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Effects of Petroleum Distillates on Amphibian Development

Kate Walker
University of Arkansas, Fayetteville

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Kate Niermeier Ireton Walker
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Dr. J.D. Willson
Thesis Co-Director

Dr. Kimberly G. Smith
Thesis Co-Director

Dr. Michelle Evans-White
Committee Member

Abstract

Petroleum distillates are widely used as an energy source and the extraction and disposal of these chemicals are done with little consideration of their effects on aquatic environments. Amphibians are considered excellent ecological indicators but little research has examined effects of petroleum distillates on aquatic species. I evaluated the lethal and sublethal effects on larval amphibians with exposure to petroleum distillates associated with various venues of pollution including hydraulic fracturing. I selected three petroleum distillates (kerosene, oil, and unleaded gasoline) that are known to have negative effects on aquatic organisms and are similar to the common constituents of mixtures used in hydraulic fracturing fluid. I examined effects of acute exposure to the water-soluble fraction of each of three distillates at four concentrations in four species: *Anaxyrus americanus, Lithobates sphenocephalus, Hyla chrysoscelis,* and *Ambystoma maculatum*. Specifically, I evaluated survival, level of narcosis, and swim time in response to a stimulus over a 72-hour period. I identified a significant distillate by time effect on response to stimulus and a distillate by concentration effect in all species except *Hyla chrysoscelis*. *Anaxyrus americanus* and *Hyla chrysoscelis* exhibited a significant three-way interaction among distillate, time, and concentration ($p<0.001$). Gasoline revealed the greatest lethal impact and oil caused the lowest levels of narcosis. My results suggest that petroleum distillate exposure through open waste ponds or leakage of petroleum distillates is a concern for amphibians. Improved knowledge of the petroleum distillates and their effects on wildlife are needed to develop policies that balance preservation of the environment with human energy needs.

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Table of Contents

Chapter 1: Introduction

There is no doubt the use of chemicals in the environment has the potential for impacts on wildlife. The widespread use of pesticides in agriculture and the drainage of these pesticides into our water have gained national attention. Pesticides have provided a huge economic advantage to the progression of the United States and their use will not be banned but is highly regulated (Cockerham & Shane 1994). Due to drainage patterns of watersheds worldwide, the water supply is often an endpoint for pollution; soil can also serve as an endpoint. In addition to watersheds, natural and manmade toxins enter the atmosphere (Cockerham & Shane 1994) and can travel great distances, requiring worldwide cooperation on regulations. Soil toxicology varies from particulate matter adsorption to land disposal units (Cockerham & Shane 1994). The particular soil type and drainage patterns are crucial when considering effects and the expanse of the effects of pollution. Humans rely on a variety of chemicals and when chemicals are spilled into the environment, watershed drainage patterns can lead them to collect in aquatic habitats in potentially high concentrations. The impact this has on wildlife of aquatic systems varies greatly among pollutants and is cause for concern.

Regulations on pollution need to be carefully maintained as the human population grows and our needs expand. Human energy needs are expanding and natural gas has been found abundantly in shale rock formations and is considered a better energy source than coal because it causes less air pollution (Rozell & Reaven 2012). Hydraulic fracturing is a drilling practice used to extract petroleum products, specifically natural gas. Hydraulic fracturing has been expanding around the world since the 1990s where shale gas deposits can be found (Entrekin et al. 2011). It includes the use of various chemicals that can

contaminant the water supply through transportation of fluids, well casing leaks, leaks through the fractured rock, drilling site discharge, and wastewater disposal (Rozell & Reaven 2012). Fluids used in hydraulic fracturing are commonly composed of water and many chemical additives (e.g., acids, chlorides, alcohols, petroleum distillates, etc.) that vary from well to well. These fluids are pumped into a geologic formation at high pressure. When the pressure exceeds the rock strength, the fluids open or enlarge fractures that can extend several hundred meters away from the well. After the fractures are created, a propping agent is pumped into the fractures to keep them from closing when the pumping pressure is released. After fracturing is completed, the internal pressure of the geologic formation causes the injected fracturing fluids to rise to the surface where they may be stored in tanks or pits prior to disposal or recycling. Disposal options include discharge into surface water with approved levels of chemical concentrations or underground injection (USEPA 2013).

Effects of hydraulic fracturing on biota are likely to include habitat loss, chemical pollution and degradation of water quality (Kiviat 2013). Fluids from hydraulic fracturing that are drained onto surrounding land, after having met regulations for quantities of other pollutants such as chloride, still killed vegetation and damaged tree leaf growth (Adams et al. 2011). In a watershed, erosion and pollution from the process of hydraulic fracturing could drain into and alter the aquatic environment. Research has focused on contaminants that threaten drinking water and groundwater, whereas data collection to address concerns associated with surface water and terrestrial ecosystems have largely been overlooked (Entrekin et al. 2011). In fact, the process of hydraulic fracturing has been studied very little for its effects on aquatic life. Onsite waste ponds could overflow, spill, or

leach into groundwater and contaminate nearby streams (Entrekin et al. 2011). Scientific studies are needed to understand the possible environmental effects caused by activities associated with natural gas extraction (Entrekin et al. 2011). As we learn more about effects of hydraulic fracturing, more regulations are put into place but regulations need to be consistent across all areas and based on research.

Use of hydraulic fracturing water in experiments does not allow one to isolate which chemicals are having the effect on aquatic species. Chemical content of the fluid used in a well is often proprietary as well. Even though companies may be required to disclose use of certain chemicals to state and federal agencies, these data are often not available to the public (Entrekin et al. 2011). Isolating and testing one chemical shows effect of each chemical, even though concentrations vary and mixes vary among companies. Petroleum distillates are used as friction reducers in the hydraulic fracturing process (Council 2011). Companies use either petroleum distillates or hydrotreated light petroleum distillates in their hydraulic fracturing mixtures. Petroleum distillates are broken up into categories (light, medium, and heavy) depending on their physical properties.

Petroleum distillates have a low solubility in water; a preparation of a water-soluble fraction was necessary in experiments (Pacheco & Santos 2001). A preparation of a water-soluble fraction isolates the strong ecological impact of the compounds, polycyclic aromatic hydrocarbons (PAHs), found in petroleum distillates (Pacheco & Santos 2001; Fedato et al. 2010). The point of using a water-soluble fraction is to account for this difference in PAH content of the distillates isolated in water and their toxicity to aquatic life. Dissolved hydrocarbons and soluble contaminants are absorbed by aquatic species through contact with the water potentially leading to serious effects in the whole ecosystem (Patrick-

Iwuanyanwu et al. 2009). The accumulation of these compounds in tissues then consumed by another organism has the potential to harm the next organism, due to biomagnifications of the pollutants. Moving up the food chain each organism absorbs the toxins from their prey potentially resulting in mortality, behavioral shifts, and impacts to the whole ecosystem. For example, Glennemeier and Begnoche (2001) demonstrated that amphibians do not accumulate as high levels of PCB's as other taxa, but could be important in pollutant transfer because they serve as prey for many species. Compounds such as PCB's and pesticides show the tendency to accumulate in the tissues of many animals (Cockerham & Shane 1994).

Polychlorinated biphenyls (PCBs) present in oil products, gasoline pipelines and hydraulic fluids belong to the same family of manmade chemicals as PAHs and are regulated by the EPA (Agency 2013). Both PCBs and PAHs are bioaccumulative pollutants that are or have been used for human energy needs. PCBs are one of the best-known groups of persistent environmental toxins (Cockerham & Shane 1994). PAHs are present in all petroleum distillates with the possibility to persist in the environment. Effects of PAHs and PCBs, include an effect on the skin of mice, causing irritation and tumors (Freeman et al. 1990). *Rana pipiens* eggs located in PCB contaminated sites demonstrated decreased hatching success (Glennemeier & Begnoche 2001), but no effect on time to metamorphosis was found. Contaminants in wastewaters affect organism's behavior at sublethal levels (Entrekin et al. 2011), in addition to lethal levels.

Amphibians are considered to be excellent environmental indicators and play an important role in ecosystems as prey or predator. Amphibians also play a role in energy transfers between aquatic and terrestrial habitats (Gillilland et al. 2001). Most North

American amphibians spend part of their life in aquatic habitats and part in terrestrial habitats. Some spend large amounts of time underground. This variation in habitat use allows us to observe various land-use changes that are affecting wildlife.

Amphibians are currently the most threatened vertebrates worldwide (Fig 1-1; IUCN 2013). Amphibians face many environmental threats and often face more than one threat simultaneously. These threats can include disease, climate change, habitat destruction and pollution (Blaustein et al. 2011). Disease, especially Chytrid fungus (*Batrachochytrium dendrobatidis*), has been linked to declines of certain species and is further exasperated by the changing climate. The changing climate is hypothesized to be creating a perfect environment for growing *Bd* and other pathogens (Pounds et al. 1994). The IUCN lists pollution as one of the top reasons for declines in all species including amphibians (IUCN 2013).

Declines in amphibians have many negative impacts on a community including loss of species diversity, species richness, and altered energy flow. Whiles et al. (2012) studied impacts of amphibian loss on streams by looking at how tadpoles reduce algal biomass on substrata. This reduced amount of algal blooms could affect the amount and ability of a system to take up nitrogen as well as alter invertebrate communities. Any change of nutrients such as nitrogen or phosphorus in aquatic environments could alter species' growth and abundance especially in algae. Algae declines could cause a severe decline in tadpoles, as algae are their main food source. Changes in organic matter alters the biotic environment changing or eliminating ecological roles for species such as anurans that play a role both as a prey and predator (Blaustein et al. 2011).

Amphibians have porous skin, rely on water or moist areas to breed, and anurans rely on a quiet environment so they can hear each other's vocalizations (Vitt & Caldwell 2014). Because of amphibian's permeable skin, which plays a key role in gas exchange, any toxin the skin is exposed to can be harmful. In the case of larval amphibians, coating of the gills, their main source of breathing could be lethal. Most amphibians undergo metamorphosis from a larva to an adult as part of their life cycle. This change affects many of the bodies' systems including respiratory, digestive, sensory, immune and skeletal. While this change is occurring amphibians are especially vulnerable.

Breeding is another vulnerable time and selection of the environment for larval growth of amphibians is critical for larval survival. Many amphibian species choose an ephemeral stream, water filled ditch or small pond to lay their eggs in (Lannoo 2005). Therefore they adapt their reproductive timing and development to the hydrology of the temporary water body (Ortiz-Santaliestra & Sparling 2007). These places are likely to see runoff or leaks from many sources including the hydraulic fracturing well chemical constituents. Another concern is the wastewater of the hydraulic fracturing well site that is stored in tanks or open pits allowing wildlife open access to pollutants (Staff 2011).

Contaminant effects are often assessed through single-species laboratory tests with standardized organisms and with single contaminants (Entrekin et al. 2011). Most toxicological research in amphibians has focused on *Anaxyrus* and *Lithobates* species (Mann & Bidwell 2001). Multiple species were chosen for study due to the application of investigating the differences in anurans versus salamanders, to cover a wide geographical distribution of amphibians as well as a difference in length of larval period affecting

exposure time (Lannoo 2005). Differences may also occur during metamorphosis, which is more drastic in anurans than in salamanders (Vitt & Caldwell 2014).

My goal in this research was to evaluate the lethal and sublethal effects that amphibian species experience due to exposure to chemicals used in hydraulic fracturing. My research included four species, two of which are not standardized test organisms, and exposure to various petroleum distillate contaminants. Species in this study include: *Hyla chrysoscelis* (Gray treefrog), *Lithobates sphenocephalus* (Leopard frog), *Anaxyrus americanus (*American toad) and *Ambystoma maculatum* (Spotted salamander). All species have a fully aquatic larval stage and breathe primarily through their skin and gills (Vitt & Caldwell 2014). In the first portion of my study, I exposed early stage larvae of the four amphibian species to water-soluble fractions of three petroleum distillates, engine oil, kerosene and unleaded gasoline in a laboratory setting. There were four concentrations of each distillate, 10%, 25%, 30%, and 50%. At four time points during the 72 h acute test, I monitored survival and measured swim time and narcosis using a standardized behavioral test (Mann & Bidwell 2001). My expectations were for higher concentrations to increase impaired response to stimuli and variance in sensitivity to pollutants between species. Among distillates, oil is expected to cause the highest mortality, since it is the heaviest distillate.

I also included in my study a chronic component examining sublethal effects over the entire larval stage through metamorphosis in outdoor mesocosms. I ran chronic tests only for *Anaxyrus americanus* exposed to two concentrations, 2.5% and 10% of each distillate. I gathered data on survival and behavioral responses every 10 days until front limb emergence, after which observations were made daily. At metamorphosis, I measured

mass, time from front leg emergence to tail re-absorption, and time from beginning the test until tail re-absorption. I expected to find delayed metamorphosis and smaller masses at metamorphosis for larvae exposed to water-soluble fractions of each distillate: gasoline, kerosene and oil.

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Figures

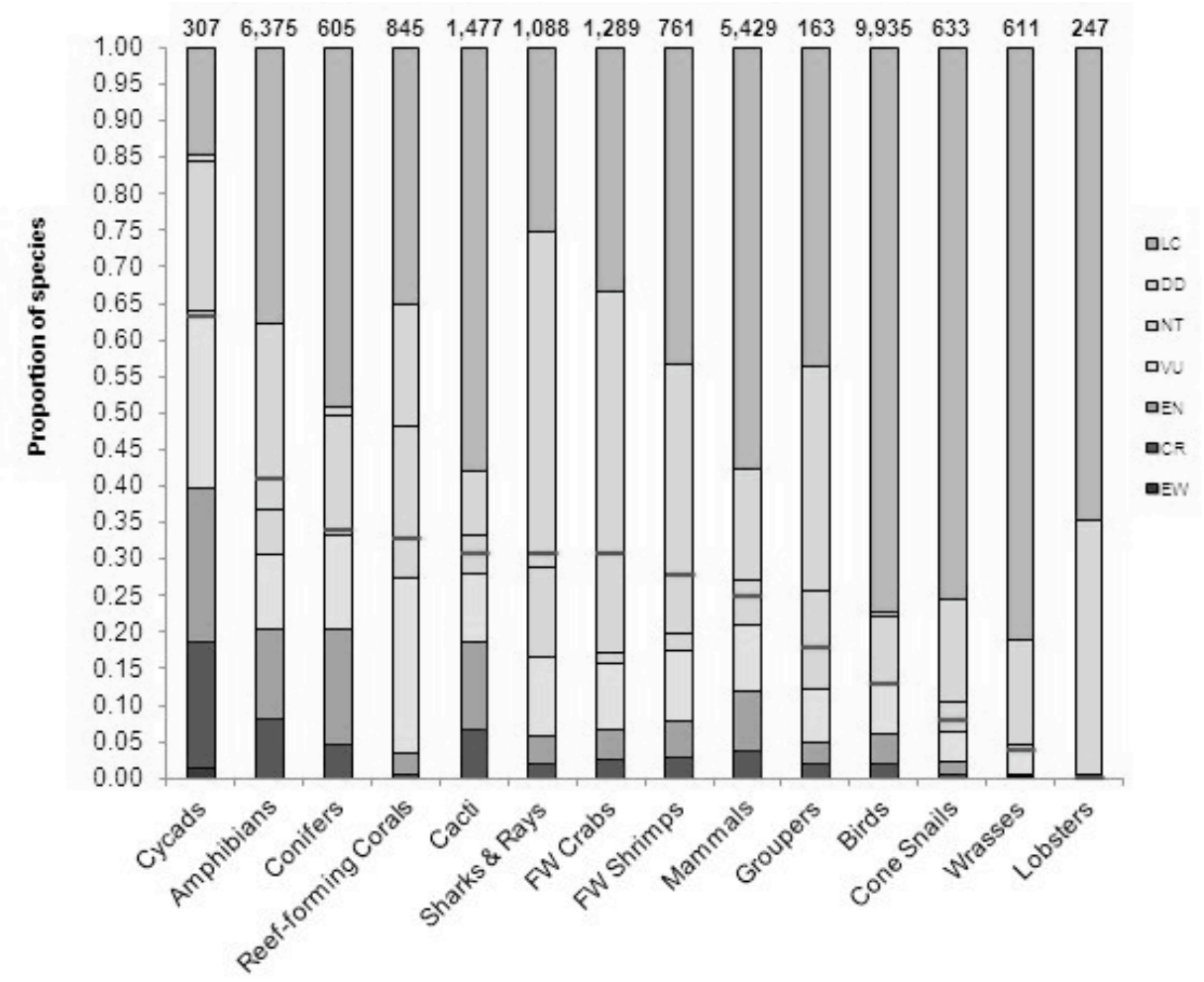

Figure 1-1. As of 2013, the conservation status of extant species according to the IUCN Red List. Out of all vertebrates, amphibians are one of the groups that have the highest numbers of critically endangered, endangered or vulnerable species. The numbers above each bar represent the total number of extant species assessed for each group. **CR** - Critically Endangered, **EN** - Endangered, **VU** - Vulnerable, **NT** - Near Threatened, **DD** - Data Deficient, **LC** - Least Concern.

