protein levels

When we examined FSHβ and LHβ protein expression in the pituitary tissue of males from the different dose regimens during experiment 2, we found FSHβ was not upregulated compared to the control group (p>0.05) in either pre-pubertal (exposure onset 21 days) or pubertal rats (exposure onset 35 days) (data not shown). LHβ content also showed no significant change within age groups (data not shown).

1.5.5 Cellular stress response

In pre-pubertal male rats (Experiment 1), cellular stress biomarkers such as HSP 70, HSP 90 and lipid peroxidation (4-HNE) were similar to the control (p>0.05; Fig 13). However, exposure to 10 μg/L and 100 μg/L of DDE resulted in significantly higher protein carbonyl derivatives compared to control (p= 0.001 and p<0.001; Fig 13C).

Similar to experiment 1, when DDE treated groups were exposed short-term (two weeks vs four weeks) to a lower range of DDE doses, there were no significant differences in HSP 90 in pre-pubertal and pubertal male rats compared to the control group (Fig 14 C, D). However, HSP 70 levels were significantly lower in pubertal but not pre-pubertal rats in the 0.1μg/L and 1μg/L dose groups compared to control (p= 0.02 and p=0.04; Fig 14B). There were no significant changes in protein lipid peroxidation in either age group when compared to control animals (P= >0.05; data not shown)

1.6 DISCUSSION

The present study demonstrated that exposure to environmentally relevant DDE levels (i.e. our doses of 0.001-1 μg/L) may adversely affect T production and mediate cellular oxidative stress, in an age dependent manner. In previous studies, effects due to

endocrine disruptors in invertebrates and mammals remain unclear because they focused on several day (i.e. up to 5 days) exposure paradigms (Diamanti-Kandarakis et al., 2009; Annamaiai and Namasivayam, 2015). This study represents the potential effects of extended exposure to environmentally relevant levels of DDE. Specifically, we explored the effects of different DDE exposure paradigms on endocrine function and oxidative stress. Although DDT has been banned, there are environmental clusters of DDT/DDE accumulations (i.e. EPA designated Superfund sites) throughout the US. For example, in a Recourse Conservation and Recovery Act Facility Investigation Report at Redstone Arsenal, AL DDE and DDT were measured in levels higher than EPA designated safe levels (i.e. 1.4 mg/kg in soil and 0.2 μg/L in drinking water). DDE in drinking water has been decreasing but the majority of US residents have detectable levels of DDE in their serum, indicating that exposure to DDE is common in the general population (CDC, 2019). In this present study, we explored the effects of DDE on pre-pubertal rats (onset PND 21-35) and pubertal rats (onset PND 35-49) which includes early stages of developmental programming where exposure to DDE and other chemicals would be expected to cause large effects on testicular function and sexual maturation (pubertal).

1.6.1 Serum and Testicular steroid hormone secretion

Environmental endocrine disruptors, such as DDE, have the ability to interrupt normal hormonal expression by either mimicking a target hormone or blocking the hormone from binding, in turn leading to an increase or decrease in hormone production (Schug et al.,2011). In experiment 1, rats treated chronically throughout the period between pre-pubertal and pubertal development (i.e. days 21-49) exhibited decreased serum T and abnormal LH-stimulated T:E2 ratios at the 20 μg/L dose, but not at higher

DDE doses. Additionally, exposure to the higher DDE doses resulted in significantly lower serum E2 levels, with a significant increase in T:E2 ratio at 100 μg/L dose when compared to controls. Since estradiol can be produced in fat (Cleary and Grossmann, 2009) and DDE is normally found at the highest levels within the fat (ATSDR, 2019), these lower estradiol levels may indicate that DDE might be inhibiting estradiol production or storage within adipose tissue. Generally, young male rats (16-25 days of age) exhibit low plasma T concentrations (≤0.5 ng/ml; *normal ranges*: ~0.66-5.4 ng/ml) with an increase in plasma T concentrations from day 26 until day 70 (~2.60- 2.95 ng/ml). At day 25-40, a steady rise of plasma LH occurs peaking at 60 ng/ml, while FSH markedly increases at day 16 with a peak occurring at day 33 (~770 ng/ml) (Gupta et al., 1975). Research has indicated that decreased levels of plasma FSH around 30 days of age with a simultaneous rise in LH plasma indicates a transition to sexual maturity in mice (Gupta et al., 1974, Lee et al., 1975). Thus, the age of approximately 26-35 PND would be considered the transition point from pre-pubertal to pubertal.

When we varied the age at onset of DDE exposure (Experiment 2), we observed a difference in steroid secretion patterns. In pubertal rats, 35-49 days of age, a dose as low as 0.1 μg/L resulted in significantly lower serum T levels, whereas pre-pubertal rats, 21-35 days of age, showed no changes in serum T regardless of DDE exposure dose (Fig 10). We also saw lower levels in testicular T explant secretion in pubertal vs. pre-pubertal rats, with pubertal rats' testicular explants exhibiting significantly lower T secretion in *both* the LH challenged and the non-challenged (basal) explants from males exposed to environmentally relevant DDE doses (Fig 10). Several studies indicate that the increased sensitivity that occurs in pubertal males is presumed to be due to higher levels of FSH

prior to maturation that increases LH receptors in the testis, therefore, increasing responsiveness or sensitivity to LH administration (Odell et al., 1973, Nazian and Mahesh, 1979, Stoker et al., 2000). Our study showed a similar trend in what naturally occurs in untreated male rats administered with LH. Similar to our pre-pubertal rats (onset PND21), immature male rats administered 100 ng/ml of LH showed no response to any doses from 7-28 days of age (Ariyaratne and Mendis-Handagama, 2000) thereby implying that pre-pubertal rats are less sensitive to LH than pubertal rats. Similar results were found when hypophysectomized immature males (21 days of age) administered up to 400 μg showed no response to any doses, whereas adult males (87 days of age) responded to LH as low as 0.66 μg. Nevertheless, if immature hypophysectomized rats were treated with both FSH and LH, they were then able to re-establish their LH responsiveness (Odell et al., 1973). This suggests that FSH plays a key role in upregulating LH receptors in the testis and thus the production of T (Stoker et al., 2000). We would expect that, as a male rat begins to mature, testosterone and LH levels would increase. Therefore, our lack of steroidogenic response is likely due to (1) changes within endogenous regulators (i.e LHRH, LH responsiveness, or production) or (2) an antiandrogenic effect preventing normal T steroidogenesis and/or secretion (Lister and Van Der Kraak, 2001; ATSDR, 2019).

1.6.2 Follicle stimulating hormone and luteinizing hormone protein levels

We did not see a significant change in either FSH or LH within the pituitary from rats exposed to any of the DDE doses (data not shown). However, pituitary FSH content was higher in pubertal rats, although not significantly, in a dose-dependent manner compared to pre-pubertal males. LH content, on the other hand, was similar within the

two age groups. LH is known to regulate Leydig-cells through cAMP-dependent and independent pathways (Dufau et al., 1987) producing T in response to LH binding to its receptor (Stoker et al., 2000). Therefore, it could be that there are fewer LH receptors at the testis causing decreased serum T levels. Altogether, a decrease in serum T and a lack of response to LH predominantly in pubertal rats indicates DDE may disrupt the establishment of sexual maturity in a dose-dependent matter (Zawatski and Lee, 2013) or indicate a lack of LH responsiveness in the testicular Leydig cells inhibiting T secretion (Fudvoye et al., 2014).

1.6.3 Cellular stress response

In experiment 1, protein carbonyl was significantly higher in livers of rats from the 10 μg/L and 100 μg/L doses indicating that DDE has the ability to induce protein carbonyls in a dose-dependent manner (Fig 13) similar to that observed after pesticide exposure causing an increase in protein carbonyls in a dose-dependent manner (Banerjee et al., 2001; Cecarini et al., 2007, Parvez and Raisuddin, 2005). Protein carbonyls have the ability to further react with α- amino acid groups on lysine residues leading to the formation of cross-links. These cross-links form protein aggregates that cannot be degraded by normal protein degradation mechanisms, leading to the accumulation of oxidative proteins that enhance cellular dysfunction (Cecarini et al., 2007). This result is important because the induction of oxidative stress is the final manifestation of a multistep process leading to an imbalance of pro- and anti-oxidant defense mechanisms (Banerjee et al., 2001), thereby influencing the function of several cellular processes (i.e. cell signaling, structure and enzymatic processes; Cecarini et al., 2007) suggesting that animals exposed chronically to DDE will exhibit altered cellular metabolism.

Since lipid peroxidation occurs as the result of oxidative stress damage to lipids causing hepatocellular injury and loss of cell viability during exposure to contaminants (Santra et al., 2000; Li et al., 2015), we suspected DDE's lipophilic nature might impact lipid peroxidation within the liver and serve as a good indicator of oxidative stress and cellular damage. For example, chronic exposure to DDE in mice decreased hepatic lipid content (i.e. significantly reduced triglycerides and *de novo* lipogenesis), despite having a high fat diet (Howell et al., 2015). Other contaminant studies have demonstrated a significant increase in liver lipid peroxidation in arsenic-treated mice with different exposure durations (Santra et al., 2000) and in a dose-dependent manner to DDT exposure in serum at 100ppm or 200 ppm for 8 weeks in mice (Koner et al., 1998). We did not see any significant changes in lipid peroxidation (4-HNE) in any of our studies despite it being a well-known marker of oxidative stress. This could be due to the lack of morphological damage in the liver (normally noted prior to lipid peroxidation), an absence of metal ions to decompose lipid peroxides, providing little secondary byproducts (i.e. 4-HNE) to be measured (Repetto et al., 2012) or DDE exposure not producing levels high enough to cause an imbalance.

Additionally, DDE has also been documented to cause apoptosis in Sertoli cells (via FAS-L pathways that induce caspase-3 and caspase-8 (Shi et al., 2009) and increase HSP 70 levels in rat brain and algae in a dose-dependent manner (Bierkens et al., 1998; Ammon-Treiber et al., 2004). HSP 70 acts as an inhibitor of apoptosis and caspase-3 to protect cells (Li et al 2000) and thus we expected to see increased levels of HSP 70 in animals exposed to higher doses or increased exposure time to DDE. We did not see this potentially protective increase even at the very low doses of DDE. Instead, HSP 70 levels

significantly *decreased* in a dose-dependent fashion but only in pubertal males (Li et al., 2000) that was not related to exposure dose or duration (experiment 1). With age, HSP 70 has a decrease in its chaperone activity, rate of synthesis and induction in response to stimuli (Bobkova et al., 2015), showing a 50% reduction in older mice hepatocytes than younger mice when exposed to heat shock (Heydari et al., 1994) and induced cellular differentiation, cell death and apoptosis in cancer cells (Tatsuta et al., 2014; Mayer and Bukau, 2005). However, the effects of declining HSP 70 with age in regards to lifespan and neurological parameters (i.e. neural density) were offset by long-term administration of intranasal human HSP 70 in aged mice with a significant increase in lifespan, improved memory and learning (Bobkova et al., 2015). Therefore, the lower levels of HSP 70 we observed in animals exposed to DDE at PND 35 (pubertal) may be due to a decreased ability with age in binding heat shock transcription factor (HSF), possibly caused by a decrease in post-translational activation of HSF (Heydari et al., 1994). Our results suggest that HSP 90 was not correlated with DDE exposure and did not change with age onset of exposure, exposure dose, or duration. Similar responses have also been observed in aquatic species exposed to cadmium, indicating that HSP 70 is a better biomarker of certain contaminants than HSP 90 (Zhang et al., 2016). Our study also supports the suggestion that HSP 70 appears to be a better biomarker than HSP 90 during DDE exposure.

1.7 Conclusion

In conclusion, this study demonstrates that exposure to DDE inhibits steroidogenic function, with lower ecologically relevant doses exhibiting an overall lower production of testosterone. Additionally, testosterone levels (either due to differential

production or secretion) are more sensitive to DDE during the pubertal life stage (as the animal is experiencing sexual maturity) vs. the pre-pubertal stage. Age at exposure to endocrine disrupting chemicals has important implications on the development of disease later in life, known as "the fetal basis of adult disease"(Schug et al., 2011). Additionally, DDE can induce up-regulation of protein carbonyl in a dose-dependent manner that may produce dysfunctional proteins, also potentially leading to a disease state. Since many DDT isomers are still used for agricultural purposes, vector control, or simply persist ubiquitously within both terrestrial and aquatic environments, the potential for low dose exposure to cause adverse effects in males merits attention. Possible mechanisms of testosterone inhibition include hypogonadism, specifically the reduction of testosterone production in Leydig cells. A further study that examines chronic exposure of low dose exposure to DDE that also examines the effects of DDE on Leydig cells would help pinpoint long-term effects of DDE on hormonal expression, specifically the effects we see on testosterone, and if these doses are able to induce oxidative stress long-term.

