Differential effects of two NSAIDs on crayfish behavior and physiology: the need for multiple assays to quantify toxicological effects

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Abstract

The evaluation of pharmaceutical compounds using both sublethal and environmentally relevant concentrations is an understudied area within ecotoxicology. Additionally, studies assessing toxicity of these compounds often use a single measure to determine impact. There exists potential for a wide variety of complex and unpredictable effects in non-target organisms due to both the design of pharmaceutical compounds and the conservation of target receptors across different phyla. This study assessed the behavioral and physiological impacts of two common non-steroidal anti-inflammatory (NSAID) medications on crayfish using environmentally relevant concentrations (0.30, 1.5, 3, 30 µg/L). Male and female crayfish were exposed by submerging them in an aqueous solution of either ibuprofen or naproxen for a 24-hr period. Following exposure to either ibuprofen or naproxen sodium, there were no significant changes to crayfish consumptive behavior, however, physiology was significantly different. Oxygen consumption by exposed female crayfish was greater and occurred faster than non-exposed control female crayfish. Impacts of sublethal exposure from pharmaceuticals may lead to reduced fitness and alter community interactions or functions. The results of this study lead us to propose that further ecotoxicological studies utilize multiple measurements to investigate the effects of chemical exposure.

Introduction

When considering how to assign priority of assessing chemicals for ecological risk, pharmaceuticals have been cited by several as “contaminants of emerging concern” in surface and drinking waters (Arnold et al., 2014; Halling-Sørensen et al., 1998; Nikolaou et al., 2007; Valavanidis et al., 2014;). This term indicates the increased awareness by the general public that a contaminant exists in the environment and is linked to human use (Sauvé & Desrosiers 2014). Pharmaceuticals are designated as an emerging contaminant of concern as production and consumption of this class of anthropogenic chemicals has increased over the past several decades, resulting in increased detection in the environment (Heberer 2002; Hughes et al., 2013; Metcalfe et al., 2003; Monteiro & Boxall 2010; Ternes 1998; Wiegel et al., 2004; Zuccato et al., 2000). In combination with long half-lives and low biodegradability (Redshaw et al., 2008), pharmaceutical compounds can persist in the environment as a result of high volumes of production and consumption, indicating the potential to be released continuously into the environment (Monteiro & Boxall 2010). This continuous low-level environmental contamination is significant since some medications are designed to be potent in low doses (Arnold et al., 2013) and others need to be administered routinely to be effective. When discharged into the environment, pharmaceuticals remain metabolically active compounds that can act on organisms they were not originally targeted to treat.

Since pharmaceuticals are designed to treat diseases or ailments, these drugs alter the physiological processes in humans or animals such that the disease is impacted, but the organism is essentially left unharmed (Boxall et al., 2012; Meredith-Williams et al., 2012). This class of anthropogenic chemicals encompasses a wide range of compounds and modes of action (i.e., anti-inflammatories, hormones, beta blockers, etc.) that all have different targets and therapeutic uses, indicating the potential for a wide variety of unintended effects on non-target organisms. Research has shown that, even at sublethal

concentrations, organisms can exhibit physiological and behavioral responses following pharmaceutical exposure (Berninger et al., 2011; Fong & Ford 2014; Handy 1994; Meredith-Williams et al., 2012; Neal & Moore 2017; Quinn et al., 2008). A possible explanation for effects in non-target organisms is the retention of target receptors across taxa. For instance, carbamazepine is a pharmaceutical that acts on well conserved systems across taxa. The typical use for this compound in humans is as an anticonvulsant medication for people suffering from conditions such as epilepsy. Carbamazepine acts as a sodium channel blocker that binds to voltage-gated sodium channels and prohibits firing of action potentials across neurons (Ambrósio et al., 2002). Decreased activity in the amphipod crustacean, *Gammarus pulex*, was observed following exposure to low concentrations of carbamazepine (DeLange et al., 2006). Additionally, Almeida et al., 2014) found that exposure to low levels ($< 10 \mu\text{g/L}$) of carbamazepine induced oxidative stress in two clam species. Carbamazepine is just one of many drugs that have a suite of unpredictable effects on exposed organisms. However, assessment of this class of contaminants is a variable process and there is little overlap when comparing impacted metrics between studies. This conservation of some receptors and effects across phyla coupled with the variable nature of an organism's physiology creates problems in assessing the entire toxicological effect of a pharmaceutical.

