CAN HEALTH OF AQUATIC WILDLIFE INDICATE THE QUALITY OF WATER RESOURCES? EFFECTS OF HARMFUL ALGAL BLOOMS ON STRESS AND IMMUNE FUNCTION IN FRESHWATER AMPHIBIANS AND REPTILES

FINAL REPORT TO THE OHIO WATER RESOURCES CENTER

29 February 2020

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Problem and Research Objectives

Harmful algal blooms (HABs) are increasing in size, frequency, and intensity worldwide (Tang et al. 2006; Wang et al. 2008). Algal blooms are caused by nutrient input from agricultural runoff, and are predicted to become more frequent and severe with climate warming (Paerl and Huisman 2008). The Western Basin of Lake Erie is becoming widely known for the algal blooms it experiences nearly every year. Lake Erie is fed by the Maumee River watershed, the largest watershed in the Great Lakes region. Most of the land within the Maumee Watershed is agricultural, and as a consequence many of the nutrients applied to farmland throughout the watershed eventually make their way into Lake Erie. The intensity of agriculture and urban fertilizer use within the Maumee River Watershed leads to excessive sediment and nutrient loading, specifically dissolved reactive phosphorus (Daloglu et al. 2012), which historically led to extremely poor water quality in Lake Erie. Following passage of the Clean Water Act in 1972, nutrient levels dropped and Lake Erie's water quality improved, but nutrient and sediment levels have continually increased in recent years, resulting in high levels of turbidity and toxic algal blooms (Michalak et al. 2013). These water quality issues that continually plague Lake Erie negatively affect the region's fisheries, recreation, drinking water, and the economy (Bridgeman et al. 2013, Manning et al. 2013). Lake Erie's water quality problems made national news when, in August 2014, a severe HAB in Western Lake Erie shut down the water supply to ~500,000 citizens of the Toledo area for two days.

Algal blooms become harmful when they are dominated by cyanobacteria species that produce toxins, most notably microcystin. Microcystin is a liver toxin that can be fatal to humans and pets if ingested (Eriksson et al. 1990). Recent research demonstrates that it can also be aerosolized, for example in boat wakes, and therefore it poses an inhalation risk as well (Backer et al. 2008). Furthermore, high concentrations of microcystin found in the tissues of shrimp, frogs, and fish suggest that humans or other animals that eat organisms exposed to HABs could face serious health risks (Papadimitriou et al. 2012). Recent research also suggests that exposure to microcystins from HABs may pose a substantial risk to humans whose livers are already compromised, such as by liver disease (Zhang et al. 2015).

Although we are making substantial inroads toward understanding how microcystin affects human health, almost nothing is known about effects of microcystin in aquatic wildlife exposed to HABs. This is an important knowledge gap because wildlife health is a critical component of ecosystem integrity and functioning; moreover, because the immune system is highly conserved across vertebrates, understanding how HABs affect wildlife health will provide valuable insight into how it is likely to affect humans as well. Because the health of aquatic wildlife is likely a key measure of the health of aquatic systems and the quality of water resources, **understanding how water quality issues such as HABs affect the health of aquatic wildlife will provide an important metric for assessing the quality of Ohio's water resources.** That is, water resources that support healthy wildlife populations are, themselves, healthy.

To date, there has been very little research into the effects of HABs on aquatic communities. In zooplankton, high concentrations of microcystin were found in consumer species, suggesting that biomagnification of algal toxins occurs in some members of aquatic communities (Kozlowsky-Suzuki et al. 2012). High concentrations of microcystin have also been found in the tissues of dead fish, turtles, and ducks exposed to severe HABs, demonstrating that microcystin can be fatal to a variety of wildlife species (Nasri et al. 2008; Chen et al. 2009). It is likely that lower concentrations and/or shorter periods of exposure to microcystin would

have sub-lethal impacts on wildlife, but to date no studies have addressed this issue. In most vertebrates, elevated levels of stress hormones lead to depressed immune function (McEwen and Wingfield 2003; Millet et al. 2007). Therefore, individuals exposed to an environmental stressor such as the toxins produced by HABs are likely to experience both direct effects of the toxins, as well as depressed immune function caused by elevated stress levels.