Acknowledgments

Animal handling and protocols were performed under Auburn University approved IACUC protocol (PRN 2018-3399). We would like to thank Rachel Knight (MS/AU), Samantha Bradley (BS/AU) and Bamidele Jeminiwa (MD/AU) for assistance with sample collection.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Work cited

Agarwal, A., G. Virk, C. Ong, and S. S. du Plessis. 2014. Effect of Oxidative Stress on Male Reproduction. World J Mens Health 32:1. http://doi.org/10.5534/wjmh.2014.32.1.1.

Alhama, J., C. A. Fuentes-Almagro, N. Abril, and C. Michán. 2018. Alterations in oxidative responses and post-translational modification caused by p,p´-DDE in Mus spretus testes reveal Cys oxidation status in proteins related to cell-redox homeostasis and male fertility. Science of The Total Environment 636:656–669. http://doi.org/10.1016/j.scitotenv.2018.04.305.

Annamalai, J., and V. Namasivayam. 2015. Endocrine disrupting chemicals in the atmosphere: Their effects on humans and wildlife. Environment International 76:78–97. http://doi.org/10.1016/j.envint.2014.12.006.

Ammon-Treiber, S., G. Grecksch, R. Stumm, U. Riechert, H. Tischmeyer, A. Reichenauer, and V. Höllt. 2004. Rapid, transient, and dose-dependent expression of Hsp70 messenger RNA in the rat brain after morphine treatment. Cell Stress Chaper 9:182. http://doi.org/10.1379/CSC-42.1.

Ariyaratne, H. B. S., and S. M. L. Chamindrani Mendis-Handagama. 2000. Changes in the Testis Interstitium of Sprague Dawley Rats from Birth to Sexual Maturity1. Biology of Reproduction 62:680–690. http://doi.org/10.1095/biolreprod62.3.680.

ATSDR. 2019. Agency for toxic substance and disease. Toxilogical profile for DDT, DDE, DDD. Available at: https://www.atsdr.cdc.gov/toxprofiles/tp35.pdf. (accessed 20 December 2019).

Banerjee, B. D., V. Seth, and R. S. Ahmed. 2001. Pesticide-Induced Oxidative Stress: Perspectives and Trends. :40. http://doi.org/10.1515/reveh.2001.16.1.1.

Bierkens, J., J. Maes, and F. V. Plaetse. 1998. Dose-dependent induction of heat shock protein 70 synthesis in Raphidocelis subcapitata following exposure to different classes of environmental pollutants. Environmental Pollution 101:91–97. http://doi.org/10.1016/S0269-7491(98)00010-4.

Bobkova, N. V., M. Evgen'ev, D. G. Garbuz, A. M. Kulikov, A. Morozov, A. Samokhin, D. Velmeshev, N. Medvinskaya, I. Nesterova, A. Pollock, and E. Nudler. 2015. Exogenous Hsp70 delays senescence and improves cognitive function in aging mice. Proc Natl Acad Sci USA 112:16006–16011. http://doi.org/10.1073/pnas.1516131112.

Bonde, J. P. 2010. Male reproductive organs are at risk from environmental hazards. Asian J Androl 12:152–156. http://doi.org/10.1038/aja.2009.83.

Braconi, D., G. Bernardini, and A. Santucci. 2011. Linking protein oxidation to environmental pollutants: Redox proteomic approaches. Journal of Proteomics 74:2324–2337. http://doi.org/10.1016/j.jprot.2011.06.029.

Breitzig, M., C. Bhimineni, R. Lockey, and N. Kolliputi. 2016. 4-Hydroxy-2-nonenal: a critical target in oxidative stress? American Journal of Physiology-Cell Physiology 311:C537–C543. http://doi.org/10.1152/ajpcell.00101.2016.

Cecarini, V., J. Gee, E. Fioretti, M. Amici, M. Angeletti, A. M. Eleuteri, and J. N. Keller. 2007. Protein oxidation and cellular homeostasis: Emphasis on metabolism. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research 1773:93–104. http://doi.org/10.1016/j.bbamcr.2006.08.039.

CDC. Washington, DC: Centers for Disease Control and Prevention; 2019. Vol 2. Fourth National Report on Human Exposure to Environmental Chemicals. Available at: http://www.cdc.gov/exposurereport/. (Accessed 18 March, 2020).

Cheek, A.O., & McLachlan, J.A. (1998). Environmental hormones and the male reproductive system. Journal of andrology, 19 1, 5-10. http://doi.org/10.1002/j.1939-4640.1998.tb02464.x.

Cleary, M. P., and M. E. Grossmann. 2009. Obesity and Breast Cancer: The Estrogen Connection. Endocrinology 150:2537–2542. http://doi.org/10.1210/en.2009-0070.

Clotfelter, E. D., A. M. Bell, and K. R. Levering. 2004. The role of animal behaviour in the study of endocrine-disrupting chemicals. Animal Behaviour 68:665–676. http://doi.org/10.1016/j.anbehav.2004.05.004.

Cochran R.C., Ewing L.L., and G.D. Niswender. 1981. Serum levels of follicle stimulating hormone, luteinizing hormone, prolactin, testosterone, 5 alpha-dihydrotestosterone, 5 alpha-androstane-3 alpha, 17 beta-diol, 5 alpha-androstane-3 beta, 17 beta-diol, and 17 beta-estradiol from male beagles with spontaneous or induced benign prostatic hyperplasia. Invest Urol 19:142–147. PMID: 6170603.

Crews D, Willingham E, Skipper JK. Endocrine disruptors: present issues, future directions. Q Rev Biol. 2000 Sep;75(3):243-60. http://doi.org/10.1086/393498.

De Jager, C. 2006. Reduced Seminal Parameters Associated With Environmental DDT Exposure and p,p'-DDE Concentrations in Men in Chiapas, Mexico: A Cross-Sectional Study. Journal of Andrology 27:16–27. http://doi.org/10.2164/jandrol.05121.

Diamanti-Kandarakis, E., J.-P. Bourguignon, L. C. Giudice, R. Hauser, G. S. Prins, A. M.

Soto, R. T. Zoeller, and A. C. Gore. 2009. Endocrine-Disrupting Chemicals: An Endocrine Society Scientific Statement. Endocr Rev 30:293–342. http://doi.org/10.1210/er.2009-0002.

Dowling, V., P. C. Hoarau, M. Romeo, J. O'Halloran, F. van Pelt, N. O'Brien, and D. Sheehan. 2006. Protein carbonylation and heat shock response in Ruditapes decussatus following p,p′-dichlorodiphenyldichloroethylene (DDE) exposure: A proteomic approach reveals that DDE causes oxidative stress. Aquatic Toxicology 77:11–18. http://doi.org/10.1016/j.aquatox.2005.10.011.

Dufau, M. L., A. Khanum, C. A. Winters, and T.-M. Chon-Hwa. 1987. Multistep regulation of Leydig cell function. Journal of Steroid Biochemistry 27:343–350. http://doi.org/10.1016/0022-4731(87)90326-8.

Fudvoye, J., J.-P. Bourguignon, and A.-S. Parent. 2014. Endocrine-Disrupting Chemicals and Human Growth and Maturation: A Focus on Early Critical Windows of Exposure. Vitamins and Hormones 94:13. http://doi.org/10.1016/B978-0-12-800095-3.00001-8.

Guillette, L. J., KelceT. S. Gross, G. R. Masson, and A. R. Woodwardff. 1994. Developmental abnormalities of the gonad and abnormal sex hormone concentrations in juvenile alligators from contaminated and control lakes in Florida. Environmental Health Perspectives 102:9. http://doi.org/10.1289/ehp.94102680.

Guillette, L. J., and M. P. Gunderson. 2001. Alterations in development of reproductive and endocrine systems of wildlife populations exposed to endocrine-disrupting contaminants. Reproduction 122:857–864. http://doi.org/10.1530/rep.0.1220857.

Gupta, D., K. Rager, J. Zarzycki, and M. Eichner. 1975. Levels of luteinizing hormone, follicle-stimulating hormone, testosterone and dihydrotestosterone in the circulation of sexually maturing intact male rats and after orchidectomy and experimental bilateral cryptorchidism. Journal of Endocrinology 66:183–193. http://doi.org/10.1677/joe.0.0660183.

Heydari, A. R., R. Takahashi, A. Gutsmann, S. You, and A. Richardson. 1994. Hsp70 and aging. Experientia 50:1092–1098. http://doi.org/10.1007/BF01923466.

Howell, G. E., C. Mulligan, E. Meek, and J. E. Chambers. 2015. Effect of chronic p , p ′-dichlorodiphenyldichloroethylene (DDE) exposure on high fat diet-induced alterations in glucose and lipid metabolism in male C57BL/6H mice. Toxicology 328:112–122. http://doi.org/0.1016/j.tox.2014.12.017.

Jeng, H. A. 2014. Exposure to Endocrine Disrupting Chemicals and Male Reproductive Health. Front. Public Health 2. http://doi.org/10.3389/fpubh.2014.00055.

Kelce, W., Stone, C., Laws, S. *et al.* 1955. Persistent DDT metabolite p,p'–DDE is a potent androgen receptor antagonist. *Nature* 375, 581–585. http://doi.org/10.1038/375581a0.

Khan, M. A. Q., S. F. Khan, and F. Shattari. 2008. Halogenated Hydrocarbons.Pages 1831–1843 in Encyclopedia of Ecology. Elsevier.

Kim, B.-M., J.-S. Rhee, C.-B. Jeong, J. S. Seo, G. S. Park, Y.-M. Lee, and J.-S. Lee. 2014. Heavy metals induce oxidative stress and trigger oxidative stress-mediated heat shock protein (hsp) modulation in the intertidal copepod Tigriopus japonicus. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 166:65–74. http://doi.org/10.1016/j.cbpc.2014.07.005.

Koner, B. C., B. D. Banerjee, and A. Ray. 1998. Organochlorine pesticide-induced oxidative stress and immune suppression in rats. Indian journal of experimental biology 36:395-8. PMID: 9717451.

Koureas, M., X. Rousou, H. Haftiki, V. A. Mouchtouri, G. Rachiotis, V. Rakitski, A. Tsakalof, and C. Hadjichristodoulou. 2019. Spatial and temporal distribution of p,p′-DDE (1-dichloro-2,2-bis (p-chlorophenyl) ethylene) blood levels across the globe. A systematic review and meta-analysis. Science of The Total Environment 686:440–451. http://doi.org/10.1016/j.scitotenv.2019.05.261.

Krause, W. 1977. Influence of DDT, DDVP and malathion on FSH, LH and testosterone serum levels and testosterone concentration in testis. Bull. Environ. Contam. Toxicol. 18:231–242. http://doi.org/10.1007/BF01686072.

Leavens, T. L., B. R. Sparrow, and M. J. Devito. 2002. Lack of antiandrogenic effects in adult male rats following acute exposure to 2,2-bis(4-chlorophenyl)-1,1-dichloroethylene (p,p′-DDE). Toxicology 174:69–78. http://doi.org/10.1016/S0300-483X(02)00072-0.

Lee, V. W. K., D. M. De Kretser, B. Hudson, and C. Wang. 1975. Variations In Serum FSH, LH And Testosterone Levels In Male Rats From Birth To Sexual Maturity. Reproduction 42:121–126. http://doi.org/10.1530/jrf.0.0420121.

Lewis, S., R. D. Handy, B. Cordi, Z. Billinghurst, and M. H. Depledge. 1999. Stress proteins (HSP's): Methods of Detection and Their Use as an Environmental Biomarker. Ecotoxicology 8, 351–368. https://doi.org/10.1023/A:1008982421299.

Li, B., C. Dorrell, P. S. Canaday, C. Pelz, A. Haft, M. Finegold, and M. Grompe. 2017. Adult Mouse Liver Contains Two Distinct Populations of Cholangiocytes. Stem Cell Reports 9:478–489. https://doi.org/10.1016/j.stemcr.2017.06.003.

Li, C.-Y., J.-S. Lee, Y.-G. Ko, J.-I. Kim, and J.-S. Seo. 2000. Heat Shock Protein 70 Inhibits Apoptosis Downstream of Cytochrome *c* Release and Upstream of Caspase-

3 Activation. J. Biol. Chem. 275:25665–25671. https://doi.org/10.1074/jbc.M906383199.

Li, S., H.-Y. Tan, N. Wang, Z.-J. Zhang, L. Lao, C.-W. Wong, and Y. Feng. 2015. The Role of Oxidative Stress and Antioxidants in Liver Diseases. IJMS 16:26087–26124. https://doi.org/10.3390/ijms161125942.

Lister, A. L., and G. J. Van Der Kraak. 2001. Endocrine Disruption: Why Is It So Complicated? Water Qual Res J Can 36:175–190. https://doi.org/10.2166/wqrj.2001.011.