Chapter 2: Acute lethal and sublethal effects of petroleum distillates on four amphibian species

Introduction

Natural and manmade toxins enter the atmosphere, soil and water (Cockerham & Shane 1994) and can travel great distances. There is no doubt the use of organic chemicals in the environment has the potential for impacts on wildlife. Some organic contaminants are extremely toxic to aquatic life, with lethal concentrations in the low parts per billion (Sparling et al. 2010). Potential effects of organic contaminants include endocrine disruption and reduced growth and developmental rates, among others. Man-made compounds remaining persistent in the environment include polychlorinated biphenyl (PCB's) and pesticides, which show the tendency to bioaccumulate in the tissues of animals and are passed from one trophic level to the next (Sparling et al. 2010). Polycyclic aromatic hydrocarbons (PAHs), another class of mad-made contaminants, also possess the potential to persist in the environment, bioaccumulate, and negatively affect organisms. For example previous research in petroleum products such as oil, exhibit higher mortality in aquatic invertebrates (Lefcort et al. 1997). A major source of environmental PAH contamination is from petroleum products.

Organic chemicals are used for economic gain to help manage and enhance agriculture or gather energy sources. The energy needs humans have are expanding as our population grows and regulations must be managed carefully. Small continuous fuel leaks from gas stations constitute one of the principal sources of soil and water contamination (Fedato et al. 2010). Widespread use in vehicles and machines make gasoline one of the most commonly spilled petroleum products in the environment (Fedato et al. 2010). Other sources of petroleum distillates include oil spills from ships at sea, urban runoffs, and

release into the atmosphere from burning of petroleum wells (Cockerham & Shane 1994). Another emerging source of petroleum pollution is hydraulic fracturing, a drilling process used in the extraction of natural gas. Natural gas has been found abundantly in shale rock formations and is thought to be a better energy source than coal because it causes less air pollution (Rozell & Reaven 2012). The chemicals used in hydraulic fracturing, which include petroleum distillates, could contaminant the water supply through transportation of fluids, well casing leaks, leaks through the fractured rock, drilling site discharge, and wastewater disposal (Rozell & Reaven 2012).

There are many classes of petroleum distillates, each of which is composed of a different arrangement of hydrocarbons and aromatic percentage. Differing boiling points in the distillation process allow components such as gasoline, oil, and kerosene to be separated. Petroleum distillates have a low solubility in water; a preparation of water-soluble fraction isolates the strong ecological impact of the compounds, PAHs, found in distillates (Pacheco & Santos 2001; Fedato et al. 2010). Dissolved hydrocarbons and soluble contaminants are absorbed by aquatic species through contact with the water. Distillates have the potential to persist in the environment, even after the insoluble parent materials are cleaned up (Patrick-Iwuanyanwu et al. 2009). Heavier distillates such as oil contain fewer compounds that will volatize out, as compounds in gasoline do, and are therefore likely to remain in the environment longer.

Amphibians are considered to be excellent environmental indicators (Gillilland et al. 2001) for several reasons. Because of amphibians' permeable skin playing a key role in gas exchange, any toxin the skin is exposed to can be harmful. In the case of larval amphibians, coating of the gills, their main source of respiration, could be lethal. Most amphibians

undergo a complex metamorphosis from an aquatic larva to a semi-terrestrial adult as part of their life cycle. This change affects many of the bodies' systems including respiratory, digestive, sensory, immune, and skeletal. While this change is occurring, amphibians are vulnerable to a variety of stressors, including pollutants (Vitt & Caldwell 2014).

Breeding is another vulnerable time for amphibians and selection of the environment for larval growth is critical for larval survival. Many species prefer ephemeral streams, water filled ditches, or small ponds for breeding (Lannoo 2005). These habitats are likely to receive runoff or spills containing petroleum distillates and the PAHs they contain. In the few studies conducted with PAHs from coal tar sealants, mortality of embryos occurred in *Xenopus laevis* (Mahler et al. 2005). A better understanding of the effects of petroleum distillates on amphibians in aquatic environments is needed.

My goal in this research was to evaluate the lethal and sublethal effects of acute exposure to petroleum distillate chemicals on amphibian larvae. I exposed four pond-breeding amphibian species to water-soluble fractions of three distillates, engine oil, kerosene and unleaded gasoline in a laboratory setting. There were four concentrations of each distillate: 10%, 25%, 30%, and 50%. At four time points during the 72 h acute test I measured survival, swim time, and degree of narcosis using a behavioral test (Mann & Bidwell 2001). Higher concentrations were expected to increase impaired response to stimuli and the sensitivity to pollutants was expected to vary between species. Among distillates, oil was expected to cause the highest mortality, since it is the heaviest distillate.

Methods

I evaluated the acute effects of petroleum distillates on three species of anurans, *Anaxyrus americanus, Lithobates sphenocephalus,* and *Hyla chrysoscelis* and one salamander,

Ambystoma maculatum. Species were selected on the basis of short but variable larval periods, abundance, and their frequency of use in research, which makes them good models for studying the adverse effects of petroleum distillates. These species typically breed in fishless ephemeral bodies of water, including farm and man-made ponds, ditches, vernal pools and wetlands (Lannoo 2005). Ephemeral bodies of water are likely to receive runoff pollution from petroleum distillates.

Anaxyrus americanus lay large clutches of eggs in strings in shallow ephemeral wetlands, pools, ponds, ditches or potholes (Lannoo 2005). The larval period is very short, approximately 50-60 days, with a rapid transformation from larvae to a terrestrial juvenile (Lannoo 2005). This species has been used in numerous studies where morphological and behavioral implications have been observed, including studies focused on pesticides (Relyea 2012), temperature (Jorgenson & Sheil 2008), heavy metals (Willson et al. 2012), and habitat alteration (Earl & Semlitsch 2013). *Lithobates sphenocephalus* lay eggs in large masses, have a 50-75 day larval period (Lannoo 2005) and are abundant throughout the southeastern United States. Studies on *L. sphenocephalus* and *L. pipiens* are various but these species have been shown to be sensitive to agrochemicals (Ortiz-Santaliestra & Sparling 2007; Relyea 2012) that may interact negatively with other factors such as competition and predation (Boone et al. 2007). *Hyla chrysoscelis/versicolor* lays eggs in tiny packets of 30-40 eggs that hatch out and have an average larval period of 42 to 112 days (Lannoo 2005). Previous studies on *H. versicolor* have had mixed results concerning the susceptibility to pollutants of this species (Beachy et al. 1999; Earl & Whiteman 2010).

A meta-analysis on experimental studies of pollutants on various amphibian species found weak differences among amphibian families (Egea-Serrano 2012). I chose an

ephemeral pond breeding salamander species to verify this meta-analysis would apply to petroleum distillates. *Ambystoma maculatum* is common and widely distributed in Arkansas; clutch sizes average 10-50 eggs. Their larval period is variable but can be as short as 45-65 days (Lannoo 2005).

Amphibian eggs were collected from various aquatic breeding habitats in northwest and central Arkansas between 15 March and 6 June 2013. Multiple clutches were obtained for each species. This allowed for clutches to be stratified across treatments to account for potential clutch effects. Following breeding phenology, eggs were gathered and experiments run for *A. americanus* first, then *L. sphenocephalus, A. maculatum* and lastly *H. chrysoscelis.* After collection, eggs were maintained in buckets of pond water in a temperature-controlled laboratory (20-22°C, 12/12 L:D) until testing.

Distillates and Preparation of Water Soluble Fractions

Three petroleum distillates were selected and purchased locally: engine oil, kerosene, and unleaded gasoline. Oil was automotive engine oil multigrade 10W-30 Rotella, considered a heavy distillate; Kerosene 1 K heater fuel and unleaded gasoline are considered light distillates. Twenty-four hours before the start of each experiment, water-soluble fractions of each distillate were prepared following the general methods of Anderson (1974). Briefly, 9 parts distilled water and 1 part distillate were spun for 24 h in a 1000 ml beaker. The beakers were tightly covered and spun at a medium speed where the vortex just reached the bottom of the beaker. They were then allowed to settle for 1 h, after which the non-soluble portion was siphoned off. To be sure the entire non-soluble portion was siphoned off, a 100 ml layer was removed including the non-soluble portion.

Based on pilot experiments, I evaluated four concentrations of the water-soluble fraction of each distillate: 10%, 25%, 30%, and 50%. Water-soluble fractions were diluted with dechlorinated tap water to create the proper concentrations and obtain 300mL of total solution. All beakers, stir bars, and equipment were used only for their respective distillate.

Experimental Procedures

A 3x4x4 factorial experiment was designed to evaluate mortality and behavioral responses of the four amphibian species to acute exposure of four concentrations of each of the three distillates. Each of the 12 treatments and a control (decholorinated tap water) were replicated ten times. Responses were monitored by measuring mortality, a narcosis test (Mann & Bidwell 2001), and swim time, at four time points over the 72 h acute test.

Experiments began when larvae were at Gosner stage 23-25. During this time external gills may still be visible and the mouthparts are beginning to develop (McDiarmid & Altig 1999). Before experimentation began, a subsample of larvae from each clutch was weighed (nearest 0.001 g), Gosner stage determined, and length measured (nearest 0.01 mm using digital calipers). Experimental setup included a random blind design where treatments were randomly assigned a cup number and grid position on a bench in a temperature controlled laboratory (20-22°C, 12/12 L:D). No covers were placed on the plastic 470 ml cups larvae were individually housed in. Larvae were not fed for the duration of the experiment.

A stimulus test was used to assess narcosis and survival of each individual at 6, 24, 48, and 72 h. At each time point each subject received a prod once to the tail by a glass rod (Mann & Bidwell 2001). If the larvae were already swimming they also received a prod.

Narcosis in response to the poke test was scored on a 1-5 scale, based on Mann & Bidwell (2001). The expected response was to swim swiftly away for more than 1 sec in a coordinated manner. If the subject met these expectations, then a value of 1 was recorded. If the subject swam in a coordinated way but failed to swim longer than 1 sec, a value of 2 was recorded. If mild narcosis was present, the larvae swam for more than 1 sec but in an uncoordinated matter, such as on its side, and received a value of 3. If mild narcosis was present and the larvae swam less than one second then a value of 4 was recorded. Often larvae in mild narcosis only were able to lie at the bottom and twitch. Larvae in full narcosis displayed a total lack of activity in response to a prod and received a value of 5. This value included dead larvae that were later removed when necrosis was evident (Mann & Bidwell 2001). Swim times were recorded to the nearest thousandth of a second. Narcosis observations were all made by the same observer, who was blind to treatments (Mann & Bidwell 2001).

Mortality was noted only when the subject was clearly deceased with observable necrosis. Some larvae initially judged to be in full narcosis were able to recover and were only 'stunned' by chemical exposure. At the conclusion of the experiments subjects were photographed and euthanized with MS-222.

An additional experiment was run to determine the persistence of the effects of the distillates on amphibians in the water soluble fractions. This experiment used only *H. chrysoscelis* and followed the same general procedures described above. However, in this experiment water soluble fractions of distillates had been aged 72-hours in uncovered cups. I used new larvae not previously in any experiments.

Analyses

In all experiments, the influence of distillates and concentrations on narcosis and swim-time for each species over time, was examined using Nonparametric Repeated-measures MANOVA in Program R (package 'nparLD'; Noguchi 2011, 2012; Team 2011). A nonparametric approach was used because neither the original data, nor their transformations, met assumptions of normality and homogeneity of variance (Bartlett and Kolmogorov–Smirnov tests; Program R). NparLD calculates an ANOVA-type statistic (ATS); ATS can be used to analyze data in many situations in which ANOVA is traditionally used, because similar hypotheses to ANOVA are tested (Erceg-Hurn & Mirosevich 2008; Shah & Madden 2004).

To evaluate the influence of distillates and their concentrations on survival of each species (i.e. test for conditional independence), I used a multiway (3x4x2) factorial contingency table in Program R (Team 2011). This approach reports a Pearson's Chi-squared test statistic for the interactions of distillate and concentration. After determining the significance of overall effects, I examined the individual influence of each distillate at each concentration as compared to the control group using chi-squared tests of independence in Program R.

The measured responses of narcosis and swim-time were analyzed separately. Before the analysis was run, each variable was standardized by subtracting the mean from each value and dividing by the standard deviation. For these analyses, narcosis or swim-time, along with distillates and concentrations were used as a between-individuals variable and time as a within-individuals repeated measure. The alpha level was set at 0.05 and main effects were evaluated along with all possible two and three-way interactions. Control

treatments were not analyzed statistically because they did not fit the factorial design, but they are included in figures for comparison.

Results

Survival

Survival of *A. americanus* was significantly influenced by the interaction between distillate and concentration (Pearson chi-square = 78, DF=11, p<0.001; Fig 2-1). No deaths were recorded in oil or kerosene, but gasoline demonstrated a strong concentration effect. Survival decreased at 25% and 30%, and dropped to zero at 50%. Mortality in the 50% treatment occurred within 24 h of the experiment for this species (Fig 2-1a). In independent contrasts, the only treatments that differed significantly from controls were 30% gasoline and 50% gasoline (30%; chi-square = 5, p<0.043; 50% chi-square = 20, p<0.001).

A two-way interaction between distillate and concentration was significant in all three other species (*L. sphenocephalus*: chi-squared=38.2, DF=11, p<0.001; Fig 2-2; *H. chrysoscelis*: chi-squared=55.9, DF=11, p<0.001; Fig 2-3; *A. maculatum*: chi-squared=52.5, DF=11, p<0.001; Fig 2-4). In the independent contrasts between distillate and control, *L. sphenocephalus* and *H. chrysoscelis* had significant values at 30% (*L. sphenocephalus*: p=0.016; *H. chrysoscelis*: p<0.002) and at 50% (*L. sphenocephalus*: p=0.043; *H. chrysoscelis*: p<0.028) gasoline. *Ambystoma maculatum* had zero larvae survive at 30 and 50% gasoline treatments versus the control (p=0.023 for both percentages). All other independent contrasts were not significant. Other than *L. sphenocephalus* experiencing unusual mortality at 10%, patterns of survival in all species were similar to *A. americanus.* All distillates decreased in survival with increasing concentration in gasoline and high survival

across concentrations of oil and kerosene. *Ambystoma maculatum* and *A. americanus* exhibited the lowest overall survival compared to *L. sphenocephalus* and *H. chrysoscelis.*

Narcosis

A three-way interaction existed between distillate, concentration, and time affected narcosis (a behavioral response test) in *A. americanus* (MANOVA=5.29, DF=11, $p<0.001$; Fig 2-5). This interaction was driven by gasoline. *Anaxyrus americanus* controls averaged a narcosis value of 1, indicating normal behavior. Mortality was high for the 50% concentration of gasoline (Fig 2-5a); all tadpoles had died 24 h into the experiment. Narcosis at the first observation (6 h) was high; tadpoles in all concentrations were in full narcosis, a rank of 5, or in mild narcosis with a rank of 4. A trend of recovery in narcosis over time in concentrations of 30%, 25% and 10% was evident. Larvae in a 50% concentration of kerosene (Fig 2-5b) recorded the highest narcosis value for this distillate. A slow recovery over time to 48 h was seen in kerosene, but at the 50% concentration narcosis remained elevated at 72 h. Concentrations of 25% and 30% had low values with little variation over time. In oil (Fig 2-5c), narcosis values show a trend of recovery over time. Initially the highest concentrations revealed higher average narcosis, as expected. By 24 h, almost complete recovery was seen, except at the 50% concentration. By 48 h, amphibians in all concentrations presented near normal behavior with a value of 1 on the narcosis scale. Although high mortality was not seen in oil and kerosene when compared to gasoline, sublethal effects were still present and concentration differences were more pronounced than in gasoline.

Lithobates sphenocephalus did not show a significant three-way interaction, but two-way interactions between distillate and time ($p<0.001$) and between concentration and

time (p=0.049) were significant. *Lithobates sphenocephalus* had an average narcosis of 1.5 for the control. In all concentrations of gasoline (Fig 2-6a), full narcosis or mild narcosis initially was prevalent in all tadpoles but overall the trend indicates tadpoles recover. As in all species, oil and kerosene responses were less severe than gasoline, and kerosene presented the weakest time effect (Fig 2-6b). Narcosis values for larvae exposed to oil were initially moderate but quickly decreased (Fig 2-6c).