Within the field of toxicology, the most commonly used methods for assessing the effects of contaminants involves testing at what concentration that contaminant impacts fifty percent of a population of test organisms (LC_{50} and EC_{50} models). The data from these studies can then be used to create values such as predicted no-effect concentrations (PNECs), which are used frequently in ecological risk assessments to determine the potential risk of the contaminant in question (Liu et al., 2020; US EPA 1998). In $\text{LC}_{50}/\text{EC}_{50}$ testing, there is often times only one metric of impairment evaluated, which is death of the test species. By only evaluating one metric, there is potential to miss equally, but longer term, deleterious effects of exposure to pharmaceuticals. No observed effect concentrations (NOEC) and lowest observed effect concentrations (LOEC) have a similar problem in that they indicate at what concentration effects occur, but do not account for any variability or uncertainty in organismal responses. While a lethal dosage is indicative of harm to an organism, $\text{LC}_{50}/\text{EC}_{50}$ assessment does not have the predictive power to explain effects from exposure below the toxic threshold. Behavioral and physiological changes that result from exposure to sublethal concentrations of contaminants can also contribute to declines in fitness and eventually lead to mortality.

Recently (within the last 20 years), more work has been done to explore non-lethal metrics of impairment, but much of the existing literature in toxicology is centered on establishing thresholds at which toxic effects exist (e.g., LC_{50} , EC_{50} , NOEC, and LOEC) for use in monitoring habitat health or quality. These types of assessments are indeed expedient in many ways, including time and cost considerations when running assays with large numbers of organisms. However, many studies that establish concentrations for risk quotient calculations and discharge limits are using test concentrations that are magnitudes higher than what is actually being detected in the environment (Chapman 2002; Liu et al., 2020). This type of testing misses more subtle changes occurring at environmentally relevant levels which may never

reach proposed effect concentrations. Changes to behavior and physiology have the potential to disrupt community and ecosystem functions, especially if exposed organisms are keystone species or ecosystem engineers.

Given the limitations of current toxicity testing in addition to the lack of literature surrounding the effects of pharmaceuticals at sublethal concentrations, the purpose of the current study was to investigate the possible behavioral and physiological changes following acute exposure to two commonly used NSAID compounds, ibuprofen and naproxen sodium. Following a 24-hour exposure to either naproxen sodium or ibuprofen, we predicted that both behavior and physiology would be significantly altered. With respect to behavior, we hypothesized that food consumption would be negatively impacted, with organisms consuming less food as NSAID concentration increased. Similarly, our hypothesis for oxygen consumption was that as NSAID concentration increased, oxygen consumption would decrease as well.

Methods

Animals

Male and female crayfish, *Faxonius rusticus*, were collected from the Portage River in Bowling Green, Ohio (41.361753, -83.500772) during the fall of 2020. All appendages, including chemosensory appendages were intact for all crayfish. Crayfish were housed in individual bins (25.2 x 16.2 x 11.8 cm) in a recirculating system in which water was delivered continuously to animals. Within this recirculating system, water temperature was kept at ambient conditions, approximately 24°C, animals were maintained on 14h:10h light-dark cycle and fed a diet of guinea pig pellets (Small World Manna Pro™) three times a week.

Experimental Design

A single crayfish was exposed to one of nine treatments of NSAIDs for 24 hours in a 37.9 L (38.5 x 20.5 x 26.2 cm; L:W:H) glass aquarium tank. A treatment consisting of non-exposed crayfish served as a control group. NSAID treatments consisted of the following nominal concentrations:

Toxicant	X	5X	10X	100X
Ibuprofen	8	8	8	8
Naproxen	8	8	8	8

Following exposure, each crayfish was run through two assays before being sacrificed. The first assay was an oxygen consumption trial to determine potential physiological impacts post-exposure. The second assay was a food consumption trial to assess potential behavioral changes following NSAID exposure. All exposed crayfish were euthanized via freezing following completion of both assays.

Chemicals and Stock Solution Preparation

NSAID compounds used for exposure trials were generic formulations of ibuprofen and naproxen, both manufactured by Kroger® and were purchased locally. Prior to use in exposures, stock solutions of each compound were prepared by finely crushing a single tablet of ibuprofen or naproxen using a mortar and pestle. The crushed tablet was then added to 1.0 L of water. The solution was then mixed thoroughly using a magnetic stir bar and stir plate for a minimum of 120 seconds. For exposures, stock solutions were delivered to tanks via micropipette of the appropriate concentration. Dilution factors from the concentration of the stock solution to the final exposure tank concentrations ranged between 4 to 6 orders of magnitude less than the stock solution, depending on treatment. Tank solutions were then mixed using a glass stir rod, which was rinsed with tap water between tanks.