Mathur, P. P., and S. C. D'Cruz. 2011. The effect of environmental contaminants on testicular function. Asian J Androl 13:585–591. https://doi.org/10.1038/aja.2011.40.

McAuliffe, M. E., P. L. Williams, S. A. Korrick, L. M. Altshul, and M. J. Perry. 2012. Environmental Exposure to Polychlorinated Biphenyls and *p,p´*-DDE and Sperm Sex-Chromosome Disomy. Environmental Health Perspectives 120:535–540. https://doi.org/10.1289/ehp.1104017.

Mitra, T., A. Mahanty, S. Ganguly, G. K. Purohit, S. Mohanty, P. K. Parida, P. R. Behera, R. K. Raman, and B. P. Mohanty. 2018. Expression patterns of heat shock protein genes in Rita rita from natural riverine habitat as biomarker response against environmental pollution. Chemosphere 211:535–546. https://doi.org/10.1016/j.chemosphere.2018.07.093.

Migliaccio, V., R. Scudiero, R. Sica, L. Lionetti, and R. Putti. 2019. Oxidative stress and mitochondrial uncoupling protein 2 expression in hepatic steatosis induced by exposure to xenobiotic DDE and high fat diet in male Wistar rats (P Mukhopadhyay, Ed.). PLoS ONE 14:e0215955. https://doi.org/10.3390/ijms21051903.

Molina, E.M., Kavazis, A.N. and Mendonça, M.T. (2021), Chronic exposure to environmental DDT/DDE in two species of small rodents: measures of contaminant load, immune dysfunction and oxidative stress. Environ Toxicol Chem. Accepted Author Manuscript. https://doi.org/10.1002/etc.5007.

Mukhopadhyay, I., A. Nazir, D. K. Saxena, and D. K. Chowdhuri. 2003. Heat shock response:hsp70 in environmental monitoring. J. Biochem. Mol. Toxicol. 17:249–254. https://doi.org/10.1002/jbt.10086.

Muralidharan, S., V. Dhananjayan, and P. Jayanthi. 2009. Organochlorine pesticides in commercial marine fishes of Coimbatore, India and their suitability for human consumption. Environmental Research 109:15–21. https://doi.org/10.1016/j.envres.2008.08.006.

Nazian, S. J., V. B., Mahesh. 1979. Alterations in End Organ Responsiveness to Testosterone during the Sexual Maturation of the Male Rat. *Biology of Reproduction* 21:465–471. https://doi.org/10.1095/biolreprod21.2.465.

Parvez, S., and S. Raisuddin. 2005. Protein carbonyls: novel biomarkers of exposure to oxidative stress-inducing pesticides in freshwater fish Channa punctata (Bloch). Environmental Toxicology and Pharmacology 20:112–117. https://doi.org/110.1016/j.etap.2004.11.002.

Patisaul, H. B., and H. B. Adewale. 2009. Long-term effects of environmental endocrine disruptors on reproductive physiology and behavior. Front. Behav. Neurosci. https://doi.org/ 10.3389/neuro.08.010.2009.

Repetto, M., J. Semprine, and A. Boveris. 2012. Lipid Peroxidation: Chemical Mechanism, Biological Implications and Analytical DeterminatioN. Catala, A., ed. InTech. https://doi.org/10.5772/45943.

Rochira, Vincenzo, Bruno Madeo, Chiara Diazzi, Lucia Zirilli, Santi Daniele, and Cesare Carani. Estrogens and Male Reproduction. In *Endotext*, edited by Kenneth R. Feingold, Bradley Anawalt, Alison Boyce, George Chrousos, Wouter W. de Herder, Kathleen Dungan, Ashley Grossman, et al. South Dartmouth (MA): MDText.com, Inc., 2000. http://www.ncbi.nlm.nih.gov/books/NBK278933/. (accessed 10 January 2020)

Sanders, B.M. 1990. Stress-proteins: Potential as multitiered biomarkers. In L. Shugart and J. McCarthy _eds. Environmental Biomarkers, pp. 165-191. Boca Raton, Florida: Lewis Publishers.

Santra, A., A. Maiti, S. Das, S. Lahiri, S. K. Charkaborty, D. N. Guha Mazumder, and D. Guha Mazumder. 2000. Hepatic Damage Caused by Chronic Arsenic Toxicity in Experimental Animals. Journal of Toxicology: Clinical Toxicology 38:395–405. https://doi.org/10.1081/CLT-100100949.

Scippo, M.-L., C. Argiris, C. Van De Weerdt, M. Muller, P. Willemsen, J. Martial, and G. Maghuin-Rogister. 2004. Recombinant human estrogen, androgen and progesterone receptors for detection of potential endocrine disruptors. Anal Bioanal Chem 378:664–669. https://doi.org/10.1007/s00216-003-2251-0.

Seki, S., Y. Habu, T. Kawamura, K. Takeda, H. Dobashi, T. Ohkawa, and H. Hiraide. 2000. The liver as a crucial organ in the first line of host defense: the roles of Kupffer cells, natural killer (NK) cells and NK1.1 Ag+ T cells in T helper 1 immune responses. Immunological Reviews 174:35–46. https://doi.org/10.1034/j.1600-0528.2002.017404.x.

Shi, Y.-Q., Y.-P. Wang, Y. Song, H.-W. Li, C.-J. Liu, Z.-G. Wu, and K.-D. Yang. 2009. p,p′-DDE induces testicular apoptosis in prepubertal rats via the Fas/FasL pathway. Toxicology Letters 193:79–85. https://doi.org/10.1002/tox.20694.

Schug, T. T., A. Janesick, B. Blumberg, and J. J. Heindel. 2011. Endocrine Disrupting Chemicals and Disease Susceptibility. J Steroid Biochem Mol Biol 127:204–215. https://doi.org/ 10.1016/j.jsbmb.2011.08.007.

Shulaev, V., and D. J. Oliver. 2006. Metabolic and Proteomic Markers for Oxidative Stress. New Tools for Reactive Oxygen Species Research. Plant Physiol 141:367–372. https://doi.org/10.1104/pp.106.077925.

Stoker, T. E., L. G. Parks, L. E. Gray, and R. L. Cooper. 2000. Endocrine-Disrupting Chemicals: Prepubertal Exposures and Effects on Sexual Maturation and Thyroid Function in the Male Rat. A Focus on the EDSTAC Recommendations. Critical Reviews in Toxicology 30:197–252. https://doi.org/10.1080/10408440091159194.

Tatsuta, T., M. Hosono, Y. Ogawa, K. Inage, S. Sugawara, and K. Nitta. 2014. Downregulation of Hsp70 inhibits apoptosis induced by sialic acid-binding lectin (leczyme). Oncology Reports 31:13–18. https://doi.org/10.3892/or.2013.2814.

U.S. Department of health and human services, 2019. Toxicological Profile for DDT, DDE, and DDD.

Wolf, C., C. Lambright, P. Mann, M. Price, R. L. Cooper, J. Ostby, and L. E. Gray Jr. 1999. Administration of potentially antiandrogenic pesticides (procymidone, linuron, iprodione, chlozolinate, p, p′-DDE, and ketoconazole) and toxic substances (dibutyl-and diethylhexyl phthalate, PCB 169, and ethane dimethane sulphonate) during sexual differentiation produces diverse profiles of reproductive malformations in the male rat. Toxicology and industrial health 15:94–118. https://doi.org/10.1177/074823379901500109.

Zala, S. M., and D. J. Penn. 2004. Abnormal behaviours induced by chemical pollution: a review of the evidence and new challenges. Animal Behaviour 68:649–664.

Zawatski, W., and M. M. Lee. 2013. Male pubertal development: are endocrine-disrupting compounds shifting the norms? Journal of Endocrinology 218:R1–R12.

Zhang, X., Y. Mo, L. Zhou, Y. Wang, Z. Wang, and B. Zhao. 2016. Induction of hsp70, hsp90, and catalase activity in planarian *Dugesia japonica* exposed to cadmium. Toxicol Ind Health 32:1373–1380. https://doi.org/ 10.1177/0748233714561488.

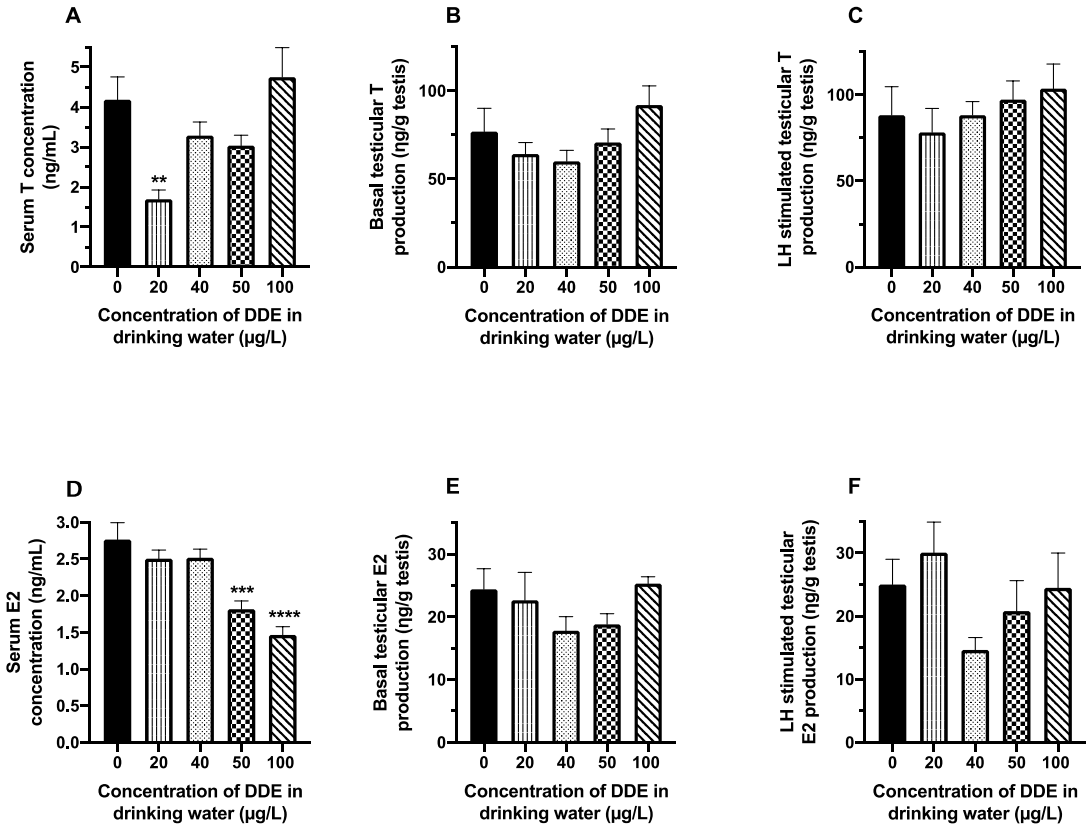

Figure 8. Effects of DDE on serum testosterone (A), serum estradiol (D) and basal (B, E) or LH stimulated testicular T explant levels (C, F) in Long-Evans male rats (PND 21-49). (n=5/group; ***p= 0.01*, ****p= 0.0001*, *****p= <0.0001*)

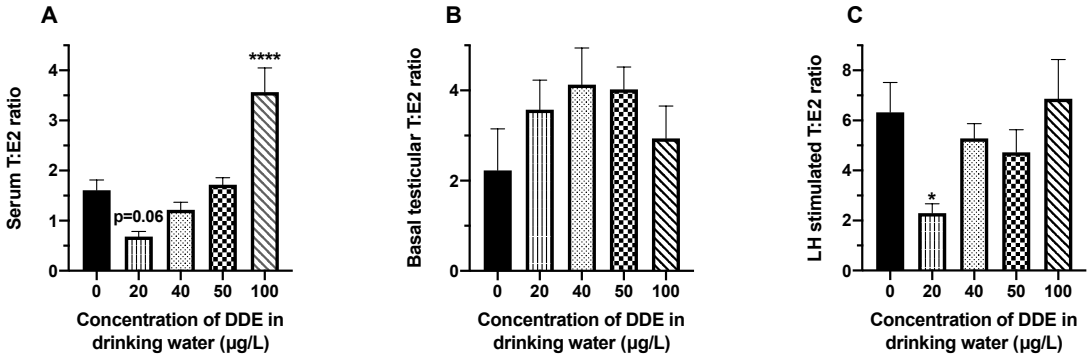

Figure 9. Effects of DDE on T:E2 ratio in serum (A), basal explants (B) or LH stimulated testicular explant levels (C) in Long-Evans male rats (PND 21-49). (*p= *<0.05*, ****p= *<0.001*)