Narcosis in *H. chrysoscelis* had a significant three-way interaction (MANOVA=3.43, DF=13, p<0.001; Fig 2-7). In the highest concentration of gasoline, *H. chrysoscelis* had full narcosis until 72 h, when slight recovery was recorded (Fig 2-7a). All other concentrations displayed relatively rapid recovery by 48 h. At 24 h, larvae in kerosene at 50% decreased down to similar narcosis values as other concentrations (Fig 2-7b). The highest concentration of oil (Fig 2-7c) resulted in consistently higher values with only a weak trend towards recovery over time. Both kerosene and oil concentrations under 30% had patterns of narcosis similar to the control.

Narcosis in *A. maculatum* revealed a significant two-way interaction between distillate and concentration (p<0.001). Initially, the two highest concentrations of gasoline (30% and 50%) produced full narcosis of all individuals (Fig 2-8a), and by 48 h survival in these treatments had dropped to zero. This mortality may be the reason for the lack of a time effect for this species (p =0.35). Gasoline concentrations of 25% and 10% both resulted in higher narcosis levels than the control and decreased in narcosis over time. Kerosene and oil (Fig 2-8b, 2-8c) did not show consistent concentration or time effects and values were similar to that of the control.

Swim time

Average swim time, measured in seconds, revealed no consistent pattern over time in all species.

Anaxyrus americanus had a significant three-way interaction between distillate, concentration, and time (MANOVA=2.32, DF=11, p=0.006). Unexpectedly, there was an overall trend for *A. americanus* tadpoles to decrease in swim speed over time. All tadpoles exposed to distillates swam for shorter periods than the control at all but the 72-hour time points, with the exception of 30% kerosene at 24 h. At 6 hours into the experiment all tadpoles in the 50% gasoline concentration were in full narcosis and recorded 0 for swim time (Fig 2-9a). As expected, exposure to higher concentrations resulted in lower average swim times. At 6 h, all but the 10% gasoline concentrations revealed tadpoles not swimming at all. All concentrations of kerosene, except in 30%, produced an initially longer swim time than controls and dropped to under 50 sec at 72 hours (Fig 2-9b). Kerosene at 50% records the lowest times as I expected. For oil, control times were higher than all concentrations except at 72 h when 10% oil concentration averages slightly above the control values (Fig 2-9c). The highest concentrations of 30% and 50% in oil represent the lowest swim times.

Lithobates sphenocephalus had a significant two-way interaction between distillate and time ($p<0.001$). At the first two time points of gasoline, 6 and 24 h, the higher concentrations recorded the lowest swim times (Fig 2-10a). No clear trend was shown in gasoline. Control values for this species in all distillates showed no clear trend. Overall in kerosene, 50%, 30%, and 10% record the lowest swim times (Fig 2-10b). No clear trend for

oil in swim times was evident (Fig 2-10c). The lowest swim times were recorded in 30%, 50% and 10% oil.

Hyla chrysoscelis had a significant three-way interaction (MANOVA=2.25, DF=13, p=0.005). This species recorded the lowest swim time of the anurans in all distillates. Through the entire experiment 30% and 50% in gasoline recorded average values of 1.5 seconds or less, the lowest of the four concentrations (Fig 2-11a). Over time swim times averaged less than five seconds in kerosene except in 30% at 48 hours and in 10% at 6 hours (Fig 2-11b). *Hyla* reacts in a similar fashion in oil and kerosene with swim times under 5 seconds except at 50% at 24 hours (Fig 2-11c). Concentrations average close to the control values.

Ambystoma maculatum did not swim in the same manner as the anurans, and generally exhibited swim times that were below 1 sec. *Ambystoma maculatum* had a significant two-way interaction between distillate and concentration ($p<0.001$) with a general trend towards increasing swim time over the course of the experiment. Initially swim times of larvae exposed to 30% and 50% concentrations of gasoline was zero (Fig 2-12a) and after 24 h all larvae had died. Other than time point 48 h, exposure to 10% and 25% concentrations of gasoline resulted in lower swim times than the control.

Aged Distillate Experiment

In the aged distillate experiment run for *H. chrysoscelis* there was an overall pattern of low narcosis values. The narcosis values of larvae in all distillates averaged 1-2, maintaining the recovery seen at the 72 h time point of the initial experiment (Fig 2-13). There was still a significant three-way interaction (MANOVA=25.78, DF=12, $p<0.001$) but

the effects of this interaction were less pronounced. Survival was >90% in all treatments (Fig 2-14).

Discussion

I evaluated the lethal and sublethal effects of acute exposure to the water-soluble fraction of three petroleum distillates in larval amphibians. I found gasoline to cause the highest mortality and highest narcosis scores and saw strong interactions between concentrations, distillates, and time. Over time, narcosis scores decreased indicating that amphibians can recover, but at high initial concentrations the sublethal effects of distillate exposure could compromise survival or growth.

Amphibians have been widely used as indicators of environmental pollution and are also recognized as a group in worldwide decline. Polycyclic aromatic hydrocarbons (PAHs) are of global concern because they cause toxicological stress in aquatic and terrestrial food webs where amphibians reside (Leney et al. 2006). Petroleum and its derivatives, such as gasoline, contain PAHs and are among the pollutants reaching aquatic ecosystems that have the greatest ecological impact. Small continuous fuel leaks from gas stations constitute one of the principal sources of soil and water contamination. Widespread use in vehicles and machines makes gasoline one of the most commonly spilled petroleum products in the environment (Fedato et al. 2010). Petroleum distillates used in the hydraulic fracturing process are occasionally applied to surrounding lands of well sites where immediate effects have been observed (Adams et al. 2011). My experiments suggested that the water-soluble fraction of petroleum distillates can be potentially harmful to amphibians.

Amphibians have highly permeable integuments and are thought to be highly susceptible to environmental contaminants (Rohr & Palmer 2005). Petroleum distillates,

such as oil, may have direct or indirect effects on amphibians. Direct effects include absorption through the skin or gills and indirect through food sources or breeding environment (Lefcort et al. 1997). I found that at high concentrations of petroleum distillates there was a higher level of mortality in amphibian larvae. Effects on organisms from petroleum distillates occur primarily from exposure to or biological metabolism of aromatic structures (Cockerham & Shane 1994). In a study of biota-sediment accumulation factors in an area affected by an oil spill, frogs were found to have higher PAH accumulation than other animals, such as mice and insects (Leney et al. 2006). Polycyclic aromatic hydrocarbons have adverse effects on biota, including cytotoxicity and genotoxicity (Fernandez & I'Haridon 1994).

Contaminants absorbed by biota from the environment come from the components of distillates that are water-soluble (Fedato et al. 2010). Living cells take up dissolved hydrocarbons and soluble contaminants readily (Patrick-Iwuanyanwu et al. 2009), so the creation of a water-soluble fraction in my study was critical. In WSFs there is an increased isolation of PAHs that have mutagenic and carcinogenic effects on the biota compared to the non-soluble WSF (Fedato et al. 2010). Water-soluble fractions of oil are greatly enriched in aromatic hydrocarbons relative to the parent oil, but appear to be less toxic than the parent oil (Anderson 1974; Hedtke & Puglisi 1982). However, in certain situations, exposure to WSFs of distillates is more relevant, because WSFs can disperse widely within the aquatic habitat and are more likely to remain after cleanup of non-soluble distillates. The lingering WSFs in aquatic systems have the potential to be taken up by biota, potentially resulting in adverse effects. Therefore, compared to other studies on petroleum products, my study focused on the hydrocarbons that are concentrated in the water-soluble

fraction solutions.

For all species, exposure to the WSF of gasoline resulted in the highest levels of mortality, contrary to my original hypothesis that oil would induce the most severe effects. Survival was highest in the lower concentrations of gasoline and most mortality occurred in the first 24 hours. Water-soluble fractions of oil and kerosene resulted in little mortality. This is in contrast to other studies that have found WSFs of oil and kerosene to be lethal. For example, exposure to a 50% WSF of kerosene resulted in total mortality in microalga on the sixth day of exposure (Phatarpekar & Ansari 2000). The difference in structure of hydrocarbons was likely the reason for high mortality in gasoline. A study on the physiological changes in fish exposed to a water soluble fraction of gasoline found adjustments in their osmotic balance and an increase in chloride cells in their gills. This possibly interfered with gas exchange, which likely occurred in the gills of the amphibians in my study (Simonato et al. 2013). In the WSFs of gasoline, mono- and polycyclic aromatic compounds are present. The pollution potential of gasoline is directly related to highly water-soluble aromatic hydrocarbons such as benzene, toluene, and xylene (BTX). High concentrations of BTX and Naphthalene are present within the gasoline WSF (Fedato et al. 2010) and it is likely that these compounds are the cause of the high mortality in my experiments as opposed to kerosene and oil, which did not contain BTX.

In addition to direct mortality, my experiments revealed the potential for amphibian larvae to experience temporary narcosis in response to exposure to petroleum distillates. Behavioral studies have shown *Lithobates catesbeiana* tadpoles to become lethargic and float to the surface when fuel oil is sprayed on water (McGrath & Alexander 1979). My studies also suggested a change in behavior, with high narcosis values comparable to

lethargy. Initial narcosis was high when larvae were exposed to the chemicals of this experiment, especially gasoline. As for survival, the significant distillate effect on narcosis was driven by gasoline in all species; kerosene and oil both had lower narcosis values in all cases. All species also had significant concentration effects, with 50% producing the highest narcosis values, as expected. Narcosis is the inability to react to a stimulus, which could inhibit the ability to escape predation. In ecosystems, amphibians are likely to also be stressed by competition and predation (Sparling et al. 2010). Multiple stressors on amphibians should be considered within the scope of ecotoxicology to reflect real-world conditions (Sparling et al. 2010). Competition for resources could lead to smaller tadpole sizes, especially if food resources are depleted from chemical pollutants (Sparling et al. 2010).

The behavior patterns observed in my experiment allowed me to assess shifts in severity of sublethal effects over time. The expected trend was for swim time to increase as amphibians recovered over 72 h. However, swim times were highly variable and few clear trends over time were discernible. Alternatively, I was able to find a pattern of recovery in narcosis scores. Over time narcosis values decreased, indicating that larvae can recover from exposure to petroleum distillates. Recovery over time could be due to chemicals volatizing out from solution, although measurements showed no more than 20 ml ever evaporated out from the 300 ml solution. Alternatively, it is possible that since amphibian larvae have the capabilities to change the composition of their membranes to adapt to temperature fluctuations they could be employing a similar tactic to recover from the distillates (Mann & Bidwell 2001). However, this type of acclimatization typically takes much more time than an acute test would provide (Mann & Bidwell 2001). Diaromatic

compounds such as naphthalene, which is found in petroleum distillates are considered most toxic and abundant during initial phases of petroleum spills or releases. This is when compounds are at their highest concentrations and acute toxic effects are most common (Cockerham & Shane 1994). Acute experiment time frame is anywhere from 6 to 96 hours. If I had not run a 72 hour acute time frame I would not have been able to record that the amphibians were recovering after conditions were considered most toxic. The results of my aged distillate experiment with *H. chrysoscelis* demonstrated that aged distillates were less potent than freshly-mixed WSFs. Fresh tadpoles exposed to aged distillated had narcosis values close to the narcosis values found at the 72-h end time point of the original acute experiment. Narcosis values continued to decrease or remain constant at 1 or 2 for the remainder of the aged distillate experiment. These results suggest that water soluble components of distillates degrade over time in solution, likely due to volatilization of toxic compounds, rather than acclimation of larvae.

My study revealed that salamander larvae experienced higher mortality than anurans. *Ambystoma maculatum* had the most deaths in gasoline with all test subjects deceased at 30% and 50% and slightly over half at 25%. *Ambystoma maculatum* have more fibrous gills than anurans and may have difficulty respiring after initial exposure to distillates. Previous studies agreeing with our results have calculated a salamander larvae LC50 of 30% for used motor oil (Lefcort et al. 1997). In addition, salamander diversity has been suggested to decline in watersheds where wastewater from hydraulic fracturing procedures has been spilled or sprayed (Gillen & Kiviat 2012). My results suggest that salamander larvae unaffected by initial exposure to distillates, were able to respond better to a stimulus than anurans in low concentrations. This is supported by a study that found *A.*

opacum and *A. tigrinum* to be fairly resistant to low concentrations of used oils (Lefcort et al. 1997).

The anuran species survival rates were similar in gasoline concentrations and almost no differences were found among species for kerosene and oil treatments. Larval *Anaxyrus americanus* have been shown to experience lethal and sublethal effects from many chemicals (Lannoo 2005), but deformities seem to be more prevalent in Ranid species than *Anaxyrus* (Lannoo 2005). A study done on stormwater that included PAHs, found *L. sylvatica* to be highly sensitive to organic pollutants (Snodgrass et al. 2008). I found *Lithobates* to have no stronger narcosis reaction than *Anaxyrus* or *Hyla*; actually *Lithobates* survived at slightly higher percentages. Snodgrass et al. (2008) examined variation in tolerance between *A. americanus* and *L. sylvatica* to sediments polluted with metals and chlorides. The authors of this study found *L. sylvatica* embryos to suffer high mortality but *A. americanus* embryos and larvae to only experience sublethal effects such as smaller sizes at metamorphosis (Snodgrass et al. 2008). My study found slight differences in resilience to the distillates among species with higher mortality in *Anaxyrus* and *Hyla* than *Lithobates*.

Not only have these experiments proposed potential harmful impacts of petroleum distillates to amphibians but they also suggest that aquatic communities may be at risk. Tadpoles consume algae, which grow more slowly in the presence of oil (Lefcort 1997). This cascading effect of fewer food resources could lead to a decrease in the survival of amphibians therefore affecting predator-prey dynamics in their community. Nutrient dynamics in streams have proven to be altered when amphibian larvae are removed, so further indirect effects on other species are possible if pollutants decrease amphibian

survival (Whiles et al. 2012). In addition to algae other potential organisms in the community affected by the distillates could include fish, mollusks, and invertebrates. Exposure to petroleum products may inhibit oxygen uptake in fish through sublethal concentrations and cause damage to the gill epithelium (Peter et al. 2007). DNA damage in hemocytes and gill cells of *Corbicula fluminea* occurred with exposure to a WSF of gasoline (Fedato et al. 2010). These studies show that not only are the distillates harmful to the individual organism, they also have the potential to alter the delicate balance of aquatic communities.

Though I found distillates to have clear effects on amphibians, additional questions remain on the distillates behavior in aquatic environments and amphibians' responses to various distillates. Further research on the chronic effects of petroleum distillates should include maternal effects, community-level effects and size of amphibians at metamorphosis. Over a longer time frame interactions of multiple stressors, such as disease or climate change, are likely to confound the problem of pollution on amphibians. Amphibians' susceptibility to disease has the possibility to harm populations over time. Pesticide exposure has shown the possibility to weaken immune systems in amphibians (Christin et al. 2003). Severe El Nino events could also increase rates of contaminant exposure in anurans. This occurs as the climate shifts out of El Nino and cloud formations allow the contaminants in the atmosphere to coalesce with the water droplets. Then, as the amphibians re-hydrate, they take in these higher level of contaminants (Pounds & Crump 1994). A thorough understanding of the threats faced by amphibian populations requires chronic studies of multiple interacting factors.

My results show that exposure to the water soluble components of petroleum distillates can have lethal and sublethal effects in larval amphibians. I found variation among distillates and concentrations, with high concentrations of gasoline producing the strongest effects. Even though recovery is possible from petroleum distillate contamination, the degree and rate of recovery depends on the chemical properties of the distillates and duration of exposure. Currently data is collected on the geology and hydrology of energy expansion and collection but consideration of environmental impacts is often lacking. As humans' energy needs have increased, ethanol has been added to gasoline, which is a growing concern. In Brazil, up to 20% ethanol is added to gasoline, which increases the solubility of hydrocarbons in water (Fedato et al. 2010). Sources of distillate pollution should be heavily monitored and studies assessed for levels that harm amphibians. My experiments are a call for further research to be done concerning energy use and its impacts to wildlife.

