# Effect of Roundup on the Release of Reproductive Hormones in Rough Shiner *(Notropis baileyi)*

by

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## Abstract

Roundup is the most used herbicide across the world, with over a billion kgs applied in the US since its introduction in 1974. Long thought to be harmless to non-target species, recent studies have called this supposition into question. Aquatic environments are particularly susceptible, as Roundup binds to the soil and can be washed into the water spiking concentrations for several days. Our research focused on the effect of Roundup on fish reproduction, looking at three common reproductive hormones (prostaglandin-F2a, 11-ketotestosterone, and estradiol) produced by leuciscids. Our research found significant impacts to fishes' ability to detect and react to the reproductive signal prostaglandin-F2a, which is essential for ensuring simultaneous breeding. After fish were exposed to Roundup for 3 days, they released significantly less of all three reproductive hormones studied in this experiment, with levels of prostaglandin-F2a and 11-ketotestoterone being too low to detect.

Acknowledgements

This thesis is dedicated to the woods of North Georgia and to my fiancée, Emily Olson, who I met amongst those trees and who followed me into the unknown. It is dedicated to my mom for teaching me the value of writing well and of stopping to look at snails in a creek and to my dad who showed me how to be a scientist and that finished is better than perfect. It is dedicated to my brother, Patrick, and the trip we took on the Altamaha that changed the way I looked at the South; and to my sister, Laura, who has always looked out for her baby brother fiercely. My uncle, Arlin Grimes, and grandfather, Lyle Grimes, both have been instrumental in fostering a respect for nature in me and the belief that it is never too late to solve a problem. I want to also extend a thank you to Adam Fox, for helping me start out on this path of science and guiding me as I started out, and to Jay Shelton for igniting a passion for our waterways and making me read *Sand County Almanac*, something that should be required of every naturalist. Additionally, I want to thank Catherine Teare-Ketter for showing how much I could do, Todd Rassmussen for being himself, and Christopher Ronald Silcox for being the best boss I ever had and a model for the naturalist I hope to become.

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## Table of Contents

List of Tables

List of Figures

List of Abbreviations

| | |
|---|---|
| PGF | Prostaglandin-F2a |
| E2 | Estradiol |
| 11KT | 11-Ketotestosterone |
| EPA | Environmental Protection Agency |
| FDA | Food and Drug Administration |
| IARC | International Agency for Research on Cancer |
| WHO | World Health Organization |
| US | United States of America |
| DW | Distilled Water |
| RU | Roundup |
| POEA | Polyethoxylated Amine |
| LH | Luteinizing Hormone |

# Introduction

The most widely used herbicide in the United States since 2001 is glyphosate, the active ingredient in the product Roundup (FDA). This trend is likely also represented globally but international herbicide use data are poorly recorded. The use of glyphosate on crops has accelerated since the introduction of genetically engineered herbicide resistant crops, so-called "Roundup Ready" crops, in 1996. Since the introduction of these engineered crops use of glyphosate has risen 15-fold globally, in the US alone more than 1.6 billion kg have been applied since 1974 (Benbrook 2016). Use is not restricted to farmland however, based on shared data from five federal agencies over a million hectares of public wildlands were sprayed with herbicide, of which glyphosate accounted for 35% of the applied active ingredients (Wagner et al. 2016). Roundup is commercially available to the public and used in suburban and urban yards and gardens, though data for this is unreported.

Glyphosate-based herbicides are applied to crops annually and are readily absorbed into the soil, especially in high-clay soils; and once entered into the soil have a very low mobility (Sprankle et al. 1975, Okada et al. 2016). Without any external treatments the accumulated glyphosate will eventually be broken down by microorganisms in the soil (Quinn et al.. 1988). However, heavy rains can transport glyphosate loaded soil into ground and surface waters, where it is able to come into contact with non-target species (Maqueda et al. 2017). Once suspended in the water column and exposed to UV light, glyphosate breaks down quickly, within 7 days of peak concentration, nearly all the glyphosate in the system will be degraded or reabsorbed into the soil (Lund-Høie and Friestad. 1986). Because of this quality, outside of runoff events, glyphosate levels in water stay relatively low; in fast flowing riverine systems similar to our

study sites the average concentration is around 10-15 μg/L (Byer et al. 2008). While the average glyphosate levels are relatively low, concentration spikes following runoff events, with a study in the Mississippi river finding levels as high as 450 μg/L following storms (Coupe et al. 2011).

The World Health Organization (WHO) and the International Agency for Research on Cancer (IARC) found in 1994 no danger to non-target organisms from glyphosate. This stance was amended in 2015 when it was linked to cancer in humans and reclassified as Category 2A (probable carcinogen for humans) (WHO 2015). These findings were specifically refuted by the EPA in 2016, with an independent study that found no link between glyphosate and cancer in humans (EPA 2021). The opposite results found by these agencies can be attributed to differences in their study designs. The EPA focused on technical glyphosate, while the IARC instead studied glyphosate-based herbicides as would be sold and used by consumers (Benbrook 2019). While glyphosate by itself is relatively inert to non-plant organisms, it is never used in its technical form; it is always applied within the Roundup formula, which increases its toxicity. This increase in toxicity can be primarily attributed to the surfactant polyethoxylated amine (POEA) within Roundup which allows the glyphosate to breach non-plant cell walls (Tsui and Chu 2005). POEA by itself can cause permanent damage to DNA at low doses (0.75 mg/L) for 24 hours in fish (Navarro et al. 2014) and the toxicity of the POEA is significantly higher than that of glyphosate by itself. POEA is largely responsible for the toxicity of the Roundup formula. The 24-hour $LC_{50}$ of rainbow trout in glyphosate is 140 mg/L while for POEA and Roundup it is only 2.1 mg/L (Folmar et al. 1979). Studies have shown that the complete Roundup formulation causes more damage to non-target species than glyphosate alone.