My lab is taking a first step towards understanding how HABs affect aquatic wildlife by studying physiological stress and immune function in several common species of reptiles and amphibians. **Our overall objective is to compare stress levels and immune function in turtles, snakes, and frogs exposed to microcystin from HABs to control, unexposed animals.** We hypothesize that animals exposed to microcystin will exhibit increased physiological stress and depressed immune function compared to unexposed, control animals. Ultimately, our research will test **whether these measures of aquatic wildlife health are correlated with quality of water resources**. This study will provide the first baseline data on sub-lethal effects of Lake Erie's HABs in aquatic wildlife, and it will test whether several measures of aquatic wildlife health are correlated with quality of water resources. The information gained during this study will substantially increase our knowledge of how HABs impact the integrity of Ohio's water resources through effects on health of aquatic wildlife.

We conducted two complimentary field- and laboratory-based studies to determine how HABs affect the health of aquatic wildlife. First, we collected blood samples from wild freshwater turtles and snakes species in sites exposed to high levels of microcystin from HABs, and also in control, unexposed sites. We compared physiological stress levels and several measures of immune function of animals from exposed vs. unexposed sites. We predicted that animals from HABs-exposed sites would exhibit higher stress levels, and lower immune function, than their counterparts from unexposed sites. Second, we conducted a controlled laboratory exposure experiment in which we exposed naïve frog larvae to microcystin concentrations reflective of those found during Lake Erie HABs. We compared stress levels, immune function, and organ histology between tadpoles experimentally exposed to microcystin vs. control, unexposed tadpoles. Similar to our hypothesis from the field study, we predicted that tadpoles exposed to microcystin during this laboratory experiment would exhibit higher stress levels, and lower immune function, than their counterparts from unexposed sites. Ultimately, we predict that these measures of aquatic wildlife health will be correlated with water quality, and therefore that health of aquatic wildlife can be a used as an indicator of the quality of water resources.

Methods

The vertebrate immune system is comprised of several different "arms" which play different roles in responding to infection. The innate immune response is the first line of defense, and responds quickly and non-specifically to infectious agents. In contrast, the adaptive immune response involves the development of specific antibodies targeting the infectious agent. While the adaptive immune response occurs more slowly than the innate immune response, it also tends to be more effective. Importantly, however, chronically-high stress levels can depress immune responses and make individuals more vulnerable to infection (McEwen and Wingfield 2003; Millet et al. 2007). Sudden environmental disasters such as crude oil spills can induce a stress response in vertebrate animals (Lattin et al. 2016), which likely also results in depressed immune function. It is unknown whether harmful algal blooms similarly induce a strong stress responses in vertebrates, but **we hypothesize that animals exposed to microcystin will exhibit**

increased physiological stress and depressed immune function compared to unexposed, control animals, and ultimately, that animals from poor-quality (i.e., HABs-exposed) water will exhibit poor health compared to animals from higher-quality (i.e., not exposed to HABs) water. To test this hypothesis, we conducted two complimentary studies: a field study on a common species of turtle and snake, and a controlled, laboratory exposure experiment on frog larvae.

Field study

We compared physiological stress levels and several measures of immune competence in a freshwater turtle and snake species between sites exposed to chronically high microcystin levels and unexposed sites. We targeted the most common freshwater reptile species at our study sites, painted turtles (*Chrysemys picta*) and Northern watersnakes (*Nerodia sipedon*; Fig. 1). Our study sites were wetlands at Ottawa National Wildlife Refuge (hereafter ONWR), Ottawa County, and Grand Lake St. Marys (hereafter GLSM), Mercer County. ONWR is adjacent to Lake Erie and contains a wide variety of wetland types, some of which exchange water with Lake Erie and some of which are isolated hydrologically. Isolated wetlands at ONWR, which would not be exposed to Lake Erie HABs because they lack water exchange with the Lake, constituted our *unexposed, control* sampling sites. Our *exposed* sampling sites were wetlands in Mercer Wildlife Management Area on the southwest side of GLSM. GLSM contains high concentrations of microcystin year-round as a result of extensive nutrient input and a lack of seasonal stratification (Walls et al. 2018). In combination, ONWR and GLSM provided ideal systems in which to test the effects of microcystin exposure in wild populations of aquatic wildlife.

