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EFFECTS OF DRUG-INDUCED HEPATOTOXICITY ON VITELLOGENESIS IN THE FATHEAD MINNOW (PIMEPHALES PROMELAS)

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EFFECTS OF DRUG-INDUCED HEPATOTOXICITY ON VITELLOGENESIS IN THE FATHEAD MINNOW (PIMEPHALES PROMELAS)

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ABSTRACT

Keding, L. T. Effects of drug-induced hepatotoxicity on vitellogenesis in the fathead minnow (*Pimephales promelas*). MS in Biology: Physiology Concentration, May 2019, 47pp. (T, King-Heiden)

The vitellogenin (VTG) assay is a test used to identify estrogen axis endocrine disruptors (EDs) in fish species. The VTG assay has been seemingly successful, though there are inherent limitations yet to be addressed. Since vitellogenin is produced in the liver, damage to the liver may inhibit vitellogenesis in the absence of any endocrine disruption. The goal of this project was to better understand the impact of liver toxicity on fathead minnow vitellogenin production. Adult female fathead minnows were exposed to a water-only control (n=56), vehicle control (0.1% ethanol (v/v); n=91), simvastatin (n=58) or simvastatin with acetaminophen (n=120) via flow-through water exposure. In a separate experiment, an estrone challenge (100 ng/L) was used to determine whether exposure rescued impacts on vitellogenin production due to liver damage. After 1, 3, 5, 6, or 9 days of exposure, VTG concentrations were measured with respect to indicators of liver toxicity (hepatosomatic index and histopathology). Impacts on liver damage were compared with VTG to determine whether this damage influences vitellogenesis. Preliminary findings suggest damage following 6 days of exposure may manifest in elevated VTG levels up to 3 days later. Furthermore, vitellogenesis may be a compensation mechanism for fat accumulation in the liver.

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INTRODUCTION

Endocrine disruptors (EDs) are a class of environmental contaminants that interfere with normal hormone function (Kabir et al., 2015). They can vary in structure and are capable of mimicking or inhibiting the effects of hormones responsible for crucial physiological processes such as development, reproduction, and general homeostasis (Baccarelli et al., 2000; Diamanti-Kandarakis et al., 2009; Sumpter and Jobling, 1995; Tyler et al., 1998). Feminization of male gonads and infertility are two commonly documented responses to EDs in fish (Balabanič et al., 2011; Parrott and Blunt, 2005), in some cases leading to the collapse of whole populations (Kidd et al., 2007). Endocrine disruptors can come from both natural and manufactured sources. Phytoestrogens found in soybeans, pesticides used in farming, steroidal androgens and estrogens found in pharmaceuticals, polychlorinated biphenyls (PCBs) in industrial coolants and lubricants, and bisphenyl A (BPA) manufactured in plastics have all been associated with endocrine-mediated dysfunction in fish and wildlife populations (Diamanti-Kandarakis et al., 2009; Hotchkiss et al., 2004; Rosenfeld et al., 2017). Endocrine disruptors often enter our rivers through domestic and industrial waste effluent (Barber et al., 2007; Diamanti-Kandarakis et al., 2009; Gomes and Lester, 2002), and current wastewater practices are insufficient at removing many of them (Gültekin and Ince, 2007). Because these contaminants enter the environment as a mixture rather than individual chemicals, simultaneous exposure to compounds may lead to additive, synergistic, or antagonistic interactions on organisms

exposed (Kortenkamp, 2008).

In order to assess the total hazardous potential of these waste effluent mixtures the national pollutant discharge elimination system (NPDES) permit program was created (EPA, 2018). The NPDES utilizes Whole Effluent Toxicity (WET) testing as a method to determine the potential risk of wastewater on animal health. These tests utilize criteria such as the LC_{50} (concentration resulting in 50% mortality), NOEC (No Observable Effect Concentration), and LOEC (Lowest Observable Effect Concentration) of effluents, as well as many other endpoints as a means of assessing survival, growth, and reproduction (EPA, 2015; EPA 2018). If an effluent is found to be toxic, the EPA has outlined a tiered protocol to assess and characterize the class of environmental contaminants present. Additionally, a variety of tests be used to identify the presence of potential EDs within water samples (Hecker and Hollert, 2011). One such test used in identifying estrogenic (estrogen mimicking) or anti-estrogenic (estrogen inhibiting) EDs is the vitellogenin assay (Hutchinson et al., 2006; OECD, 2011).

Vitellogenin (VTG) is a dimeric yolk protein precursor made in fish hepatocytes, the primary nutrient source for developing embryos (Mann et al., 1999). VTG synthesis begins with estrogen binding to an estrogen receptor (ER) in the liver (Lai et al., 2000; Sellin et al., 2009). Due to their high levels of estrogen, VTG is made primarily in females; however, production may also be induced in males exposed to estrogenic compounds (Parks et al., 1999; Scott and Sumpter, 1983). Once translated, vitellogenin is transported to the oocyte for uptake and cleavage into yolk proteins (Patino and Sullivan, 2002). Through the use of the fish VTG assay, researchers can quantify the amount of vitellogenin produced in fish and therefore assess a compound's potential as an ED. A

significant increase in VTG concentrations within a male fish suggests it was exposed to an estrogenic compound, while significant reductions of VTG concentrations within females suggests exposure to an anti-estrogenic compound (Sumpter and Jobling, 1995). Although the VTG assay has been verified and is seemingly successful at identifying estrogenic and anti-estrogenic compounds in effluent samples, there are inherent limitations that have yet to be addressed (Hecker and Hollert, 2011; Wheeler and Coady, 2016; Wu et al., 2017; Zacharewski, 1998).

Since the water samples being evaluated are mixtures of environmental contaminants, identification of EDs can be difficult to ascertain (Barber et al., 2011; Hayes et al., 2006; Zacharewski, 1998). For example, a chemical analysis of a water sample may indicate that endocrine disruptors are present within a mixture, but vitellogenin assays do not detect them (Baker et al., 2014). Pesticides, industrial chemicals, and pharmaceuticals found in waste effluent can affect liver function through several mechanisms, including necrosis (cell death) or steatosis (lipid retention) (Cullen, 2005). Since the liver produces vitellogenin, contaminants that damage the liver (hepatotoxicants) may alter vitellogenin concentrations in the absence of endocrine disruption. For example, general liver necrosis may lead to a reduction in the number functional liver cells, leading to lowered vitellogenin concentrations. In addition, heavy lipid accumulation and oxidative stress (a common effect of hepatotoxicants) in the liver is generally accompanied by inflammation (Bacon et al., 1994; Day et al., 1998). An inflammation-induced stress response could lead to the down regulation of parasympathetic function and lower the expression of hepatic enzymes crucial for

vitellogenesis, which would also lead to a decrease in vitellogenin production (Chrousos et al., 1998).

Damage to the liver could lead to two potential errors in interpretation of the VTG assay. The first error is a type I error (a false positive), a decrease in vitellogenin levels due to liver damage would mask increases in VTG in males exposed to an estrogenic compound. VTG reductions due to liver damage could also result in a type II error (a false negative) – low VTG levels in female fish could be mistaken for the effects of an anti-estrogenic compound. Inaccurate interpretation of WET test data of this type would result in wasted time and resources, or failure to identify potentially harmful endocrine disruptors entering our environment.

Research Objective

The goal of this project was to describe the impacts of two known liver toxicants on the liver of adult female fathead minnows and determine whether damage to the liver was associated with a reduction in plasma vitellogenin concentrations. With a greater understanding of liver damage's effect on vitellogenesis we can better interpret results of tests such as the VTG assay and make more informed regulatory decisions - saving time, money, and the lives of countless animals.

Hypotheses

H_0: Drug-induced hepatotoxicity will cause no change in blood vitellogenin levels of female fathead minnows.

H_A: Drug-induced hepatotoxicity will cause a reduction in blood vitellogenin of female fathead minnows.

METHODS

Exposure Chemicals

Simvastatin (SIM) (≥97% [HPLC]; CAS 79902-63-9) was obtained from Sigma Aldrich Co. (St. Louis, MO) and dissolved in an ethanol vehicle. SIM is a common cholesterol-lowering drug that has been known to induce liver damage in organisms exposed at high concentrations (Bao et al., 2018; Horsmans et al., 1990). Dosing solutions were diluted into well water from 1000X stock solutions for final nominal concentrations of 0.05, 0.5, 5, or 50 µg/L, ensuring ethanol comprised 0.1% (v/v) of each exposure mixture.

Acetaminophen (APAP) (4-Acetamidophenol, 98%, CAS 103-90-2) was obtained from Fisher Scientific (Waltham, MA). APAP is an over-the-counter analgesic, also known to induce hepatotoxicity at high concentrations. Acetaminophen was dissolved directly into water resulting in concentrations of 2.5, 5, 7.5, or 10 mg/L. Both drugs were selected for their ability to effectively induce hepatotoxicity while not interacting directly with either the ER or estrogen. Concentrations of hepatotoxicants were established based on results of previous literature (Bao et al., 2018; Kim et al., 2012; Ribero et al., 2015).

Estrone (E1) (≥99%; CAS 53-16-7) was obtained from Sigma Aldrich Co. (St. Louis, MO). Estrone is an estrogen shown to reliably induce vitellogenesis in fish (Van den Belt et al., 2004). E1 was dissolved directly into the ethanol vehicle alone or in conjunction with simvastatin. Solutions were then added directly to treatment water or

combined with acetaminophen, and then added to treatment water. Each estrone addition resulted in a final nominal concentration of 100 ng/L.