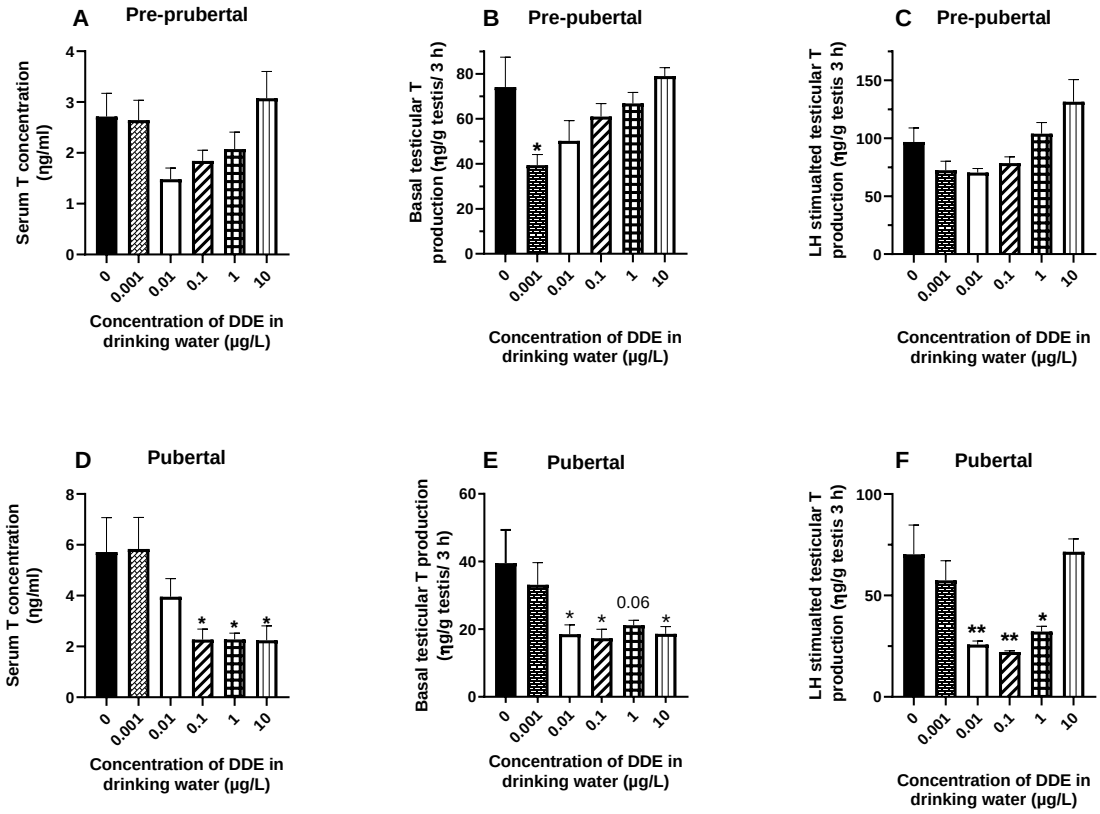

Figure 10. Effect of age at onset of exposure on differential androgen secretion. Effects of DDE on serum testosterone (A, D), and basal (B, E) or challenged testicular explant levels (C, F) in Long-Evans male rats, pre-pubertal, PND 21-35 (A, C) and pubertal, PND 35-49 (D, F; *$p= 0.05$, **$p = 0.01$).

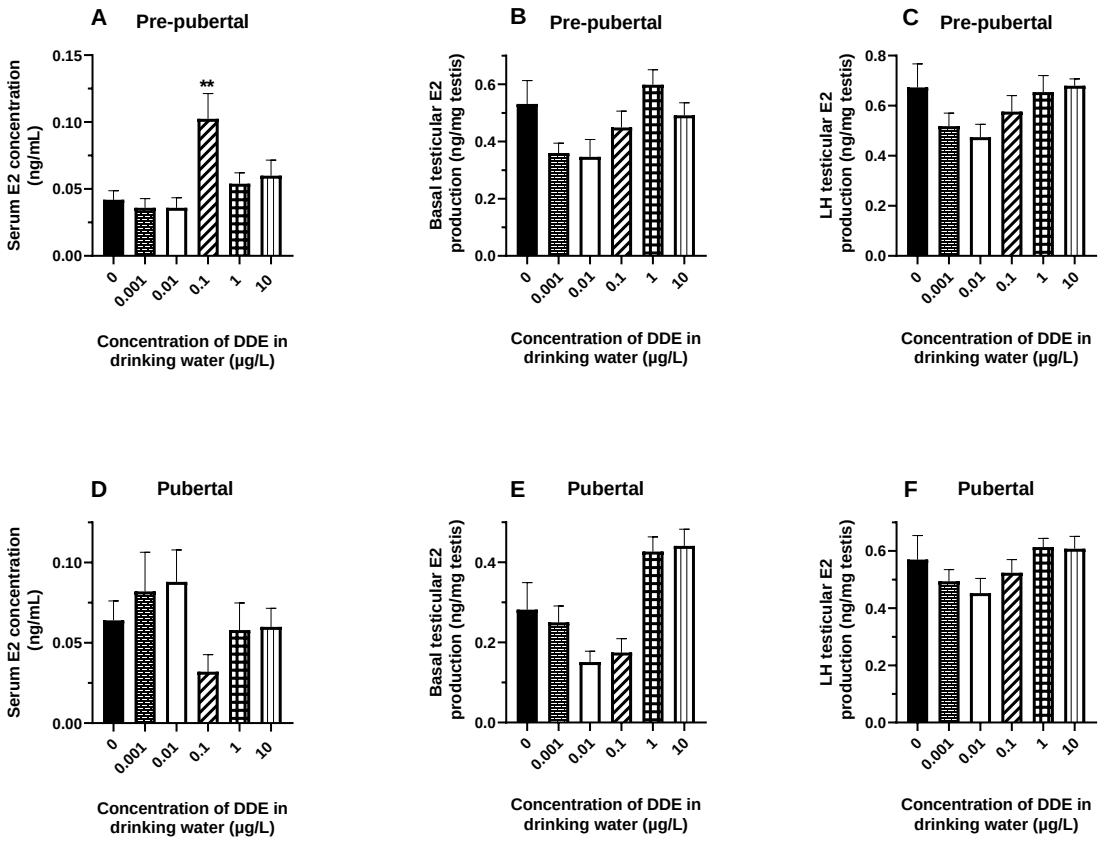

Figure 11. Effect of age at onset of exposure on differential E2 secretion. Effects of DDE on serum estradiol (A, D), and basal (B, E) or LH stimulated testicular explant levels (C, F) in Long-Evans male rats, pre-pubertal, PND 21-35 (A-C) and pubertal, PND 35-49 (D-F;***p = 0.01*).

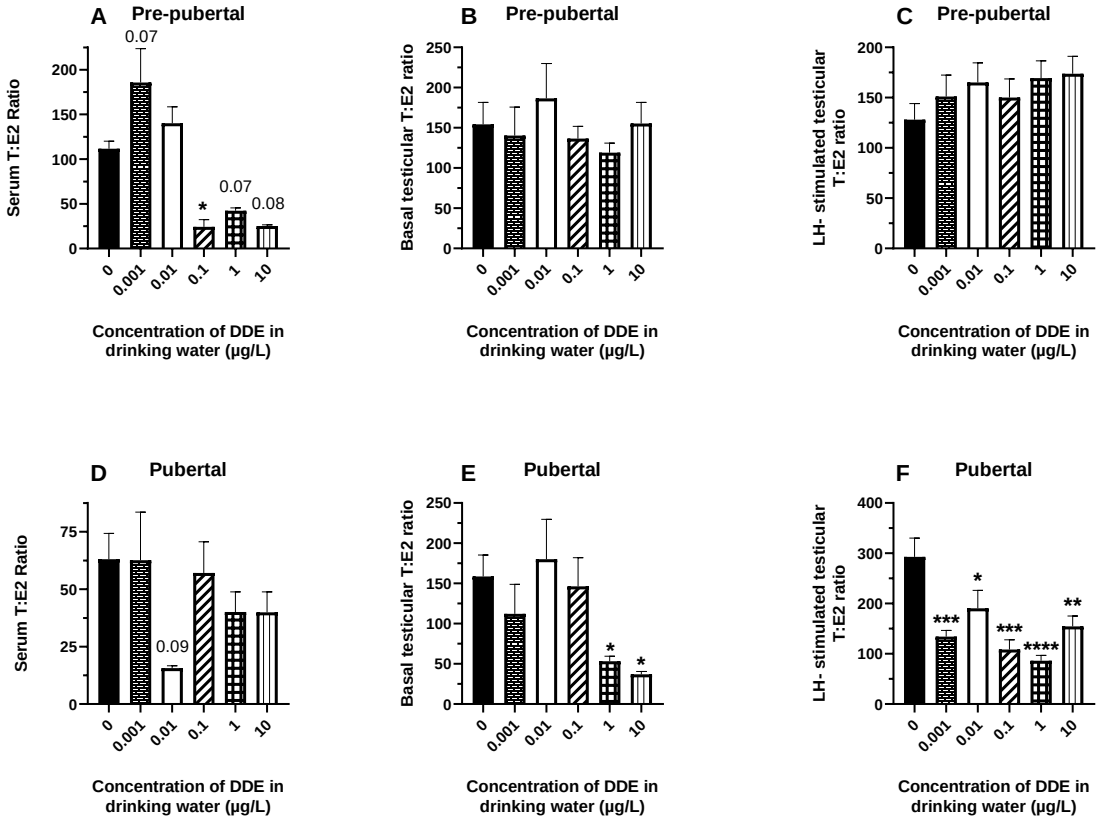

Figure 12. Effects of DDE on T:E2 ratio in pre-pubertal and pubertal basal explants (A, C) or LH-stimulated testicular explant levels (C, D) in Long-Evans male rats. There was no significant differences in serum T:E2 for either age group (data not shown; *p= *<0.05*, **p= *0.01*, ***p= *0.0001*, ****p= *<0.001*).

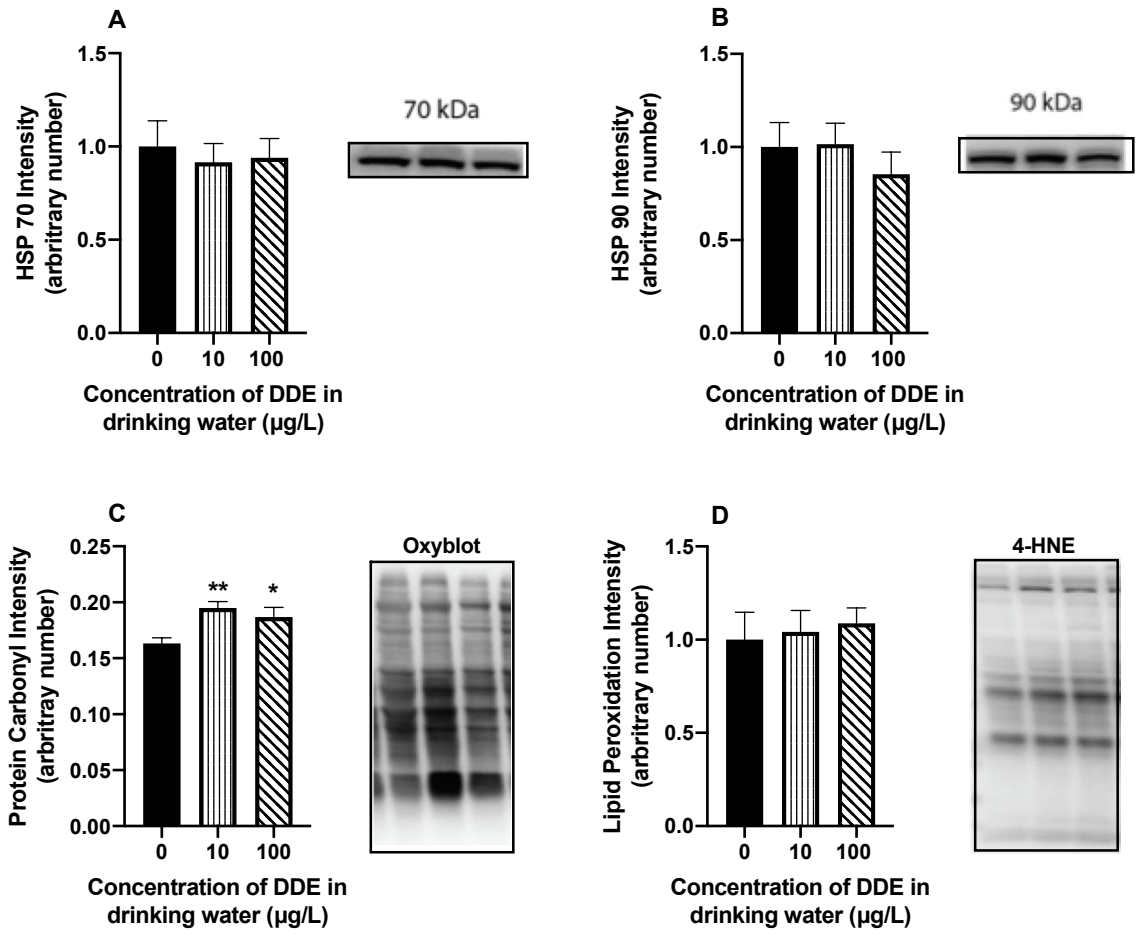

Figure 13. Effects of DDE on oxidative stress markers in the liver after DDE exposure. (A) HSP 70 (B) HSP 90 (C) Oxyblot and (D) Lipid peroxidation (4-HNE) protein was analyzed in liver obtained from animals exposed to DDE from 21-49 days of age. Protein levels were normalized to total protein using a Ponceau stain on the same membrane prior to antibody incubation. (n=6/group; *$p= 0.05$, **$P = 0.01$).