We sampled turtles and snakes in April – early June of 2018 and 2019, when capture efficiencies are highest. We captured turtles using a variety of aquatic traps, including basking traps, hoopnet traps baited with sardines and corn, and fyke nets. We captured snakes along wetland edges, either by hand or in minnow traps baited with sardines. We collected standard morphological measurements (snout-vent length, mass, and sex. All captured turtles and snakes were individually marked to ensure that no animals were sampled more than once. We marked turtles by filing an individual combination of notches in the shell margin (Cagle 1939), and we will marked snakes by clipping a unique combination of ventral scales (Blanchard and Finster 1933).

We collected a blood sample from the caudal vein using a heparinized, 28-ga syringe from each individual following capture. Blood samples never exceeded 5% of an individual's total mass. For each individual, we made a blood smear on a glass slide, and then immediately centrifuged the blood sample to separate the plasma from the packed blood cells. The plasma was subsequently drawn off using a pipette, aliquoted into separate tubes for subsequent immune assays, and flash-frozen in the field. All plasma samples were then stored at -80^{o}C in our lab at UT. Snakes were released at the site of capture immediately following collection of a blood sample.

For turtles only, we measured adaptive immune competency using a phytohemagglutinin (PHA)-challenge assay, which measures localized skin swelling in response to an infection (here, PHA injected into the toe webbing of a hind foot; Martin et al., 2006). For the PHA-challenge only, turtles were maintained in captivity for 48 hours and the skin-swelling response was measured at four times points: prior to injection with PHA, and again at 6, 24, and 48 h post-injection. The change in foot web thickness at each of these four times indicates the degree of

immune response to the PHA challenge (as in Schwanz et al. 2011; Sanchez and Refsnider, *in press*). Immediately after the 48-h measurement, all turtles were transported back to their capture site and released.

Following field sampling, we used a variety of laboratory assays to quantify physiological stress level and immune function in turtles and snakes using the blood smears and frozen plasma samples (as in Refsnider et al. 2015; Sanchez and Refsnider, *in press*). First, we measured physiological stress levels by quantifying ratios of heterophils to lymphocytes (H:L ratios) in the blood smears. Heterophils and lymphocytes are two types of white blood cells important in mounting an immune defense. An individual's H:L ratio becomes elevated when animals are exposed to a stressor; therefore, the higher the H:L ratio, the higher an individual's baseline level of physiological stress (Davis et al. 2008). Importantly, in contrast with corticosterone concentrations that can rise immediately upon handling by a researcher, H:L ratios in reptiles may take hours to days to increase in response to a stressful event, allowing for a more accurate assessment of an individual's baseline level of physiological stress than can be measured by quantifying corticosterone levels (Davis et al., 2008).

Second, we quantified innate immune competency using two assays. We conducted a bacteria-killing (BK) assay, which measures the bactericidal capacity of complement proteins in the blood plasma to kill *E. coli* (Tieleman et al. 2005). In this assay, diluted plasma samples are applied to cultures of *E. coli* and given time for bactericidal activity in the plasma to kill the bacteria. The proportion of the *E. coli* inoculum killed compared to the number of *E. coli* colonies present in control samples represents the bactericidal capacity of an individual at the time of plasma collection. We also conducted a natural antibody agglutination (NABs) assay (Matson et al. 2005). Natural antibodies are produced constitutively and function by agglutinating and lysing foreign cells. This second measure of immune function assessed individuals' constitutive innate immunity in terms of ability to adhere to and lyse foreign red blood cells.

We compared H:L ratios, per cent of bacteria killed, natural antibody agglutination titers, and PHA-induced skin-swelling response within species between the HABs-exposed and -unexposed sites using chi-square tests (for H:L ratios) and *t*-tests (all immune measures). We predicted that individuals from GLSM would exhibit significantly higher stress levels, and significantly lower immune responses, than individuals from ODNR across both species.

Laboratory exposure experiment

This experiment was conducted using naïve bullfrog (*Rana catesbeiana*) tadpoles, the most abundant amphibian species present in the wetlands at our study site. We captured 20 bullfrog larvae from ONWR (i.e., from isolated wetlands where animals are not exposed to Lake Erie HABs) in June 2018. We collected blood samples from the tadpoles as described above for baseline measures of physiological stress and immune function. We then randomly assigned tadpoles two groups: a treatment group, which was exposed to concentrations of microcystin reflective of those found during Lake Erie HABs, and a control, unexposed group. Tadpoles were housed in groups of five in five-gallon aquaria with filter tops in secure Biosafety Level II cabinets in the University of Toledo's Department of Laboratory Animal Research facility (Fig. 2). Tadpoles were maintained at 24^{o}C and 60% relative humidity, and ambient light:dark cycles. We housed these tadpoles for seven days in pond water collected from the site of capture at ONWR, along with natural aquatic vegetation for cover. We monitored and fed the tadpoles daily during this experiment.