NSAID Exposures

In each exposure trial, a single crayfish was placed into a 37.9 L (38.5 x 20.5 x 26.2 cm; L:W:H) glass aquarium tank filled with aged tap water and dosed with a sublethal concentration of either ibuprofen or naproxen for a total of 24 hours. Crayfish were free to move about the tank throughout the duration of the trial. Dosages were randomly assigned to tanks prior to the addition of a crayfish in order to prevent any bias or confounding factors with treatments. Concentrations used in exposure trials consisted of the following:

Toxicant	X (µg/L)	5X (µg/L)	10X (µg/L)	100X (µg/L)
Ibuprofen	0.30	1.5	3.0	30
Naproxen	0.30	1.5	3.0	30

Oxygen Consumption Assay

Following exposure to one of the above treatments, a single crayfish was placed in a Ball glass mason jar (1.8 L) filled with aged tap water. The lid of the mason jar was modified by drilling a hole (1 cm in diameter) through the center and then fitted with a rubber O ring. This ensured an airtight seal around the oxygen probe for the duration of the assay. Once a crayfish was inside the chamber, a NeuLog oxygen sensor probe (NUL-205) was inserted into the chamber to measure the amount of oxygen consumed. All oxygen consumption trials were run for 600 s at a sampling rate of 10 Hz, using the NeuLog software (version 7.113.125).

Food Consumption Assay

Following the oxygen consumption assay, each crayfish also underwent a food consumption assay as well. A single crayfish was placed into a 37.9 L (38.5 cm x 20.5 cm x 26.2 cm L:W:H) glass aquarium tank filled with aged tap water. A 1.88 cm³ plastic cap filled with sardine gelatin was placed in the arena and crayfish were able to feed from the cap for a total of 4 hours. After the trial ended, the cap was reweighed to measure the amount of food consumed.

$$MassConsumed = Mass_{final} - Mass_{Initial}$$

Sardine gelatin was prepared by combining 13 g of sardines, 7 g of Knox unflavored gelatin, and 175 ml of boiling water in a blender. The mixture was then poured into the plastic caps and covered with plastic wrap before refrigerating for a minimum of 12 hours at 2° C.

Data Analysis

Oxygen Consumption

From the oxygen consumption assay, the data collected from the probe was exported into Microsoft Excel. From there, raw curves (saturation vs. time) were imported into OriginPro ver 9.8 (OriginLab). Within Origin, saturation vs. time graphs were constructed, and an exponential decay was fit to each curve using the nonlinear curve fitting module. All curves were successfully fit using an exponential decay ($p < 0.001$) with r^2 ranging from 0.64 to 0.87.

$$y = A1 * \exp_{t1}^{-x} + y_0$$

For each curve three coefficients (Y_0 , $A1$, $t1$) were extracted. These coefficients are the offset (Y_0), the time constant ($t1$), and the amplitude ($A1$).

Statistical analysis of coefficient data took place in RStudio (R Core Team, 2021). First, histograms, qqplots, and normality tests for each extracted coefficient were performed. All statistical models were performed using generalized linear mixed models (Zuur et al., 2009). All models run in R used the lmer function from the lme4 package in R (Kuznetsova et al., 2017, R Core Team 2021). Outputs from the models were extracted using the anova function from the car package in R (Fox et al., 2013). For the models, the coefficients served as the dependent variable and the concentrations of the ibuprofen and naproxen treatments served as the independent measure. Additionally, the tank number of each trial was used as a random factor within each model.

Food Consumption Assay

For each food consumption assay, the mass of sardine gelatin eaten was calculated from the final and starting masses of the plastic cap containing the gelatin. Statistical analysis of sardine gelatin data took place within RStudio (R Core Team, 2021). Data conditioning procedures followed those typically done in mixed model analysis (Zuur et al., 2009). First, histograms, qqplots, and normality tests were performed on the mass of sardine gelatin consumed. Food consumption data was not normally distributed (Shapiro-Wilks $p = 0.043$). The data was then transformed using a Yeo-Johnson transformation, which created a normal data set. All subsequent statistical tests were performed on the transformed food consumption data.