**Non-target Effects**

The ability of glyphosate to breach non-target cell walls in the Roundup formulation helps explain why it causes oxidative stress and genetic damage to organisms exposed to it. Generally, the most vulnerable species are those that rely on permeable membranes and those that live at least partially in water where they can be submerged in chemical-laden waters. Glyphosate and its formulations were found to be a cause of both cytotoxicity and genotoxicity in frog species from North America, South America and Australia with the larval forms being particularly vulnerable (Howe et al. 2009, Meza-Joya et al. 2013, Mann and Bidwell 1999). One study, simulating conditions in the wild, found the lethality of Roundup was doubled in one species of tadpole when they were concurrently exposed to predator stress (Relyea 2005).

Reptiles are usually more tolerant to environmental pollution than amphibians. However, studies of aquatic reptiles (turtles and caiman) found signs of oxidative stress in turtles after only a 96-hour treatment and reduced complement system activity and lower numbers of leukocytes in caimans, leaving them immunocompromised (Héritier et al. 2017, Siroski et al. 2016, and Latorre et al. 2013). In terrestrial reptiles, it was found that exposure to Roundup caused genetic damage in embryonic tegus and resulted in molecular damage to the liver and gonads of field lizards (Schaumburg et al. 2016, Chianese et al.. 2023).

Generally, birds are documented to be affected by herbicides in agricultural areas, since they feed on the plants and seeds that have been sprayed by herbicides. Studies in North America found that 67-72 million birds a year were killed from pesticide toxicity in the US (Pimentel et al. 1992, Pimentel 2005). Studies on Japanese Quail (*Coturnix quail*), a common model avian, found that the offspring of females who had consumed glyphosate laced seeds had a higher rate

of embryonic failure, molted later, and male offspring had reduced testosterone (A-Ruuskanen et al. 2020, B-Ruuskanen et al. 2020, C-Ruuskanen et al. 2020).

The effects of glyphosate and its formulations on mammals is of particular interest for our understanding of its effects on humans. It was found that mice that had been fed Roundup at a rate of 50 mg/kg of body weight exhibited lower weight as compared to control groups and significant liver damage after fifteen days of treatment (Jasper et al. 2012). Mammalian sperm exposed to Roundup suffered reduced motility at doses lower than 5ug/mL and worsened viability at higher doses (Nerozzi et al. 2021). Chronic low dose exposure (5 weeks at 1/250 $LD_{50}$) in male rats resulted in significant oxidative stress to the testes and inhibition of testosterone production (Astiz et al. 2013). In female rats, exposure to Roundup during development disrupts uterine development and alters morphology, increasing the sensitivity of the uterus to estradiol (Schimpf et al. 2017, Schimpf et al. 2018).

Lymphocytes from humans and bovines exhibited oxidative stress following exposure to Roundup in vitro (Lioi et al. 1998, Vigfusson and Vyse 1980). Furthermore, at doses of only .06% Roundup inhibited aromatase activity in both equine and human placental tissues after 18 hours exposure (Richard et al. 2005). Additional studies on the effects of glyphosate and Roundup on human cells have produced contradictory results. Studies in the United States and Europe have linked the use of glyphosate to the emergence of non-Hodgkin lymphoma in exposed population (De Roos et al. 2003, Eriksson et al. 2008), later studies found no significant linkage between exposure and cancers in agricultural workers (De Roos et al. 2004, Andreotti et al. 2017). Long-term human trials are lacking in the data however, most studies rely on statistical analysis of cancer data in an area and self-report data for herbicide exposure. A small number of studies found no toxic effects of glyphosate and Roundup on humans, though both of these

papers acknowledge funding and data coming from Monsanto, the producer of Roundup (Williams et al. 1999, Williams et al. 2011) suggesting a potential conflict of interest.

The toxicity of the surfactant and the whole Roundup formulation were found to be almost the same to a variety of aquatic vertebrates and invertebrates while glyphosate by itself was significantly less toxic (Folmar et al. 1979). The effects of exposure to the whole formulation depend on a number of factors, such as exposure time and dose concentration.

Glyphosate-based herbicides have wide ranging effects on aquatic organisms. Following chronic, low concentration exposure (1.8 ppm for 15 days) fishes' food intake were drastically reduced and did not return to normal even after cessation of chemical dosing (Giaquinto et al. 2017). Acute exposures at a slightly higher concentrations (2.5-20 mg/L) showed that after 96 hours of exposure doses of 10 mg/L and higher caused oxidative stress in the kidney, liver and brain tissue of goldfish (Lushchak et al. 2009). Exposures above 10 mg/L reduces fertilization rates, permanently damages gene expression of developing fish embryos, and causes neurological damage to adult fish (Webster et al. 2014, Zhang et al. 2017, Roy et al. 2015). For a 96-hour exposure to the full Roundup formulation the LC50 for rainbow trout fingerlings was 8.3 mg/L (Folmar et al. 1979).

**Fish Interactions**

The bluehead chub (*Nocomis leptocephalus*) is a freshwater leuciscid found throughout most of the Southeastern United States. Similar to other *Nocomis*, *N. leptocephalus* builds stone nests in rivers by piling up small rocks to form a mound. These mounds are incorporated into many other riverine minnow's reproductive strategies (Johnston 1994, Pendelton et al. 2012). For example, our target species, *Notropis baileyi* (Rough Shiner) is a nest associate, spawning in

chub nests. The relationship between *N. leptocephalus* and its nest associates is a mutualistic one, increasing all species' reproductive potential by utilizing the dilution effect, i.e., individual survival rate increases in proximity to a greater number of heterospecifics (Johnston 1994). Many nest associate minnows, are unable to spawn without the presence of chub nests and will withhold spawning until they have found a *Nocomis* nest (Johnston 1991, Wallin 1992). Approximately 20% of North American minnows are imperiled. Many of these requiring the mutualist benefits of chub nests to spawn, as such *N. leptocephalus* are considered a keystone species in Southeastern riverine systems. Conservation efforts must incorporate both *Nocomis spp.* and their nest associates (Johnston 1994, Johnston 1999). Some species of nest associates are able to detect an active vs inactive *Nocomis* nest and choose to spawn on the active one; they will synchronize their spawning to occur at the same time as the *Nocomis* (Wallin 1994). It is difficult to tell what precise form this communication takes but it is likely a mix of chemoreception of *Nocomis* pheromones (Hunter and Hasler 1965), visual cues (Miller 1964), and auditory cues from rock shuffling (Steele 1978).