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Figures

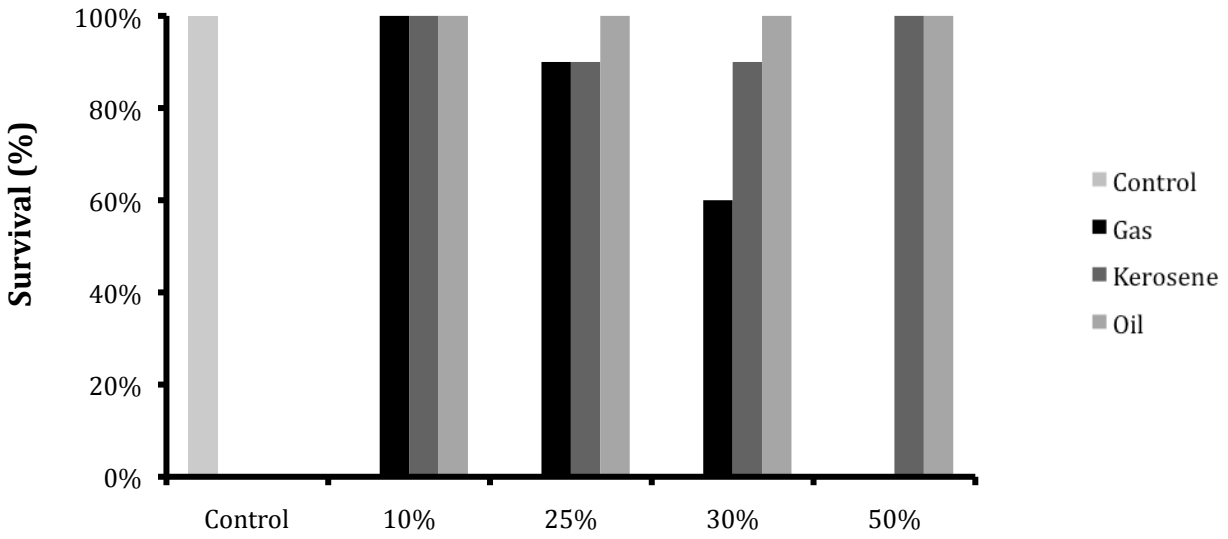

Figure 2-1. Survival of *Anaxyrus americanus* over 72 hours exposure to treatments of four concentrations of water-soluble fractions of three distillates and a control (Distilled H_20). Absent bars are equivalent to death of all replicates in a concentration.

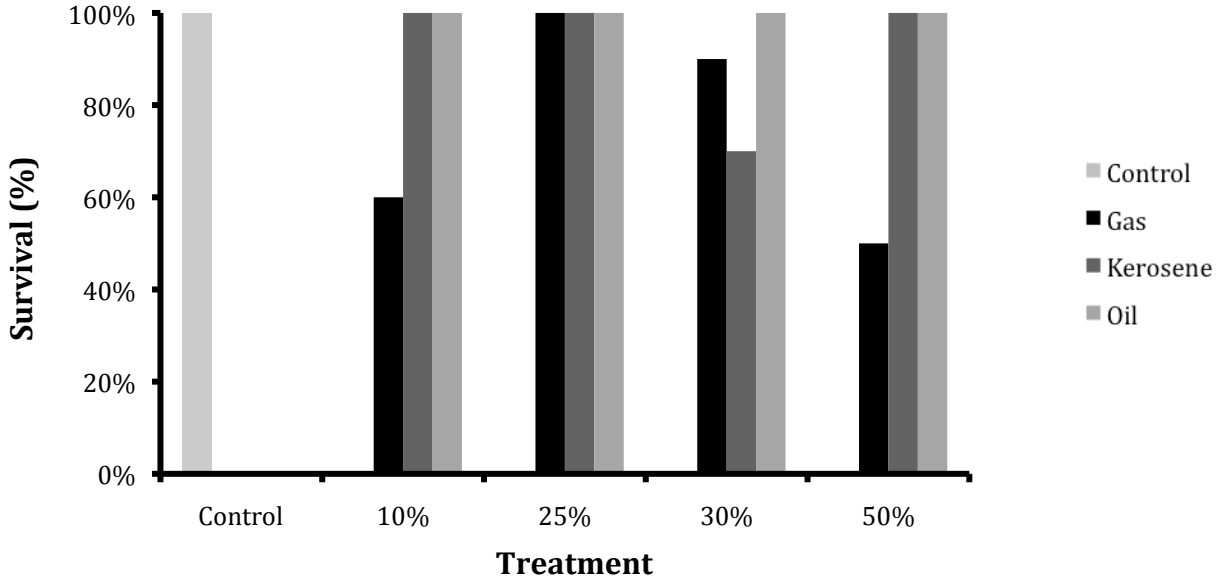

Figure 2-2. Survival of *Lithobates sphenocephalus* over 72 hours exposure to treatments of four concentrations of water-soluble fractions of three distillates and a control (Distilled H_20). Absent bars are equivalent to death of all replicates in a concentration.

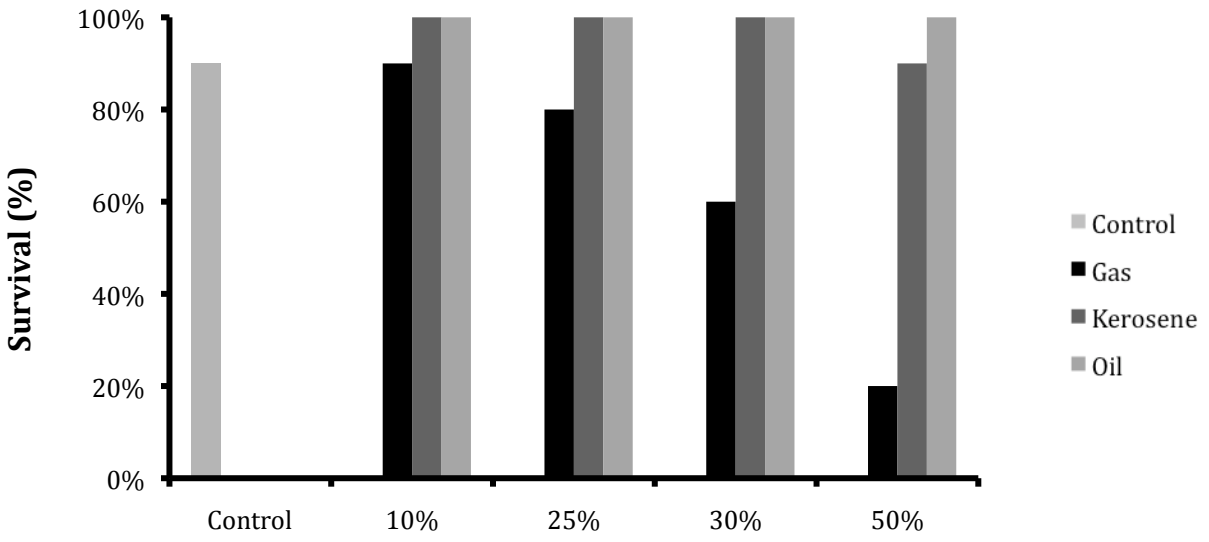

Figure 2-3. Survival of *Hyla chrysoscelis* over 72 hours exposure to treatments of four concentrations of water-soluble fractions of three distillates and a control (Distilled H_20). Absent bars are equivalent to death of all replicates in a concentration.

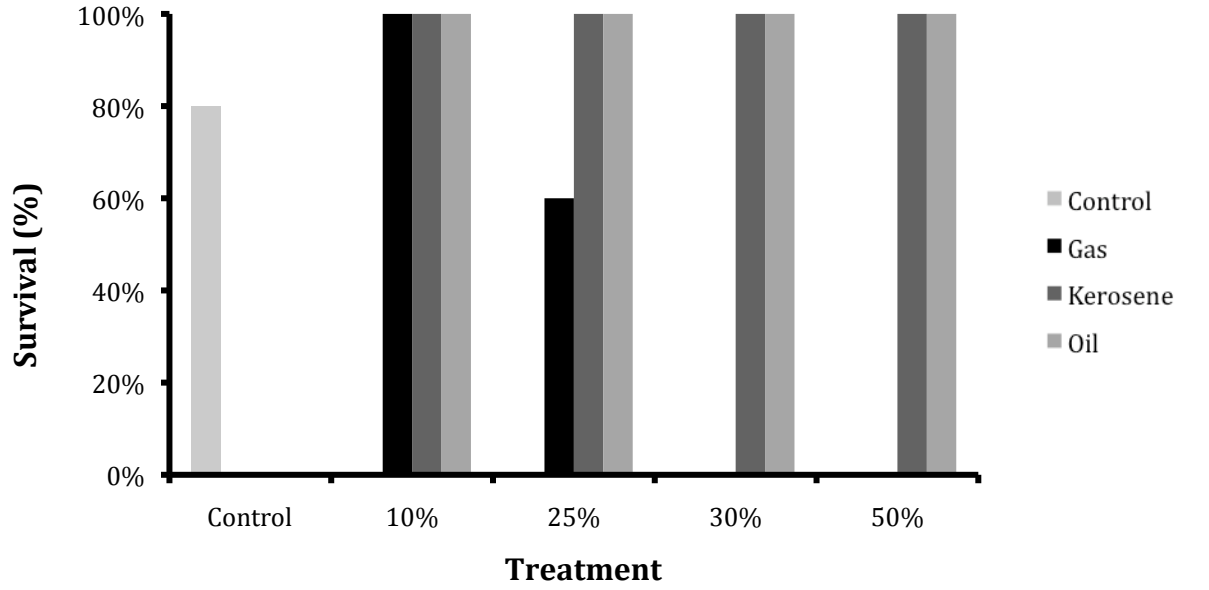

Figure 2-4. Survival of *Ambystoma maculatum* over 72 hours exposure to treatments of four concentrations of water-soluble fractions of three distillates and a control (Distilled H_20). Absent bars are equivalent to death of all replicates in a concentration.

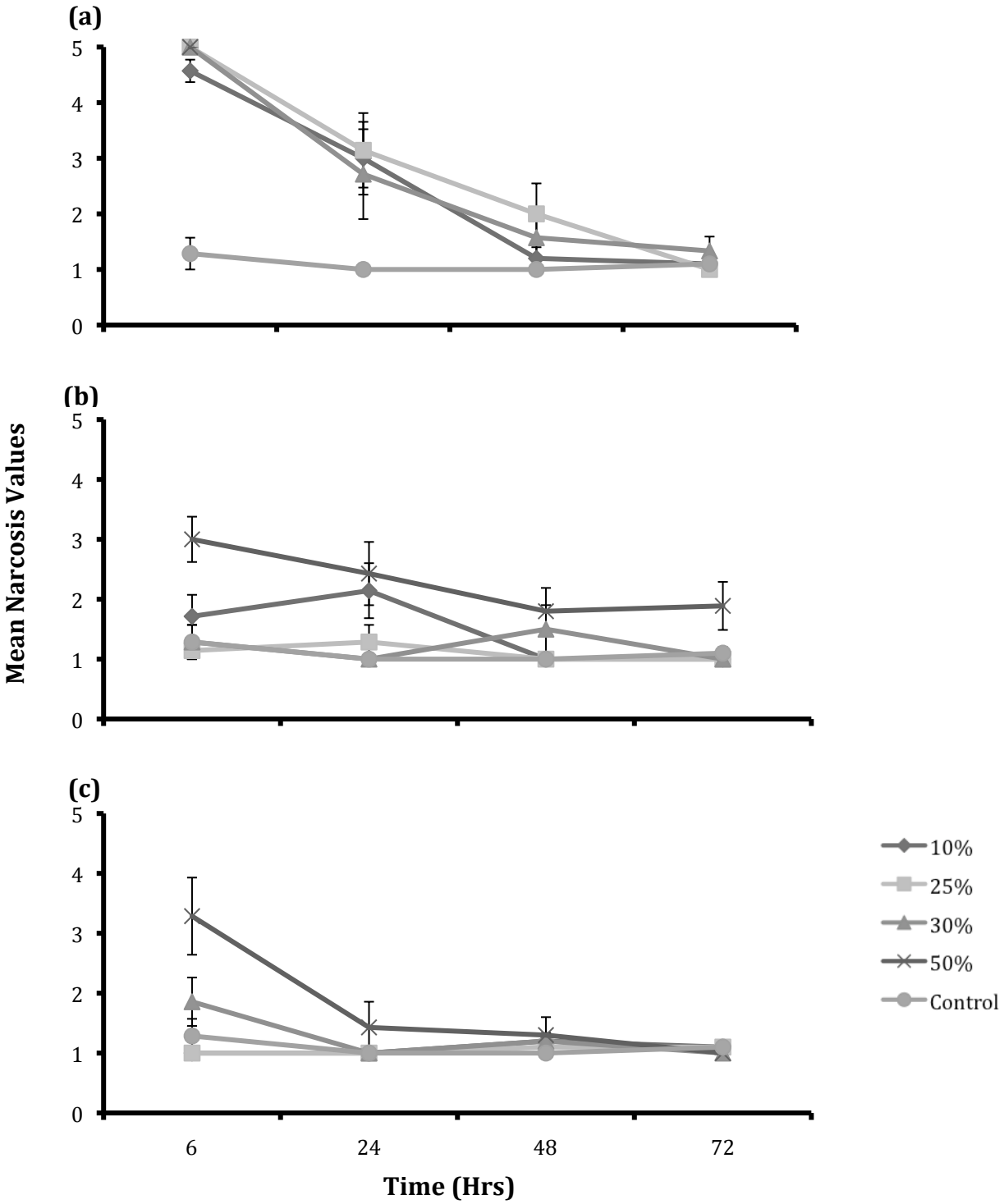

Figure 2-5 Mean narcosis values ± 1SE of *Anaxyrus americanus* at four time points(6, 24, 48, 72 hours) in a control (Distilled H_20) and 4 concentrations of water soluble fractions of gasoline(a) of kerosene (b) and of oil (c). Narcosis values range from 1 (normal tadpole behavior) to 5 (full narcosis, meaning no movement). Missing lines are equivalent to death of all replicates in a concentration. Controls were not analyzed statistically but are included for comparison.

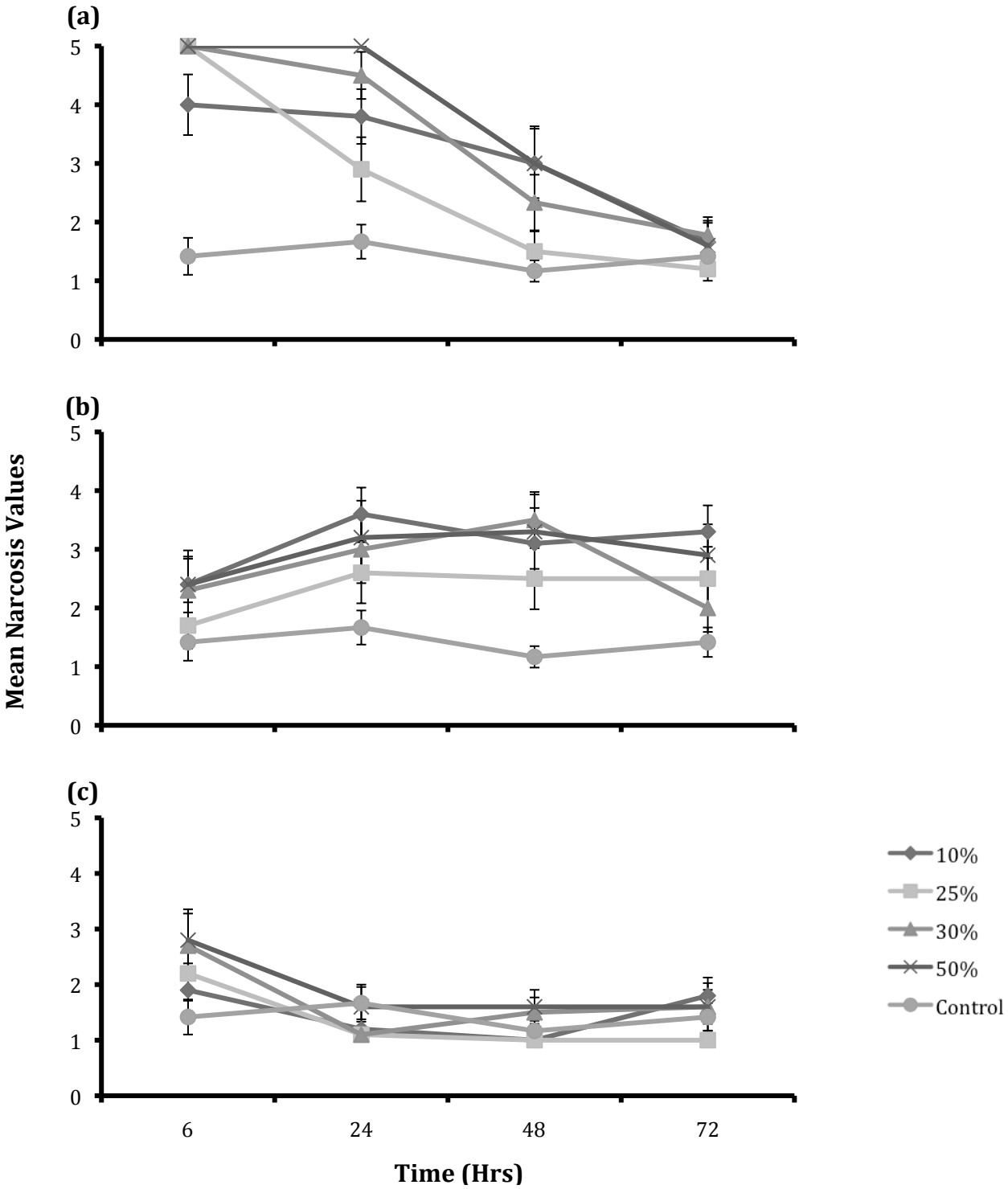

Figure 2-6. Mean narcosis values ± 1SE of *Lithobates sphenocephalus* at four time points (6, 24, 48, 72 hours) in a control (Distilled H_20) and 4 concentrations of water soluble fractions of gasoline(a) of kerosene (b) and of oil (c). Narcosis values range from 1 (normal tadpole behavior) to 5 (full narcosis, meaning no movement). Controls were not analyzed statistically but are included for comparison.