All statistical models were performed using generalized linear mixed models (Zuur et al., 2009). All models run in R used the lmer function from the lme4 package in R (Kuznetsova et al., 2017, R Core Team

2021). Following model construction, the outputs were extracted using the anova function from the car package in R (Fox et al., 2013). For the models, the mass of sardine gelatin consumed served as the dependent variable and the concentrations of the ibuprofen and naproxen treatments served as the independent measure. Tank number was used as a random factor within each model.

Results

Oxygen Consumption

Coefficients

Mixed model analysis of the oxygen consumption data indicated significant differences for all three coefficients extracted from nonlinear curves. Analysis of the A1 and t1 coefficients indicated significant interaction effects between treatment type and crayfish sex ($F_{8,51} = 2.5755$, $p = 0.02$; $F_{8,51} = 6.7389$, $p < 0.0001$ respectively; Tables 1, 2). A Tukey post-hoc analysis revealed which test groups were impacted (Tables 3, 4). Models indicate a one-way effect on Y0 (initial amount of oxygen in water at start of trial) due to drug treatment ($F_{8,51} = 2.1602$; Fig. 1).

Table 1
Output from the mixed model run for the A1 coefficient. Statistical significance ($p < 0.05$) is indicated by an asterisk (*).

A1 Model Output			
Factor	Degrees of Freedom	F value	p value
Treatment	8	1.1905	0.32325
Sex	1	2.6547	0.10940
Treatment * Sex	8	2.5755	0.01923 *

Table 2
Output from the mixed model run for the t1 coefficient. Statistical significance ($p < 0.05$) is indicated by an asterisk (*).

t1 Model Output			
Factor	Degrees of Freedom	F value	p value
Treatment	8	0.8004	0.60497
Sex	1	4.3415	0.04222 *
Treatment * Sex	8	6.7389	05.383×10^{-6} ***

The A1 coefficient corresponds to the amplitude of the nonlinear curve and can be equated to how much oxygen was being consumed at a given sampling point. For this coefficient, female exposed crayfish had higher values than that of female crayfish in the control group ($p < 0.05$), indicating greater consumption of oxygen. This was true for females within the ibuprofen 0.30 and 30 $\mu\text{g/L}$ treatments, as well as females in the naproxen 0.30 and 3.0 $\mu\text{g/L}$ treatments. Amplitudes between female and male crayfish were also significantly different, with male control animals having higher amplitudes than females ($p < 0.05$; Table 3).

Table 3
The Tukey post-hoc comparisons for the oxygen coefficient A1, also known as the amplitude. The column magnitude lists the difference between the values of the comparison listed in the leftmost column. p-value column lists the p value resulting from the Tukey test.

A1 Tukey Results		
Comparison	Magnitude	p value
Control Female – IBU0 Female	-48.16	0.0436
Control Female – IBU3 Female	-55.48	0.0086
Control Female – NPX0 Female	-56.97	0.0079
Control Female – NPX2 Female	-60.77	0.0112
Control Female – Control Male	-46.96	0.0439

For the t1 coefficient, which corresponds to the time rate at which oxygen was consumed, female exposed crayfish had higher coefficient values than females in the control group ($p < 0.0001$). These differences were seen in all concentrations of both ibuprofen and naproxen treatments. Similar to the A1

coefficient, male crayfish in the control group had significantly higher t1 values than females in the control group ($p < 0.0001$; Table 4).

Table 4

The Tukey post-hoc comparisons for the oxygen coefficient t1, also known as the time rate. The column magnitude lists the difference between the values of the comparison listed in the leftmost column. p-value column lists the p value resulting from the Tukey test.

t1 Tukey Results		
Comparison	Magnitude	p value
Control Female – IBU0 Female	-802.2	< 0.0001
Control Female – IBU1 Female	-805.7	< 0.0001
Control Female – IBU2 Female	-715.0	< 0.0001
Control Female – IBU3 Female	-835.5	< 0.0001
Control Female – NPX0 Female	-844.9	< 0.0001
Control Female – NPX1 Female	-851.2	< 0.0001
Control Female – NPX2 Female	-814.5	< 0.0001
Control Female – NPX3 Female	-831.1	< 0.0001
Control Female – Control Male	-852.0	< 0.0001

Coefficients of Variation

In addition to analyzing the coefficients, a coefficient of variation (COV) was calculated

for A1, t1, and Y0 using the standard error of the mean (SEM). For each coefficient, all COV's showed significant differences based on the interaction between treatment type and crayfish sex ($p < 0.05$; Tables 4, 5, 6).