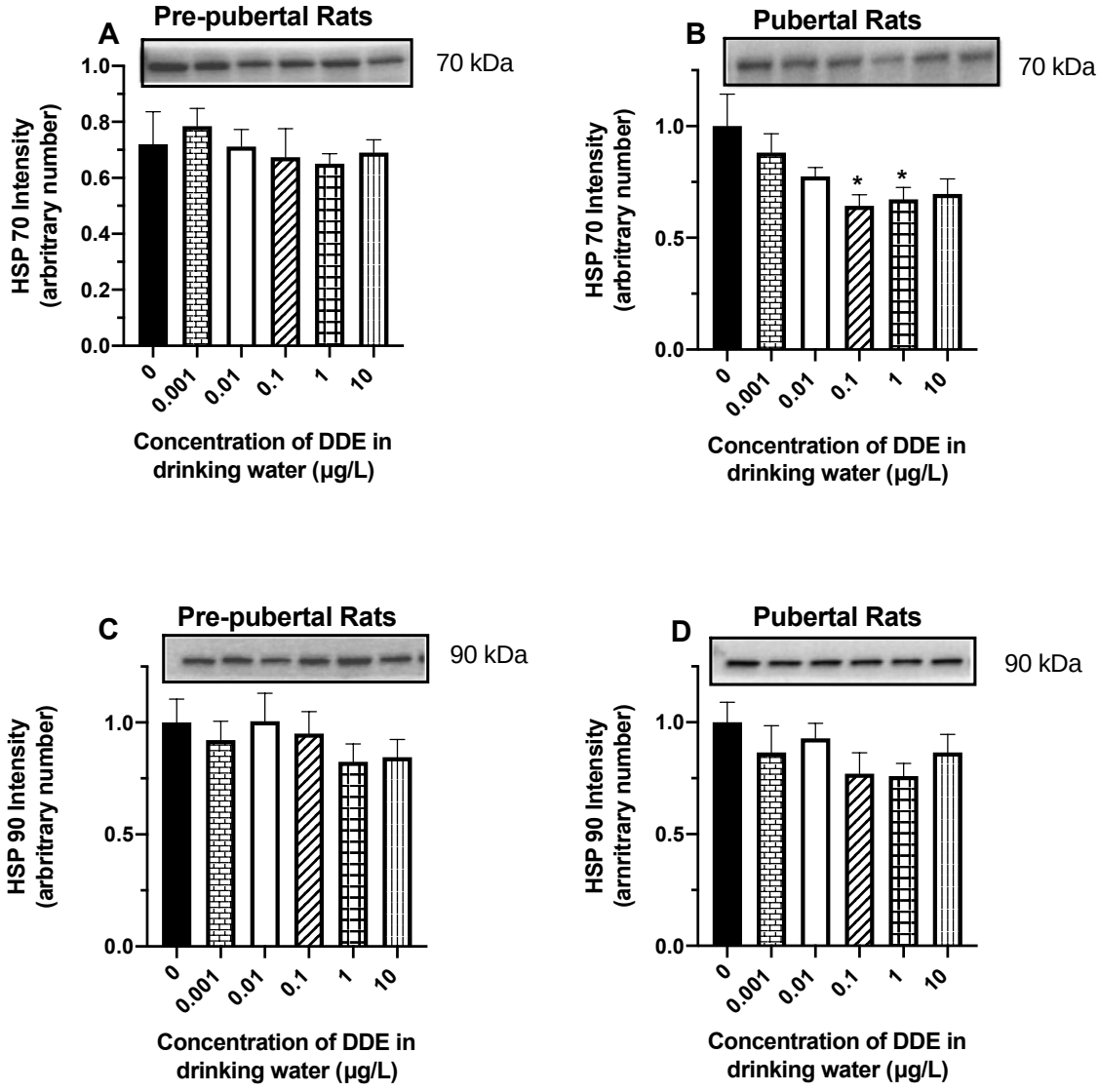

Figure 14. Effects of differential age at exposure (PND 21 vs. PND 35) and low environmentally relevant DDE doses on HSP 70 and HSP 90 in the liver. After sacrifice livers were obtained and analyzed for in HSP 70 (A, B), and HSP 90 (C, D) in pre-pubertal (A, C) and male rats pubertal (B, D). Protein levels were normalized to total protein using a Ponceau stain on the same membrane prior to antibody in

Chapter 4: Effects of chronic DDE exposure on testicular steroid hormone secretion and the steroidogenic pathway in male Long-Evans rats

Erica M Molina[a], Andreas N Kavazis[b], Mary T Mendonça[a], Benson T Akingbemi[c]

[a]Department of Biological Sciences, Auburn University, 331 Funchess Hall, 350 South College St, Auburn, AL 36849, USA. Email: emm0044@tigermail.auburn.edu (Corresponding author)
[b]School of Kinesiology, Auburn University, 287 Kinesiology Building, 301 Wire Road, Auburn, AL 36849, USA. Email: ank0012@auburn.edu
[c]Department of Anatomy, Physiology and Pharmacology, Auburn University, 1130 Wire Road, Auburn, AL 36849, USA. Email: akingbt@auburn.edu

ABSTRACT

Endocrine disruptors, such as DDE, are still found around the world and target androgen production, affecting reproductive capacity. Previously we found that male rats chronically exposed to environmentally relevant doses of DDE had significantly lower levels of serum testosterone (T) which caused abnormal T to estradiol (E2) ratios. Additionally, although pituitary levels of Luteinizing Hormone (LH) and Follicle Stimulating Hormone (FSH) levels were not affected by this exposure, testicular explants had significantly reduced capability of producing T. These results suggested that DDE may not just interfere with androgen receptor binding but have a direct effect on testicular LH receptors or on the steroidogenic pathway within the Leydig cells or on the number of Leydig cells themselves. For successful spermatogenesis and sexual differentiation to occur, there must be proper production and secretion of T by the Leydig cells within the testes. Long-Evans male rats were used to determine the effect of chronic DDE (doses- 0.1 and 10 μg/L) exposure on Leydig cell T production, the steroidogenic pathway (17β-hydroxysteroid dehydrogenase (17 β-HSD), 3β-hydroxysteroid dehydrogenase (3β-HSD), steroidogenic acute regulatory protein (StAR), aromatase and the LH receptor (LH-R))

and markers of apoptosis ((poly-(ADP-ribose) polymerase protein (PARP) and caspace-3). We found that LH-stimulated Leydig cell T production was significantly decreased at both 0.1 and 10 µg/L DDE dose when compared to control. Additionally, steroidogenic enzymes within the Leydig cells showed a biphasic dose response with lower levels at the 0.1 µg/L DDE dose but higher levels at the 10 µg/L DDE dose, with T being driven towards E2, probably due to commensurate increases in the aromatase levels within the Leydig cells. Apoptotic markers (Caspase 3 ratio, pro-caspase 3, PARP and cleaved PARP (cPARP)) also showed elevated levels at the highest DDE dose (10 µg/L) when compared to controls. Overall, this study showed that DDE has the ability to prevent T production through alterations of the steroidogenic pathway.

INTRODUCTION

The correct timing of the production and secretion of testosterone (T) by the testes is essential for sexual differentiation, spermatogenesis, and expression of male secondary sex characteristics (Dohle et al., 2003, Payne and Youngblood, 1995). Since gonadal steroids regulate many physiological processes, including reproduction, they are a prime target for endocrine disrupting chemicals, particularly by estrogenic compounds (Clotfelter et al., 2004). Exposure to endocrine disrupting chemicals (i.e., DDE) during critical developmental windows of life can produce permanent alterations of vertebrate reproductive function (Wolf et al., 1999). DDT and its metabolite DDE have hormonal effects in males by blocking androgen receptors and testosterone induced cellular responses (Sikka and Wang, 2008). In our previous study on the effects of chronic exposure of male rats to environmentally relevant doses of DDE, we found exposed individuals had significantly lower levels of serum T and serum T to estradiol

(E2) ratio. We also found that Luteinizing Hormone (LH) and Follicle Stimulating Hormone (FSH) levels within the pituitary were not affected by exposure to the DDE treatments, but testicular explants had significantly diminished ability to secrete T in response to LH stimulation. These results suggest that DDE may a have direct effect on testicular LH receptors or androgen receptor binding or on gonadal steroid production dynamics within the Leydig cells themselves.

The steroidogenic capacity of individual Leydig cells and Leydig cells per testis determines the concentration of circulating testosterone (Hardy et al., 2005), which in turn, is the hormone that is the primary determinant of the male phenotype (Nanjappa et al., 2012). Any disruption in testicular testosterone production by Leydig cells can not only alter the male phenotype but also spermatogenesis.

Previously we found that acute DDE exposure of male rats (age 21-35 and 35-49 days of age: a period encompassing both pre-pubertal and pubertal developmental stages) resulted in significantly lower levels of circulating serum T and testicular T production in a dose dependent manner, with our results exhibiting a nonmonotonic dose response that is common for endocrine disruptors (Molina et al, 202x, Hill et al., 2018). In order to further elucidate the mechanisms behind our significantly lower T levels, we investigated the possibility of changes in individual Leydig cell's ability to produce testosterone (i.e., a disruption in the steroidogenic pathway, thus inhibiting the conversion of precursor steroids into androgens) or Leydig's cells ability to respond to LH as a result of a downregulation of the testicular LH receptors (LHR). DDE has primarily been documented to have an anti-androgenic effect, by inhibiting the ability of androgen to bind to the androgen receptor (AR), decreasing hormone production.

Leydig cell isolation and culture allow the assessment of steroid hormone production within individual Leydig cell's (Chen and Zirkin, 1999; Hardy et al., 2005). Lower T levels could be due to a decrease in the ability of individual Leydig cells to produce testosterone, in turn causing decreased levels of measured T production after DDE treatment. Therefore, the purpose of this study is to determine if chronic exposure to environmentally relevant doses of DDE 1) affects T concentration at the level of individual Leydig cell collections, which in turn could result in decreased serum T levels (2) causes a decrease in LH receptor levels in Leydig cells, lowering T production and/or secretion and (3) alters steroidogenic enzyme levels in the steroid production pathway within Leydig cells or functional Leydig cell number (perhaps due to apoptosis).

MATERIALS AND METHODS

Animal study

Male Long–Evans rats at post-natal day (PND) 21 were obtained from Harlan-Teklad, (Madison, WI). Animals were allowed to acclimatize for 3 days at the College of Veterinary Medicine Division of Laboratory Animal Health Housing Facility. Depending on size, animals were placed in groups of 3 per cage (length, 0.47 m; width, 0.25 m; height, 0.22 m) (Snyder Manufacturing Company, Centennial, CO).

Animals were given water, *ad libitum* containing environmentally relevant doses 0, 0.1, and 10 μg/L of DDE from 21-49 days of age (pre-pubertal; n =12/ group). These doses were found to be effective in causing decreased serum and testicular explant testosterone in a previous study (Molina et al 202x). Animals were maintained under constant conditions of light (12 L:12 D) and temperature (20–23.38 ˚C) with free access to pelleted food (soy- and alfalfa-free diet, 2020, Harlan-Teklad, Indianapolis IN) during chemical exposure. Body weights (to 1 gram) were taken prior to being euthanized using

a Mottler Toledo analytical balance (Rite-weight, Inc, Model 8104). Animals were euthanized within 24 h of terminating chemical treatment to obtain tissues for analysis, including blood and testis. Animal euthanasia procedures were performed in accordance with a protocol approved by the Auburn University Institutional Animal Care and Use Committee based on recommendations of the panel on Euthanasia of the American Veterinary Medical Association.