For the microcystin treatment group only, we added microcystin to a final concentration of 20 μg/L to the aquarium water. This concentration represents the recreational threshold limit imposed by the Ohio Environmental Protection Agency, and is within biologically realistic levels measured during a HAB (Michalak et al. 2013). The microcystin for this experiment was collected from the Maumee River during the 2017 HAB, was stored frozen to maintain toxicity, and was thawed immediately before application to the water in which treatment group tadpoles were housed. Following the seven-day exposure experiment, we collected a second blood sample from all tadpoles to measure stress and immune responses to the experimental treatments. After seven days, the tadpoles were euthanized in MS-222, and the intestines and livers from each tadpole were removed, immediately fixed in 10% neutral buffered formalin for 24 hours, and subsequently transferred to 70% ethanol.

Following the laboratory exposure experiment, we quantified H:L ratios in blood smears to measure physiological stress levels, and conducted a bacteria-killing assay to measure immune competency, as described above, in pre- and post-treatment plasma samples from the tadpoles. We compared changes in individuals' H:L ratios and percent of bacterial colonies killed pre- and post-treatment using repeated-measures analysis of variance. We compared the pre- to post-treatment change in stress levels and immune function between the exposed and control groups to determine how exposure to microcystin affects these physiological parameters.

Organ histopathological analyses were conducted by our collaborators in the laboratories of Dr. Stephen Haller and Dr. David Kennedy (Su et al. in review). Briefly, the formalin fixed intestinal and liver tissues were embedded in paraffin, then sectioned into 5 um sections, placed on glass slides, and stained with hematoxylin and eosin and Periodic acid-Schiff. We photographed histology slides using an Olympus VS120 slide scanner. We measured intestinal diameter and counted the number of intestinal folds as indicators of an inflammation response. We also measured the perimeter of liver cells and determined the number of binucleated liver cells as indicators of potential liver damage. Finally, we measured protein oxidation by reactive oxygen species in both intestine and liver cells, which is an indicator of oxidative cell damage and which is a key mechanism of microcystin toxicity.

All research was conducted in accordance with approved animal care protocols (University of Toledo's Institutional Animal Care and Use Committee protocols 108657, 108742, 108743, 108802, and 108803), University of Toledo's Institutional Biosafety Committee (protocol #108801), and collection permits (Ohio Department of Natural Resources permits 18-155 and 21-016 and U.S. Fish and Wildlife Service permit #2017018).

Principle Findings and Results – Preliminary

Field study: Turtles

We quantified physiological stress levels and several measures of immune functioning between 11 painted turtles collected from a harmful algal bloom, and 11 turtles collected from a control, unexposed site. The average H:L ratios of exposed vs. control turtles were 0.37 and 0.34, respectively, and were not significantly different ($\chi^2 = 0.98$, $P = 0.32$), demonstrating that microcystin-exposed turtles did not have higher physiological stress levels than control turtles (Garcia 2018). Control turtles had significantly higher bacteria-killing capacity than microcystin-exposed turtles (control: 36%; exposed: -22%; $t = 4.79$; $P < 0.0001$; Garcia 2018). Natural antibody agglutination titers did not differ between microcystin-exposed or control turtles ($t = 1.45$; $P = 0.16$; Garcia 2018). Finally, painted turtles' peak skin swelling in response

to PHA occurred six hours post-injection (also see Sanchez & Refsnider, 2017). The average peak skin-swelling response was 0.035 mm in the microcystin-exposed group and 0.107 mm in the control group, but was not significantly different between groups (t = -1.44; P = 0.18; Garcia 2018).

Field study: Watersnakes

We quantified physiological stress levels and several measures of immune functioning between 14 Northern watersnakes collected from a harmful algal bloom, and 24 watersnakes collected from a control, unexposed site. The average H:L ratios of exposed vs. control watersnakes were 0.072 and 0.047, respectively, and was greater in watersnakes from the microcystin-exposed site (χ^2 = 4.79, P = 0.029), demonstrating that microcystin-exposed snakes had higher physiological stress levels than control snakes. Microcystin-exposed watersnakes had significantly higher bacteria-killing capacity than control snakes (control: 8%; exposed: 44%; t = -2.26; P = 0.037). Natural antibody agglutination titers tended to be higher in microcystin-exposed watersnakes compared to control watersnakes (control: 2.90; exposed: 3.92; t = -1.90; P = 0.07).