Chemoreception is the pathway most readily interrupted by the introduction of foreign chemicals (Fisher et al. 2006) and is the focus of our study. We selected three representative reproductive pheromones as study targets: 11-ketotestosterone (11KT), prostaglandin-$F_{2\alpha}$(PGF), and 17β-estradiol (E2). They were selected because of their importance in reproduction and the ability to test waterborne levels using commercially available ELISA kits (Kidd et al. 2010). While fish have three different methods of chemoreception (respiratory, gustatory, and olfactory) they perceive pheromones primarily through the olfactory pathway. Many fish taxa exhibit olfactory sensitivity to steroid hormones and prostaglandins. These chemicals serve as 'hormonal pheromones' for their action as internal hormones and external signaling pheromones. Because

most fish are fully aquatic, they have been able to adapt the hormones normally used for internal functions to be expelled and used as pheromones targeted to nearby conspecifics. One of our tested metabolites, PGF, is an excellent example of this: during the post-ovulatory stage, female fish experience a surge of luteinizing hormone (LH) which triggers oviductal oocytes to begin synthesis of PGF which initiates reproductive behavior. As PGF increases in the female's body and begins initiating behavioral changes and reproduction, the body naturally expels some of the hormone through urine. This trace PGF in the water is detected by the male olfactory sensors to trigger their own reproductive behaviors and milt production. Furthermore, female fish nearby who detect the PGF will begin to ovulate (Kitamura et al., 1994). It is suggested that PGF acts to synchronize male and female reproductive behavior and subsequent gamete release. For fishes with external fertilization, such the *N. baiyeli*, synchronizing gamete release increases the likelihood of successful fertilization (Sorenson et al., 2018).

During the pre- and post-ovulatory period of reproduction, female fish are experiencing rapid changes to the types and levels of reproductive hormones within their bodies. The hypothalamic-pituitary-gonadal (HPG) axis is primarily responsible for regulating the levels of luteinizing hormone (LH) during ovulation and the levels of LH in males through chemical and visual cues. Inside the body, LH triggers males to develop milt and initiates reproductive behavior in females (Kobayashi et al., 2002).

11KT is produced by both male and female teleost fish, though in greater quantities in males where it determines both primary and secondary sex characteristics (Grober and Bass 2002). 11KT is a steroid hormone, synthesized from cholesterol and secreted primarily from the gonads and to a lesser extent the adrenal glands. In leuciscids, higher levels of 11KT in males have been linked to brighter and more coverage of nuptial coloration, as well as increased

development rate and size of gonads (Schade and Stallsmith 2011). Additionally, concentrations of 11KT in the external mucus of teleosts have been found to be representative of internal concentrations (Schultz et al. 2005). Because of its biological importance and ability to sample to both male and female fish, 11KT was selected as one of our study pheromones.

E2, another steroid hormone, is also found in both males and female teleosts but is found in much higher quantities in female fish compared to males. E2 plays an important role in all stages of female reproduction, though it is at its peak in the body during the periods of vitellogenesis and fertilization (Ramsey et al. 2011). Though most commonly considered in the context of reproduction, E2 performs a wide variety of functions across body systems. Notably E2 is an important molecule in adult neurogenesis and responds to brain damage; when a lesion was developed on the brain of a fish levels of E2 proliferation increased in the area, and peaked after 7 days (Diotel et al. 2013). Similar results are found across taxa, finding estradiol has a surprising capacity for neurogenesis and brain protection, while also playing a role in cytophagy (McCarthy et al. 2009, and Brown et al. 2009). During reproduction E2 increases until the release of LH, at which point the body ceases production of E2, transferring instead to the production of PGF. As such, E2 expresses a negative correlation with PGF, lower levels of E2 in the water indicate females are detecting the PGF.

The final metabolite we are studying, PGF, is essential to initiating reproduction in fish and acts as a stimulating signal to both male and female teleosts (Sorenson and Goetz, 1993). Leuciscids are particularly sensitive to waterborne PGF (Kitamura et al. 1994). Prostaglandins are not technically hormones in the body, as they are synthesized by the arachidonic acid pathway from lipids, however they behave and signal in much the same way hormones do, and their actions are comparable. After ovulation is completed, internal levels of PGF rise, initiating

reproductive behavior PGF is then released into the water through urine signaling conspecifics to induce reproduction (Sorenson et al. 2018, and Stacey 2015). Male fish detect the waterborne PGF, inducing reproductive behavior, and sperm production (Kobayashi et al. 2002). PGF is an excellent study pheromone as it is received by both males and females and release into the water is a good indicator of reproductive activity.

Simultaneous spawning is essential for the success of oviparous teleosts. These pheromones, particularly PGF, ensure the simultaneous initiation of spawning behavior and are of particular interest to managers. As such, any impacts to production or reception of PGF could have serious impacts on fish reproduction. Because it affects males and females, it is a good to monitor changes in with the addition of Roundup. The effect of Roundup on hormone expression in fish is not well understood; but in other vertebrate species it has been found to cause hormonal disruption (Gill et al. 2018). Our study seeks to explore the impacts of Roundup on both of these key routes in fish reproduction: sensory and hormonal. Based upon the work of previous studies, it is our hypothesis that reproductive hormone (PGF, 11KT, E2) secretion and olfactory chemoreception will be negatively impaired in fish following exposure to Roundup.