Leydig cell Isolation

Animals were sacrificed by CO_2 asphyxiation. Isolation of Leydig cells was done by collagenase digestion of testis followed by Percoll density centrifugation. Pooled testes from each group of rats were preserved in cold PBS for transfer from the surgical room to the hood for isolation under aseptic conditions. No more than 4 to 8 testes were decapsulated into test tubes containing 5 ml of dissociation buffer containing M-199: Gibco #31100-035, 0.71gm NaHCo3: Sigma S-5761, 2.1gm HEPES: Sigma H-4034, 1gm BSA Fraction V: ICN #103703, 25mg SBTI: Sigma T-9003, 0.25 mg/mL collagenase, 46μg/mL dispase and 6μg/mL DNase. Each test tube containing the decapsulated testes in DB was incubated in a water bath at a temperature of 34° C and shaking speed of 70 RPM for 45 to 60 minutes. After testis digestion, digested testicular contents were separated from seminiferous tubules by filtration with Spectra mesh microporous filters (Spectrum Lab Inc.) into 250 mL Falcon tubes. Digested testicular contents in 250mL Falcon tubes were concentrated by centrifuging at 4° C, 3000 RPM for 20 minutes. Cell fractions from the concentrate were loaded on to a Percoll gradient tube containing 16ml of Percoll solution containing 44ml Percoll: Sigma #SLBW 5969, 4ml of HBSS: Gibco #1297749 and 6μg/mL DNase and centrifuge at a temperature of 4°

C, and speed of 14000 RPM for 60 min to isolate bands of Leydig cells. Leydig cell yield was estimated by hemocytometer counting. The purity of cell fractions was assessed by histochemical staining for HSD3B using 0.4 mM etiocholan-3β-ol-17-one as the enzyme substrate (catalog no. E-5251, lot no. 11K4058; Sigma)

Measurement of testicular and Leydig cell steroid hormone secretion

Immediately after euthanasia, testicular explants were obtained from males in both Experiments 1 and 2. Explants were incubated in 1 ml DMEM/Ham's F-12 culture medium in triplicate without (basal) or containing 100 ηg/ml ovine LH (NIDDK, NIH) and put into a shaking water bath at 34° C for 3 h. Explants were then centrifuged at 3000 RPM for 15 min and the supernatant was transferred into fresh tubes and stored at -20° C until aliquots of spent media were analyzed for estradiol and T concentrations using a tritium-based RIA (Cochran et al., 1981). In testicular explants, hormone production was normalized to nanograms per testicular mass (milligrams) and Leydig cell hormone production was normalized to 10^6 cells.

Western blot analysis

The relative concentration of proteins involved in the steroidogenic pathway (17β-hydroxysteroid dehydrogenase (17 β-HSD), 3β-hydroxysteroid dehydrogenase (3β-HSD), steroidogenic acute regulatory protein (StAR), aromatase and the LH receptor (LH-R) were quantified in Leydig cells via Western blot analysis. We also quantified biomarkers of apoptosis, in Leydig cells, (poly-(ADP-ribose) polymerase protein (PARP) and caspace-3). To accomplish this, Leydig cells were homogenized 250 µl of in 5 mM Tris HCl (pH 7.5) and 5 mM EDTA (pH 8.0), and protease inhibitor cocktail (14224–396, VWR, Radnor, PA, USA) and centrifuged at 1500 g for 10 min at 4 °C. The protein

content of the supernatant was quantified by the method of Bradford (Sigma). Equal amounts of proteins were loaded and separated by polyacrylamide gel electrophoresis via 4-15% polyacrylamide gels (BioRad, Hercules, CA, USA). After electrophoresis, the proteins were transferred to polyvinylidene fluoride membranes. The resulting membranes were stained with Ponceau S to determine equal loading and transfer. Nonspecific sites were then blocked by incubation of membranes in phosphate-buffered saline (PBS) solution containing 0.1% Tween 20 and 5% nonfat milk. Membranes were then incubated for 1 hour with primary antibodies directed against 17β HSD (Santa Cruz, sc376719 1:1000), 3β HSD (Santa Cruz, sc515120, 1:1000), StAR (Santa Cruz, sc16682, 1:1000) aromatase (Gene Tex, GTX32456, 1:1000), PARP (cell signaling, 9542S, 1:1000), caspace-3 (Cell signaling, 9961S; 1:1000 dilution), and LHR (Protein tech, 19968-1-AP; 1:500).

Following incubation with primary antibodies, membranes are washed with PBS-Tween (5 min, 3x) and then incubated with secondary antibodies for 1 h at room temperature. After washing (5 min, 3x), a chemiluminescent system was used to detect labeled proteins (GE Healthcare Life Sciences, Pittsburgh, PA). Images were taken and analyzed with the ChemiDoc-It Imaging System (Biorad, Hercules, CA). Western blots were normalized to total protein using a Ponceau stain on the same membrane prior to antibody incubation.

Measurement of steroid hormone concentrations

Serum was collected from trunk blood at the end of each experiment from animals at sacrifice and frozen at – 20 C until the time of assay. We measured basal serum testosterone (T) and 17β-estradiol concentrations from all treatment groups using a

tritium-based radioimmunoassay protocol (Cochran et al., 1981), with an interassay variation of 7%–8%.

STATISTICAL ANALYSIS

Data were analyzed by one-way analysis of variance (ANOVA) followed by the Dunnett test for post-hoc multiple group comparisons (GraphPad Prism software, San Diego, CA). Bar graphs are presented using SEM. Weights are expressed as mean ± SEM. The differences were considered significant when a $p \leq 0.05$ was obtained.

RESULTS

General observations. Treatment with DDE did not significantly affect body weights in either of the treatment dose groups when compared to control group (control vs. 0.1 µg/L vs 1.0 µg/L: 245 ± 4 vs 245 ± 3 vs 240 ± 4 grams, respectively).

Effect of DDE on steroid hormone secretion. Compared to the control group, serum T was significantly decreased in rats dosed with 0.1 µg/L of DDE ($p=0.03$) but T did not differ significantly from controls in the 10 µg/L group (figure 15A). In testicular explants, LH stimulated testicular T did not differ from control in the 0. µg/L group but was significantly higher in the group exposed to 10 µg/L of DDE ($p=0.003$; figure 15C). Basal T levels did not differ among the dose groups (figure 15B). In contrast, Leydig cell preparations exhibited a different pattern: while basal T did not differ among the three dose groups (Figure 16 A), LH stimulated Leydig cells responded by having a significantly lower levels of T, in a dose response pattern (0.1 µg/L DDE: $p=0.005$ and 10 µg/L DDE: $p=0.0002$; Figure 16 B). Altogether, it appears that DDE has the greatest effect on LH stimulated Leydig cell T production at both the 0.1 µg/L and 10 µg/L doses. Serum E2 and LH stimulated testicular E2 secretion was increased in animals exposed to

10 µg/L of DDE compared to control (p=0.01, figure 17A; p=<0.0001, figure 17C), but not for the 0.1 µg/L group. Basal testicular E2 production showed no significant changes when compared to controls (figure 17B). DDE doses also had no effect on basal or LH stimulated Leydig cell E2 production (figure 18A-B).

DDE effects on steroidogenic enzyme and LH receptor levels in Leydig cells. Analysis of candidate enzymes in Leydig cell cultures involved in the steroidogenic pathway showed that StAR, 3β HSD, and 17β HSD all exhibited a biphasic response to DDE exposure. At the low dose of 0.1 µg/L, the mean levels of these three proteins were all significantly lower than the mean seen in the control group, while all three proteins exhibited significantly higher levels than those observed for the control and 10 µg/L dose (p= <0.05; figure 19). Aromatase protein levels showed a similar pattern: aromatase levels were significantly higher in the 10 µg/L exposure group, but no differences were noted in the 0.1 µg/L group (figure 19E). LHR levels did not differ in either DDE exposure dose compared to control (figure 19A).

DDE effect on Leydig cell apoptosis. Overall, the expression of proteins related to the apoptotic pathway showed elevated levels at the highest DDE dose (10 µg/L) when compared to controls. Caspase 3 ratio and pro-caspase 3 exhibited significantly higher levels in Leydig cells after rats were exposed to 10 µg/L DDE dose compared to controls (figure 20A; p=<0.05). PARP and cleaved PARP (cPARP) also showed significantly higher levels at the 10 µg/L DDE dose when compared to controls (figure 20B-C; p=<0.05).

DISCUSSION

The present study demonstrated that exposure to environmentally relevant levels

of DDE may adversely affect T production by disruption of the steroidogenic pathway and increased apoptosis in testicular Leydig cells. In contrast, LHR levels in Leydig cells were not different between the different DDE groups, indicating that these results are not due to LH receptor deficiency but rather due to effects of DDE on the enzymatic function in the Leydig cells themselves. However, it remains possible that the observed differential response patterns to different DDE doses may not just be disruption of steroidogenic pathway but differences in apoptosis or anti-androgenic effects of DDE.

Adult Leydig cells originate through postnatal differentiation from progenitor cells (PND 14-28) and immature Leydig cells (PND 28-56; Svechnikov et al., 2010). Immature Leydig cells express high levels of steroidogenic enzymes involved in the biosynthesis of testosterone (i.e. 3β HSD) and as male rats approach puberty, testes become more sensitive to LH and serum T levels increase. A lack of significant differences in FSH or LH levels within the pituitary in our animals treated previously at environmentally relevant levels of DDE, for two weeks, provided evidence that although pituitary LH and FSH levels are not different from those of control animals, the Leydig cells of DDE exposed rats may not be responding to LH (Molina et al., 202x).

In this study, we investigated the effects of DDE on the reproductive axis. Serum T concentrations were significantly lower after exposure to 0.1 µg/L DDE dose when compared to control (Figure 15). LH stimulated testicular T was significantly higher at the 10 µg/L DDE exposure dose when compared to the other treatments (Figure 15). In contrast, LH stimulated Leydig cell T levels were significantly *lower* at both 0.1 µg/L and 10 µg/L DDE doses when compared to control (Figure 16). It has long been established that intratesticular testosterone concentrations are much higher than serum

testosterone (Le et al., 2014). The testes serum gradient has been shown to persist despite the exogenous administration of testosterone to animals, indicating the possibility of the testes functioning as a reservoir for testosterone, partially due to the presence of androgen binding proteins (Jarrow et al., 2005), which may be occurring at our 10 μg/L dose. Sertoli synthesize androgen-binding protein that helps maintain high levels of T within the testis that is essential for spermatogenesis (Hinson et al., 2010). Additionally, studies in men with hypogonadism have shown that deficiencies of circulating T are predominantly due to decreased responsiveness of Leydig cells to LH, rather than central deficiencies in the hypothalamus (i.e., primary hypogonadism) (Zirkin and Papadopoulos, 2018).

Other hypotheses would be a disruption in the steroidogenic pathway, increased apoptosis within the testis or inhibition of androgen binding to the androgen receptor within the testis. Although androgens are being produced, it is possible that dehydroepiandrosterone (DHEA) is converted by 3β HSD to estradiol by aromatase instead of to testosterone. Hypophysectomized rats (PND 23) that were treated with FSH + E2 (0.5 μg and 5μg) suppressed LH stimulated T production in Leydig cells after 3 and 5 days of E2 treatment, suggesting that estrogen inhibits LH stimulation of Leydig cells (van Beurden et al., 1978; Schulster et al., 2016). These observations are similar to results from the present study with an increase in serum E2 and LH stimulated testicular E2 secretion at the10 μg/L DDE dose (Figure 17), which corresponded to a decrease in Leydig cell T production. Additionally, incubation of immature Leydig cells with 30μl of etomidate (an antiandrogenic anesthetic agent), Leydig cell T secretion related to decreased levels of the 3β HSD1 and CYP11a1 enzymes (Liu et al., 2015).

The development of normal testicular function in males depends on the maturation of the steroidogenic pathway to support Leydig and Sertoli cell development which are necessary for subsequent development of the reproductive tract (De Falco et al., 2015). Although not always reflected in measurements of T secretion, exposure to DDE affected the development of steroidogenic capacity in Leydig cells. Aromatase, StAR, 3β-HSD, 17β-HSD levels were increased at the 10 µg/L DDE dose but with a decrease in StAR, 3β-HSD, 17β-HSD levels at the 0.1 µg/L DDE dose. Previous research has shown that rats dosed with bisphenol A (BPA), another endocrine disruptor, exhibited decreased Leydig cell steroidogenesis (Akingbemi et al., 2004). Our results indicate that DDE has a biphasic nonmonotonic dose response, reducing steroidogenesis in Leydig cells at lower levels of DDE exposure while increasing steroidogenesis at higher doses of DDE. The biphasic pattern seen in steroidogenesis may be due to the slight decrease in LHR availability at the 0.1 µg/L DDE dose, in turn diminishing the ability of LH, the primary regulator of steroidogenesis in Leydig cells, to stimulate testosterone production through steroidogenesis (Sherrill et al., 2010; Svechnikov et al., 2010). Also, the coupling of decreased LH stimulated Leydig cell T production with upregulation of steroidogenesis at the 10 µg/L DDE dose may be an attempt to alleviate decreased T production and maintain reproductive endocrine homeostasis. DDE has been previously shown to induce aromatase activity in the liver after seven daily oral treatments of DDE at 100 mg/kg in male rats (You et al., 20010). Despite higher levels of steroidogenic enzymes to produce testosterone, the increase in aromatase levels was most likely causing the conversion of testosterone to estradiol at the 10 µg/L DDE dose (Figure 19E).