Laboratory exposure experiment: Bullfrog tadpoles

We exposed 10 bullfrog tadpoles to 20 μg/L of microcystin for 7 days, and 10 bullfrog tadpoles were housed in unaltered pond water as controls for 7 days (Fig. 2). There was no difference in H:L ratios or bacteria-killing capacity between microcystin-exposed or control tadpoles following the 7-day experiment, demonstrating that physiological stress levels and immune function did not noticeably change in one week of exposure to microcystin (Garcia 2018).

Microcystin-exposed tadpoles exhibited greater intestinal diameters (Fig. 3) and shorter intestinal fold heights (Fig. 4) than control tadpoles, both of which indicate intestinal inflammation (Su et al. *in review*). Microcystin-exposed tadpoles also had hepatocytes with greater surface area (Fig. 5), and a greater number of binucleated hepatocytes (Fig. 6), than control tadpoles, which indicate liver damage and cell repair (Su et al. *in review*). Finally, both intestinal and liver sections in microcystin-exposed tadpoles demonstrated a greater degree of staining than sections from control tadpoles, signifying greater oxidative damage in microcystin-exposed tadpoles (Su et al. *in review*).

<u>Finding Significance</u>

Our results demonstrate that aquatic wildlife exposed to harmful algal blooms demonstrate several sublethal effects. Northern watersnakes exhibited higher physiological stress levels when exposed to harmful algal blooms than did control animals (a pattern also seen in a parallel study on several wetland-associated songbird species). Painted turtles exposed to HABs did not exhibit increased physiological stress levels compared to control turtles, but this is perhaps not surprising because painted turtles generally do not exhibit increases in physiological stress levels in response to several different environmental stressors (such as novel climatic conditions; Refsnider et al. 2015). The effects of harmful algal blooms on immune functioning differed between watersnakes and painted turtles: bactericidal capacity decreased in microcystin-exposed turtles, whereas bactericidal capacity increased in microcystin-exposed watersnakes. No other measure of immune functioning differed between microcystin-exposed or control animals. If immune functioning is depressed in turtles from sites experiencing a HAB event,

then individuals could be at greater risk of contracting infections from parasites or pathogens. Conversely, in watersnakes which demonstrated an increase in immune functioning in individuals from a site experiencing a HAB event, the increased energy expenditure required to maintain enhanced immune activity could come at an energetic cost, for example by decreasing reproductive output or growth rates.

Our laboratory exposure experiment demonstrated that even 7 days of exposure to a HAB resulted in organ damage in exposed tadpoles. The intestinal and liver damage evident in our study raises the important point that our concern for aquatic wildlife exposed to HABs should not be limited to those species suspected of chronic exposure, such as fish, but we should also take into consideration the health risks in species suspected of acute exposure to microcystin (Su et al. *in review*).

Overall, we have found that even when HABs do not cause direct mortality of exposed wildlife, they can act as a physiological stressor across several different taxa, which may lead to other sublethal effects such as organ damage and depressed immune functioning in some groups. Therefore, the health of aquatic wildlife does appear to be a useful indicator of water quality and of the health of aquatic systems. However, because different measures of individual health were affected differently by exposure to microcystin in different wildlife taxa, the use of wildlife health as an indicator of water quality should be used with caution, depending on the species and health measure used.

Publication citations (all journal articles, proceedings and presentations at conferences)

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- Refsnider, J.M. "Can turtles serve as bio-indicators of environmental health? Applications of conservation physiology in Midwestern herpetology." Midwest Fish and Wildlife Conference, Milwaukee, WI, 30 January 2018.
- Su, R., C. Meyers, J. Garcia, A. Lad, J. Breidenbach, J. Refsnider, D. Malhotra, S. Haller, and D. Kennedy. *In review*. Microcystin-LR toxicity in *Lithobates catesbeiana* tadpoles. Toxins.

