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EFFECTS OF THE HERBICIDE IMAZAPYR ON JUVENILE OREGON SPOTTED FROGS

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Abstract—Conflict between native amphibians and aquatic weed management in the Pacific Northwest is rarely recognized because most native stillwater-breeding amphibian species move upland during summer, when herbicide application to control weeds in aquatic habitats typically occurs. However, aquatic weed management may pose a risk for aquatic species present in wetlands through the summer, such as the Oregon spotted frog (OSF, *Rana pretiosa*), a state endangered species in Washington. Acute toxicity of herbicides used to control aquatic weeds tends to be low, but the direct effects of herbicide tank mixes on OSFs have remained unexamined. We exposed juvenile OSFs to tank mixes of the herbicide imazapyr, a surfactant, and a marker dye in a 96-h static-renewal test. The tank mix was chosen because of its low toxicity to fish and its effectiveness in aquatic weed control. Concentrations were those associated with low-volume (3.5 L/ha) and high-volume (7.0 L/ha) applications of imazapyr and a clean-water control. Following exposure, frogs were reared for two months in clean water to identify potential latent effects on growth. Endpoints evaluated included feeding behavior, growth, and body and liver condition indices. We recorded no mortalities and found no significant differences for any end point between the herbicide-exposed and clean-water control frogs. The results suggest that imazapyr use in wetland restoration poses a low risk of direct toxic effects on juvenile OSFs. Environ. Toxicol. Chem. 2013;32:228–235. © 2012 SETAC

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INTRODUCTION

The leading causes of amphibian decline in the Pacific Northwest are habitat loss and deterioration [1]. The introduction and spread of invasive species can lead to extinctions of native species [2]. Wetlands may be particularly vulnerable to invasion by nonnative plant species, especially when surrounding landscape changes alter wetland hydrology and nutrient levels [3]. Invasive plants can alter habitats, reduce the abundance and diversity of animal species, alter nutrient cycles, and potentially change food-web dynamics (reviewed in Zedler and Kercher [3]). Many invasive wetland plants form monocultures, establishing and maintaining dominance in wetlands through a combination of factors including tolerance to variable hydrologic conditions, high seed production or viability in wet conditions, and ability to spread vegetatively by rhizome expansion or movement of stem or root fragments [3]. The ability of these plants to dominate wetlands coupled with increasing restrictions on chemical control complicates habitat restoration for native species, including amphibians.

The Oregon spotted frog (OSF, *Rana pretiosa*) is a federal candidate for listing under the U.S. Endangered Species Act [4], listed as vulnerable on the International Union for Conservation of Nature Red List (www.iucnredlist.org, accessed August 2012), and listed as endangered in Canada (www.cosewic.gc.ca/, accessed August 2012) and in Washington State (wdfw.wa.gov/, accessed August 2012). Habitat loss and degradation are considered among the most likely causes of the decline and extirpation of the species from 70 to 90% of its former range [5]. Loss and alteration of shallow breeding wetlands are of particular concern [5], including degradation caused by invasive reed canarygrass (*Phalaris arundinacea*) [6].

The Washington Department of Fish and Wildlife is responsible for OSF conservation in Washington State. Habitat enhancement/recovery for the OSF is a top priority of the U.S. Fish and Wildlife Service. Washington State's Wildlife Action Plan makes habitat enhancement and recovery a top priority for at-risk species. Washington State lists wetlands among the priority habitats most at risk (wdfw.wa.gov/conservation/cwcs/, accessed August 2012), making them the most deserving of recovery efforts. The relatively recent spread of reed canarygrass into areas formerly occupied by the OSF has led to a focus on reed canarygrass control in efforts to restore OSF habitats.

Control of invasive aquatic plants is often difficult because mechanical or manipulative approaches used to date show limited efficacy or are restricted in application because of local conditions. Reed canarygrass is particularly difficult to manage, leading to the establishment of the Reed Canarygrass Working Group within the Northwest Chapter of the Society for Ecological Restoration. A publication from The Nature Conservancy details control options in the Pacific Northwest [7]. Based on available options, effective reed canarygrass control apparently can be achieved only through a long-term commitment using a combination of several different methods, including herbicide application [7].