# Methods

## Collection and Housing

Our target species, *N. baileyi*, is a common leuciscid native to the Chattahoochee drainage. We collected 120 individuals using a 3 m seine net during the months of May and June, during the spawning season. Fish were transported to the Auburn University Fish Biodiversity lab in coolers containing site water and an airstone. Once in the lab, fish were housed in 20gal tanks maintained at 23˚ C and kept on a 12-hour photoperiod to simulate spawning conditions. Length and weight data were collected following experimentation, all fish had an average total length of 62.97 mm (SD ±6.66) and an average weight of 2.23 g (SD ±0.73).

## Exposure

A 96-hour exposure period was selected to reflect the period of time glyphosate stays in the environment following storm events (Brovini et al. 2021). Fish were then randomly assigned to the control group (0.0mg/L) or the Roundup exposure (3.0 mg/L). Two 100L coolers were prepared for both exposure groups and were prepared with aerators one week prior to exposure. Rough shiners were sorted into the coolers in equal groups (10 per cooler) for their 96-hour treatment period.

## Trials

Following the 96-hour exposure period, fish were exposed to one of two chemical stimuli in a test tank: 0.1 mg/L PGF mixed into 10 mL of distilled water or distilled water (control stimulus). The test tank consisted of a glass aquarium containing 75.7 L of water with 3/16”

gauge plastic tubing running down one side and onto the bottom of the tank. A syringe containing the chemical stimulus was attached to the tubing and delivered pulses of either PGF or DI Water during exposure trials over the course of 1 minute. In both coolers, half of the fish were randomly sorted to receive the PGF stimulus, the remaining fish would receive the DI water stimulus. For every trial, five fish would be added to the test tank and allowed to acclimate for 5 minutes. After acclimation, the appropriate chemical stimulus was injected into the tank. All trials were recorded on a video camera for later analysis.

Water samples were collected from the tank at four timepoints: following the 10-minute acclimation period pre-stimulus, 5 minutes after chemical stimulus, 15 minutes after stimulus and 30 minutes after stimulus. A 400 ml water sample was removed from the test tank to measure waterborne hormone levels. After each collection, a fresh aliquot of 400 ml water was added to replace the volume removed for hormone analysis. Raw water samples underwent coarse filtration to remove large particulates, such as scales and mucus. Following coarse filtration, water samples were filtered through C18 cartridges primed with two 2ml washes of methanol and two 2ml washes of DI water. C18 cartridges were stored at -20°C until extraction and analysis.

**Pheromone Extraction and Analysis**

Hormones were eluted from C18 cartridges with two 2ml washes of ethyl acetate. This solution was then evaporated to dryness under nitrogen gas in a dry bath. The dried residues were reconstituted with 300 µl of the ELISA buffer provided with the kit. 2-fold serial dilutions were performed for each hormone to determine the appropriate dilution factor and validate the

kit for use with our target species. Hormone samples were loaded into Cayman Chemical ELISA plates in accordance with kit instructions. Sensitivity of assays varied based on hormone, E2 plates measured from 20-10,000 pg/mL, 11KT plates detected from 1.3-100 pg/mL, and PGF plates measured from 10-500 pg/mL.

Following two-fold serial dilution, individual dilution factors for each treatment type and hormone were determined. For the control treatment groups: 11KT and E2 were diluted to a ratio of 1:10 in both exposure types, PGF samples needed to be separated into those under the control exposure and those with PGF challenge. Control treatment PGF exposed to control DI Water was diluted to 1:16 while the PGF group exposed to the PGF challenge was diluted to 1:300. Neither PGF nor 11KT had high enough levels in the Roundup treatment group to be detected by the ELISA kits. For E2, under the Roundup treatment group, a dilution factor of 1:4 was determined.

**Statistical Analysis**

Each sample point was assigned four variables: treatment group (DI water or Roundup), test exposure group (DI water as control and PGF as positive control), time point sampled, and hormone type sampled. Thus, each sample point can be categorized as: DW-DW (Control-Control), DW-PGF (Control-Test), RU-DW (Test-Control), or RU-PGF (Test-Test). For those samples too low to detect, the minimum detection value for the ELISA plates was used in analysis. Statistical analysis was completed in RStudio, utilizing a multiple linear regression to determine significance of interactions between each variable. Figures were created in RStudio using the ggplot package. Length and weight data was assembled in Excel where standard deviation was also calculated.

## Results

Across averages, E2 was measured at its highest at time point 2 for the DW-DW group (Figure 1), with little variation across the time points for DW-DW. At all time points the E2 concentrations in the DW-DW group was higher than in the DW-PGF group, notably this trend was reversed in the RU groups (Table 1). 11KT was found in higher concentrations at nearly all time points in the DW-PGF group (165.0-241.7 pg/mL) with the exception of time point 3 (153.0 pg/mL) (Table 2). PGF was found in higher concentrations across all time points in the DW-PGF group (4505.2-36981.5 pg/mL) as compared to the DW-DW group (2314.2-3873.2 pg/mL) (Table 3).

Time point 2 is the sample taken shortly after stimulus had been added to the experimental tank, meaning the levels at this time point and following are of particular interest. For 11KT DW-DW and DW-PGF, there is a slight increase between time point 1 and 2, 32.9 pg/mL for DW-DW and 37.5 pg/mL for DW-PGF (Figure 2). This difference is significant in the PGF hormone analysis, there is 284.1 pg/mL decrease between time points 1 and 2 for the DW-DW group, while there is a 32,476.3 pg/mL increase in the DW-PGF group (Figure 3).

Because the RU treatments for 11KT and PGF were too low to be detected by the ELISA kits only E2 was able to have the complete multiple linear regression run. This statistical test found that the only variable with significant impact on the amount of E2 sampled was treatment with Roundup (Table 4). For the PGF hormone test the exposure to test-PGF was significant as well (Table 4), to a greater degree than could be accounted for by the artificial injection. No variables were found to have significance in the case of 11KT.