Students Supported

- Jessica Garcia – M.S. in Ecology (graduated January 2018)

- Brittany Holliker – University of Toledo undergraduate in Biology (Directed Research project, 2 semesters)
- Austin Hulbert – ongoing M.S. in Ecology (expected graduation August 2020)
- Casey Meyers – undergraduate (Wittenburg University, Biology major) in NSF-funded Research Experience for Undergraduates Program (summer 2019)
- Ashley Nunez – undergraduate (Ursinus College, Biology and Environmental Studies major) in NSF-funded Research Experience for Undergraduates Program (summer 2019)

Profession Placement of Graduates

- Jessica Garcia – private sector; Hazardous Materials Shipping Supervisor for UPS (Cleveland, OH)

Awards or Achievements

Nothing to report.

Any additional funding for this project

The University of Toledo's Lake Erie Center received a three-year National Science Foundation grant (NSF grant DBI-1852245) for a Research Experience for Undergraduates (REU) program, on which I am the lead Principal Investigator. This program brings ten highly qualified undergraduate student researchers from universities across the U.S. to the Lake Erie Center for ten weeks each summer. REU students are paired with a faculty and graduate student mentor, and work with this mentoring team to design and conduct independent research projects broadly exploring solutions to Lake Erie's environmental challenges. In 2019, one the ten REU students, Casey Meyers, worked with me and Drs. Stephen Haller and David Kennedy; Dept. of Medicine on the histology of microcystin exposure on liver and gut histology of bullfrog tadpoles. The tadpole histology research was further supported funding from the Harmful Algal Bloom Research Initiative from the Ohio Department of Higher Education, the David and Helen Boone Foundation Research Fund, the University of Toledo Women and Philanthropy Genetic Analysis Instrumentation Center, and the University of Toledo Medical Research Society (all to Dr Haller and Dr. Kennedy). An additional NSF-funded REU student, Ashley Nunez, and her faculty mentor, Dr. Henry Streby, helped with field sampling of turtles and watersnakes in 2019 and conducted a parallel study on the effects of microcystin on wetland-associated songbirds.

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Figure 1. Aquatic wildlife species sampled at microcysin-exposed (Grand Lake, Mercer County, OH) and control (Ottawa Natl. Wildlife Refuge, Ottawa County, OH) sites. Left to right: painted turtle (*Chrysemys picta*), Northern watersnake (*Nerodia sipedon*), and bullfrog tadpole (*Rana catesbeiana*; photo by J. Garcia).

Figure 2. Controlled laboratory experiment exposing bullfrog tadpoles to microcystin.