After performing a Tukey post-hoc analysis, we see consistency between the results of the pairwise comparisons for the A1 coefficient and the COV generated for A1. Similar to the A1 coefficient, exposed female crayfish had significantly higher variations in amplitude than the non-exposed female crayfish in the control group ($p < 0.0001$; Table 5). This is true for females across all treatment groups compared to the control females. Additionally, males in the control group had higher variations in amplitude than females in the control group ($p < 0.0001$, Table 5).

Table 5

The Tukey post-hoc comparisons for the coefficient of variance (COV) for the A1 coefficient. The column magnitude lists the difference between the values of the comparison listed in the leftmost column. p-value column lists the p value resulting from the Tukey test.

A1 COV Tukey Results		
Comparison	Magnitude	p value
Control Female – IBU0 Female	-0.285	< 0.0001
Control Female – IBU1 Female	-0.304	< 0.0001
Control Female – IBU2 Female	-0.272	< 0.0001
Control Female – IBU3 Female	-0.295	< 0.0001
Control Female – NPX0 Female	-0.296	< 0.0001
Control Female – NPX1 Female	-0.294	< 0.0001
Control Female – NPX2 Female	-0.300	< 0.0001
Control Female – NPX3 Female	-0.306	< 0.0001
Control Female – Control Male	-0.298	< 0.0001

Following post-hoc analysis, there were significant differences for the COV generated for the t1 coefficient. Exposed females had more variation of the time rate than did female crayfish in the control group ($p < 0.0001$; Table 6). As with the COV for A1, the t1 COV was higher for females across all treatment groups than the COV for control group females. Finally, we see again that male crayfish in the control had higher COV values for the t1 coefficient than control group females.

Table 6

The Tukey post-hoc comparisons for the coefficient of variance (COV) for the t1 coefficient. The column magnitude lists the difference between the values of the comparison listed in the leftmost column. p-value column lists the p value resulting from the Tukey test.

t1 COV Tukey Results		
Comparison	Magnitude	p value
Control Female – IBU0 Female	-0.253	< 0.0001
Control Female – IBU1 Female	-0.265	< 0.0001
Control Female – IBU2 Female	-0.238	< 0.0001
Control Female – IBU3 Female	-0.254	< 0.0001
Control Female – NPX0 Female	-0.256	< 0.0001
Control Female – NPX1 Female	-0.253	< 0.0001
Control Female – NPX2 Female	-0.259	< 0.0001
Control Female – NPX3 Female	-0.276	< 0.0001
Control Female – Control Male	-0.263	< 0.0001

Post-hoc analysis revealed significant differences for the COV related to the Y0 coefficient. Female crayfish in the control group had higher variation among offset values than female crayfish in any exposure treatment ($p < 0.0001$; Table 6). Females in the control group also had higher variation in offset values than males in the control group ($p < 0.0001$).

Table 6

The Tukey post-hoc comparisons for the coefficient of variance (COV) for the Y0 coefficient. The column magnitude lists the difference between the values of the comparison listed in the leftmost column. p-value column lists the p value resulting from the Tukey test.

Y0 COV Tukey Results		
Comparison	Magnitude	p value
Control Female – IBU0 Female	5.38×10^{-2}	< 0.0001
Control Female – IBU1 Female	5.44×10^{-2}	< 0.0001
Control Female – IBU2 Female	4.54×10^{-2}	< 0.0001
Control Female – IBU3 Female	5.41×10^{-2}	< 0.0001
Control Female – NPX0 Female	5.36×10^{-2}	< 0.0001
Control Female – NPX1 Female	5.38×10^{-2}	< 0.0001
Control Female – NPX2 Female	5.42×10^{-2}	< 0.0001
Control Female – NPX3 Female	5.37×10^{-2}	< 0.0001
Control Female – Control Male	5.36×10^{-2}	< 0.0001

Food Consumption

Crayfish consumption was not significantly affected by either drug type or concentration ($F_{8,63} = 1.3414$ $p > 0.1$; Fig. 1). Analysis also revealed no significant differences in consumption as a result of an interaction between treatment type and crayfish sex ($(F_{8,54} = 1.5760$ $p > 0.1)$). Control crayfish that were not exposed to either ibuprofen or naproxen consumed an average of 1.53 ± 0.22 g of fish gelatin. Consumption within ibuprofen exposures ranged from 0.84 ± 0.39 g up to 1.78 ± 0.21 g (3.0 $\mu\text{g/L}$ and 30 $\mu\text{g/L}$ treatments, respectively). For the naproxen exposures, crayfish consumed an average of 1.00 ± 0.23 g up to 1.96 ± 0.30 g of fish gelatin (30 $\mu\text{g/L}$ and 3.0 $\mu\text{g/L}$ treatments, respectively).