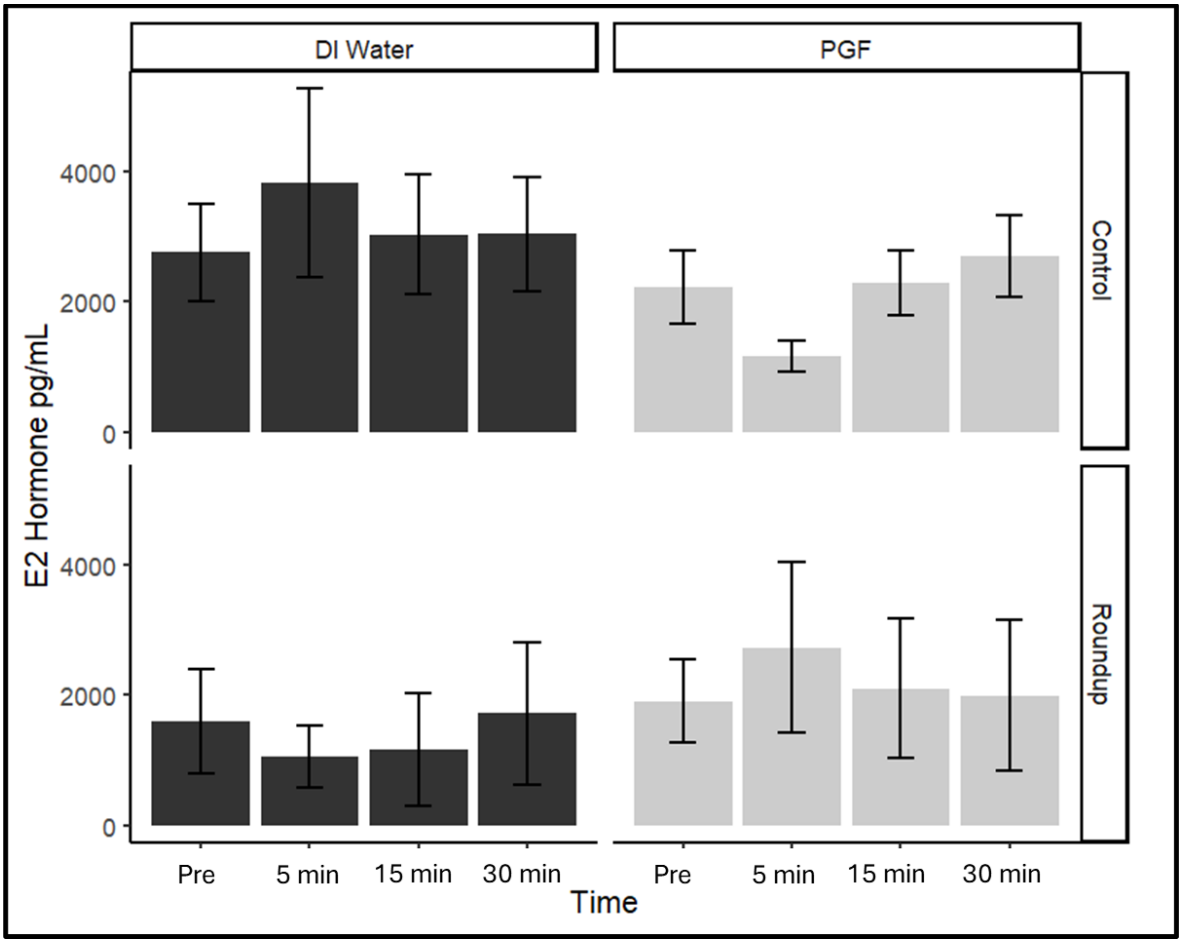


**Figure 1.** Estradiol hormone analysis: four different bar charts representing the average concentration (pg/mL) of waterborne E2 at the corresponding time point across all trails. The top two bar charts represent the DW-DW and DW-PGF trails (left and right respectively) while the bottom two represent RU-DW and RU-PGF respectively. Additional lines represent standard error and are included for each time point.