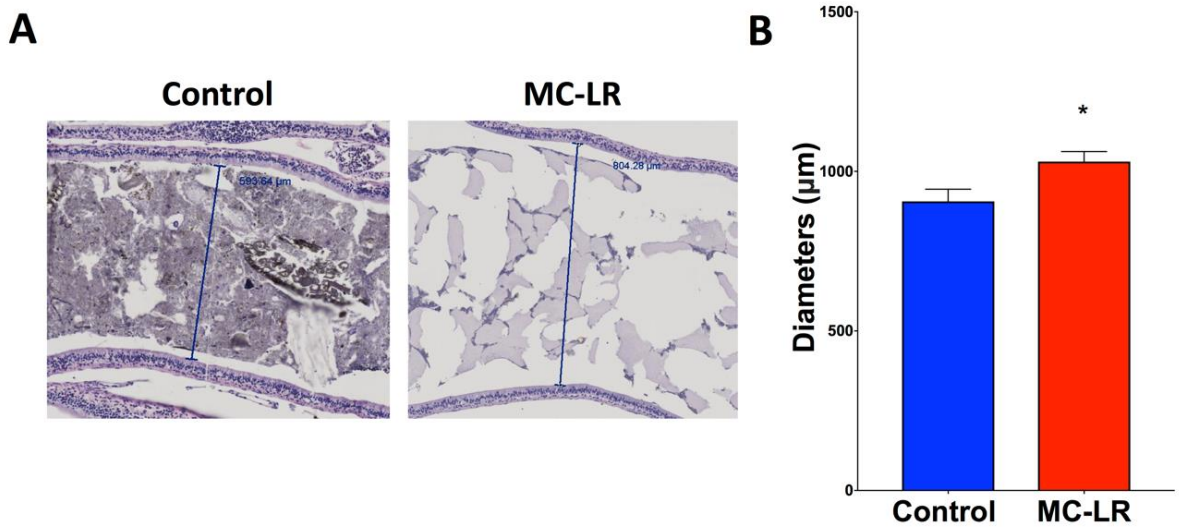

Figure 3. Tadpole intestinal diameters. (**A**) H&E stained intestinal sections reveal visibly larger intestinal diameters in the MC-LR exposed tadpoles as compared with the control tadpoles. (**B**) Quantitative analysis reveals significantly greater intestinal diameters in the MC-LR exposed tadpoles as compared with the control tadpoles. Data presented indicate the mean ± SEM (n = 10 tadpoles per group; 10 measurements taken per tadpole). *$p < 0.05$ vs. control group. (From Su et al. *in review*)

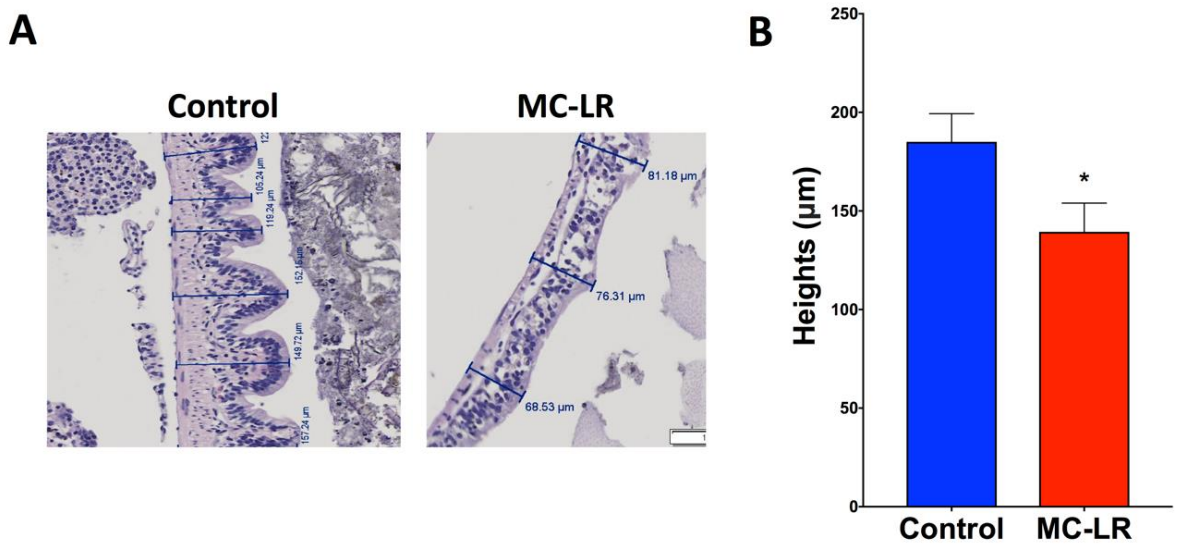

Figure 4. Tadpole intestinal fold heights. (**A**) H&E stained intestinal sections reveal visibly shorter intestinal fold heights in the MC-LR exposed tadpoles as compared with the control tadpoles. (**B**) Quantitative analysis reveals significantly shorter intestinal fold heights in the MC-LR exposed tadpoles as compared with the control tadpoles. Data presented indicate the mean ± SEM (n = 10 tadpoles per group; 20 measurements taken per tadpole). *$p < 0.05$ vs. control group. (From Su et al. *in review*)