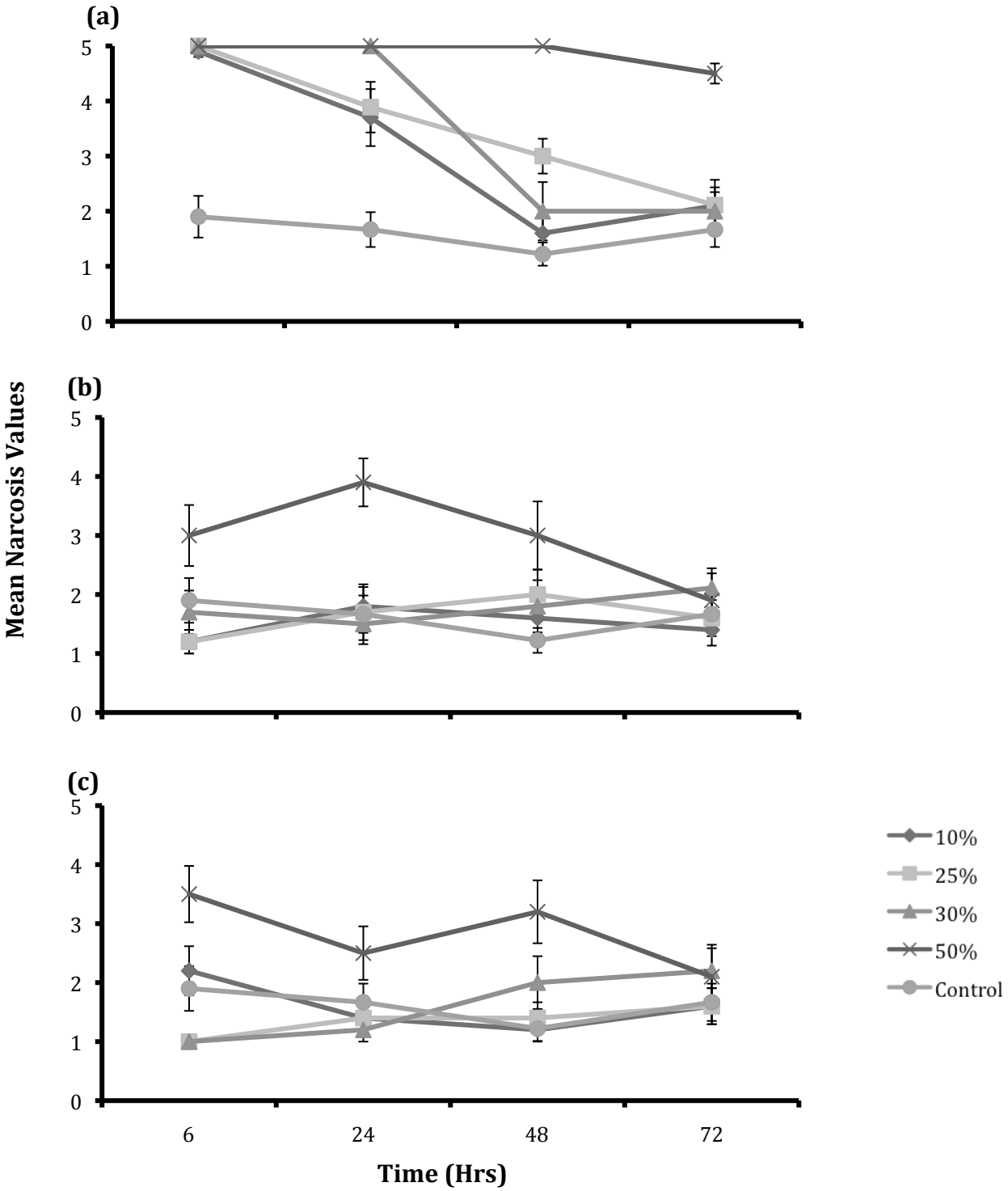

Figure 2-7. Mean narcosis values ± 1SE of *Hyla chrysoscelis* at four time points (6, 24, 48, 72 hours) in a control (Distilled H_20) and 4 concentrations of water soluble fractions of gasoline(a) of kerosene (b) and of oil (c). Narcosis values range from 1 (normal tadpole behavior) to 5 (full narcosis, meaning no movement). Missing lines are equivalent to death of all replicates in a concentration. Controls were not analyzed statistically but are included for comparison.

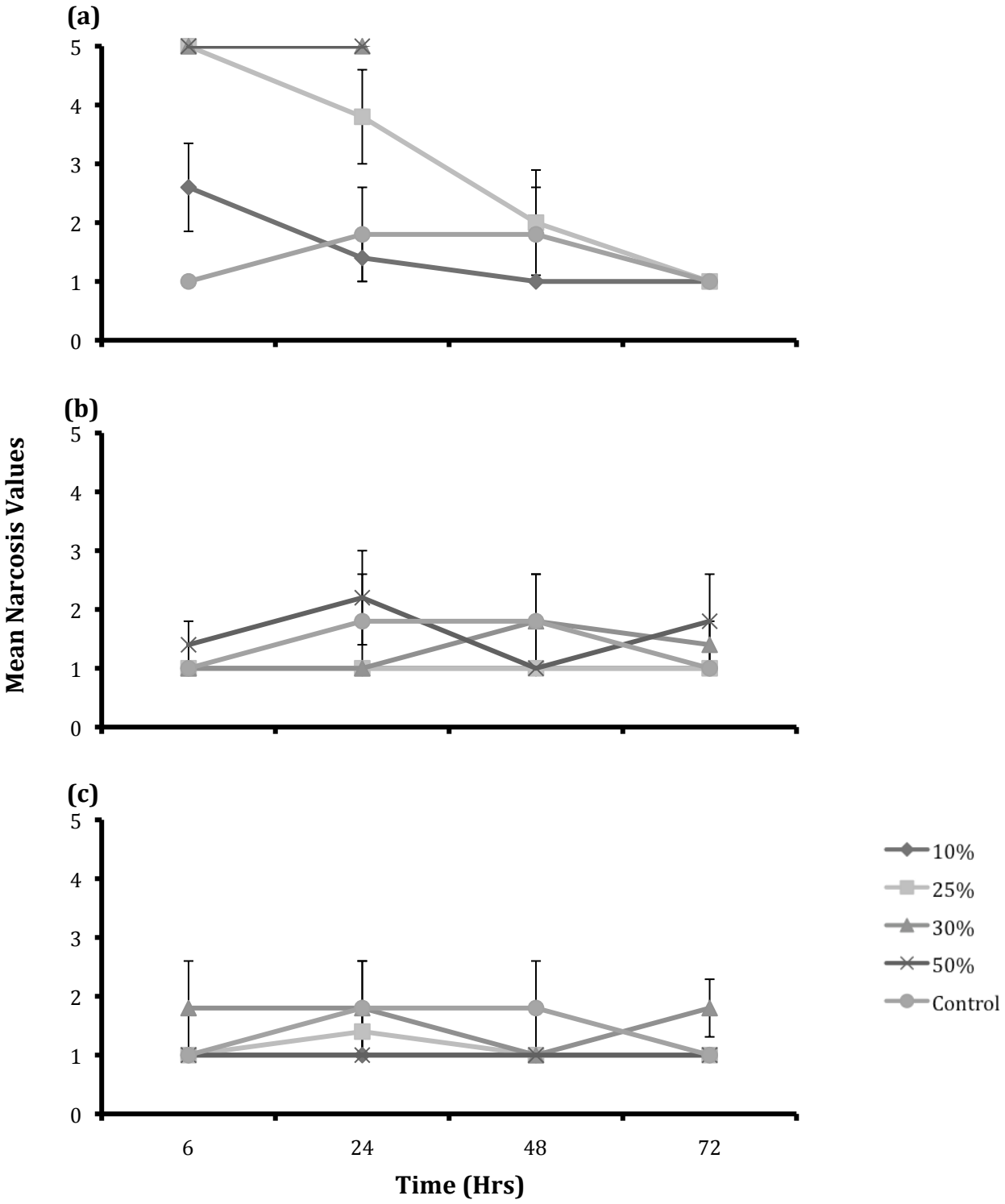

Figure 2-8. Mean narcosis values ± 1SE of *Ambystoma maculatum* at four time points(6, 24, 48, 72 hours) in a control (Distilled H_20) and 4 concentrations of water soluble fractions of gasoline(a) of kerosene (b) and of oil (c). Narcosis values range from 1 (normal tadpole behavior) to 5 (full narcosis, meaning no movement). Missing lines are equivalent to death of all replicates in a concentration. Controls were not analyzed statistically but are included for comparison.

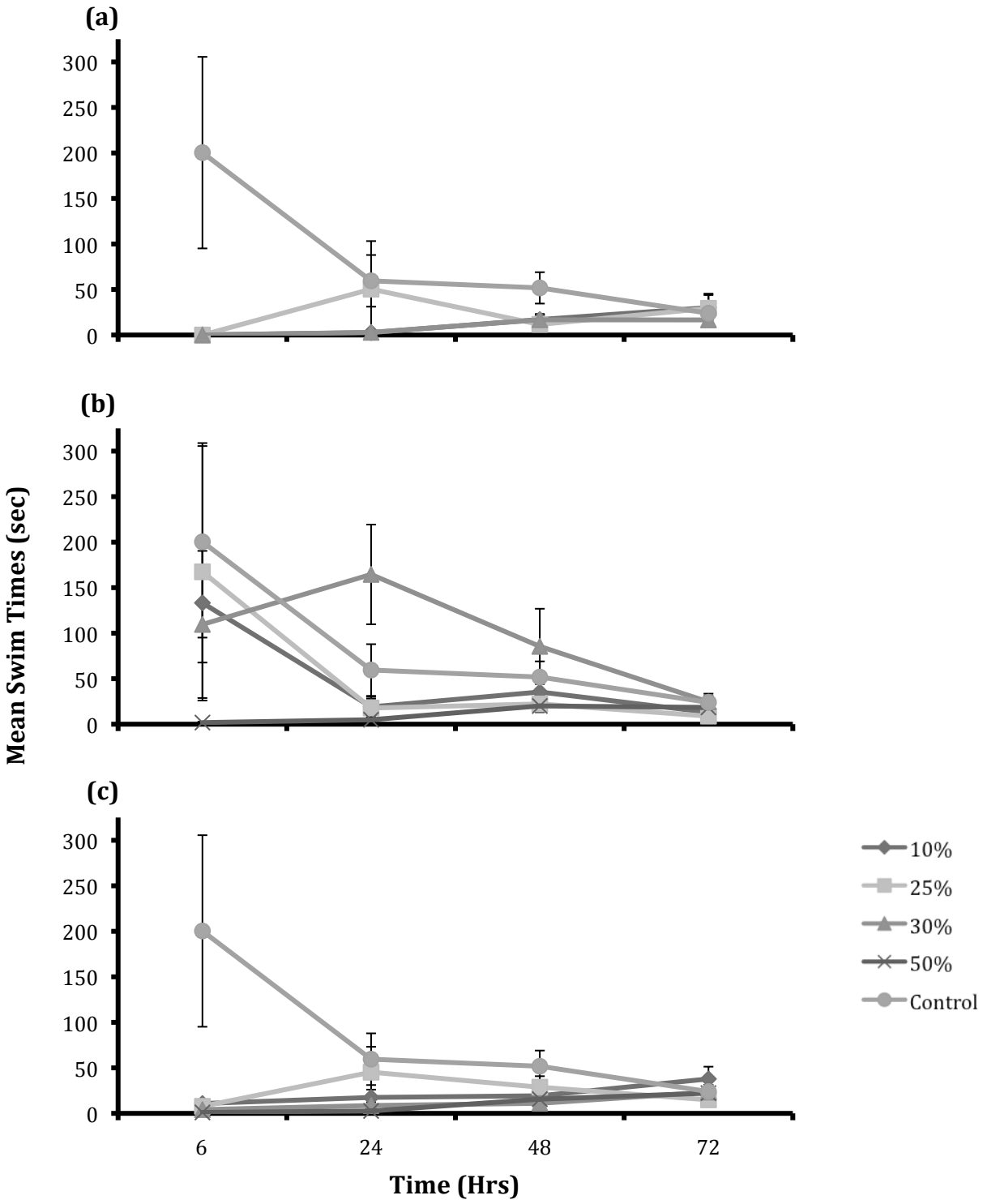

Figure 2-9. Mean swim time times (sec) ± 1SE of *Anaxyrus americanus* at four time points(6, 24, 48, 72 hours) in a control (Distilled H_20) and 4 concentrations of water soluble fractions of gasoline(a) of kerosene (b) and of oil (c). Missing lines are equivalent to death of all replicates in a concentration. Controls were not analyzed statistically but are included for comparison.

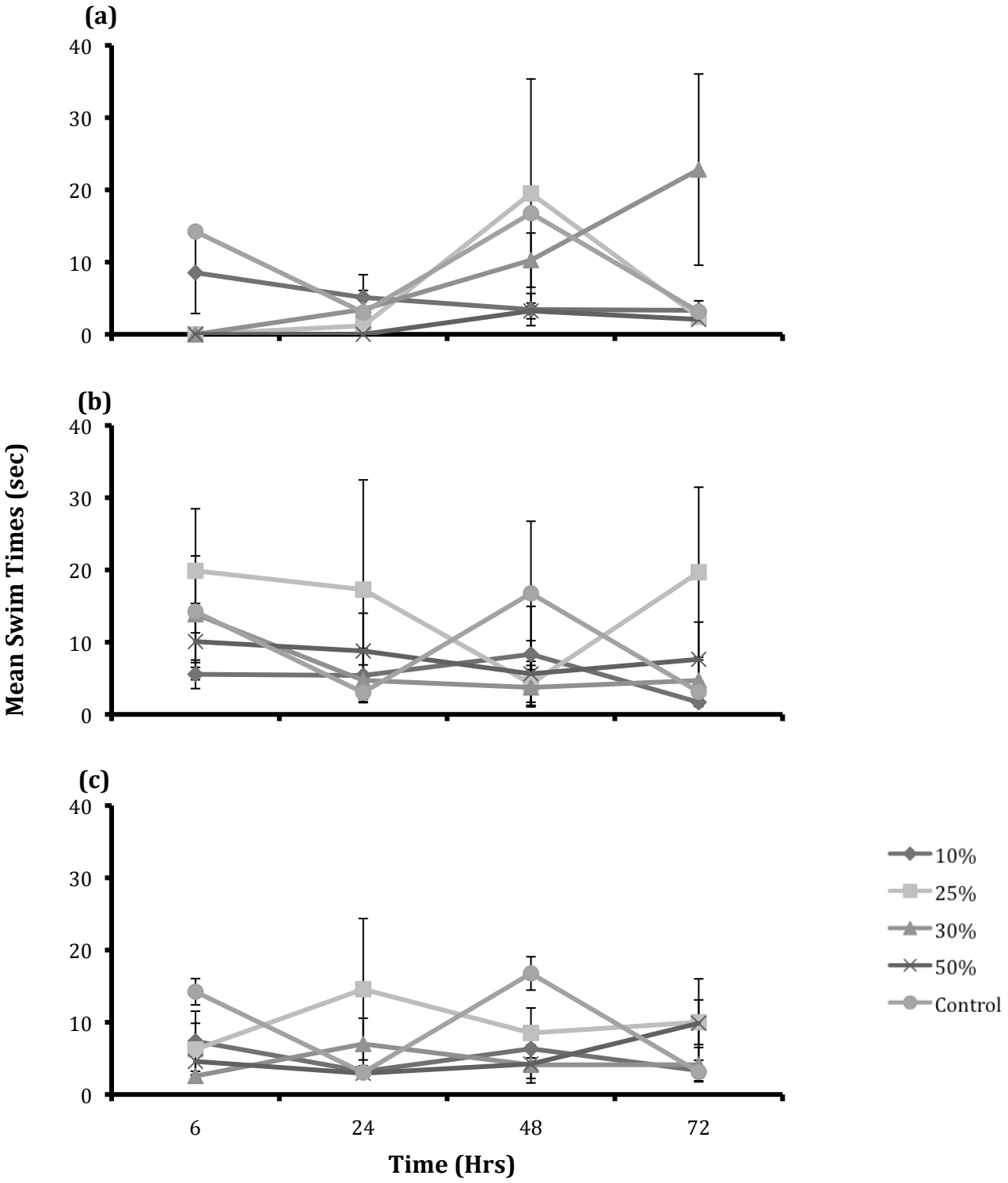

Figure 2-10. Mean swim time values ± 1SE of *Lithobates sphenocephalus* at four time points (6, 24, 48, 72 hours) in a control (Distilled H_20) and 4 concentrations of water soluble fractions of gasoline(a) of kerosene (b) and of oil (c). Missing lines are equivalent to death of all replicates in a concentration. Controls were not analyzed statistically but are included for comparison.