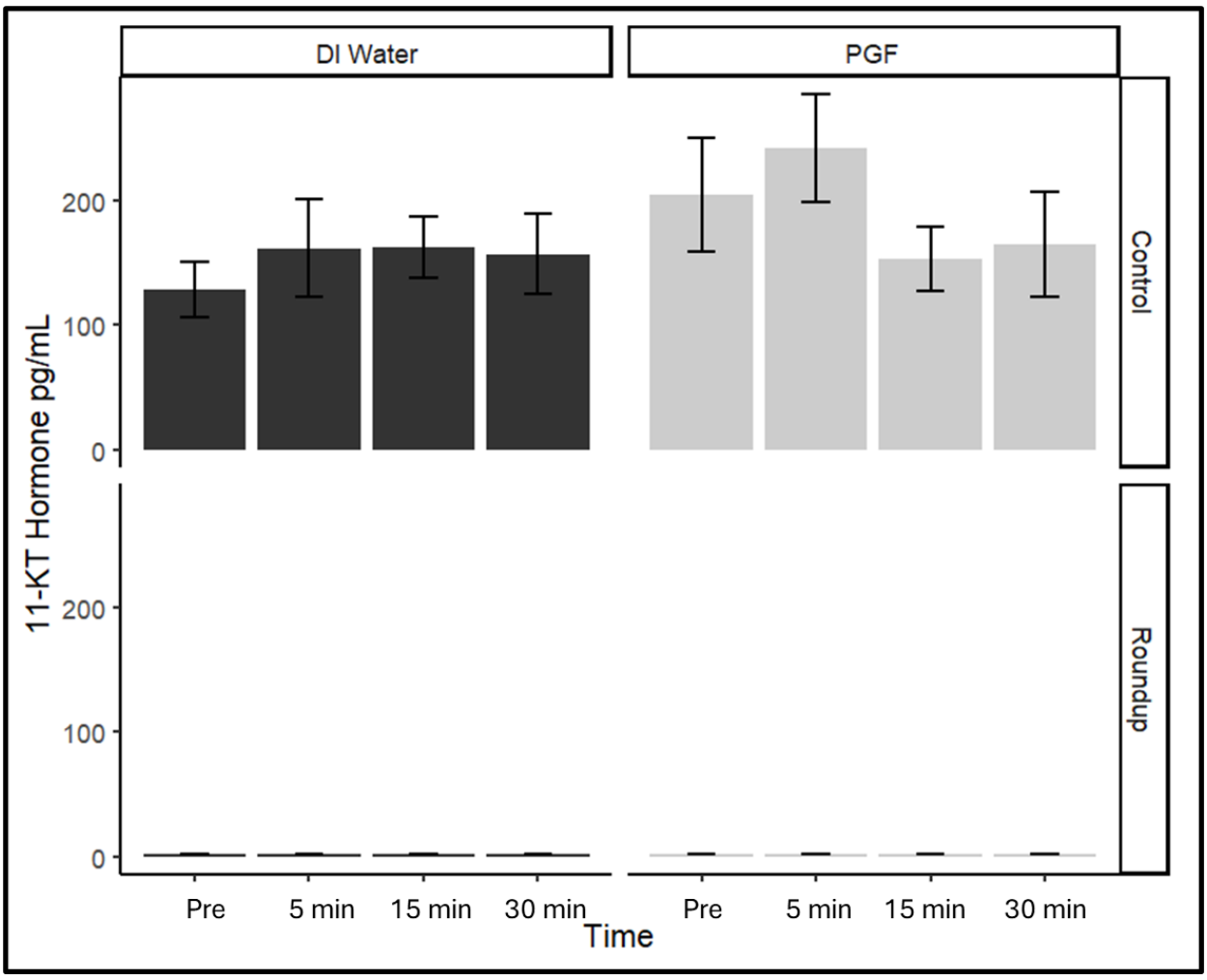


**Figure 2**. 11KT Hormone analysis: Average concentrations (pg/mL) of 11KT for each time point, with those exposed to DI Water displayed on the left in black, and those exposed to PGF displayed on the right in grey. Additional lines represent standard error and are included for each time point.

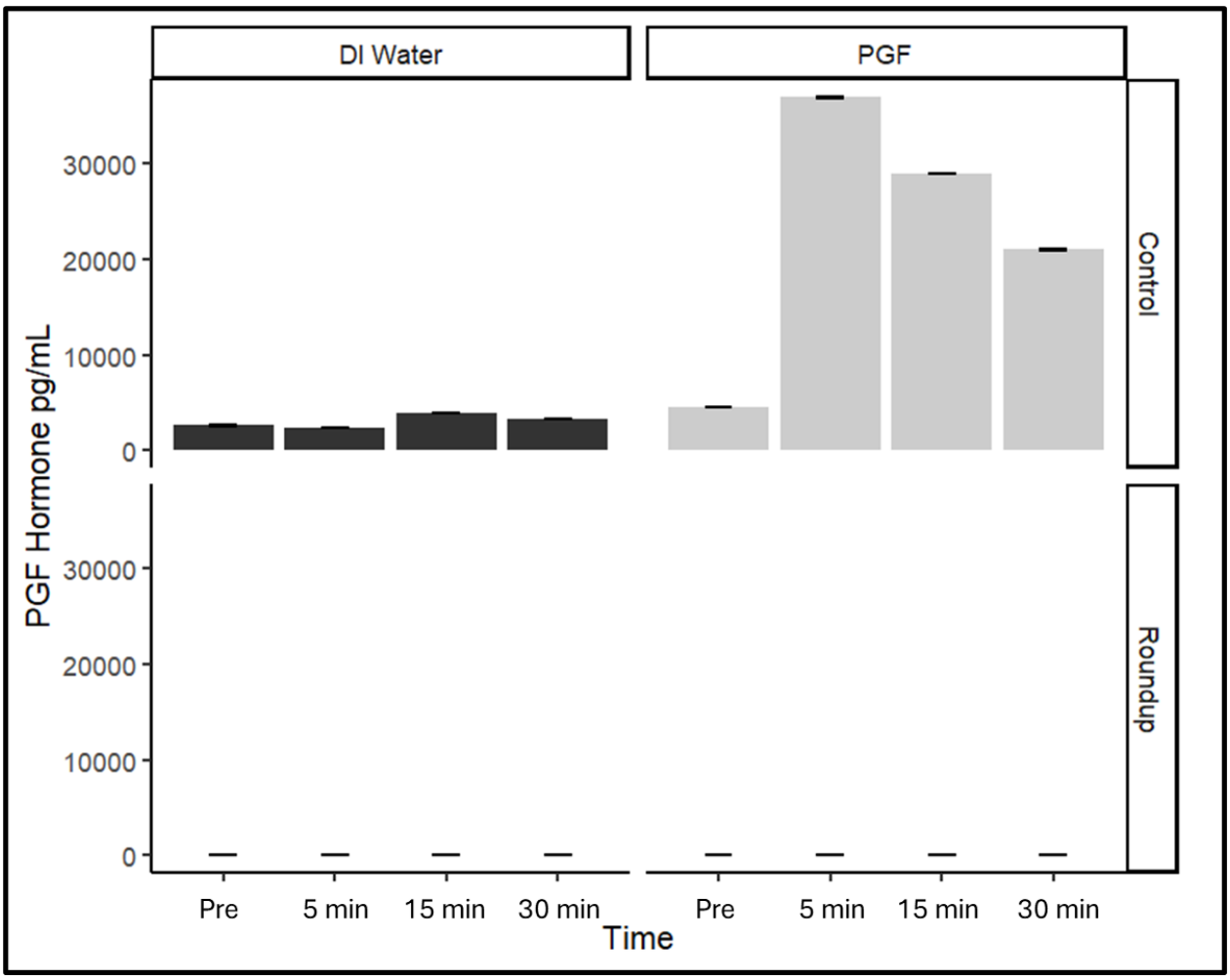


**Figure 3**. PGF Hormone analysis: Average concentrations (pg/mL) of PGF for each time point, with those exposed to DI Water displayed on the left in black, and those exposed to PGF(test) displayed on the right in grey. Additional lines represent standard error and are included for each time point.