Few herbicides are approved for use in aquatic habitats in Washington State (www.ecy.wa.gov/programs/wq/plants/management/aqua028.html, accessed August 2012). For those that are approved, data on their effects on native amphibians are lacking. This limitation effectively restricts herbicide use in most habitat enhancement/recovery efforts that could benefit at-risk species. Imazapyr and glyphosate are two nonselective

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herbicide active ingredients that are recommended for control of emergent aquatic weeds such as reed canarygrass in the Pacific Northwest [8] and allowed for use in aquatic habitats of Washington (www.ecy.wa.gov/programs/wq/pesticides/regpesticides.html, accessed August 2012). Imazapyr is considered to be among the least toxic herbicides available for use in aquatic environments, with a 96-h median lethal concentration (LC50) > 100 mg/L for fish and aquatic invertebrates; but few data exist on its toxicity to amphibians [9]. Depending on the chemical and life stage, amphibians may be more or less sensitive than fish [10]. The only study available on the toxicity of imazapyr to amphibians is as yet unpublished but shows very low toxicity (www.cal-ipc.org/symposia/archive/pdf/2008/7Trumbo.pdf, accessed August 2012). The 96-h LC50 of Habitat (28.7% imazapyr IPA salt) for American bullfrog (*Lithobates catesbeianus*) tadpoles was 1,739 mg/L (95% confidence interval [CI] 990.6–2256.7 mg/L). This suggests lower toxicity than triclopyr TEA, the active ingredient of a selective herbicide also allowed for use in aquatic environments (96-h LC50 814.1 mg/L, 95% CI 769.6–847.1 mg/L; www.cal-ipc.org/symposia/archive/pdf/2008/7Trumbo.pdf, accessed August 2012).

Herbicide tank mixes include the formulated product and additional carriers (e.g., water) and may also contain a surfactant and marker dye. Herbicide products labeled for use in aquatic systems are generally formulated without the addition of surfactants, which can increase the efficacy of the product but also its toxicity (e.g., glyphosate-based products with the surfactant polyethoxylated tallow amine labeled for terrestrial application [11]). Surfactants allow the herbicide to penetrate the leaf cuticle, thereby increasing its efficacy. The addition of a surfactant approved for use in aquatic environments is recommended for emergent aquatic weed control. One of the least toxic surfactants (based on LC50s) approved for use in Washington is Agri-Dex, which consists of a mixture of paraffin-based petroleum oil, polyoxyethylene, and sorbitan fatty acid ester (www.ecy.wa.gov/programs/wq/pesticides/regpesticides.html, accessed August 2012). It represents the surfactant of choice for control of reed canarygrass in the OSF habitat, yet no data exist for effects of tank mixes containing imazapyr products + Agri-Dex on amphibians.

A key to understanding the potential effects of herbicides on amphibians is identification of the life stages at risk of exposure to herbicide at the time of weed control. For reed canarygrass, herbicide application in September may achieve the greatest control with the least amount of herbicide [12]. Postmetamorphic juveniles are the youngest OSF life stage (i.e., potentially the most vulnerable to herbicide toxic effects) present in September (A. Yahnke, unpublished data). For management agencies to proceed with reed canarygrass control in OSF habitats, it will be critical to demonstrate that harm to the species targeted for conservation does not occur from habitat-restoration efforts.

The present study was designed to examine the acute and latent effects of operational imazapyr tank mixes on juvenile OSFs under laboratory conditions. Frogs were exposed for 96 h and then reared in clean water for two months to assess latent effects on growth. In addition, because physiological effects can manifest through stress in the metabolic or detoxification pathways such as fatty inclusions or enlargement of the liver [13], liver condition indices were compared across treatments. Finally, because of concerns about the potential for endocrine disruption from exposure to environmentally relevant concentrations of some herbicides (e.g., atrazine [14]), the gonads were visually inspected for gross anomalies and discrepancies between primary and secondary sexual characteristics.

MATERIALS AND METHODS

Experimental design

Postmetamorphic juvenile OSFs were exposed to two tank mixes with different herbicide concentrations associated with low- and high-volume applications and a clean-water control in a 96-h static-renewal test. Five aquaria were assigned to each herbicide tank mix and the control. Three frogs were randomly placed in each aquarium. Replicate tanks were randomly distributed on three sides of a water table (used to maintain constant temperature in the test aquaria), five tanks per side. After the 96-h exposure period, frogs were reared in clean water for two months.

Study animals

Oregon spotted frogs were reared from eggs collected from Conboy Lake National Wildlife Refuge (Glenwood, WA, USA). Forty-five juveniles were obtained from the rearing facility (Woodland Park Zoo, Seattle, WA, USA) on August 18, 2010, within four weeks postmetamorphosis. Prior to transfer, the rearing facility confirmed that the frogs were not infected by the amphibian chytrid fungus *Batrachochytrium dendrobatidis* and certified them in good health. At the start of the 96-h exposure, on August 23, frog masses averaged 3.3 g (± 1.3 standard deviation [SD]) and body sizes (snout–vent length [SVL]) averaged 29.0 mm (± 3.4 SD). Frogs were too young to be sexed at the time of distribution to treatment tanks. At the end of the experiment, gender assessment revealed male to female ratios of 5:10 in the control group, 7:8 in the low group, and 8:7 in the high group.