Discussion

Despite the lack of behavioral changes following exposure to NSAID pharmaceuticals, crayfish had significant physiological impacts. Female crayfish exposed to ibuprofen and naproxen had higher amplitude and time constant values for their oxygen consumption models than non-exposed females indicating an altered physiology. Amplitude in this context corresponds to the amount of oxygen being

consumed at any given point whereas the time constant corresponds to the rate at which oxygen was consumed. In the lowest (0.30 µg/L) and highest (30 µg/L) concentrations of both ibuprofen and naproxen treatments, females consumed more oxygen and at a faster rate than the control females. These results indicate that assessing the biological impacts of some toxicants should be done at multiple levels of biological organization because at one level (behavioral), organisms may not show effects that are present at a secondary level (physiology).

This study utilized measurements at two different biological organizational levels to assess impairment from pharmaceutical exposure. By including both behavioral and physiological assays, there is an increase in the potential to detect more subtle effects of exposure at sublethal concentrations, especially in non-target organisms. A singular assay, particularly at higher levels of biological organization, may not be sensitive enough to determine the toxicity of a contaminant, especially when delivered in concentrations that are below established NOEC or LOEC standards. While behavioral assays may be easier to perform, as well as EC₅₀ or LC₅₀ assays, smaller physiological effects could remain undetected, producing effect concentrations that may be incorrect. Additionally, because animal behavior can change as a result of physiological effects, including a physiological measurement of impairment may aid in predicting what types of behavioral impairments can follow contaminant exposure.

While changes in oxygen use and consumption may seem like subtle toxicological effects, these small changes can add up to larger population and ecosystem effects. Chronic exposure to contaminants that change oxygen usage or food consumption could eventually disrupt a crayfish's ability to maintain homeostasis as well as perform more energy intensive behaviors important to their ecology, such as burrow construction and aggression. Crayfish that consume more oxygen to create shelters and defend themselves are more likely to exhaust energy supplies. If so, other physiological processes related to crayfish reproduction can also be negatively impacted by changes to oxygen use and food consumption. Exoskeleton molting and regeneration are both tasks that require a significant amount of energy and are crucial for growth and mating. If animals are hindered in their oxygen consumption, these critical functions may become lethal. By modulating oxygen use, crayfish may not be able to successfully perform a suite of behaviors essential for sustaining their populations. Shifting the population dynamics of crayfish has implications to aquatic communities. In freshwater habitats, crayfish occupy a substantial ecological niche, as they can alter macrophyte abundance (Chambers et al., 1990), aid in nutrient cycling through processing of detritus (Parkyn et al., 2001; Usio & Townsend 2004, Creed & Reed 2004), and redistribute sediment along stream banks (Creed & Reed 2004). While we are unclear of the toxic mechanism by which NSAIDs act to change oxygen use in crayfish, these impacts add to the growing body of literature concerning effects of pharmaceuticals on non-target organisms.

As a broad class of chemicals, pharmaceuticals have an even broader range of physiological effects than just oxygen consumption (Arnold et al., 2014). Given that there is still growing evidence of unintended consequences on target organisms (primarily humans), there should be unexpected and unpredictable effects on non-target organisms (Arnold et al., 2014; Boxall et al., 2012). One example of unpredictable effects on non-target organisms related to pharmaceutical exposure is the major decline of the *Gyps*

vulture populations on the Indian subcontinent (Swan et al., 2006). Veterinary use of diclofenac (another common NSAID compound) in livestock led to vultures ingesting diclofenac through feeding on the carcasses of treated animals. Even relatively small doses of the anti-inflammatory drug ($LC_{50} = 0.1-0.2$ mg/kg) were found to be toxic and caused kidney failure in the birds (Swan et al., 2006). Another commonly cited occurrence of effects on non-target organisms is the feminization of male fish and production of viable eggs following estradiol exposure, which has been linked to residue from oral contraceptives in freshwater (Kidd et al., 2007). At the invertebrate level, there is a wide variety of effects, physiological and behavioral, that can result from exposure to selective serotonin reuptake inhibitors (SSRIs), the most commonly prescribed class of antidepressant medications (Fong & Ford 2014). Both freshwater and marine species of gastropods exhibited foot detachment from substrates (Fong & Hoy 2012; Fong & Molnar 2013) whereas amphipod crustaceans altered their light preferences and activity levels (Guler & Ford 2010). In the present study, we found little effect at the behavioral level, but varied changes at the physiological level that targeted oxygen consumption and use.