| Time Point | 1 | SE | 2 | SE | 3 | SE | 4 | SE |
|---|---|---|---|---|---|---|---|---|
| DW-DW | 2761.5 | ±744.3 | 3831.4 | ±1449.3 | 3038.8 | ±927.4 | 3048.7 | ±876.8 |
| DW-PGF | 2227.2 | ±567.6 | 1170.0 | ±241.3 | 2294.2 | ±505.2 | 2701.1 | ±625.9 |
| RU-DW | 1594.8 | ±792.4 | 1051.6 | ±485.3 | 1161.4 | ±862.8 | 1714.5 | ±1092.5 |
| RU-PGF | 1905.5 | ±640.7 | 2727.9 | ±1316.2 | 2101.9 | ±1082.2 | 1994.4 | ±1161.9 |

**Table 1:** E2 hormone levels: each treatment-exposure group represented along left edge, each column represents the average hormone concentration collected, in pg/mL, at each corresponding time point. SE is the standard error as calculated for each time point's average.

| Time Point | 1 | SE | 2 | SE | 3 | SE | 4 | SE |
|---|---|---|---|---|---|---|---|---|
| DW-DW | 128.5 | ±22.0 | 161.4 | ±39.2 | 161.9 | ±24.5 | 156.9 | ±32.1 |
| DW-PGF | 204.2 | ±45.3 | 241.7 | ±43.3 | 153.0 | ±25.6 | 165.0 | ±41.9 |
| RU-DW | 1.3 | 0 | 1.3 | 0 | 1.3 | 0 | 1.3 | 0 |
| RU-PGF | 1.3 | 0 | 1.3 | 0 | 1.3 | 0 | 1.3 | 0 |

**Table 2:** 11KT levels: each treatment-exposure group represented along left edge, each column represents the average hormone concentration collected, in pg/mL, at each corresponding time point. SE is the standard error as calculated for each time point's average. Cells with hyphens represent points where concentrations were not able to be detected by the ELISA kits.

| Time Point | 1 | SE | 2 | SE | 3 | SE | 4 | SE |
|---|---|---|---|---|---|---|---|---|
| DW-DW | 2598.3 | ±624.9 | 2314.2 | ±370.5 | 3873.2 | ±1322.6 | 3245.8 | ±803.6 |
| DW-PGF | 4505.2 | ±1187.9 | 36981.5 | ±14886.9 | 29009.9 | ±6319.3 | 21003.5 | ±4341.0 |
| RU-DW | 10 | 0 | 10 | 0 | 10 | 0 | 10 | 0 |
| RU-PGF | 10 | 0 | 10 | 0 | 10 | 0 | 10 | 0 |

**Table 3:** PGF levels: each treatment-exposure group represented along left edge, each column represents the average hormone concentration collected, in pg/mL, at each corresponding tcime point. SE is the standard error as calculated for each time point's average. Cells with hyphens represent points where concentrations were not able to be detected by the ELISA kits.

| | | Treatment | Exposure | Time Point |
|---|---|---|---|---|
| **E2** | *p-value* | 0.035* | 0.817 | 0.719 |
| | *adjusted r^2* | 0.0182 | 0.0182 | 0.0182 |
| | *Std error* | ±434.8 | ±435.07 | ±194.48 |
| **11KT** | *p-value* | <2e-16** | 0.0778 | 0.566 |
| | *adjusted r^2* | 0.6996 | 0.6996 | 0.6996 |
| | *Std error* | ±12.63 | ±12.64 | ±5.65 |
| **PGF** | *p-value* | 2.66e-6** | 0.000193** | 0.286 |
| | *adjusted r^2* | 0.291 | 0.291 | 0.291 |
| | *Std error* | ±2542 | ±2544 | ±1137 |

**Table 4:** Statistical results of Multiple Linear Regression. The results for p-value, $r^2$, and the standard error are presented, as were generated from a multiple linear regression. Each row corresponds to one of the hormones sampled as indicated, the column represents the variables measured for significance in relation to manipulating the hormone levels in the sample. Those results marked with a dash (11KT and PGF Treatment column) denote those variables unable to be assessed due to lack of data. Those p-values marked by * indicate significance (*p<.05, **p<.001).

# Discussion

Roundup is applied in the millions of gallons every year to farmland, suburban gardens, and public lands across the United States. While the direct toxic effects of these chemicals are being studied heavily, there are less obvious secondary effects to all taxa that are only now being understood. Urgent work to discover and mitigate these effects needs to be taken. This study reveals one such concern: the reduction of waterborne reproductive hormones following exposure. The blocking of these key reproductive hormones could have major deleterious effects on our nation's fish species, many of which are already imperiled or limited to a small range.

Our results show the significant effect Roundup can have on the release and reception of reproductive hormones. We have shown that a 96-hour exposure to Roundup significantly reduces the amount of reproductive hormones released into the water both passively and in response to chemical stimulus. While the pathway by which Roundup impairs chemoreception and dispersal is still unknown, our results indicate that Roundup causes endocrine disruption in Rough Shiners. Because these compounds function as both hormones and pheromones, they are susceptible to internal and external disruption via impairment of hormone synthesis or detection by sensory organs.