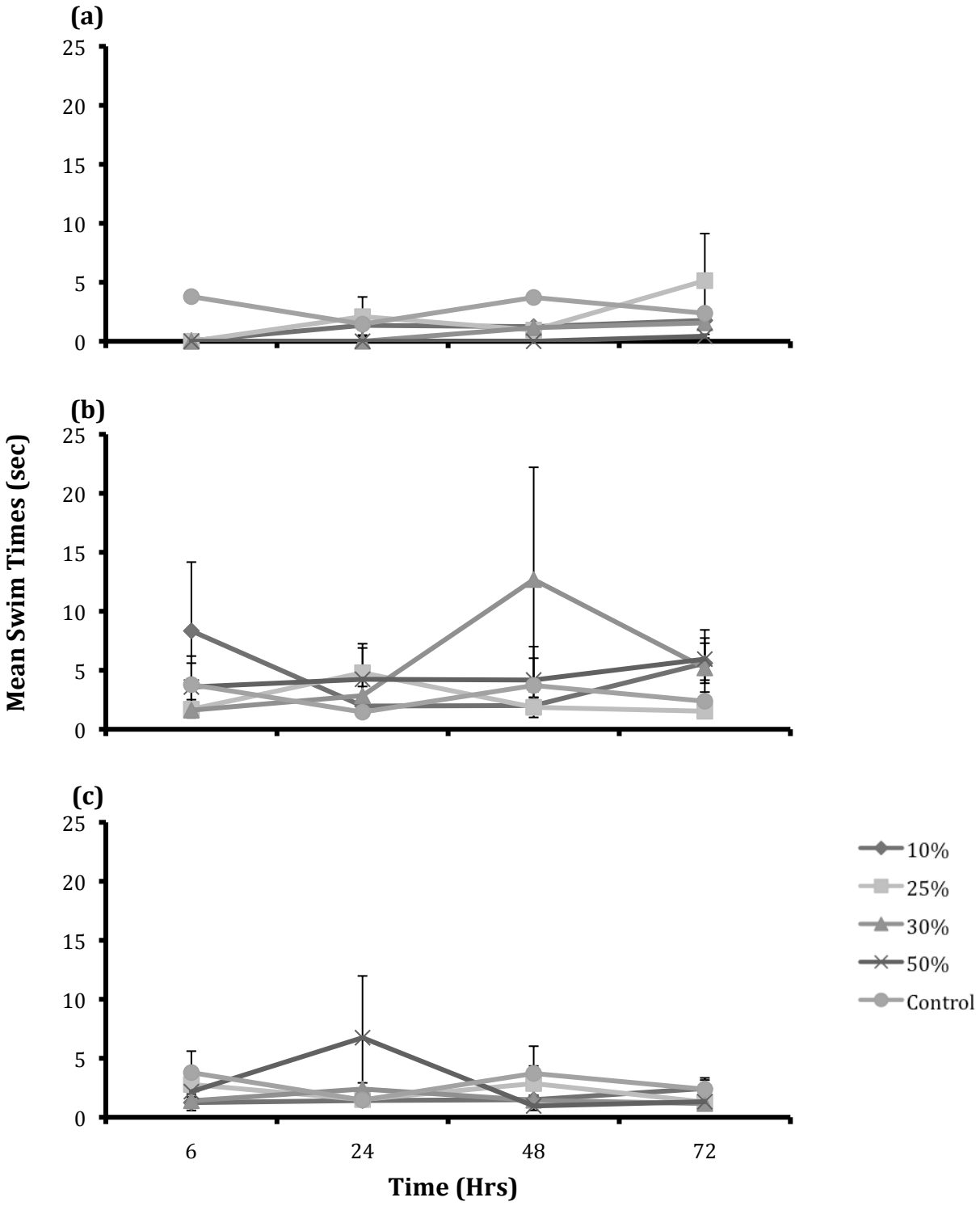

Figure 2-11. Mean swim time values ± 1SE of *Hyla chrysoscelis* at four time points(6, 24, 48, 72 hours) in a control (Distilled H_20) and 4 concentrations of water soluble fractions of gasoline(a) of kerosene (b) and of oil (c). Missing lines are equivalent to death of all replicates in a concentration. Controls were not analyzed statistically but are included for comparison.

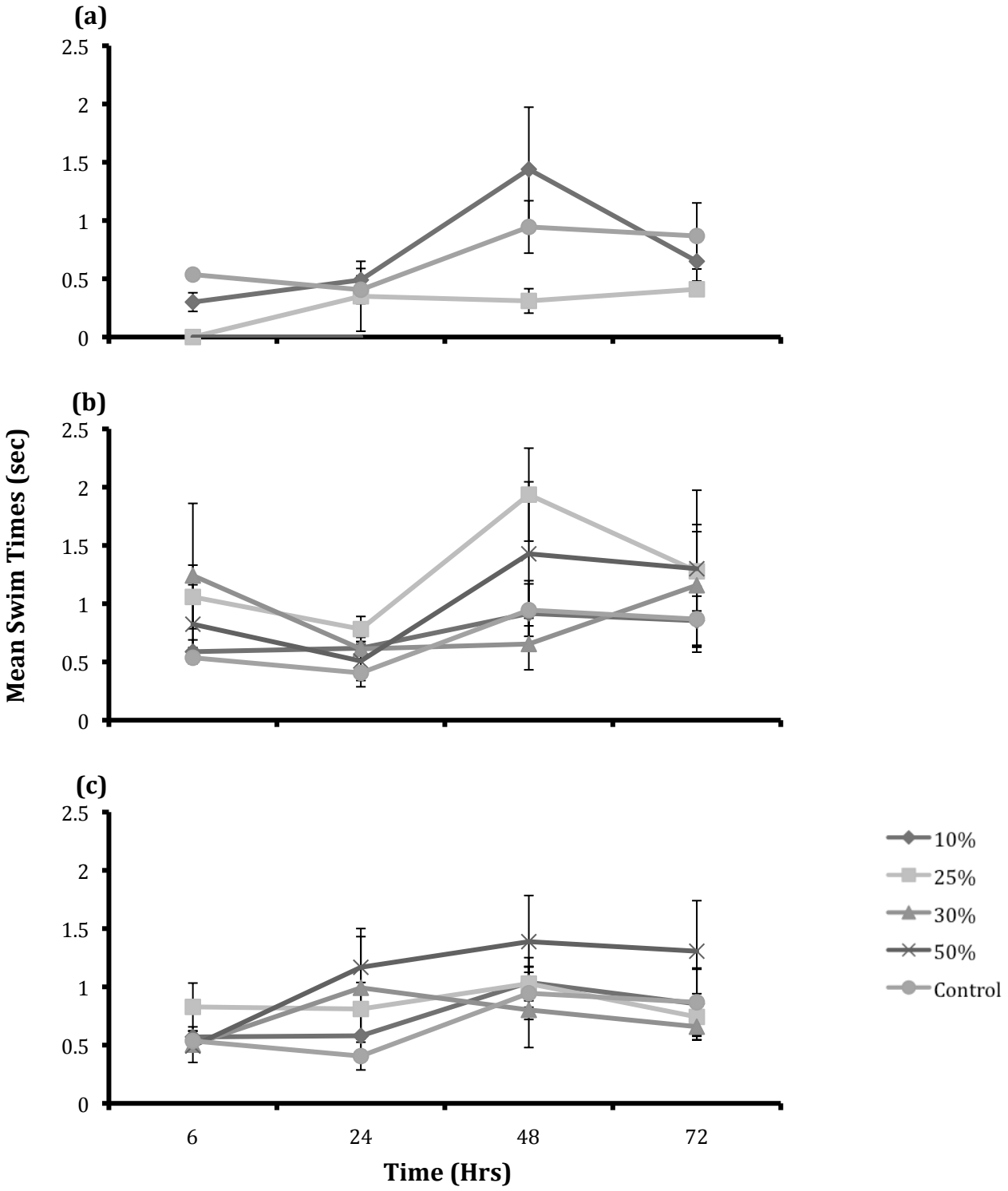

Figure 2-12. Mean swim time values ± 1SE of *Ambystoma maculatum* at four time points(6, 24, 48, 72 hours) in a control (Distilled H_20) and 4 concentrations of water soluble fractions of gasoline(a) of kerosene (b) and of oil (c). Missing lines are equivalent to death of all replicates in a concentration. Controls were not analyzed statistically but are included for comparison.

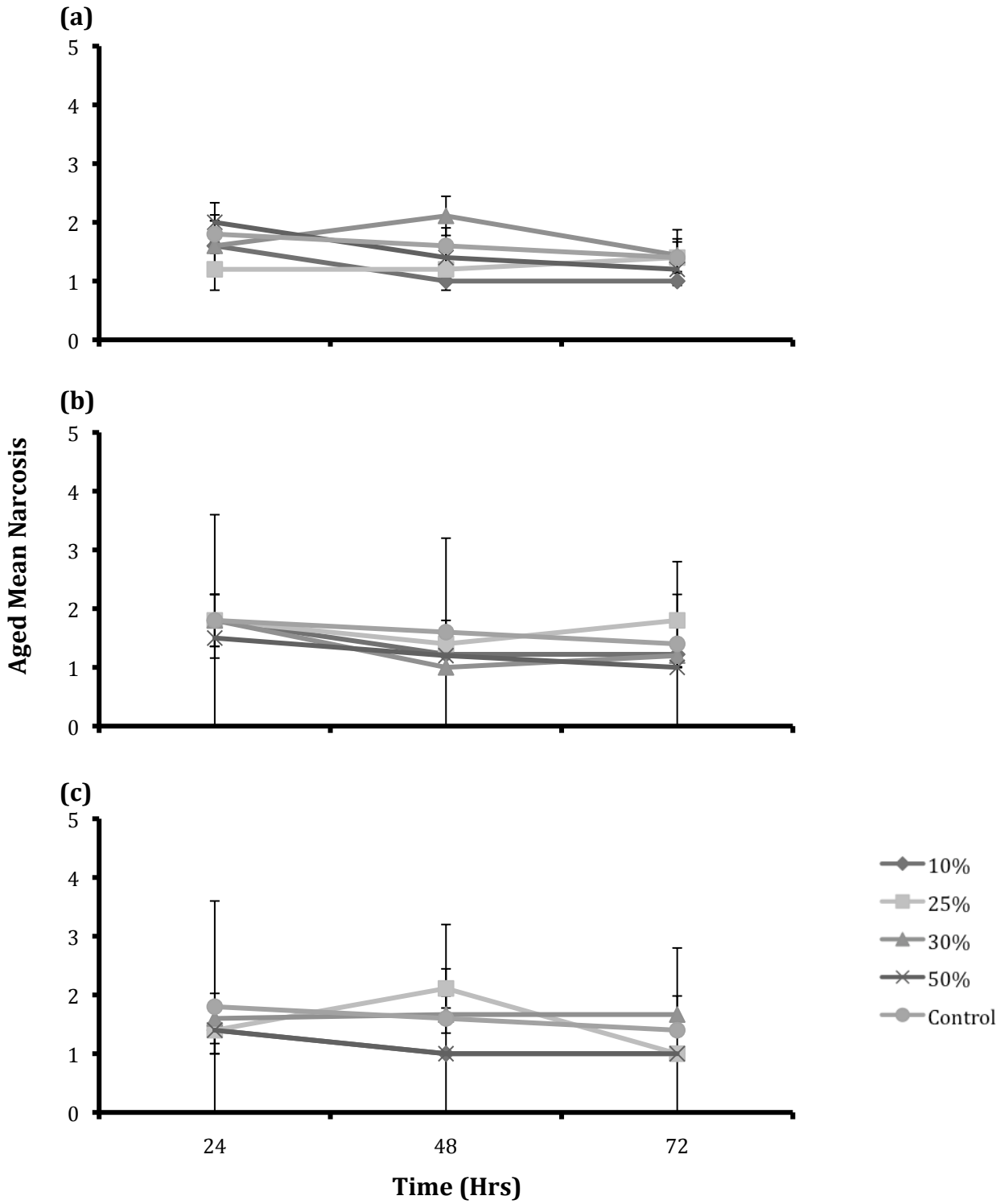

Figure 2-13. Aged mean narcosis values ± 1SE of *Hyla chrysoscelis* at three time points(24, 48, 72 hours) in a control (Distilled H_20) and 4 concentrations of water soluble fractions of gasoline(a) of kerosene (b) and of oil (c). Narcosis values range from 1 (normal tadpole behavior) to 5 (full narcosis, meaning no movement). Missing lines are equivalent to death of all replicates in a concentration. Controls were not analyzed statistically but are included for comparison.

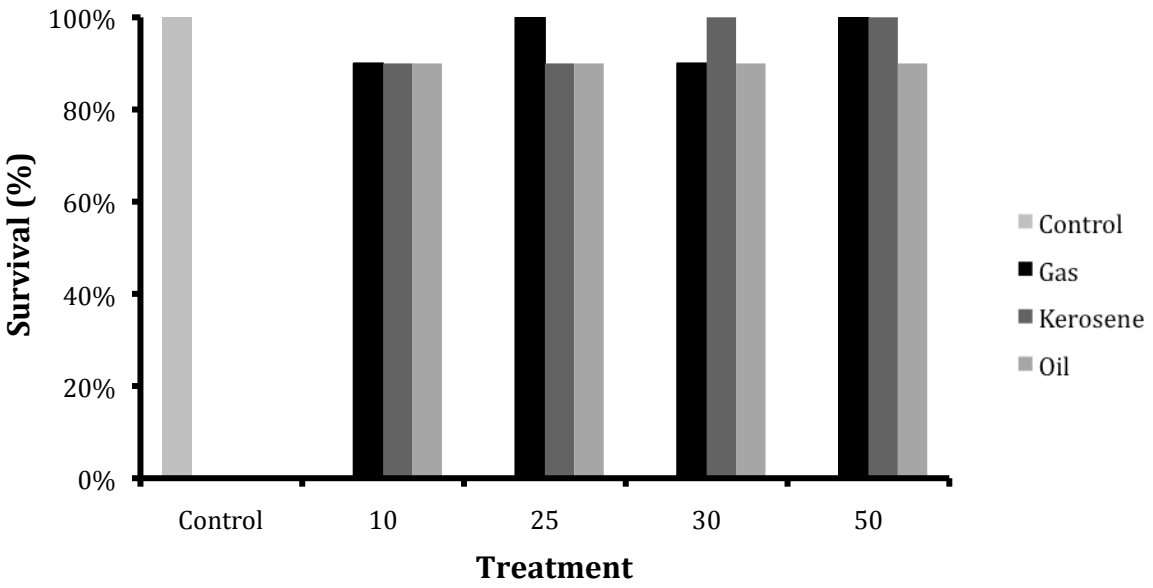

Figure 2-14. Aged experiment survival of *Hyla chrysoscelis* over 72 hours exposure to treatments of four concentrations of 72-hour aged water-soluble fractions of three distillates and a control (Distilled H_20).

Appendix 1: Research Compliance Copy

UNIVERSITY OF ARKANSAS

Office of Research Compliance

MEMORANDUM

TO: J. D. Willson

FROM: Craig N. Coon, Chairman
Institutional Animal Care
And Use Committee

DATE: February 6, 2013

SUBJECT: IACUC Protocol **APPROVAL**
Expiration date : **July 1, 2015**

The Institutional Animal Care and Use Committee (IACUC) has **APPROVED** Protocol **#13032 - "Effects of Hydraulic fracturing chemicals on anuran development**". You may begin this study immediately.

The IACUC encourages you to make sure that you are also in compliance with other UAF committees such as Biosafety, Toxic Substances and/or Radiation Safety if your project has components that fall under their purview.