Due to the variety of effects that can occur as a result of exposure to pharmaceuticals, risk assessment for this class of contaminants should include more than a single assay to assess impairment and to determine acceptable levels for discharge into the environment. Previous work has shown that environmentally relevant concentrations of naproxen dosed in flowing water are sufficient to induce changes in crayfish behavior (Neal & Moore 2017), however, similar concentrations used in this study produced no significant changes in behavior. This conflicting evidence suggests that one assay may not be sensitive enough to encapsulate the full scope of deleterious effects that this pharmaceutical can have on a non-target organism. Additionally, this evidence further supports that the mode of toxicant delivery plays a crucial role in shaping chemical exposure. As mentioned above, changes to behavior can arise from seemingly subtle changes to physiology (Kravitz 1988). For instance, modulating serotonin levels in decapod crustaceans is known to affect aggressive behavior (Kravitz 2000). One could hypothesize that exposure to pharmaceuticals that regulate serotonin release (SSRIs) could consequently impact aggressive behavior. A study that utilizes both a behavioral component that directly measures aggression, as well as a physiological measurement, such as tissue sampling for serotonin, would provide more information into this phenomenon than a behavioral assay by itself. By adding on the tissue sampling, one may be able to determine more exact concentrations at which aggressive behavior is significantly altered instead of approximations used for exposure treatments.

Moving forward, toxicity testing and environmental risk assessment of pharmaceuticals should seek to incorporate several levels of biological organization in their analysis as well as more realistic pulsed exposure patterns. Our results indicate that while an organism's behavior may not significantly be altered by low levels of pharmaceuticals, acute exposure can still produce unexpected changes to physiology. By including more sensitive assays, we can more thoroughly elucidate the effects of more complex toxicants, such as pharmaceuticals and their mixtures, on non-target organisms. The standards used in toxicology today (LC_{50} , EC_{50} , NOEC, LOEC, etc.) are useful but limited in application, as the determining criteria for harm is mortality among test organisms. Additional standards that describe effects of

toxicants in sublethal concentrations are one of several missing links in a comprehensive picture of chemical exposure.

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Author Contributions:

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Alexandra Neal and Paul Moore. The first draft of the manuscript was written by Alexandra Neal and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data Availability:

Data will be made public and available through the University of Michigan Biological Station data hub, [mfield](#) upon publication of this manuscript.

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Figures

Figure 1

Averages of Y0 (offset) in increasing concentrations of ibuprofen (IBU; black) and naproxen (NPX; hatched lines) treatments. Error bars indicates standard error of the mean (SEM)

Figure 2

Averages of A1 coefficient (amplitude) in increasing concentrations of ibuprofen (IBU; black) and naproxen (NPX; hatched lines) treatments. Error bars indicates standard error of the mean (SEM)

Figure 3

Averages of t_1 coefficient (time constant) in increasing concentrations of ibuprofen (IBU; black) and naproxen (NPX; hatched lines) treatments. Error bars indicates standard error of the mean (SEM)

Figure 4

Averages of COV for the A1 coefficient in increasing concentrations of ibuprofen (IBU; squares) and naproxen (NPX; triangles) treatments. Error bars indicates standard error of the mean (SEM)

Figure 5

Averages of COV for the t1 coefficient in increasing concentrations of ibuprofen (IBU; squares) and naproxen (NPX; triangles) treatments. Error bars indicates standard error of the mean (SEM)

Figure 6

Averages of COV for the Y0 coefficient in increasing concentrations of ibuprofen (IBU; squares) and naproxen (NPX; triangles) treatments. Error bars indicates standard error of the mean (SEM)

Figure 7

Comparing the average masses of fish gelatin consumed (grams) by crayfish in increasing concentrations of ibuprofen (IBU; black) and naproxen (NPX; hatched lines) treatments. Error bars indicates standard error of the mean (SEM)