Animal husbandry

All materials (aquaria, floats, nets, buckets) to which the frogs were exposed were presoaked in a buffered, polyvinylpyrrolidone iodine solution (1:200 dilution, Ovadine; Western Chemical), then rinsed and soaked in dechlorinated water before use. All nets and any other reused materials were separated by treatment, held in the same solution of iodine (in separate buckets by treatment), and rinsed in dechlorinated water immediately prior to use.

Frogs were housed in enclosed 37.9-L glass aquaria held in a flow-through water table, with water temperatures maintained at 21.9°C (± 1.0 SD). Light was provided along the edge of one short side of the tanks from 2% UVB fluorescent bulbs (Reptisun 2.0 UVB; ZooMed) located within 45 cm of the bottom of the tank and filtered through the nylon mesh screen top [15]. Ultraviolet lights and overhead room lights were synchronized to a 13:11 light:dark cycle, the approximate duration of daylight at the time of the study. Upon arrival from the rearing facility, frogs were acclimated to the aquaria for 3 d prior to the start of the 96-h exposure. During the acclimation and exposure periods, aquaria were filled with 4 kg of clean water or clean water with treatment solution, respectively, corresponding to a fluid depth of approximately 2 cm. That depth was sufficient for submersion of the frogs and to allow them to maintain an energy-conserving, semifloating position with the hind limbs contacting the bottom. All water changes used dechlorinated City of Seattle water.

Each frog was offered five three-week-old crickets (Fluker Farms) twice per day, at approximately 0900 and 1700 h. Frogs were allowed to forage undisturbed for 20 min, after which the

remaining crickets were counted and removed, along with any other waste, to minimize effects on water quality. During the grow-out in clean water, crickets were dusted with calcium (Tetrafauna Reptocal; Tetra Werke) and vitamins (Reptivite without D_3; Zoo Med Laboratories) prior to feeding on days when full water changes were scheduled (after feeding), to minimize frog exposure to conductivity changes in the water from the vitamins.

Floats were used as feeding platforms throughout the experiment and as haul-outs and refugia during grow-out. Floats were constructed from 1.6-cm diameter chlorinated PVC pipes and 15 $cm^2 \times 0.45$ cm clear plastic (Lucite International) plates. Fifteen-centimeter pipes were connected with 90° chlorinated PVC elbows using drinking water–grade PVC cement (Rain-R-Shine PVC Cement 30890; Oatey) to make a square float. A 0.45-cm-deep feeding well was created by cutting an 11.5-cm-diameter hole into the center of the plastic plate and attaching a second plastic plate, 12 cm diameter × 0.20 cm, with aquarium silicone sealant (All-Glass Aquarium). The plastic was sand-blasted to create a more opaque plate that the frogs could also use as a refuge during grow-out. The corners of the plastic plates were attached to the middle of each pipe on the chlorinated PVC floats with aquarium silicone (Fig. 1). The attachment created holes at the corners of the chlorinated PVC pipe squares, where frogs could emerge from the opaque refuge while maintaining a sense of cover, thereby providing some habitat complexity in an effort to minimize stress in the laboratory environment. Floats were provided for only 20 min at the 0900- and 1700-h feedings during the 96-h tank mix exposure but were placed in aquaria continuously during the two-month grow-out.

At the end of the 96-h exposure, frogs from herbicide treatments were transferred to new aquaria and new floats were provided. All aquaria initially received 5.25 L of clean, dechlorinated water. Water volume was increased during the grow-out period to account for frog growth and the associated increase in ammonia levels. Full water changes were made every 2 to 4 d during grow-out, and partial water changes were made as needed based on water quality.

Temperature (°C) in the water bath was monitored daily, with current, minimum, and maximum values recorded approximately every 24 h. Water pH, dissolved oxygen (DO; mg/L), and conductivity (μS/cm) levels were monitored daily during the 96-h exposure, prior to renewal, and at full water changes during the grow-out. Waterproof electronic testers were used to determine instantaneous pH and conductivity (PCTester 35 and ECTester 11; Oakton Instruments) as well as DO and temperature (HQ-10; Hach). Water-quality measurements were collected from mixing buckets prior to distribution of treatment solutions. Ammonia (ppm) levels were monitored daily with API Freshwater/Saltwater Ammonia Test Kits (Mars Fishcare) during the 96-h exposure and at full and partial water changes during the grow-out.

Frogs were killed at the end of the experiment by submersion in MS-222 at 3 g/L with equal sodium bicarbonate for 90 min. Livers were extracted and weighed, and primary sex organs were observed to confirm sex determination from secondary sex characteristics and to check for any overt abnormalities. All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Washington, protocol 2185-42.