Levels of T production are dependent on the number of Leydig cells and T

production rate per Leydig cell (Akingbemi et al., 2007). The present study demonstrated increased levels of apoptosis via PARP, cPARP and Caspase-3 ratio at 10 µg/L DDE dose (Figure 20). Caspase-3 and PARP are both considered markers of apoptosis with cPARP being documented in higher levels in infertile men (Agarwal et al., 2009). Apoptosis within the testes was previously demonstrated in rats treated with DDE, male rats exposed to 60 mg/kg DDE for 10 days showed a significant increase in caspase -8,-3 and -9 (Shi et al., 2013). Activated caspase-3 interacts with a large number of targets in an affected cell to bring about its destruction through apoptosis (Shi et al., 2013). In this study, caspase 3 ratio, PARP and cPARP showed significantly increased levels at our highest dose (10 µg/L). This suggests that despite the body trying to neutralize the damage caused by DDE through the upregulation of PARP, the concurring upregulation of caspase-3 was inhibiting repair by cleaving PARP neutralizing their ability to repair any damage incurred (Figure 20). Cleavage of PARP by caspase-3 inhibits PARP's DNA repairing ability, which commonly occurs during oxidative stress (Agarwal et al., 2009). The upregulation of these apoptotic markers indicates a possible increase in Leydig cell apoptosis within the testis with a decreased ability to repair the damage, consequently causing the corresponding decreases in T production.

In conclusion, the present data demonstrated that DDE has the capacity to directly target and/or alter specific proteins in the male steroidogenic pathway which may alter reproductive capacity. Despite the persistent decrease in LH stimulated Leydig cell T production in our treated animals, we found that steroidogenic regulatory enzymes were significantly increased in 10 µg/L dosed rats but decreased at our lowest dose (0.1 µg/L) possibly due to increased apoptosis in our higher dose and attempts to maintain

reproductive endocrine homeostasis. Despite the increase in regulatory enzymes/proteins to produce T, T levels were still affected with T going towards E2 most likely due to the induction of aromatase pathway by DDE. In our study it appears that T is still able to be produced but may be unable to bind AR. Additionally, DDE is targeting the steroidogenic enzyme pathway, by possibly disrupting enzyme utilization or activity. A study to further investigate the proliferative effects of DDE on Leydig cells will be helpful in determining the rate of apoptosis or if DDE may have a proliferative effect on Leydig cell numbers, which enhanced testicular T concentrations even as androgen biosynthesis was declining in individual Leydig cells at the 10 µg/L DDE dose. Overall, exposure to environmentally relevant levels of DDE has implications for reproductive capacity through disruption of T and E2 secretion which in turn may cause abnormal male reproductive development and sperm production.

Work Cited

Agarwal, A., R. Z. Mahfouz, R. K. Sharma, O. Sarkar, D. Mangrola, and P. P. Mathur. 2009. Potential biological role of poly (ADP-ribose) polymerase (PARP) in male gametes. Reprod Biol Endocrinol 7:143.

Akingbemi, B. T., C. M. Sottas, A. I. Koulova, G. R. Klinefelter, and M. P. Hardy. 2004. Inhibition of Testicular Steroidogenesis by the Xenoestrogen Bisphenol A Is Associated with Reduced Pituitary Luteinizing Hormone Secretion and Decreased Steroidogenic Enzyme Gene Expression in Rat Leydig Cells. Endocrinology 145:592–603.

Akingbemi, Benson T., Tim D. Braden, Barbara W. Kemppainen, Karen D. Hancock, Jessica D. Sherrill, Sarah J. Cook, Xiaoying He, and Jeffrey G. Supko. 2007. Exposure to Phytoestrogens in the Perinatal Period Affects Androgen Secretion by Testicular Leydig Cells in the Adult Rat. Endocrinology 148, no. 9: 4475–88.

Chen, Haolin, and Barry R. Zirkin. 1999. Long-Term Suppression of Leydig Cell Steroidogenesis Prevents Leydig Cell Aging. Proceedings of the National Academy of Sciences 96(26): 14877–81.

Clotfelter, E. D., A. M. Bell, and K. R. Levering. 2004. The role of animal behaviour in the study of endocrine-disrupting chemicals. Animal Behaviour 68:665–676.

De Falco, Maria, Maurizio Forte, and Vincenza Laforgia. 2015. Estrogenic and Anti-Androgenic Endocrine Disrupting Chemicals and Their Impact on the Male Reproductive System. Frontiers in Environmental Science 3.

Dohle, G. R., M. Smit, and R. F. A. Weber. 2003. Androgens and male fertility. World J Urol 21:341–345.

Guillette, L.J., and M.P, Gunderson. 2001. Alterations in development of reproduction and endocrine systems of wildlife populations exposed to endocrine-disrupting contaminants. Reproduction 122:857-864.

Hardy, M. P., H.-B. Gao, Q. Dong, R. Ge, Q. Wang, W. R. Chai, X. Feng, and C. Sottas. 2005. Stress hormone and male reproductive function. Cell Tissue Res 322:147–153.

Hill, Corinne E., J. P. Myers, and Laura N. Vandenberg. 2018. Nonmonotonic Dose–Response Curves Occur in Dose Ranges That Are Relevant to Regulatory Decision-Making. Dose-Response 16(3).

Hinson, Joy, Peter Raven, and Shern Chew. 2010. 8 - Hormonal Control of Reproduction Part I: Male Reproductive System. The Endocrine System (Second Edition), edited by Joy Hinson, Peter Raven, and Shern Chew, 87–98. Churchill Livingstone.

Jarow, J. P. 2005. The Androgen Microenvironment of the Human Testis and Hormonal Control of Spermatogenesis. Annals of the New York Academy of Sciences 1061(1): 208–20.

Le, B., H. Chen, B. Zirkin, and A. Burnett. 2014. New Targets for Increasing Endogenous Testosterone Production: Clinical Implications and Review of the Literature. *Andrology* 2(4): 484–90.

Liu, H.-C., D. Zhu, C. Wang, H. Guan, S. Li, C. Hu, Z. Chen, Y. Hu, H. Lin, Q.-Q. Lian, and R.-S. Ge. 2015. Effects of Etomidate on the Steroidogenesis of Rat Immature Leydig Cells (D Ma, Ed.). PLoS ONE 10:e0139311.

Nanjappa, M. K., L. Simon, and B. T. Akingbemi. 2012. The Industrial Chemical Bisphenol A (BPA) Interferes with Proliferative Activity and Development of Steroidogenic Capacity in Rat Leydig Cells. Biol Reprod 86 Available at https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3364919/.

Payne, Anita H., and Geri L. Youngblood. 1995. Regulation of Expression of Steroidogenic Enzymes in Leydig Cells1. Biology of Reproduction 52, no. 2: 217–25.

Schulster, M., A. Bernie, and R. Ramasamy. 2016. The role of estradiol in male reproductive function. Asian J Androl 18:435.

Sherrill, Jessica D., Morgan Sparks, John Dennis, Mahmoud Mansour, Barbara W. Kemppainen, Frank F. Bartol, Edward E. Morrison, and Benson T. Akingbemi. 2010. Developmental Exposures of Male Rats to Soy Isoflavones Impact Leydig Cell Differentiation1. Biology of Reproduction 83, no. 3: 488–501.

Shi, Y.-Q., H.-W. Li, Y.-P. Wang, C.-J. Liu, and K.-D. Yang. 2013. p,p'-DDE induces apoptosis and mRNA expression of apoptosis-associated genes in testes of pubertal rats. Environmental Toxicology 28:31–41.

Sikka, S. C., and R. Wang. 2008. Endocrine disruptors and estrogenic effects on male reproductive axis. Asian J Andrology 10:134–145.

Svechnikov, K., G. Izzo, L. Landreh, J. Weisser, and O. Söder. "Endocrine Disruptors and Leydig Cell Function." Review Article. Journal of Biomedicine and Biotechnology. Hindawi, August 25, 2010. van Beurden WM, Roodnat B, Mulder E, van der Molen HJ. 1978. Further characterization of the effects of hypophysectomy, FSH and estrogen on LH stimulation of testosterone production in Leydig cells isolated from immature rats. Steroids 31: 83–98.

van Beurden WM, Roodnat B, Mulder E, van der Molen HJ. 1978. Further characterization of the effects of hypophysectomy, FSH and estrogen on LH

stimulation of testosterone production in Leydig cells isolated from immature rats. Steroids 31: 83–98.

Wolf, C., C. Lambright, P. Mann, M. Price, R. L. Cooper, J. Ostby, and L. E. Gray Jr. 1999. Administration of potentially antiandrogenic pesticides (procymidone, linuron, iprodione, chlozolinate, p, p′-DDE, and ketoconazole) and toxic substances (dibutyl-and diethylhexyl phthalate, PCB 169, and ethane dimethane sulphonate) during sexual differentiation produces diverse profiles of reproductive malformations in the male rat. Toxicology and industrial health 15:94–118.

You, Li, Madhabananda Sar, Erika Bartolucci, Stephen Ploch, and Mary Whitt. 2001. Induction of Hepatic Aromatase by p,P′-DDE in Adult Male Rats. Molecular and Cellular Endocrinology 178, (1): 207–14.

Zirkin, B. R., and V. Papadopoulos. 2018. Leydig cells: formation, function, and regulation. Biology of Reproduction 99:101–111.

FIGURES

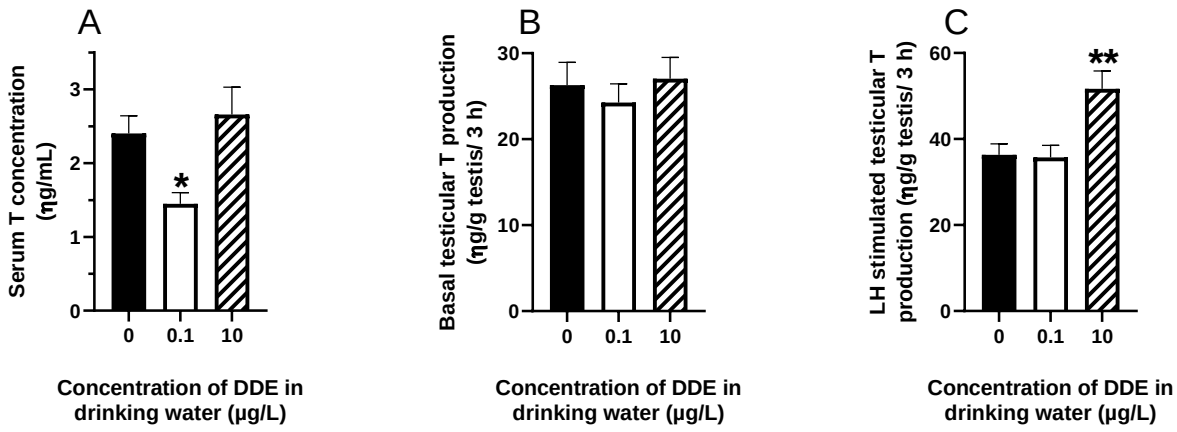

Figure 15 Effects of DDE on testicular (T) androgen secretion. Male rats at 21 days of age (n=12) were maintained on a control or with water containing DDE for 28 days. After sacrifice, serum was separated from blood to measure T concentration (A). Testicular explants were obtained and incubated in DMEM/ Ham's F-12 culture media for 3 hours without (B) or containing 100 ηg/ml ovine LH (NIDDK, NIH) (C). Aliquots of serum were analyzed to measure T concentrations measured by RIA. (*p= 0.05, **p = 0.01)

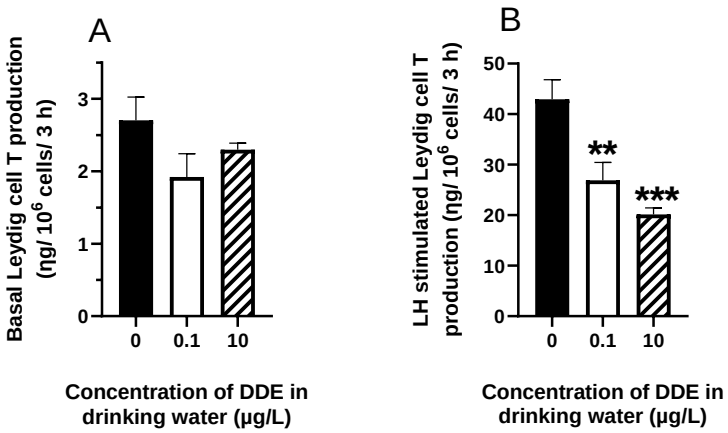

Figure 16 Effects of DDE on androgen secretion by Leydig cells. Male rats at 21 days of age (n=12) were maintained on control or water containing DDE for 28 days. After sacrifice, testes were pooled to isolate Leydig cells which were then incubated in DMEM/ Ham's F-12 culture media for 3 hours without (A) or containing 100 *η*g/ml ovine LH (NIDDK, NIH) (B). Aliquots of spent media were analyzed to measure T concentrations by RIA. (**p = 0.01, ***p= 0.001)