Test Organisms

Fathead minnows were selected as the test organism due to their tolerance of a wide range of water parameters (pH, alkalinity/hardness, and temperature) along with their documented use in endocrine disruptor studies (Ankley and Villeneuve, 2006; Brungs, 1971; McCarraher and Thomas, 1968). Five-month-old female fathead minnows were purchased from the Environmental Consulting and Testing Inc. (Superior, Wisconsin). Upon arrival, fathead minnows were allowed to acclimate for a minimum of 2 hours, then randomly placed into an aquarium (6 total) that was sub-divided into 6 individual tanks (3 fish/tank, 18 fish/treatment). Treatments were administered by flow-through exposure at a rate of 17.5 L/day to respective aquaria. Fathead minnows were maintained in 13.5 L of water (pH of 8.0 ± 0.5, D.O. of 6.0 ±2.0 mg/L) at a temperature of 22.5 ± 1.5 °C, for photoperiods of 16:8 hours (light: dark) and fed twice daily (80:20, brine shrimp:bloodworms). All experiments were conducted in accordance with the St. Cloud State University IACUC Animal Care and Use Protocol for Live Vertebrates (IACUC #8-82 and #8-107).

Overview of Exposure

To induce liver damage, fathead minnows were exposed to known hepatotoxicants (simvastatin alone, or simvastatin with acetaminophen). Three replicate experiments were performed, resulting in n=3-18/concentration/day. Fish were exposed to 0, 0.05, 0.5, 5, and 50 µg/L of SIM or mixtures of SIM + APAP: 0.05 µg/L SIM + 2.5

mg/L APAP, 0.5 µg/L SIM + 5 mg/L APAP, 5 µg/L SIM + 5 mg/L APAP, 5 µg/L SIM + 7.5 mg/L APAP, and 50 µg/L SIM + 10 mg/L APAP. In an attempt to elucidate the role of estrogen's potential rescue effects on vitellogenin with exposure to hepatotoxicants, an estrone (E1) challenge was administered to the vehicle control and two hepatotoxic treatments, resulting in three exposures: vehicle control + 100 ng/L E1, 5 µg/L SIM + 5 mg/L APAP + 100 ng/L E1, and 50 µg/L SIM + 10 mg/L APAP + 100 ng/L E1. For all exposures, 3 - 40 fish were subsampled following 1, 3, 5, 6, or 9 days of exposure to assess impacts on general health, liver toxicity, and vitellogenin concentrations as described below.

General Health

During exposures, general health and mortality were monitored daily. Following 1, 3, 5, 6, or 9 days of exposure, a sub sample of n=3-9 fathead minnows were removed and anesthetized with a tricaine mesylate (MS-222, Sigma, St. Louis, MO) and sodium bicarbonate (Fisher Scientific, Waltham, MA) mixture. Condition factor (CF) was calculated as an indicator of general health (Kavanagh et al., 2013). Each fish was then patted dry, measured for total and standard lengths, and weighed to the nearest 0.01 gram. The standard length and body weight were used to calculate the condition factor (CF = 100 × total weight [g]/ standard length^3 [cm]). Ovary weights were used to calculate the ovosomatic index (OSI = 100 × ovary weight [g]/ body weight [g]) as an indicator of maturation status (Arcand-Hoy and Benson, 1998).

Serum glucose concentrations were collected as a measure of general stress (Hattingh, 1977). Blood (~0.5 µL) was collected from the caudal vein and glucose readings were determined using the True Metrix® Air Blood Glucose Meter, measured to

the nearest 1 mg/dL. The proportion of abnormal blood glucose readings (%) was determined by values ± 1 standard deviation from mean water control values. Remaining blood was collected with a heparinized micro hematocrit tube and centrifuged (5,000 g for 5 min). Blood plasma was isolated and stored at -80°C.

Hepatotoxicity

Liver weights were obtained to calculate the hepatosomatic index (HSI = 100 × liver weight [g]/ body weight [g]) as an indicator of general liver health (Goede, and Barton, 1990). After obtaining weights, half the liver was stored in 0.5 mL RNA later and the other half was processed for histomorphological assessment. Liver tissues were fixed in 10% neutral buffered formalin. After a 24-hour fixation, liver tissues were dehydrated in a graded series of ethanol, cleared in xylene, and embedded in paraffin using a Leica ASP3005 Tissue Processor. Liver tissues were sectioned (5 μm thick), mounted on slides, and stained with hematoxylin and eosin.

Histopathology was performed blind to the observer. Liver damage was assessed according to the presence or absence of five common pathological markers: lipid vacuolization (fat accumulation), glycogen vacuolization (carbohydrate accumulation), necrosis (cell death), hyalinization (breakdown of hyaline fibers), and karyopyknosis (condensation of nuclear contents). Two separate sections at two levels (100 μm apart) were assessed for each fish, for a total of four observations; observations were made at 40x magnification. Tissue samples were scored qualitatively for each pathological category using a 1-4 scale rating (l meaning lowest observed level of pathology, 2 meaning low/intermediate pathology, 3 meaning high/intermediate pathology, and 4

meaning highest observed level of pathology). All 5 pathology categories were then added together for a total histopathology score (lowest score of 5 [all 5 pathology categories scored a “1” and had minimal damage], highest of 20 [all pathology categories scored a “4” and had high damage]). The 4 total histopathology scores from a single sample liver were then averaged for a final histopathology score ranging from 5-20 for each sampled fathead minnow.

Vitellogenin

Plasma vitellogenin concentrations were determined by a colorimetric, competitive antibody-capture ELISA assay using a species-specific polyclonal antibody and purified vitellogenin standard. The antibody was produced in rabbits, following estradiol-exposed fathead minnow plasma injections (Parks et al., 1999). Purified vitellogenin standards were made from estradiol-exposed fathead minnows and purified through anion-exchange chromatography (Parks et al., 1999). Samples of fathead minnow plasma were run in triplicate at 3 dilutions ([50, 250, and 1000] or [100, 500, and 2000]) and analyzed on a Multiskan EX (Thermo Electron) at a wavelength of 412 nm (Shappell et al., 2010). Sample analysis was repeated and averaged when volumes were sufficient (>10 uL). Standard curves were constructed via Multiskan Ascent software. Within each experiment, vitellogenin concentrations were normalized to control (% of control) to account for inter-assay variability.

Data Analysis

Survival rates among exposures were determined using Kaplain-Meier survival analysis with Gehan-Breslow significance analysis. The proportion of abnormal blood

glucose readings were analyzed using the Fisher Exact test. The proportion contribution to total histopathology of combined treatments was assessed by chi-square analysis. All other parametric data was assessed through one or two way analysis of variance (ANOVA) for multiple groups, and t-tests when comparing two groups. The tukey multiple comparisons technique was used to assess significant results of ANOVA tests. Kruskal-Wallis analysis on ranks was implemented for non-parametric data with multiple groups, and Dunn's method was implemented to assess significant non-parametric results. The Mann-Whitney Rank Sum Test was used when comparing two, non-parametric groups. Significance was established at $\alpha = 0.05$. All error bars represent SEM.

RESULTS

Effects of Exposure to Hepatotoxicants on Overall Health

Following 9 days of exposure, the 50 µg/L SIM caused 16% mortality (Fig. 1) and the addition of 10 mg/L of APAP to the highest SIM concentration caused a 34% increase in mortality in fish (Fig 1).

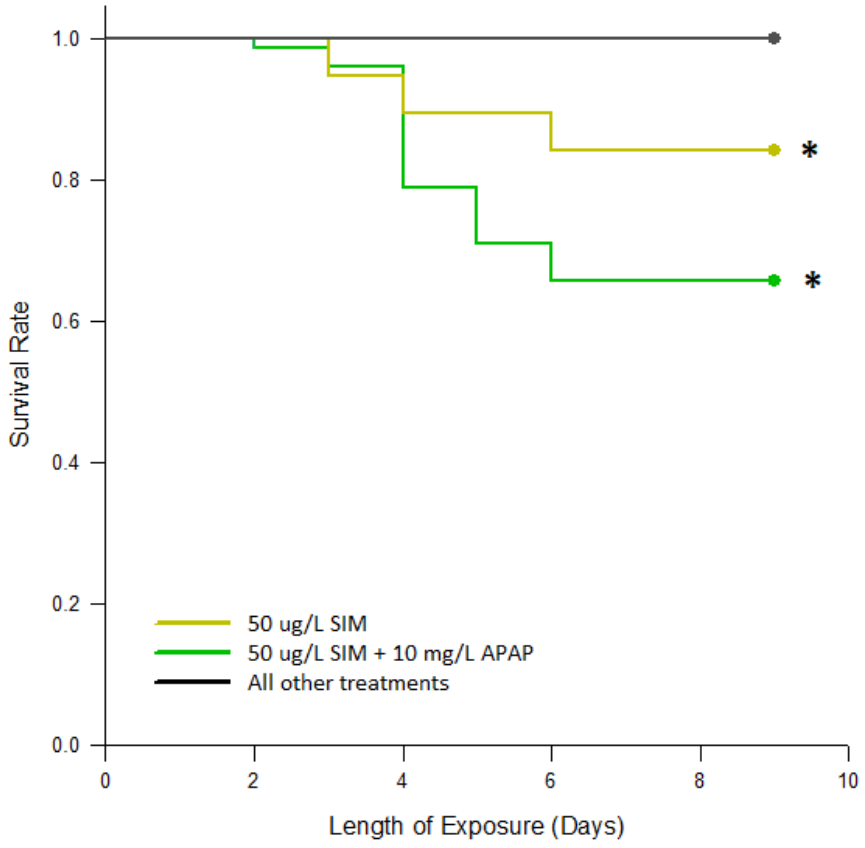

Figure 1. Dose-related impacts of exposure to hepatotoxicants on survival rate. "*" denotes a significant difference from control ($p < 0.05$).