The one pheromone that did not fall below detectable levels following exposure to glyphosate was E2. For this hormone, exposure to Roundup caused a significant decline in E2 excreted by fish, but concentration of excreted E2 did not change significantly over time. While it was hypothesized that E2 would decrease with time in the PGF exposed groups, this was not entirely the case. While E2 decreased by 1057.2 pg/mL following stimulus in the DW-PGF

group, the levels of E2 increased by 1124.2 pg/mL going into time point 3 and stayed level. A possible explanation is that E2 was not breaking down in the water, so even if fish ceased production and release of the hormone, we would not detect that cessation for some time. E2 has variable breakdown time in water, depending on UV exposure and water purity, but its half-life is generally between 0.5-5 hours, with more UV and untreated water causing it to degrade faster (Lin et al., 2005, Liu et al., 2017). While this is a possible explanation, the decrease in E2 levels between time points 1 and 2 for the DW-PGF group and between time points 2 and 3 for the RU-PGF group implies this is not the case. A more likely explanation is that all of the PGF stimulus in the tank was rapidly taken up by the fish and following this initial response fish returned to normal levels. This is supported by the noted decrease in time point 2 just after stimulus injection, and the return to pre-stimulus levels at time point 3. An interesting finding in the E2 hormone analysis is that in the RU-PGF group levels of E2 increased at time point 2 when exposed to stimulus, which is the opposite reaction to what is expected. The cause of this is unknown at this point, but future research investigating this effect would be beneficial.

The inability to measure either 11KT or PGF in the Roundup treated group is important. Potentially the lack of 11KT detected could be attributed to a low number of representative males in the test group, as females release far less 11KT in the water than male fish. The relatively low levels (>500 pg/mL) of 11KT in the waterborne samples as compared to E2 and PGF could support this, particularly as E2 was found in such high quantities (~2000 pg/mL). But, even with skewed sex ratios the control treatment groups both had detectable levels of 11KT, indicating the Roundup treatment is responsible for the lack of data. Because females initiate the PGF cascade, and this initial release of PGF is essential to ensure synchronous spawning, they are the most vulnerable stage of the process. If the females are unable to release

PGF or detect it in the water when released from others then the following cascade of pheromones is disrupted, preventing males from producing milt and initiating reproductive behavior. An interruption in this one stage could have strong, detrimental effects with respect to the timing of gamete release, which is essential to the success of oviparous minnow species, particularly those that breed over *Nocomis* nests.

The lack of detectable PGF in the Roundup treated group implies a breakdown of this cascade occurred. PGF was detectable even in the DW-DW group, indicating fish were releasing it into the water without any external stimulus. Sampling and testing occurred during and just after the *N. baileyi* breeding season, so a natural level of expelled PGF during this time is expected. The lack of this baseline level of PGF in the RU-DW group signifies that exposure to Roundup reduced this natural level of release. In addition, there is a significant difference in PGF levels detected between the DW-DW group and DW-PGF group, this indicates that release of a low dose of lab-synthesized PGF elicits a strong reaction in study groups. The lack of this strong reaction in the RU-PGF group serves to further underscore the impact Roundup treatment has on the reproductive ability of these fish.

*Nocomis*-nest breeding leuciscids may have natural redundancies to protect from the individual hormone suppression crashing their breeding. Because there are so many species gathering around these nests should one species prove more resilient to the effects of Roundup their release of PGF into the water has the potential to trigger reproduction in nearby heterospecifics. Because of how widely represented PGF is across taxa, and the clear and significant reaction to lab synthesized PGF in this study, it is likely that non-species PGF released by other nearby minnows would trigger reproductive behavior in impacted species. However, this contingency relies on at least some leuciscid species living in the same area being

unevenly impacted by Roundup. The relative sensitivity of different leuciscid species to Roundup is unknown at this time.

This work explores a novel avenue of research previously understudied, displaying results that are significant and imply a greater degree of damage from Roundup than previously expected. A key limitation to this research is a causal link between Roundup exposure and disruption of chemoreception. While with the results we can say that Roundup is impacting hormone production; the mechanism by which this occurs is unclear. It may be due to an interruption in the chemosensory organs, blocking the body's production of response hormones, or a more cryptic explanation. Future studies investigating the mechanisms by which Roundup disrupts the endocrine system would give vital information into how reproduction is being impacted. Another avenue to investigate is sex-specific differences. Our research focused on community assemblage, losing the ability to detect sex-specific issues. The difference in effect on males and females may be extreme, as our data showed Roundup treated groups with significantly less 11KT in the water as compared to E2 it may be the case that males are unevenly affected by chemical pollutants.

Understanding the non-target effects of the chemicals we use is essential, particularly when the use of Roundup is so prevalent on a national and global scale. Considering only the direct effects on fields where it is applied gives only a partial understanding. Research being undertaken on the effects of Roundup across non-target taxa is showing very similar results to this, organisms of all types are being impacted and damaged by exposure to environmental Roundup. Because of how long Roundup stays active in the environment for and the nature of its acute exposure following storms, it is essential to understand the full range of its non-target effects.

Another unknown is how climate change will affect how Roundup enters and behaves in the environment. Under a model representative of our current action, surface temperatures are expected to increase by 3.2˚ by 2100, while precipitation is expected to increase in intensity, with a larger number of high-intensity storms across much of the world (IPCC, 2023). The toxicity of Roundup in aquatic ecosystems has been found to increase with temperature, a change in temperature from 7˚ to 15˚ C corresponded with a 10% increase in fish mortality (Drechsel et al., 2024). As high-intensity storms grow more common the rate of runoff from these herbicide-laden lands will proportionally increase as well. The effects climate change has on agricultural erosion and runoff is dependent on what the existing environment in that area is like and how changing precipitation will impact the region. Generally speaking, those areas that currently receive higher than average rainfall will receive even more, while dryer areas will get even less. From the standpoint of erosion this means in wet areas, like the Mississippi delta erosion will increase by 9-12% from precipitation, while in a dry area like Eastern Europe there is a predicted 60% reduction in runoff (Yasarer et al., 2017, Krajewski et al., 2021). Much of the US's agricultural land will likely experience more rainfall as climate change intensifies, so the effects of runoff Roundup will only become more pronounced in the future.

This research is especially topical now as the EPA is currently reviewing the health and environmental safety of glyphosate-based herbicides. While the EPA has already made their position clear in that they believe the economic benefits of glyphosate outweighs the danger posed, a change to the formulation or standards of use could still greatly reduce the potential damage these pesticides can cause (EPA 2022).

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