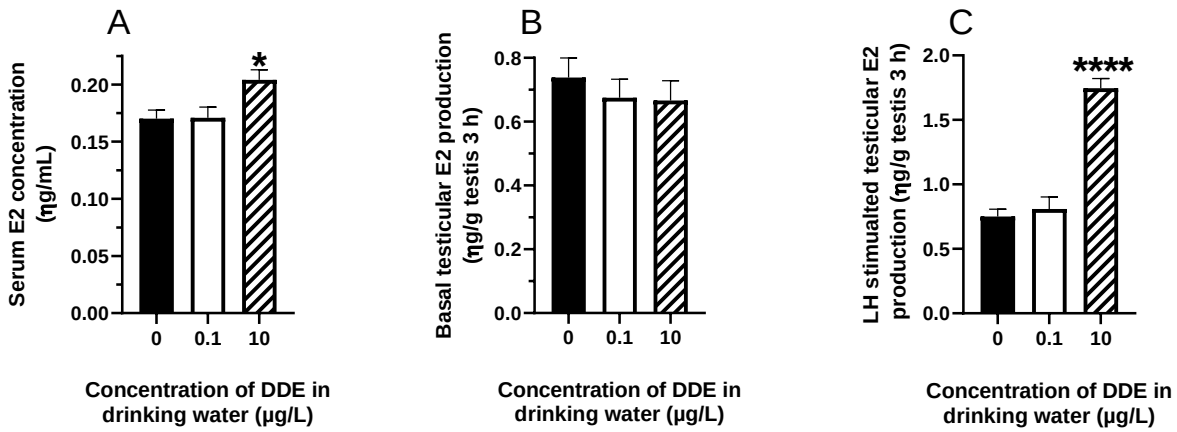

Figure 17 Effects of DDE on testicular 17β- estradiol (E2) secretion. Male rats at 21 days of age (n=12) were maintained on control or water containing DDE for 28 days. After sacrifice, serum was separated from blood to measure E2 concentration (A). Testicular explants were obtained and incubated in DMEM/ Ham's F-12 culture media for 3 hours without (B) or containing 100 ηg/ml ovine LH (NIDDK, NIH) (C). Aliquots of serum were analyzed to measure E2 concentrations by RIA. (*p= 0.05, ****p = 0.0001)

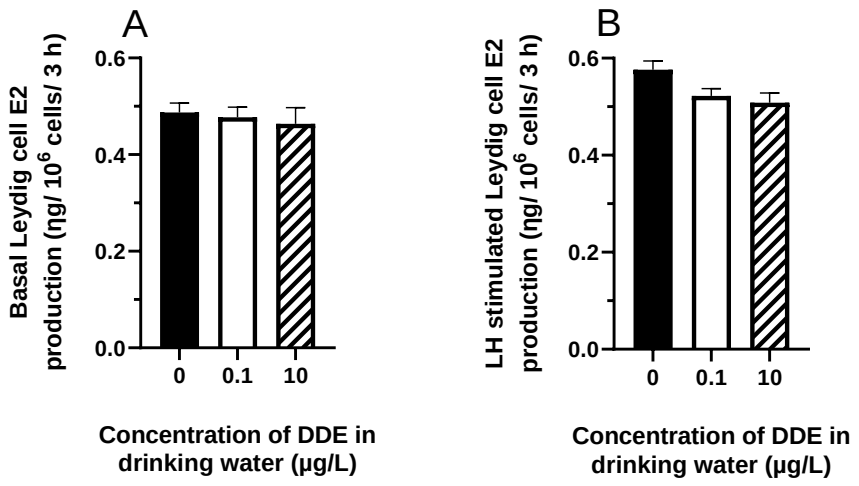

Figure 18 Effects of DDE on 17β- estradiol (E2) secretion by Leydig cells. Male rats at 21 days of age (n=12) were maintained on a control drinking water or with DDE-containing water for 28 days. After sacrifice, testes were pooled to isolate Leydig cells which were then incubated in DMEM/ Ham's F-12 culture media for 3 hours without (A) or containing 100 ηg/ml ovine LH (NIDDK, NIH) (B). Aliquots of spent media were analyzed to measure E2 concentrations by RIA.

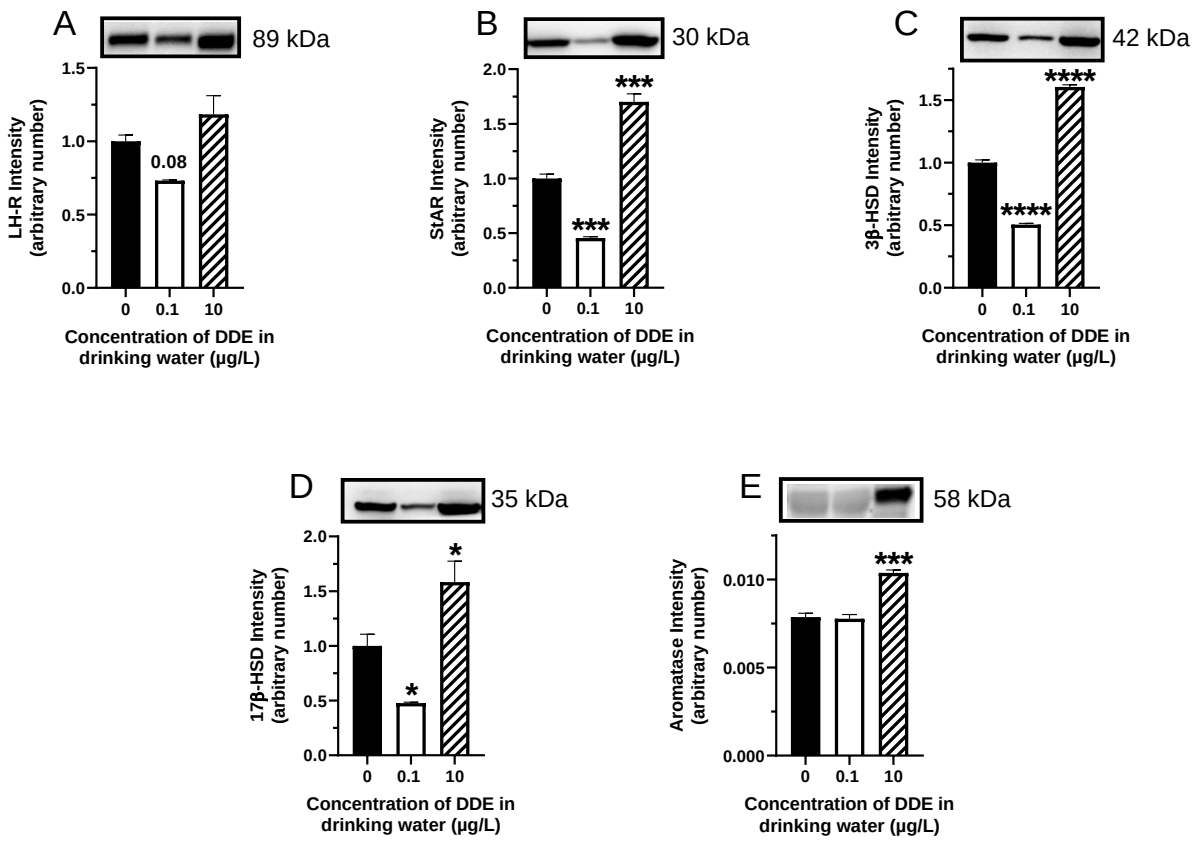

Figure 19 Effects of DDE on steroidogenic gene expression in Leydig cells. Male rats at 21 days of age (n=12) were maintained on a control drinking solution or with DDE treated water for 28 days. After sacrifice, testes were pooled to isolate Leydig cells and processed by western blotting to measure luteinizing hormone receptor (LH-R) (A), steroidogenic acute regulatory protein (StAR) (B), 17β-hydroxysteroid dehydrogenase (17 β-HSD) (D), 3β-hydroxysteroid dehydrogenase (3β-HSD) (C) and aromatase protein expression (E) as described in materials and methods. (*p= 0.05, ***p= 0.001, ****p = 0.0001)

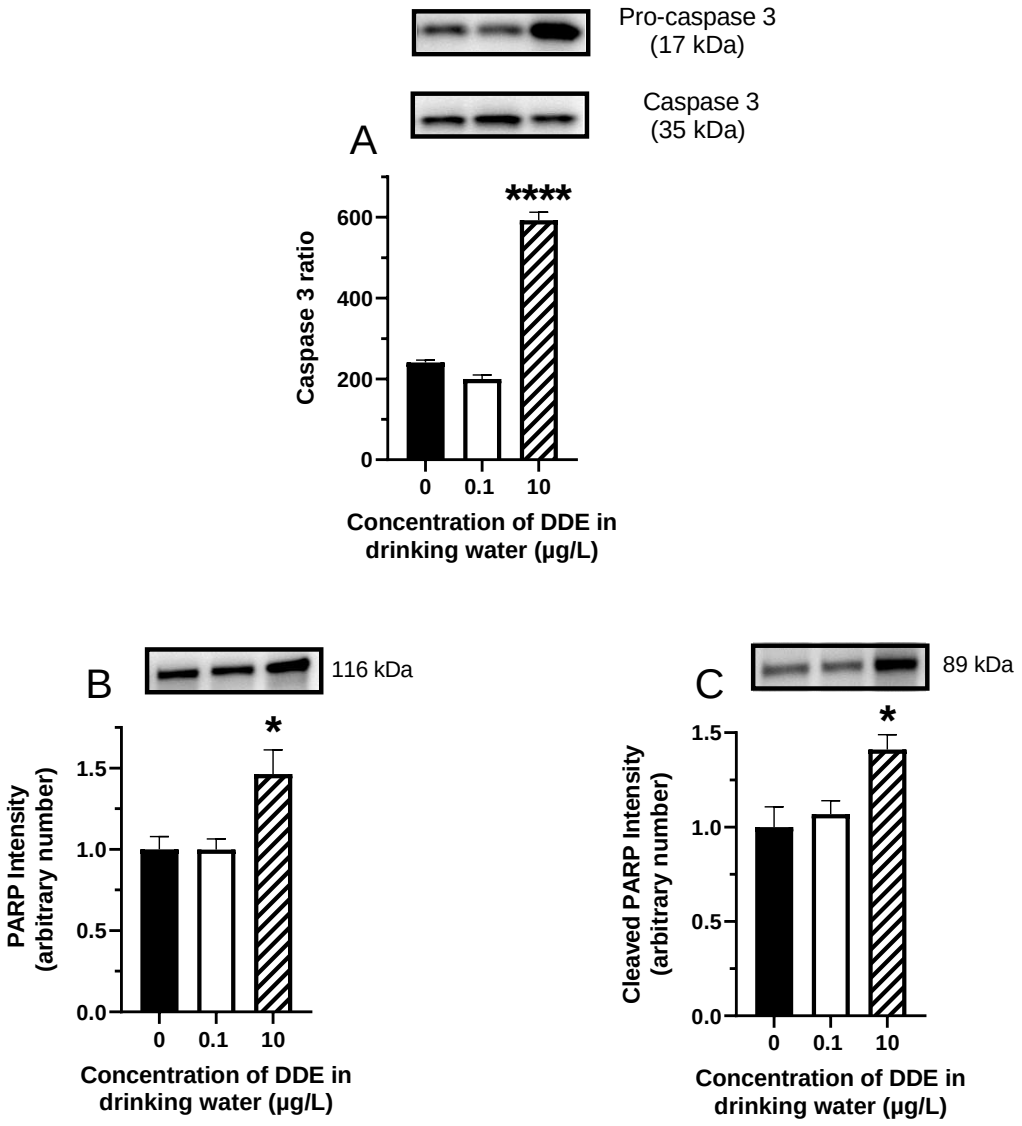

Figure 20 Effects of DDE on steroidogenic gene expression in Leydig cells. Male rats at 21 days of age (n=12) were maintained on a control drinking solution or with DDE treated water for 28 days. After sacrifice, testes were pooled to isolate Leydig cells and processed by western blotting to measure Caspase 3 ratio (A), full length (B) and cleaved Poly (ADP-ribose) polymerase (PARP) protein as described in materials and methods. (*p= 0.05, ****p = 0.0001)