Surviving fish exposed to ≤0.5 µg/L SIM showed no signs of overt toxicity (Fig. 2A and B). Fish exposed to 5 µg/L SIM for 6 days showed a 22% reduction in condition factor and fish exposed to 50 µg/L SIM after 9 days of exposure showed a 43% reduction compared to vehicle controls (Fig. 2A). Fathead minnows exposed to SIM + APAP appeared healthy with no overt signs of toxicity (Fig. 2C). There were no significantly altered serum glucose concentrations in fish exposed to SIM alone (Table 1; Fig 2B). Fish exposed to ≤0.5 µg/L SIM + <5 mg/L APAP also showed no altered serum glucose concentrations (Fig. 2D). Within the 50 µg/L SIM + 10 mg/L APAP treatment, serum glucose concentrations increased significantly from day 3 to day 5 (+130 mg/dL) (Table 1). Following 6 days of exposure to 5 µg/L SIM + 5 mg/L APAP or 50 µg/L SIM + 10 mg/L, a ≥55% increase of abnormal glucose concentrations was observed compared to the water control (Fig. 2D).

Table 1. Dose-related impacts on serum glucose concentrations.

	Exposure (days)				
Treatment	1	3	5	6	9
Water Control	69 ± 10	51 ± 2	-	49 ± 3	51 ± 4
Vehicle Control	53 ± 4	47 ± 7	-	51 ± 3	54 ± 4
SIM Only Exposures					
0.05 µg/L SIM	-	55 ± 9	-	58 ± 7	30 ± 6
0.5 µg/L SIM	-	52 ± 7	-	52 ± 11	59 ± 9
5 µg/L SIM	-	51 ± 9	-	33 ± 8	37 ± 6
50 µg/L SIM	-	70 ± 21	-	76 ± 33	43 ± 5
SIM + APAP Exposures					
0.05 µg/L SIM + 2.5 mg/L APAP	45 ± 3	49 ± 5	-	39 ± 1	28 ± 4
0.5 µg/L SIM + 5 mg/L APAP	50 ± 5	50 ± 8	-	46 ± 8	40 ± 14
5 µg/L SIM + 5 mg/L APAP	-	-	-	27 ± 4	42 ± 6
5 µg/L SIM + 7.5 mg/L APAP	51 ± 6	52 ± 8	-	51 ± 9	37 ± 7
50 µg/L SIM + 10 mg/L APAP	46 ± 4	**35 ± 5[A]**	**165 ± 43[B]**	92 ± 74	44 ± 6
E1 Addition Exposures					
Vehicle Control + 100 ng/L E1	-	-	-	38 ± 5	51 ± 6
5 µg/L SIM + 5 mg/L APAP + 100 ng/L E1	-	-	-	42 ± 7	43 ±7
50 µg/L SIM + 10 mg/L APAP + 100 ng/L E1	-	-	-	46 ± 8	59 ± 12

[A/B] and bolded text denotes a significant difference across days, within a single treatment group (one-way ANOVA) ($p < 0.05$).
Data is presented as the average [mg/dL] ± SEM.

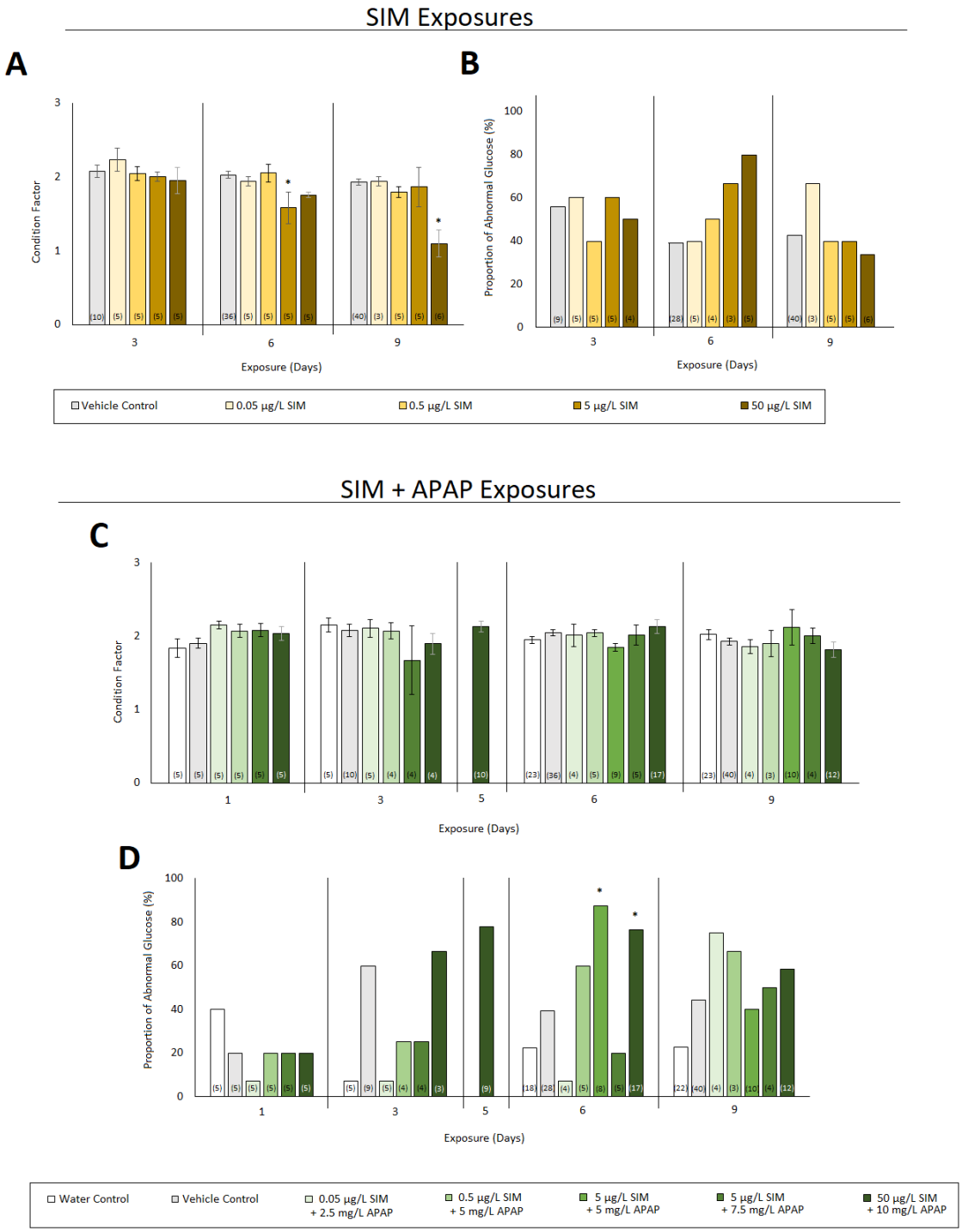

Figure 2. Dose-related impacts on health. Average condition factors and the proportion of abnormal blood glucose (%) are shown for SIM only (A and B) and SIM + APAP (C and D) exposed fish. Numbers in bars denote number of fish sampled. "*" denotes a significant difference from control within days (1-way ANOVA) ($p < 0.05$).

Hepatotoxicity of Simvastatin Alone and in Conjunction with Acetaminophen

Gross Hepatotoxicity Assessments

Fish exposed to SIM alone and SIM + APAP showed minimal signs of overt liver toxicity (Fig. 3A and B). Days 6 and 9 of exposure in SIM alone treatments produced higher HSIs collectively compared to day 3; however no dose-related increases in HSI were observed (Fig 3A). SIM and APAP exposures resulted in a lower average HSI in the 5 µg/L SIM + 5 mg/L APAP treatment in comparison to the water control on day 9 (Fig 3B).