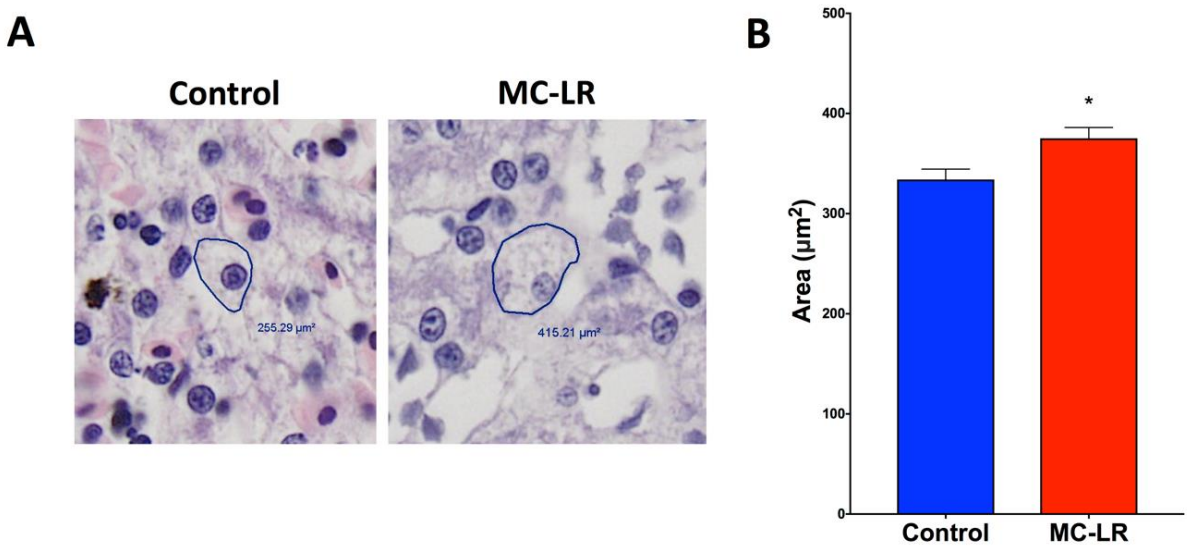

Figure 5. Hepatocyte sizes of tadpole liver sections. (**A**) H&E stained liver sections reveal visibly smaller hepatocytes in the MC-LR exposed tadpoles as compared with the control tadpoles. (**B**) Quantitative analysis reveals significantly smaller hepatocytes, as measured by surface area, in the MC-LR exposed tadpoles as compared with the control tadpoles. Data presented indicate the mean ± SEM (n = 10 tadpoles per group; 50 hepatocytes measured per tadpole). *$p < 0.05$ vs. control group. (From Su et al. *in review*)

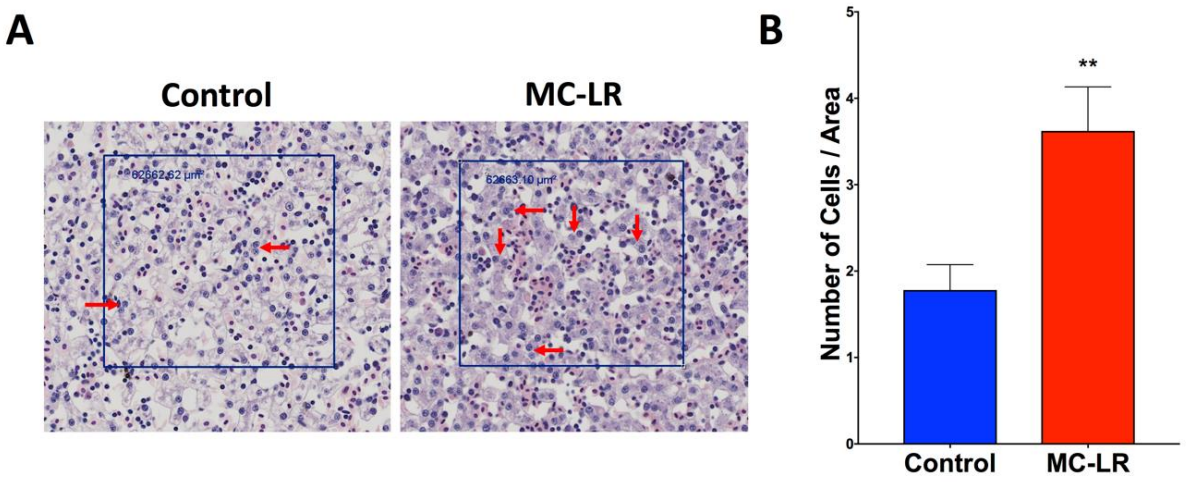

Figure 6. Hepatocyte binucleation. (**A**) H&E stained liver sections reveal a visibly greater number of binucleated hepatocytes in the MC-LR exposed tadpoles as compared with the control tadpoles. (**B**) Quantitative analysis reveals a significantly greater number of binucleated hepatocytes, in the MC-LR exposed tadpoles as compared with the control tadpoles. Data presented indicate the mean ± SEM (n = 10 tadpoles per group; 5 areas measured from each quadrant the liver of each tadpole). $^{**}p < 0.01$ vs. control group. (From Su et al. *in review*)