In granting its approval, the IACUC has approved only the protocol provided. Should there be any changes to the protocol during the research, please notify the IACUC in writing [via the Modification Request form] **prior** to initiating the changes. If the study period is expected to extend beyond **07-01-2015**, you may request an extension [up to 02-03-2016] via the Modification Request form. By policy the IACUC cannot approve a study for more than 3 years at atime.

The IACUC appreciates your cooperation in complying with University and Federal guidelines for research involving animal subjects.

cnc/car

cc: Animal Welfare Veterinarian

Administration Building 210 • 1 University of Arkansas • Fayetteville, AR 72701-1201 • 479-575-4572
Fax: 479-575-3846 • http://vpred.uark.edu/199

Chapter 3: Chronic effects of petroleum distillates on *Anaxyrus americanus*

Introduction

There is no doubt the use of chemicals in the environment has the potential for impacts on wildlife. Chemicals enter the environment as human energy needs expand and the practices of acquiring these energy resources are inconsistently managed. Natural gas has been found abundantly in shale rock formations and is thought to be a better energy source than coal as it causes less air pollution (Rozell & Reaven 2012). Hydraulic fracturing is a process used to extract natural gas and includes the use of various chemicals, including petroleum distillates. The chemicals used in hydraulic fracturing could contaminate the water supply through transportation of fluids, well casing leaks, leaks through the fractured rock, drilling site discharge and wastewater disposal (Rozell & Reaven 2012).

Because of their permeable skin, amphibians are considered to be excellent environmental indicators (Gillilland et al. 2001). Most amphibians must undergo a complex metamorphosis, a particularly sensitive stage of the life history, (Sowers et al. 2009) which affects many of the bodies systems including respiratory, digestive, sensory, immune and skeletal (Vitt & Caldwell 2014). Breeding is another vulnerable time and selection of the environment for larval growth is critical for larval survival. Places likely to see runoff or spills containing petroleum distillates include areas where anurans choose to breed. These areas include ephemeral streams; water filled ditches or small ponds (Lannoo 2005).

Because petroleum distillates have a low solubility in water, a preparation of a water-soluble fraction was necessary (Pacheco & Santos 2001). The point of using a water-soluble fraction is to isolate polycyclic aromatic hydrocarbons (PAHs), a particularly strong compound with the ability to persist in the environment similar to polychlorinated biphenyl (PCB) (Pacheco & Santos 2001; Fedato et al. 2010). Dissolved hydrocarbons and

soluble contaminants such PAHs or PCBs are taken up by living cells (Patrick-Iwuanyanwu et al 2009). Negative effects from petroleum distillates or the WSF of petroleum distillates have been found in a variety of organisms (Fernandez & I'Haridon 1994; Phatarpekar & Ansari 2000; Lefcort et al. 1997). However, petroleum distillates have no effect on time to metamorphosis in *Rana pipiens* in a PCB study conducted by Glennemeier and Begnoche (2002). Concentrations of PCB's were low in *Hyla cinera* in Michigan and due to this low accumulation, few deformities were found (Gillilland et al. 2001).

There is little information concerning the long term lethal and sublethal effects of distillates in the aquatic environment. The persistence of these compounds in the environment may alter amphibian size at metamorphosis, survival through metamorphosis, or behavior (narcosis). Metamorphic climax is a sensitive period in the development of amphibians and is triggered by the thyroid hormone (Bergeron et al. 2011; Ortiz & Sparling 2007). Some contaminants can alter thyroid function and reduce amphibian population reproductive success (Ortiz & Sparling 2007). Effects can occur through different phases of development including sublethal effects to swimming behavior, timing of development and physical abnormalities.

I conducted a mesocosm experiment examining chronic effects of prolonged exposure to two low concentrations (10% and 2.5%) of three petroleum distillates on *Anaxyrus americanus*. The mesocosm design allowed us to evaluate interactions between the chemicals and environmental conditions (Sparling et al. 2010). I expected to see a delayed response the longer the exposure time, also survival is expected to be high and abnormalities are not expected. I expected to find delayed metamorphosis and smaller mass at metamorphosis.

Methods

Anaxyrus americanus breed and lay large clutches of eggs in strings in shallow ephemeral wetlands, pools, ponds, ditches or potholes (Lannoo 2005). The larval period is very short, approximately 50-60 days, with a rapid transformation from larvae to a terrestrial juvenile (Lannoo 2005). This species has been used in numerous studies where morphological and behavioral implications have been observed, including studies focused on pesticides (Relyea 2012), temperature (Jorgenson & Sheil 2008), heavy metals (Willson et al. 2012), and habitat alteration (Earl & Semlitsch 2013).

Anaxyrus americanus eggs were collected from various aquatic-breeding habitats in northwest Arkansas between 15 March and 31 March 2013. Multiple clutches were obtained which allowed for clutches to be stratified across treatments to account for potential clutch effects. After collection, eggs were maintained in pond water in buckets in a temperature-controlled laboratory (20-22°C, 12/12 L:D) until beginning the experiment.

Distillates and Preparation of Water Soluble Fractions

Three petroleum distillates were selected and purchased locally: engine oil, kerosene, and unleaded gasoline. Oil was automotive engine oil multigrade 10W-30 Rotella, considered a heavy distillate; Kerosene 1 K heater fuel and unleaded gasoline are considered light distillates. Twenty-four hours before the start of each experiment, water-soluble fractions of each distillate were prepared following the general methods of Anderson (1974). Briefly, 9 parts distilled water and 1 part distillate were spun for 24 h in a 1000 ml beaker. The beakers were tightly covered and spun at a medium speed where the vortex just reached the bottom of the beaker. They were then allowed to settle for 1 h,

after which the non-soluble portion was siphoned off. To be sure the entire non-soluble portion was siphoned off, a 100 ml layer was removed including the non-soluble portion. All beakers, stir bars, and equipment were used only for their respective distillate.

Based on acute experiments, I selected concentrations that would allow us to evaluate long-term sublethal effects including time to metamorphosis, mass and behavioral changes. I chose two concentrations of the water-soluble fraction of each distillate: 10%, and 2.5%. Total solution per each bucket equaled 5 L. This solution was given a half water change every 9 days. The water change included dropping the water level down by half to 2.5 liters and adding in dechlorinated water and the WSF of distillate to maintain original concentrations.

Experimental Procedures

A 3x2 factorial experiment was designed to evaluate mortality and chronic behavioral responses of *A. americanus* to two concentrations of each of the three distillates. Ten replications of each treatment and a control (dechlorinated water) were monitored by taking measurements of mortality, narcosis, and swim time, up to and through metamorphosis.

Experiments began when larvae were at Gosner stage 23-25. During this time external gills may still be visible and the mouthparts are developing (McDiarmid & Altig 1999). Before experimentation began, a subsample of larvae (not used in the experiment) from each clutch was weighed (nearest 0.001 g), Gosner stage determined, and length measured (nearest 0.01 mm using digital calipers). The first trial (10% concentration) lasted seven weeks, from 31 March to 20 May; water temperatures ranged from 5 to 26°C.

The second trial (2.5% concentration) lasted approximately 12 weeks, from 9 April to 27 June; bucket temperatures ranged from 11 to 36°C.

Experiments were conducted at an outdoor mesocosm facility at the University of Arkansas, which was paved with asphalt and allowed full sunlight. Larvae were housed individually in 19 L white plastic buckets, each of which contained 5 L of solution, including a one time inoculation of pond water, and were covered with a screen lid. Buckets were evenly spaced, numbered, and positioned randomly with respect to treatment. Water temperature was assessed every 9 days in two separate buckets containing 5 L of water. Solutions were changed and bucket positions were rotated as well at this time. Every 9 days, larvae were subjected to a stimulus test, weighed (nearest .001 g), assessed for Gosner stage, and fed. This process was done quickly to minimize stress; tadpoles were also checked for limb emergence and occasionally photographed. Tadpoles were fed goldfish granules (Aqueon Goldfish Granules; Central Aquatics Franklin, WI), beginning with 3 granules (.009g) until the first weighing. After the first weighing, tadpoles were offered 6% of the average body weight of all individuals every three days for the remainder of the experiment (Bergeron et al. 2011). Waste and excess food were removed in conjunction with water changes.

A stimulus test was used to assess narcosis and survival of each individual at 6, 24, 48, and 72 h. At each time point each subject received a prod once to the tail by a glass rod (Mann & Bidwell 2001). If the larvae were already swimming they also received a prod. Narcosis in response to the poke test was scored on a 1-5 scale, based on Mann & Bidwell (2001). The expected response is to swim swiftly away for more than 1 sec in a coordinated manner. If the subject met these expectations, then a value of 1 was recorded. If the subject

swam in a coordinated way but failed to swim longer than 1 sec, a value of 2 was recorded. If mild narcosis was present, the larvae swam for more than 1 sec but in an uncoordinated matter, such as on its side, and received a value of 3. If mild narcosis was present and the larvae swam less than one second then a value of 4 as recorded. Often larvae in mild narcosis only were able to lie at the bottom and twitch. Larvae in full narcosis displayed a total lack of activity in response to prod and received a value of 5. This value included dead larvae that were later removed when necrosis was evident (Mann & Bidwell 2001). Swim times were recorded to the nearest thousandth of a second. Narcosis observations were all made by the same observer, who was blind to treatments (Mann & Bidwell 2001). Mortality was noted only when the subject was clearly deceased with observable necrosis.

Survival was also checked daily and as subjects neared metamorphic completion, front limb emergence time was recorded as well as time to complete metamorphosis, judged by full tail re-absorption. When tadpoles approached metamorphosis and the emergence of forelimbs was recorded, substrate (plastic dish covered in paper towels) was added and they were checked daily. At this time, the water level was dropped down so the metamorph could easily climb up the paper towels onto the dish out of the water.

At the conclusion of the experiments subjects were euthanized with MS-222 Triacaine Methanesulfonate, labeled and frozen for future study.

Results

No statistical tests were run, the sample size was too small due to the high mortality occurring in each of the trials. I completed both trials with surviving tadpoles even though sample sizes were undesirable. One major weather event in April and another in May impacted my chronic studies and was the cause of many deaths.

Survival

The trends I observed in the first trial of exposure to 10% distillate included earlier mortality than the trends of the second trial of exposure to 2.5% distillate. At the beginning stages of the 10% concentration trial many died on Week 1, April 6th during a cold rain and a water temperature of 5° C. By the second week into the experiment, only half the tadpoles remained and survival of the controls was low. The unusual cold snow event, dropping water temperatures to 11° C around May 6th was 3 weeks into the second trial of 2.5% concentrations and killed 15 out of the 40 tadpoles. In the 10% treatment, survival to metamorphosis (Fig 3-1) was highest in the control group. Tadpoles' exposed to kerosene and oil had low survival percentages and tadpoles in gasoline had zero survival. In the 2.5% concentration trial, tadpoles in the gasoline treatment had the highest survival percentage. Control, kerosene and oil were all under 20% survival.

In the 10% concentrations of distillates it took 46 to 51 days for surviving tadpoles to complete metamorphosis. Mass at metamorphosis averaged 0.16 g. The treatments that survived through metamorphosis included 3 control, 1 oil and 2 kerosene. Exposure to 10% suggested gasoline as having the strongest impact to the tadpoles.

The trends in the 2.5% trial included higher survivorship with 4 control, 5 gasoline, 2 kerosene and 1 oil treatment reaching metamorphosis. It took 45 to 80 days for all remaining tadpoles to complete metamorphosis. Mean mass at metamorphosis was 0.08 g.

Narcosis

All larvae exposed to the 10% concentration of gasoline exhibited narcosis values of 5 at week 1 (Fig 3-2a), indicating full narcosis, and all individuals in this treatment were deceased by week 2. Surviving larvae exposed to 10% concentrations of kerosene and oil

appeared to show a slow recovery over time, but this pattern was not appreciably different from controls. Larvae exposed to a 2.5% concentration of gasoline appeared to increase somewhat in narcosis level over the course of the experiment (Fig 3-2b). Larvae exposed to 2.5% concentrations of kerosene and oil revealed variable patterns of narcosis that did not differ in a consistent way from controls.

Swim time

Swim time displayed a decrease over time in both trials (Fig 3-3). Although small sample sizes prevented statistical analyses, no clear differences in swim time were evident between distillates or concentrations.

Discussion

I evaluated the lethal and sublethal effects of chronic exposure to the water-soluble fraction of three petroleum distillates on larval amphibians. High mortality resulted in sample sizes that were too small to complete a definitive analysis.

Petroleum and its derivatives, such as gasoline, are among the pollutants reaching aquatic ecosystems that have the greatest ecological impact. Small continuous fuel leaks from gas stations constitute one of the principal sources of soil and water contamination. Widespread use in vehicles and machines make gasoline one of the most commonly spilled petroleum products in the environment (Fedato et al. 2010). Petroleum distillates used in the hydraulic fracturing process are occasionally applied to lands surrounding well sites where immediate effects have been observed (Adams et al. 2011). I find the benefits of doing chronic studies to be pertinent since these chemicals persist in the environment and can be readily taken up by amphibians, which are widely used as indicators of environmental pollution.

Contaminants absorbed by the environment or by tissues of biota in the environment come from the compounds of chemicals that are water-soluble (Fedato et al. 2010). Due to the fact that living cells take up dissolved hydrocarbons and soluble contaminants readily (Patrick-Iwuanyanwu et al. 2009), the creation of a water-soluble fraction (WSF) in my study was critical. Monocyclic aromatic hydrocarbons benzene, toluene, and xylene (BTX) concentrated in the WSF of gasoline are among the most damaging compounds to the environment, due to their high chronic toxicity (Fedato et al. 2010). Heavier aromatic structures are more persistent in the environment and have the potential for chronic toxicological effects (Cockerham & Shane 1994). Polycyclic aromatic hydrocarbons (PAHs) interacting with sunlight have the potential to be more genotoxic through photochemical production of its derivatives from the parent compounds (Fedato et al. 2010). The lingering WSFs in aquatic systems that have the potential to be taken up by the biota are likely to produce sublethal effects including impacts to growth, mass, and amount of time spent in the larval stage. Therefore, compared to other studies on petroleum products, my study focused on the hydrocarbons that are concentrated in the water-soluble fraction solutions.

In a mesocosm study by Smith and Dibble (2012) ammonium nitrate increased the mass at metamorphosis of *A. americanus* as was generally consistent with other studies. In the 10% concentration I saw an increase in average mass at metamorphosis and in the 5% concentration a decrease in average mass and therefore can draw no conclusions to how petroleum distillates may affect mass.

Studies at the critical developmental period surrounding metamorphosis have found increased mortality rates just before completion of metamorphosis (Glennemeier &

Begnoche 2002). Also some chemicals, such as perchlorate, actually inhibited metamorphosis, leading to higher mortality (Ortiz-Santaliestra 2007), indicating that pollutants can inhibit thyroid functions. Studies in fish have already found thyroid hormones to be affected by WSFs of distillates (Peter et al. 2007). Growth and timing to metamorphosis has been reduced in studies on *Hyla cinera* in used crankcase oil (Sparling et al. 2010). These delays in development could negatively impact populations of amphibians through reducing juvenile recruitment (Ortiz-Santaliestra & Sparling 2007). Due to high mortality events in my experiment, I was unable to ascertain that metamorphosis may be inhibited by the distillates. For mortality, the trends I observed in the 10% concentration suggested that this concentration over a longer time frame has the potential to be lethal or the trends may have shown that the unusually cold weather around April 6th caused early mortality. Gasoline had the highest mortality, which agrees with the results of my acute experiments. Studies clearly indicate chronic exposure to chemicals could have lethal and sublethal effects on amphibians and should be a high priority for future research.

Weather had a strong impact in my experiments. I found a difference in time to metamorphosis between my two concentrations. In the 10% concentration tadpoles surviving began the process of metamorphosis earlier than the 2.5% concentration. The cooler temperatures seen more often in the 10% concentration could have put pressure on the tadpoles to grow and metamorphose quickly to survive. Whereas with the warming temperatures and lack of pressures in the 2.5% concentration there was likely less environmental stress on the remaining tadpoles to grow or metamorphose quickly. Problems associated with metamorphosis can be exasperated by the interactions of

multiple stressors. Disease, competition, predation or climate change, may confound the problems of pollution on amphibians over a longer time frame, especially as they are undergoing metamorphosis. These environmental stressors on individual amphibians can lead to physiological changes (Sparling et al. 2010).