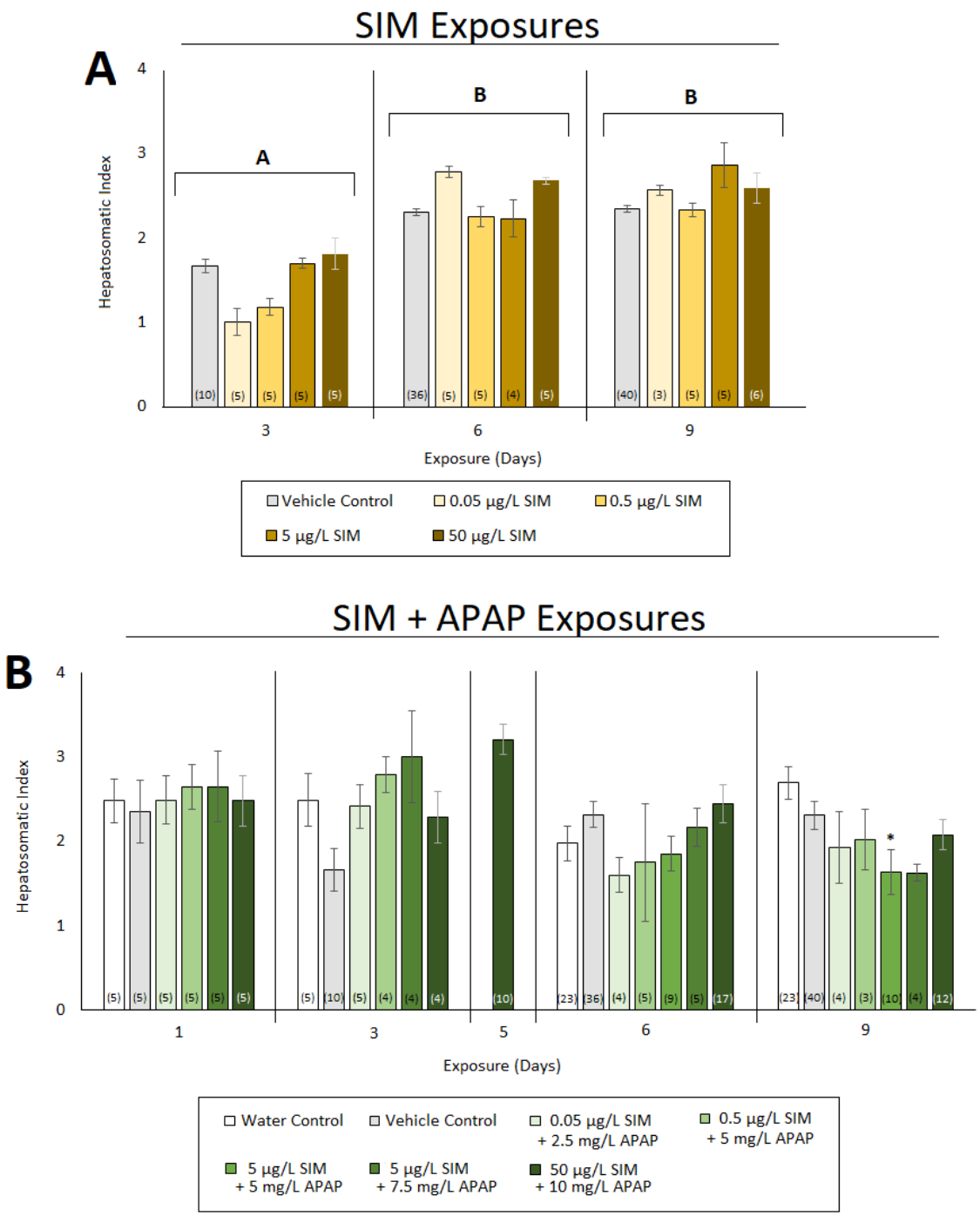

Figure 3. Dose-related impacts on hepatosomatic index (HSI). Average HSIs are shown for SIM only (A) and SIM + APAP (B) exposures. Numbers in bars denote number of fish sampled. "*" denotes a significant difference from control within days (1-way ANOVA). Bracketed letters denote significant differences between days (2-way ANOVA) ($p<0.05$).

Histopathology Assessments

Liver tissues exhibiting health and pathology after exposure to hepatotoxicants are shown (Fig. 4). Lipid vacuolization, glycogen vacuolization, hyalinization, karyopyknosis, and necrosis of liver tissues were all present in fathead minnow sampled (Fig. 4B, C, and D). Combined scores across all concentrations SIM and SIM + APAP show primary pathology consisted of lipid vacuolization and necrosis (Fig. 5). Liver damage in the vehicle control was not proportionally different from the water control (Fig. 5) In SIM treatments, necrosis was the primary pathology observed, while hyalinization was the least (Fig. 5). With the addition of APAP, increased necrosis, glycogen vacuolization, and karyopyknosis, and decreased hyalinization and lipid vacuolization relative to water control were observed (Fig. 5). Liver tissues had a 15% decrease in lipid vacuolization in E1 exposure compared to control (Fig. 5).

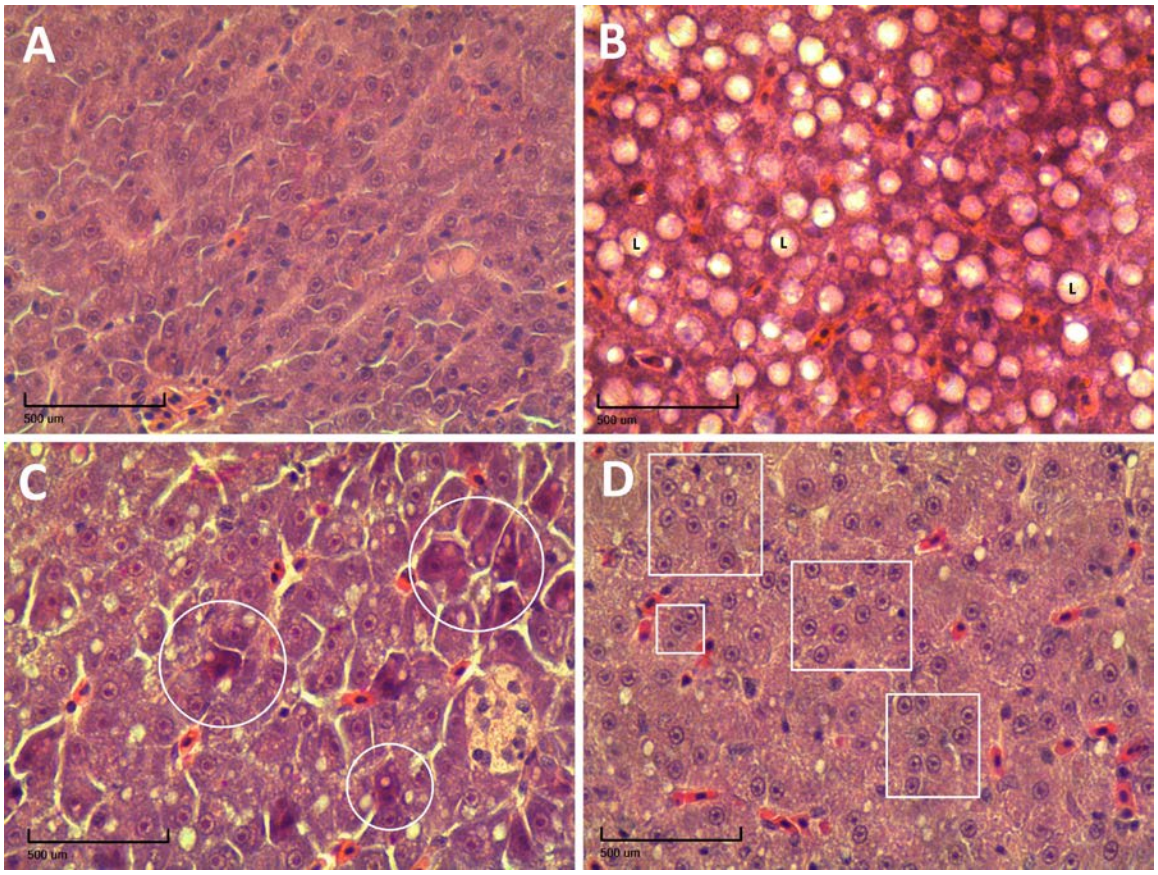

Figure 4. Liver damage in female fathead minnows. (A) A representation of a healthy fathead minnow liver. (B) High lipid (clear, spherical) vacuolization "L" occurring in hepatocytes. (C) Liver displaying high levels of necrosis, circled areas indicate breakdown of nuclear membrane and loss of cellular detail in hepatocytes - moderate glycogen (pink spherical) and lipid vacuolization were also present. (D) Condensed chromatin within nuclear membranes outlined in boxes are indicative of karyopyknosis. Images were captured at 40X magnification, bars indicate 500 µm.

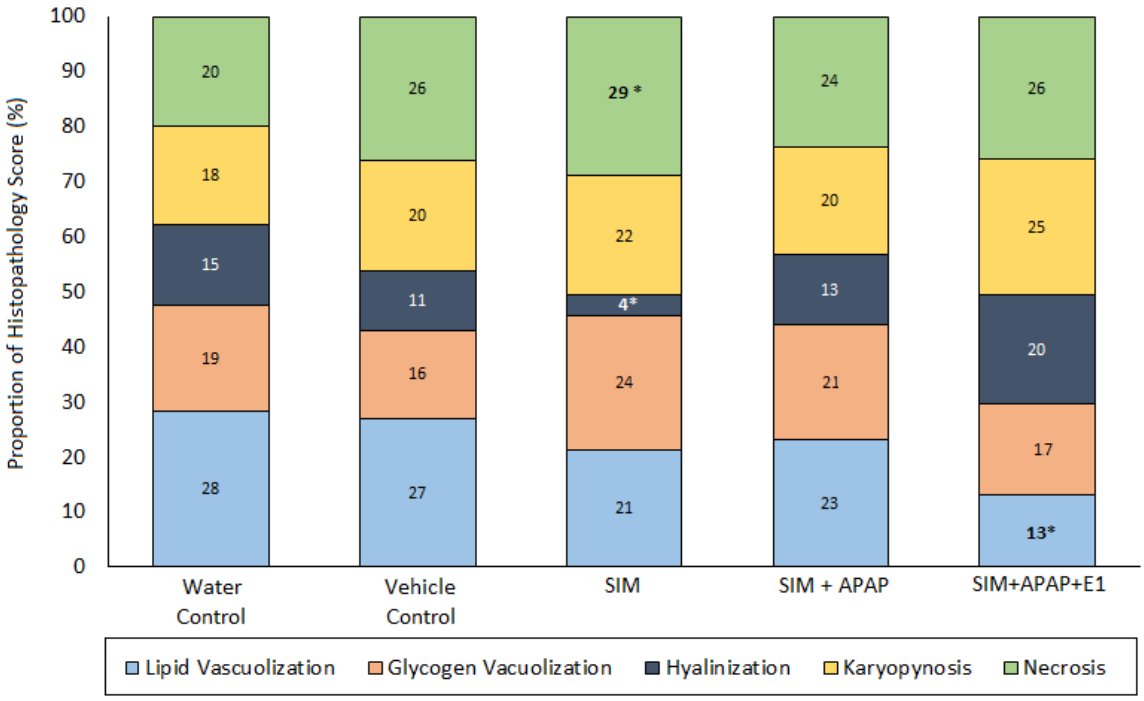

Figure 5. Proportion composition (%) of histopathology scores in treatment exposures. Bolded text and * denote significant differences from water control (chi-square analysis) ($p<0.05$).

Fish exposed to SIM alone showed no overt liver histopathology, while exposure to SIM + APAP resulted in significant liver damage (Fig. 6). Increased histopathology was observed in fish following 1, 3, 6, and 9 days of exposure to SIM + APAP. Vehicle control produced the highest pathology (11.5) after one day of exposure (Fig. 6B). At 1, 3, and 6 days of exposure, SIM + APAP treatments induced significant histopathology in a dose-independent manner (Fig. 6B). After 9 days of exposure, significant liver damage was observed in 5 µg/L SIM + 7.5 mg/L APAP (11.9) and in 50 µg/L SIM + 10 mg/L APAP (10.9) treatments compared to the water control (7.8) (Fig. 6B).

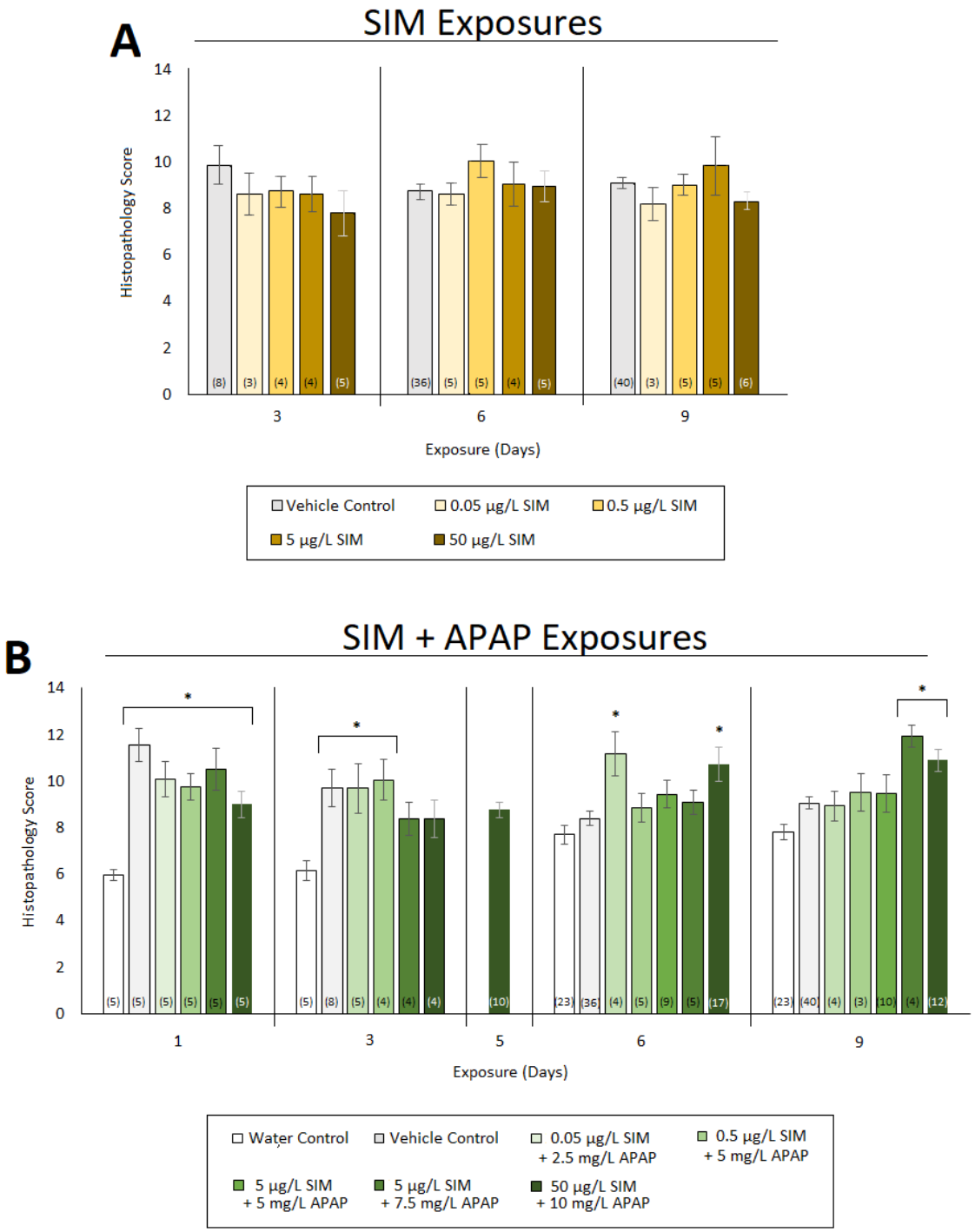

Figure 6. Dose-related impacts on histopathology score. Average scores are shown for SIM only (A) and SIM + APAP (B) exposures. Numbers in bars denote number of fish sampled. "*" denotes a significant difference from control within days (1-way ANOVA) ($p<0.05$).

Hepatotoxicity Effects on Vitellogenesis

There was no direct effect on vitellogenin concentrations in fish exposed to varying concentrations of SIM alone or SIM + APAP (Fig. 7, 8, and 9). When assessing relative VTG as a function of histopathology scores in controls (water control [n = 49, r^2 = 0.0095, p = 0.506], vehicle control [n = 78, r^2 = 0.0481, p = 0.054]), across SIM alone treatments (0.05 µg/L SIM [n = 7, r^2 = 0.0281, p = 0.720], 0.5 µg/L SIM [n = 10, r^2 = 0.0180, p = 0.712], 5 µg/L SIM [n = 8, r^2 = 0.0102, p = 0.812], and 50 µg/L SIM [n = 15, r^2 = 0.0679, p = 0.348]) and in SIM + APAP exposures (0.05 µg/L SIM + 2.5 mg/L APAP [n = 13, r^2 = 0.0208, p = 0.638], 0.5 µg/L SIM + 5 mg/L APAP [n = 16, r^2 = 0.162, p = 0.123], 5 µg/L SIM + 5 mg/L APAP [n = 15, r^2 = 0.0008, p = 0.918], 5 µg/L SIM + 7.5 mg/L APAP [n = 18, r^2 = 0.0437, p = 0.405], and 50 µg/L SIM + 10 mg/L APAP [n = 45, r^2 = 0.0006, p = 0.873]) no relationship was found (Fig. 7B and 8B). A pattern of increased VTG is shown across days in SIM + APAP exposures (Fig. 8A), as well as a positive correlation between day 6 histopathology scores and day 9 relative vitellogenin (n = 5, r^2 = 0.239, p = 0.404) (Fig. 9), though both were found to be non-significant.

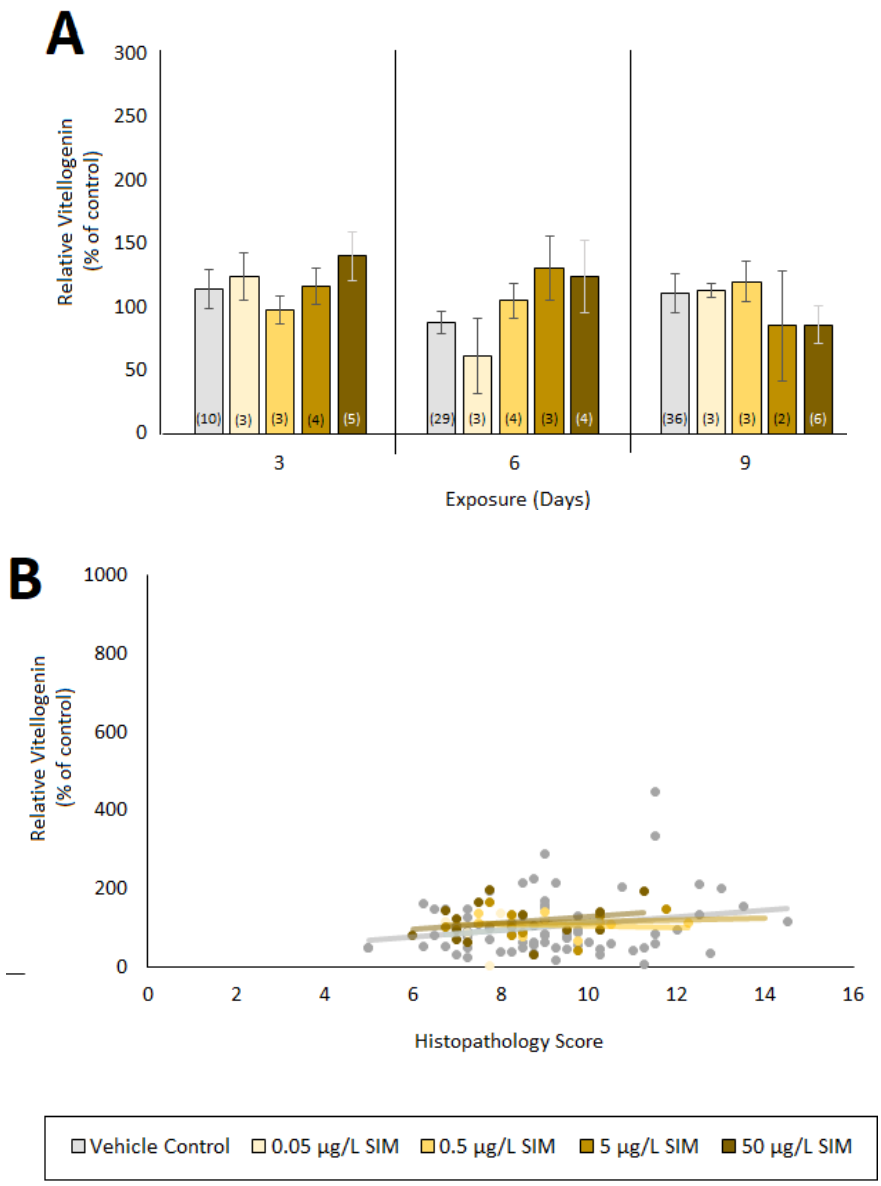

Figure 7. Impacts on relative vitellogenin (% of control) following SIM exposures. Average relative vitellogenin in treatments (A) and the relationship between relative vitellogenin and histopathology score (B) in SIM exposures are shown.