Sources of distillate pollution should be heavily monitored and studies assessed for levels that harm amphibians, among other wildlife. Pollutants have the ability to impose chronic effects through air, water or soil. Distillate pollution occurs from urban area runoff or extraction of energy sources from the earth, such as fracking. Further research testing fracking well fluid requires legal issues to be tackled and approval from landowner and gas companies but could tell scientists much more about how the costs of using this form of energy is impacting the environment. My experiments are a call for further research on energy use and its impacts to wildlife.

Future chronic studies concerning sources of distillate pollution issues should consider a mesocosm design with higher replication and possibly a location in a greenhouse where extreme weather events will not hinder the experiment. Also a chronic study concerning reproductive and maternal effects from petroleum distillates and their associated water-soluble components could allow us to better understand physiological changes associated with exposure and their implications for amphibian populations.

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Figures

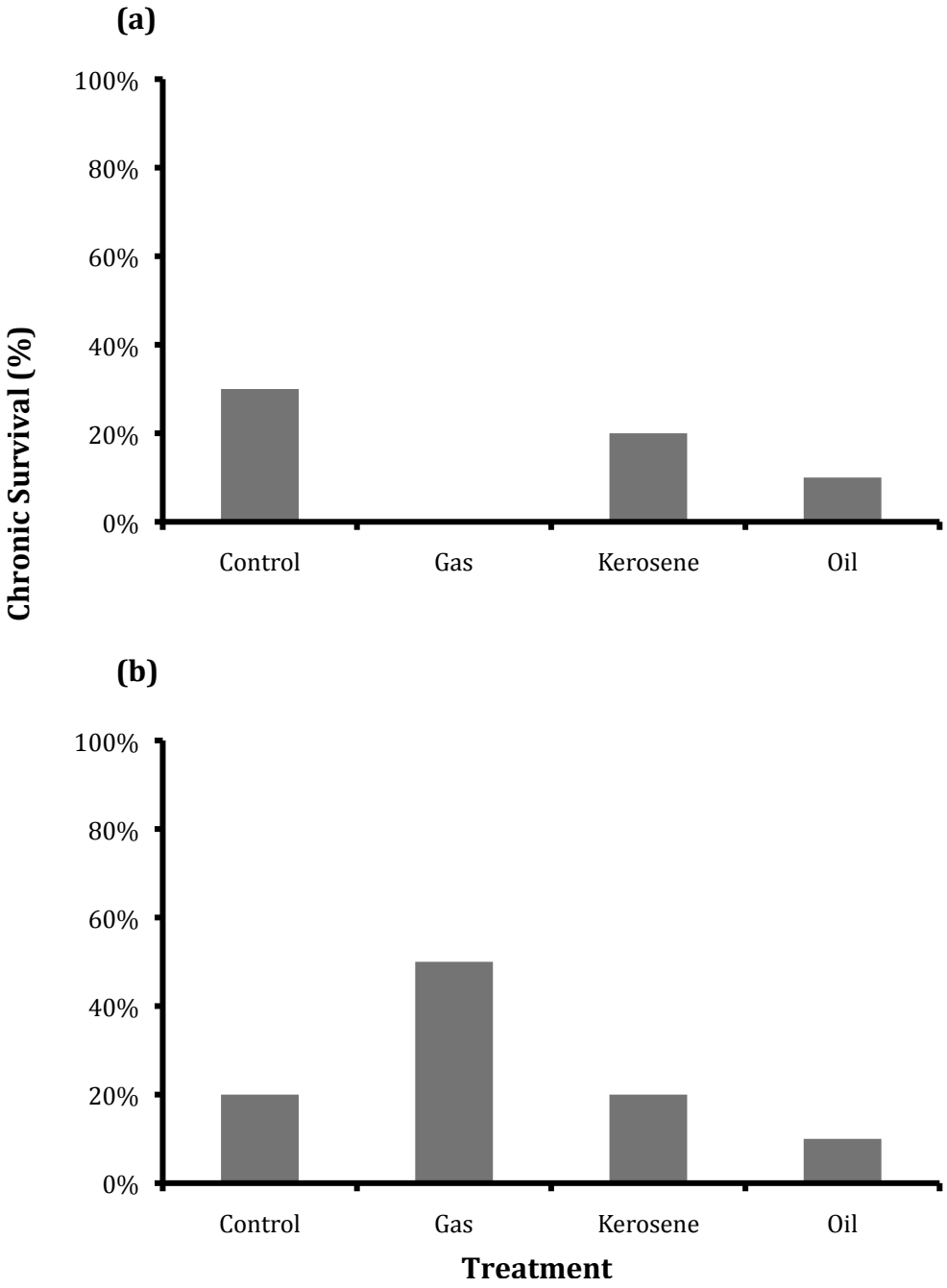

Figure 3-1. Survival of *Anaxyrus americanus* exposed to treatments of water soluble fractions of three distillates and a control (Distilled H_20) in 10% concentrations(a) and 2.5% concentrations(b). Absent bars are equivalent to death of all replicates in a treatment.

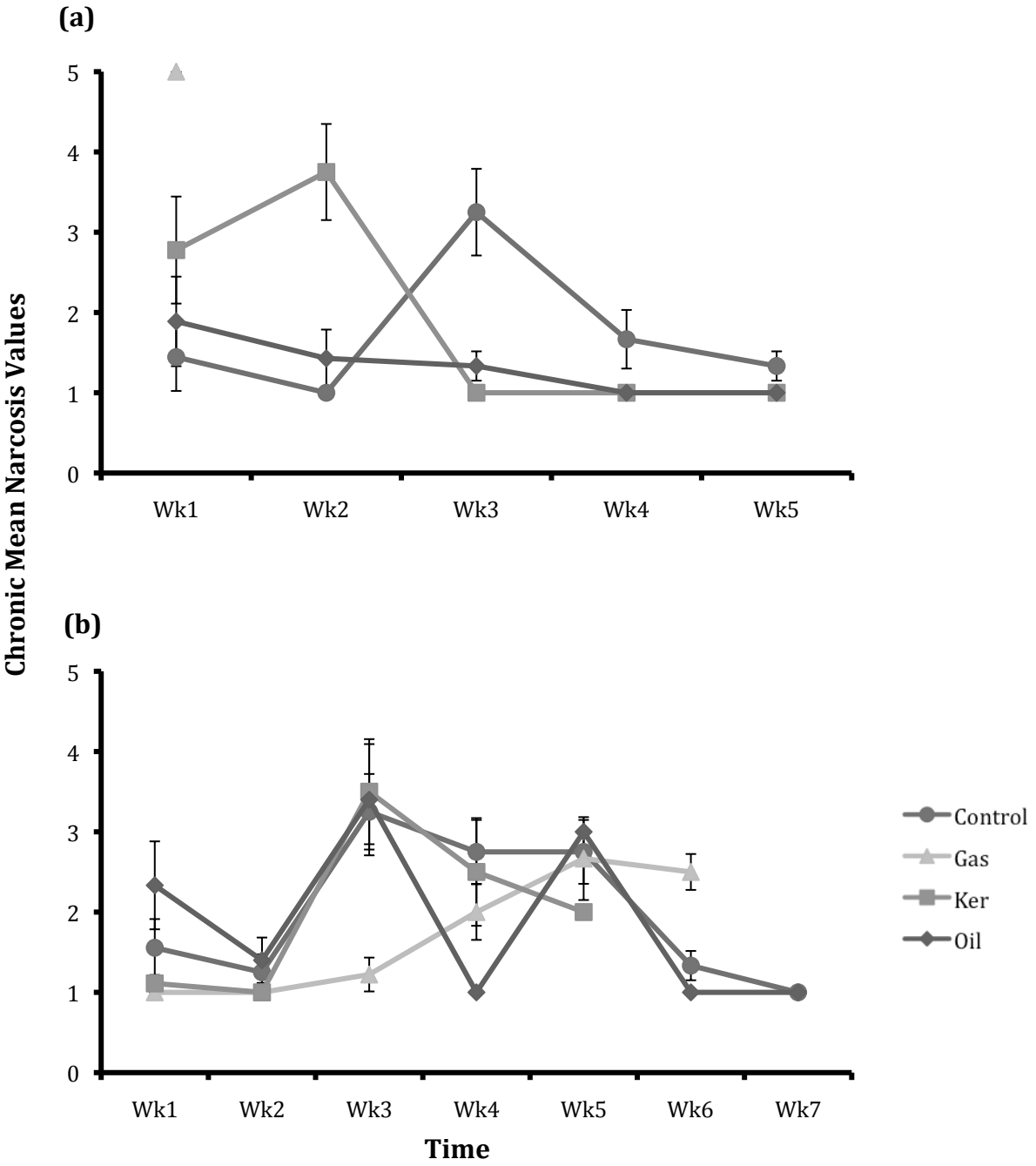

Figure 3-2. Mean narcosis values ± 1SE of *Anaxyrus americanus* for each week 10% concentrations (a) and 2.5% concentrations (b) in a control (Distilled H_20) and water-soluble fraction of 4 concentrations of gasoline of kerosene and of oil. Narcosis values range from 1 (normal tadpole behavior) to 5 (full narcosis, meaning no movement). No data at late weeks indicates metamorphosis. Small and unbalanced sample sizes precluded statistical analyses.

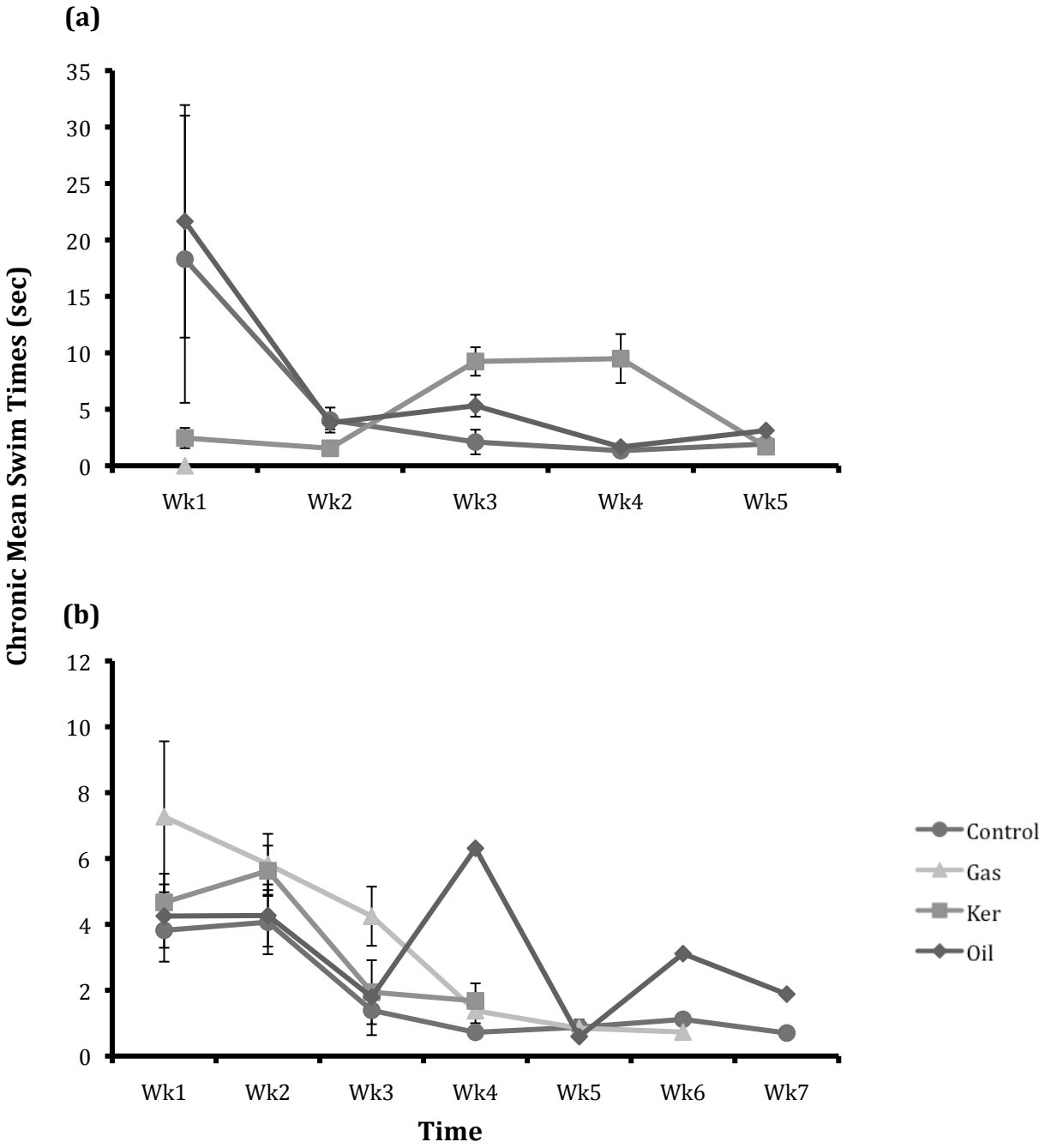

Figure 3-3. Mean swim time values ± 1SE of *Anaxyrus americanus* for each week in trial 1(a) and trial 2(b) in a control (Distilled H_20) and water-soluble fraction of 4 concentrations of gasoline, of kerosene, and of oil. No data at late weeks indicates metamorphosis. Small and unbalanced sample sizes precluded statistical analyses.

Appendix 1: Research Compliance Copy

UNIVERSITY OF ARKANSAS

Office of Research Compliance

MEMORANDUM

TO: J. D. Willson

FROM: Craig N. Coon, Chairman
Institutional Animal Care
And Use Committee

DATE: February 6, 2013

SUBJECT: IACUC Protocol **APPROVAL**
Expiration date : **July 1, 2015**

The Institutional Animal Care and Use Committee (IACUC) has **APPROVED** Protocol **#13032 - "Effects of Hydraulic fracturing chemicals on anuran development**". You may begin this study immediately.

The IACUC encourages you to make sure that you are also in compliance with other UAF committees such as Biosafety, Toxic Substances and/or Radiation Safety if your project has components that fall under their purview.

In granting its approval, the IACUC has approved only the protocol provided. Should there be any changes to the protocol during the research, please notify the IACUC in writing [via the Modification Request form] **prior** to initiating the changes. If the study period is expected to extend beyond **07-01-2015**, you may request an extension [up to 02-03-2016] via the Modification Request form. By policy the IACUC cannot approve a study for more than 3 years at atime.

The IACUC appreciates your cooperation in complying with University and Federal guidelines for research involving animal subjects.

cnc/car

cc: Animal Welfare Veterinarian

Administration Building 210 • 1 University of Arkansas • Fayetteville, AR 72701-1201 • 479-575-4572
Fax: 479-575-3846 • http://vpred.uark.edu/199

Ch. 4 Conclusions

I evaluated the lethal and sublethal effects of acute and chronic exposure to the water-soluble fraction of three petroleum distillates on larval amphibians. I found gasoline to cause the highest mortality and highest narcosis scores likely due to the high BTX content present only in gasoline (Fedato et al. 2010). My experiments also revealed the potential for tadpoles to experience temporary narcosis in response to exposure to petroleum distillates, which can increase predation risks, slow growth and possibly delay metamorphosis. In many aquatic ecosystems amphibians are likely to be further stressed by competition and predation (Sparling et al. 2010). Over time narcosis values decreased and a pattern of recovery from petroleum distillate contaminants was present. The aged distillate experiment with low narcosis values suggested that distillates continue to degrade over time in solution and possibly volatized out of solution. My chronic experiments had the goal of looking at the effects during metamorphosis including mass and time to complete tail re-adsorption. These effects are likely only to be seen if chemicals persist in the environment.

Petroleum and its derivatives, such as gasoline, are among the pollutants reaching aquatic ecosystems that have the greatest ecological impact. Sources of petroleum reaching aquatic environments include road runoff, leaks from tanks and vehicles transporting distillates, and the use of petroleum products in hydraulic fracturing, (Fedato et al. 2010; Adams et al. 2011). Water-soluble fractions of petroleum distillates are one of the contaminants of aquatic environments causing a strong ecological impact, due to the increased isolation of PAHs (Fedato et al. 2010). An ecological effect to food webs, communities, and ecosystems can occur as the PAHs may potentially biomagnify in

organisms through predator prey interactions. My results suggest that lingering WSFs of petroleum distillates in aquatic systems are likely to produce sublethal effects including impacts to individual growth or predator avoidance.

My studies have revealed potential negative effects to wildlife from chemicals used in the process of hydraulic fracturing. Further research should include understanding more about how the process of hydraulic fracturing is impacting the environment in chronic and acute timeframes. This research would require legal issues to be tackled but could provide critical knowledge in the extraction of this energy source. Multiple factors, such as disease, climate and habitat change, over a longer time frame are likely to confound the problem of pollution on amphibians and should be incorporated into experiments as well. My experiments raise awareness concerning energy use and its impacts to our wildlife.

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