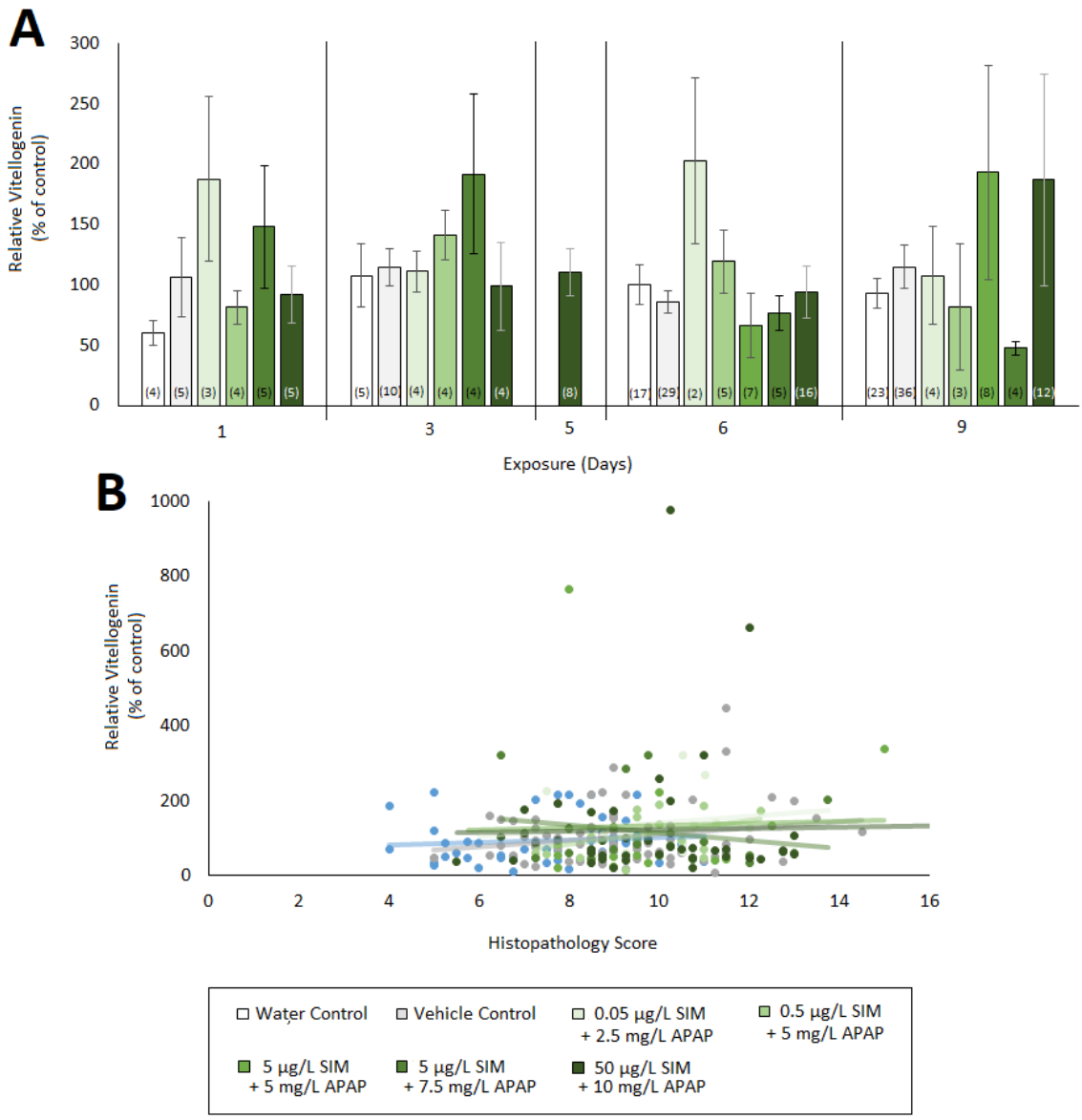

Figure 8. Impacts on relative vitellogenin (% of control) following SIM + APAP exposures. Average relative vitellogenin in treatments (A) and the relationship between relative vitellogenin and histopathology score (B) in exposures are shown.

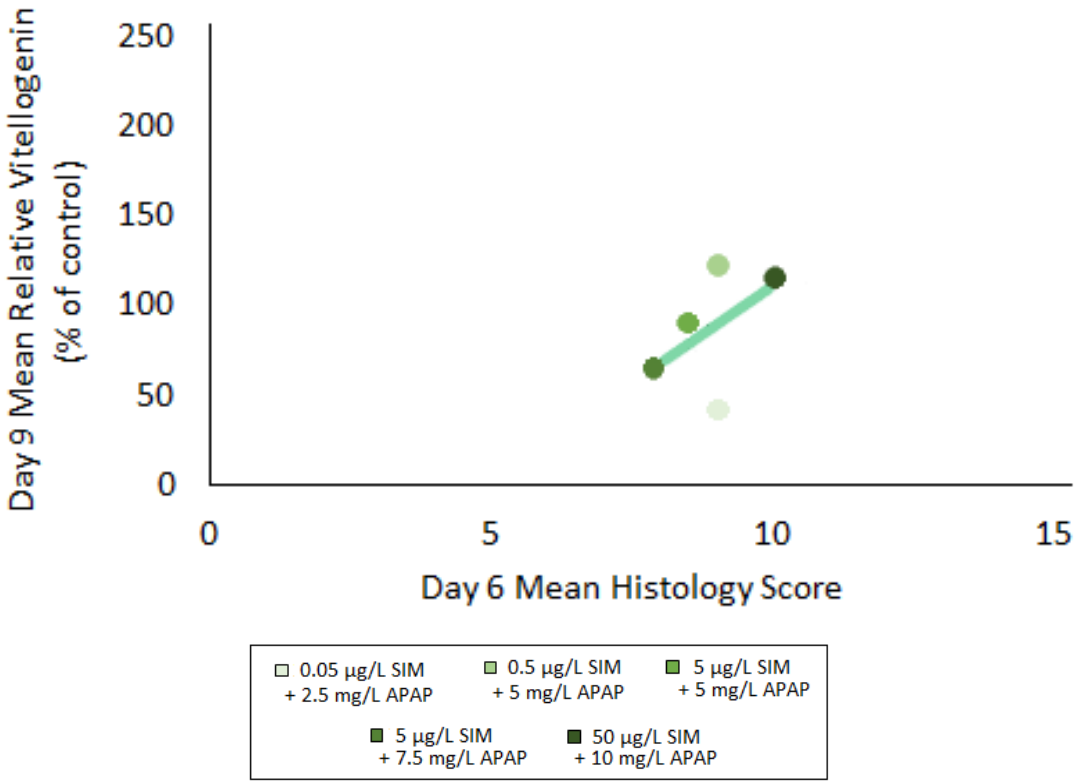

Figure 9. Relationship between day 6 average histopathology scores and day 9 average relative vitellogenin (% of control) in SIM + APAP exposures.

Effects Of An Estrone Challenge

There were no signs of general toxicity in fish exposed to E1 treatments (Fig. 1, 10A, and B). Non-significant mortality of 6% was observed in the vehicle control + 100 ng/L E1 treatment, with a single death occurring following 2 days of exposure. Altered serum glucose observed in 5 µg/L SIM + 5 mg/L APAP and 50 µg/L SIM + 10 mg/L APAP treatments after 6 days of exposure was significantly higher compared to the same exposure concentrations with the addition of 100 ng/L E1 (Fig. 10B).

No apparent liver toxicity occurred in vehicle, SIM + APAP, or E1 addition exposures, as indicated by HSI (Fig. 10C). An increase in histopathology was observed with the addition of 100 ng/L E1 to the vehicle control on day 6 (+3.0) and day 9 (+1.8) (Fig. 10D). The opposite effect was observed in the 50 µg/L SIM + 10 mg/L APAP treatment, with decreased histopathology was observed with the addition of 100 ng/L E1 on day 9 (-3.1) (Fig. 10D). Relative vitellogenin levels did not differ between treatments within the same day, however total VTG levels on day 9 were found to be higher than day 6 (Fig. 10E). The addition of 100 ng/L E1 to treatments had no effect on OSI values (Fig. 10F). There was also no relationship observed in any of the combined exposure treatments of relative VTG as a function of OSI (water control [$n = 47$, $r^2 = 0.0057$, $p = 0.616$], vehicle control [$n = 80$, $r^2 = 0.0197$, $p = 0.214$], SIM exposures [$n = 43$, $r^2 = 0.0134$, $p = 0.459$], SIM + APAP exposures [$n = 107$, $r^2 = 0.0035$, $p = 0.546$]) (Fig. 11).

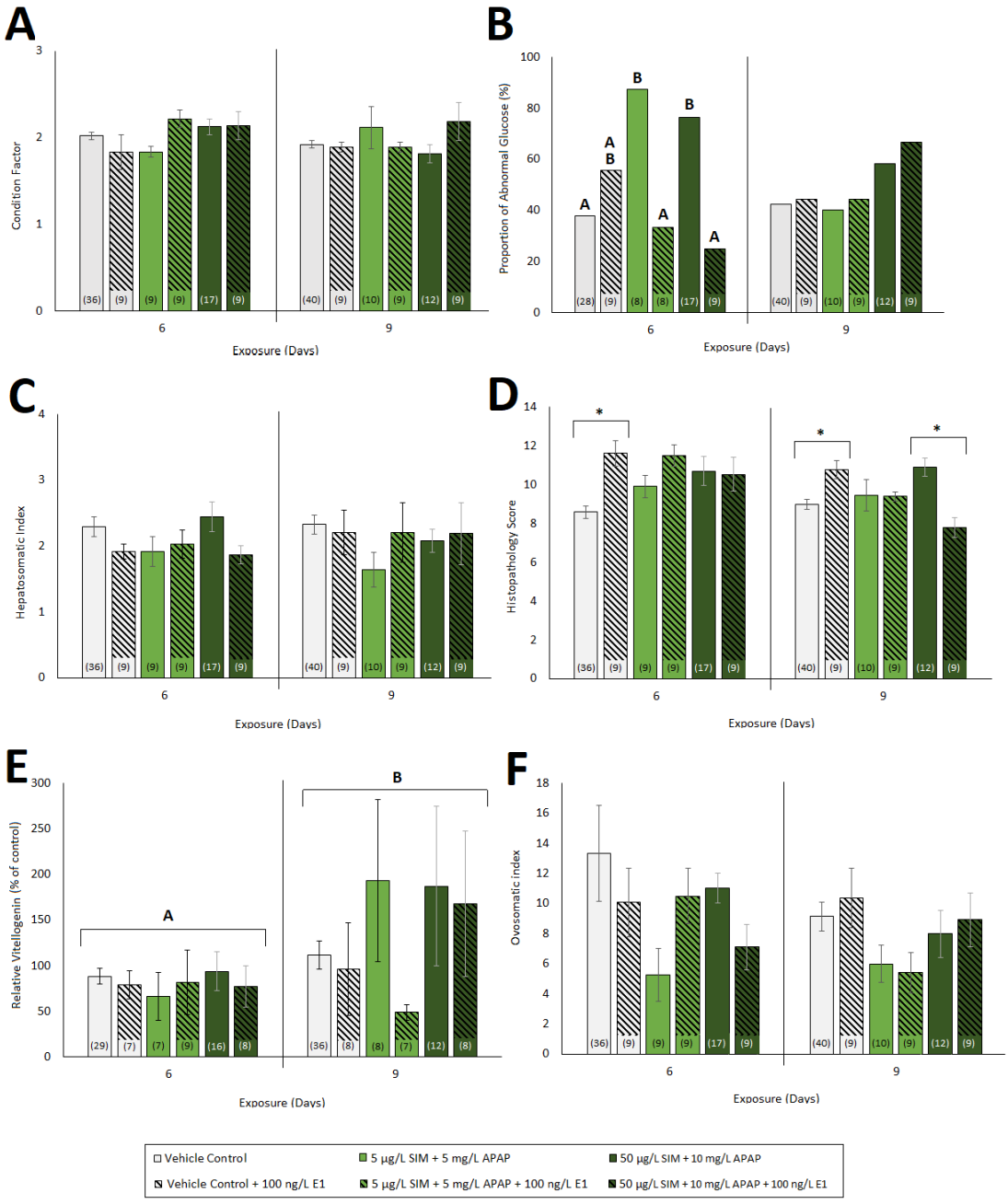

Figure 10. Estrogenic impacts on general health and hepatotoxicity. Average condition factor (A), proportion of abnormal glucose (%) (B), average HSI (C), average histopathology scores (D), relative vitellogenin (% to control) (E) and average OSI (F) are shown by exposure condition. Numbers in bars denote number of fish sampled. Letters denote differences between treatments within days (1 – way ANOVA). Bracketed "*" denotes a significant difference between two treatments (t-test). Bracketed letters denote significant differences between days (2-way ANOVA) ($p<0.05$).

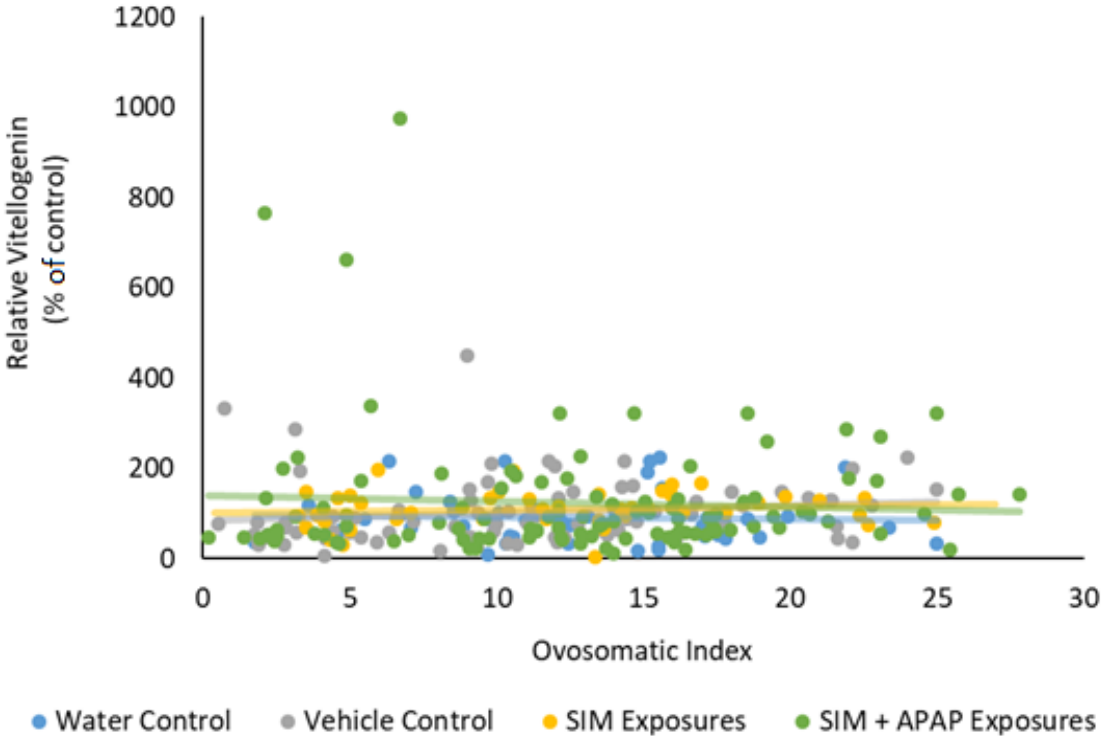

Figure 11. Relationship between OSI and relative vitellogenin across exposures.

DISCUSSION

Toxicity of Simvastatin

Limited research has been done on the effects of simvastatin on aquatic animals (Bao et al., 2018; Ribero et al., 2015). Its mechanism of action on cholesterol involves inhibition of hepatic - 3 - hydroxy - 3 - methylglutaryl - coenzyme A (HMG-CoA) reductase, though there is evidence that its hepatotoxic effects may be due to mitochondrial oxidative stress induction (Bao et al., 2018; Endo et al., 2004). Ribero et al. (2015) described mortality of 6.3 ± 3.8 % in zebrafish embryos after an 80 hour exposure to 5 µg/L simvastatin, and Bao et al. (2018) observed no deaths in adult mosquito fish following 7 days of exposure to concentrations as high as 500 µg/L simvastatin. The mortality observed in fathead minnows (Fig. 1) and evidence of general toxicity following 6 and 9 days of exposure (Fig. 2A) may be an indication of the fathead minnow's greater sensitivity to the toxic effects of simvastatin.

A low CF has been traditionally reported in fish displaying general toxicity (Baer et al., 2009), and fathead minnows exposed to 5 and 50 µg/L SIM exhibited this trend. However, fish did not appear to be under major stress in SIM exposures (Fig. 2B), as assessed via altered serum glucose levels (Barton et al., 1986; Jentoft et al., 2005). Future experiments should use serum cortisol concentrations to more accurately assess acute stress levels in fish.

Data depicted general increases in the HSI of fish exposed to simvastatin over a 9-day period. Increased hepatosomatic indices may be indicative of general hepatotoxicity occurring from both the vehicle control and simvastatin concentrations due to inflammation and hypertrophy of the liver (Fig. 3A). Hepatocyte swelling and increased HSIs have commonly been observed in fish following exposure to sub-lethal concentrations of hepatotoxicants (Arnold, 1995; Goede, and Barton, 1990). Observed pathology supports this idea. Although SIM exposure histopathology did not differ from the vehicle control (Fig. 6A), in comparison to fish exposed to water control, 1 and 3 days of exposure led to increased liver damage in vehicle exposures (Fig. 6B). Histopathology shows that SIM exposure primarily induced necrosis (Fig. 5), as was previously observed in rats (Kaufman et al., 2006). Necrotic swelling could be the basis for HSI increases across treatments (Fig. 3A).

Though we assessed lipid vacuolization increase as a sign of hepatotoxicity in tissues, liver damage has also been found to decrease lipid and glycogen vacuolization within hepatocytes (Bao et al., 2018; Wolf and Wheeler, 2018; Wolf and Wolfe, 2005). As seen in mosquito fish exposed to 5 µg/L of simvastatin (Bao et al., 2018), fathead minnows exposed to SIM showed a 7% reduction in lipid vacuolization compared to water controls (Fig. 5). It makes sense that lipid storage may decrease, considering simvastatin's mechanism of action, blocking cholesterol production through mevalonic acid inhibition (Bao et al., 2018; Istvan, 2001). Future histological examination should take into account to possibility of decreased lipid and glycogen vacuolization as an indication of pathology in fathead minnow livers.

Toxicity of Simvastatin and Acetaminophen

Significant mortality (34%) occurring as a result of SIM + APAP exposures was attributed to acute toxicity, as all significant death occurred in the highest SIM + APAP treatment groups (Fig. 1). All surviving fish showed no decrease in CF values across all SIM + APAP treatments, supporting the lack of overt toxicity occurring in SIM + APAP treatments (Fig. 4C). The general toxic effects on body shape and size (CF) observed with SIM alone seemed to not manifest with the addition of APAP.

The significant alterations in serum glucose concentrations observed in the SIM + APAP treatments could be evidence of hepatotoxic effects in fathead minnows. An increase in histopathology coincided with an increase in abnormal serum glucose levels on day 6 in the 50 µg/L SIM + 10 mg/L APAP treatment (Fig. 2D and 6B). Although classically an indicator of general stress, abnormal blood glucose in fish can be due to several environmental, hormonal, and toxicological conditions (Polakof et al., 2012). Liver damage has been shown to be associated with abnormally high and low levels of glycogen vacuolization in the liver, and both simvastatin and acetaminophen have been shown to produce inflammation within necrotic hepatocytes (Bao et al., 2018; Hinson et al., 2010; Jaeschke, 2015; Waters et al., 2001; Wolf and Wheeler, 2018). General stresses, like inflammation, have been found to induce glycogenolysis (the release of glucose from glycogen) in the livers of freshwater fish, resulting in hyperglycemia (high glucose levels) (Hattingh, 1977; Lazaro-Côté et al., 2018). It is possible that SIM + APAP-induced inflammatory stress occurring on day 6 may have led to an increase in glycogen breakdown, and therefore increased serum glucose levels.

Acetaminophen's derivative, NAPQ, has been found to induce mitochondrial oxidant stress and dysfunction, an inflammatory response (the release of cytokines and immune cell activation), and necrotic cell death (Jaeschke et al., 2012). Present experiments coincide with past findings, with necrosis contributing most to total histopathology, and karyopyknosis following close behind (Fig. 5). Pathology in liver tissue following 1, 3, 6, and 9 days of SIM + APAP exposure indicates significant hepatotoxicity of the chemicals in combination, though the hepatotoxic effect of the drugs together do not appear to increase with dose (Fig. 6B) as SIM and APAP alone have both been individually shown to do (Jaeschke, 2015; Kaufmann et al., 2006).

A trend of decreasing lipid vacuolization was observed occurring in SIM + APAP exposures relative to water control (Fig. 5). Along with evidence of simvastatin's lipid-reducing mechanism (Bao et al., 2018), studies have also associated acetaminophen toxicity with lipid peroxidation (lipid oxidative degradation), leading to a general decrease in lipid levels (Hinson et al., 2010; Wendel et al., 1979). Both simvastatin and acetaminophen have been shown to cause mitochondrial oxidation, leading to necrosis and inflammation (Jaeschke, 2015; Kaufmann et al., 2006). Significant increases in histopathology present intermittently on all days at varying concentrations suggest to hepatotoxic effects of SIM + APAP on fathead minnows (Fig. 6B). High levels of cell death paired in combination with high levels of karyopyknosis cell regeneration suggest that the liver is repairing damaged cells (Fig. 5).

Effects of Hepatotoxicants on Vitellogenesis

There appears to be no indication that exposure to the concentrations of simvastatin alone or in conjunction with acetaminophen caused sufficient damage to the

liver that resulted in altered serum VTG concentrations in the fathead minnow (Fig. 7 and 8). While the negative feedback mechanisms for vitellogenesis are not well understood, it is possible that lowered VTG concentrations caused by the minimal liver damage observed here was sufficient to trigger the HPG axis to allow for increased estrogen-induced vitellogenesis. Also, as liver damage increases there may be a reduced functional capacity to metabolize hormones, potentially leading to increased circulating estrogen levels and corresponding vitellogenin levels (Wheeler and Coady, 2016). Given these potential complications, the impacts of vitellogenin concentrations on the HPG axis in fish warrants further exploration.

It is also possible that there is a latent physiological effect in fish exhibiting liver pathology that would not manifest in altered VTG concentrations until days later. We observed a slight positive trend between day 6 histopathology and day 9 vitellogenin levels in SIM + APAP treated fish (Fig. 9). This pattern is reinforced when examining trends in day 6 altered serum glucose (Fig. 2D) and day 9 vitellogenin (Fig. 8A) – spikes in abnormal glucose corresponded well with increases of VTG. Increased stress and histopathology on day 6 could be manifested days later in the form of altered vitellogenin expression.

Finally, it has also been hypothesized that increases in VTG production may be a protective measure against a surplus of intracellular lipids within tissues (Ma et al., 2009), as would occur in a damaged liver. This could lead to increased VTG production as a consequence of liver damage - a compensatory mechanism for removing lipids from hepatocytes and thereby minimizing inflammation that is induced by heavy lipid vacuolization in the liver. Increased vitellogenesis as a tool to combat high lipid

vacuolization could also help explain the paradoxical high and low levels of fat accumulation observed in the literature as a result of liver damage. Increasing the subsampling time points, as well as extending the exposure period to 12 days may help elucidate latent effect trends in VTG observed. Measuring the relative levels of VTG in blood directly as well as monitoring VTG mRNA synthesis at the liver during hepatotoxic and healthy conditions could also shed light on the possible compensatory role of vitellogenesis.

Effects of an Estrone Challenge on Vitellogenesis

Fathead minnows experiencing hepatotoxicity did not exhibit the rescue effect commonly observed when introduced to an estrogen (Fig. 10E). Vitellogenin levels have been shown to increase in the presence of estrogenic compounds (Chakravorty et al., 1992), and the non-significant increases and decreases in treatments between E1 treated and non-treated hepatotoxic exposures suggests a possible disruption in vitellogenesis occurring.

It is also possible that additional estrogen in hepatotoxic treatments lowered the hepatotoxic stresses on the liver. The addition of 100 ng/L E1 appeared to combat the effects of exposures on serum glucose, significantly lowering the abnormal percentage in both SIM + APAP treatments (Fig. 10B). There was also a significant decrease in observed lipid vacuolization in E1 exposed treatments (Fig. 5), a trend previously described in male fathead minnows when exposed to estradiol (Pawlowski et al., 2004). High lipid vacuolization in the liver has been correlated with the down regulation of glycogenolysis and gluconeogenesis, resulting in abnormally low blood glucose levels

(Konopelska et al., 2011). With the additional estrogen in treatments, more lipids may have been recruited for VTG synthesis, therefore minimizing stress (altered serum glucose) from the effects mentioned in Konopelska et al. (2011). Reducing fat levels in the liver would also theoretically lower inflammation induced by lipid vacuolization as described in Bacon et al. (1994), leading to reduced stress and reduced altering of blood glucose.

Estrone exposure also appeared to induce further pathology in vehicle controls following 6 and 9 days of exposure, while reducing pathology in the 50 µg/L SIM + 10 mg/L APAP group following 9 days of exposure (Fig. 10D). This contradicts previous findings in rats, demonstrating synthetic estrogen's (EE2's) effect in reducing signs of liver damage like necrosis (Xu et al., 2004). One explanation could be the ethanol vehicle possible role in combating vitellogenesis. Harris et al. (2001) demonstrated that an alcohol vehicle control (methanol) had been shown to suppress circulating E2 concentrations. The decrease in pathology observed in the 50 µg/L SIM + 10 mg/L APAP + E1 group (Fig. 10D) could be attributed to the significant increase in VTG synthesis observed on day 9.

Estrogenic compounds have been demonstrated to increase vitellogenin concentrations while decreasing OSIs and reducing egg production in fish (Kramer et al., 1998; Pawlowski et al., 2004; Van den Belt et al., 2004). OSI was not affected by E1 additions (Fig 10C and F). A typical OSI range of 8 – 13 in adult female fathead minnows has been reported (Jensen et al., 2001), comparable to our vehicle control data (Fig. 10F). With an increase in SIM + APAP concentration, OSI values begin to fall below this range. It is possible that there is not an increase in VTG with hepatotoxicity,

but rather an issue with VTG uptake at the oocyte during liver damage. This would explain increasing VTG levels, while slightly decreasing OSI values when exposed to hepatotoxicants.

Conclusion

Endocrine disruptors are a particularly concerning class of contaminants due to their large potential impact on development and reproduction in organisms exposed. Measuring changes in serum vitellogenin concentrations is a staple biomarker in identifying EDs, and a thorough understanding of hepatotoxic effects on VTG production is necessary for proper interpretation of EDs during WET testing. Our data suggests simvastatin and acetaminophen in combination produce dose-independent hepatotoxicity in adult female fathead minnows. Induced liver damage also appears to have no direct effect on vitellogenesis, though latent effects of liver damage on vitellogenesis may be occurring as suggested by increases in vitellogenin observed on day 9 following significant pathology observed on day 6. Larger sample sizes and longer exposures may help shed light on the validity of this trend, as well as the incorporation of VTG mRNA monitoring at the liver as an indicator of vitellogenesis.

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