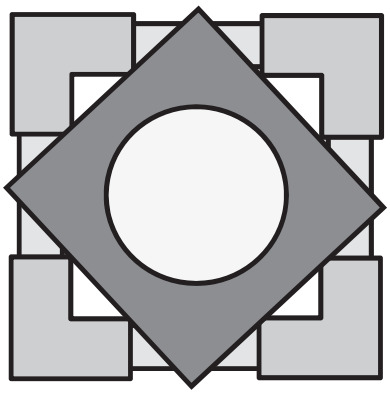

Fig. 1. Diagram of feeder float. The chlorinated PVC pipes, 1.5 cm diameter, were connected with 90° chlorinated PVC elbows to serve as a float. A 15-cm^2 plastic platform with a 0.45-cm deep, 11.5-cm diameter well was attached to the CPVC pipes. Floats served as feeding and haul-out platforms as well as refuges.

Tank mixes and analytical chemistry

The tank mix included the formulated imazapyr product Polaris AQ (Nufarm Americas), the surfactant Agri-Dex (Helena Chemical), and the marker dye Hi-Light (Becker Underwood). Concentrations of tank mixes were determined based on the estimated worst-case field-exposure scenario for direct overspray to 2 cm of standing water with no intervening vegetation. The test concentrations of Polaris AQ were based on label recommendations for low-volume, 3.5 L/ha, and high-volume, 7.0 L/ha, applications (hereafter low and high). Polaris AQ is formulated with 28.7% isopropyl amine salt of imazapyr (active ingredient [a.i.]) by weight. Exposure concentrations were 4.8 ppm and 9.7 ppm a.i. for low and high treatments. These exposure concentrations are 24 to 48 times higher than maximum field concentrations of 0.2 ppm measured 1 h posttreatment in Washington (www.ecy.wa.gov/programs/wq/pesticides/final_pesticide_permits/noxious/monitoring_data/monitoring_index.html, accessed August 2012). Agri-Dex and Hi-Light were included at equal rates in both treatments. Agri-Dex exposure of 44.4 ppm was based on a calculation of 1% volume/volume and an application rate of 9.4 L/ha. The Hi-Light concentration of 11.2 ppm was based on an application rate of 2.3 L/ha.

For the 96-h exposure test, the tank mixes were premixed by weight with deionized water for each treatment in an amber glass stock bottle to make 500 g of stock solution and stored at 2 to 4°C. At each 24-h renewal, fresh treatment solutions were made from 10 ml of each treatment stock mixed with 24 kg dechlorinated water in a 26.5-L plastic bucket with a clean, food-grade plastic liner. The dechlorinated water was the same temperature in which the frogs were maintained. For each replicate tank, renewal treatment solutions were distributed by weight from the stock buckets. Prior to distribution, a sample was collected from the 26.5-L mixing buckets of each treatment solution. Another sample was collected at the time of treatment solution renewal (24, 48, 72, 96 h) from one randomly selected aquarium in each of the high and low treatments. Two additional samples were collected from high aquaria 24 h after frogs were transferred to clean water at the end of the 96-h exposure period. Samples were held up to 48 h at 2 to 4°C until shipment to Pacific Agricultural Laboratory (Portland, OR, USA) for imazapyr analysis. Samples were analyzed for imazapyr using the American Cyanamid Method (liquid chromatography–mass spectrometry), with a method reporting limit of 1.0 ppm.

Test endpoints

Endpoints included growth, body condition, behavior, number of crickets consumed, an index of liver to body condition, and sexual traits. Growth was measured as weight (to the nearest 0.01 g) and SVL (to the nearest 0.5 mm) and converted to body condition for analysis. Frogs were measured before and after the 96-h exposure test, twice during the two-month grow-out, and just prior to death at the end of the experiment (0, 4, 32, 49, and 60 d after the start of the 96-h exposure).

Body condition was estimated using the scaled mass index ($M_I = M_i[L_0/L_i]b_{SMA}$), which is calculated using the mass (M_i) and SVL of the *i*th individual (L_i), mean SVL (L_0 is an arbitrary value of SVL to standardize the SVL), and slope (b_{SMA}) from the standardized major axis (SMA) regression of linearized mass on SVL [16]. Scaled mass index is preferred over other measures of body condition because it is insensitive to size differentials that may occur between genders in sexually dimorphic species like the OSF [16] and it has been shown to perform better than alternative body condition estimates [17]. We calculated b_{SMA} using the software for Reduced Major Axis Regression (synonymous with SMA), JAVA Version (http://www.kimvdlinde.com/professional/rma.html, accessed August 2012). Body condition was also used to estimate an index of liver health by taking the ratio of liver mass (to the nearest 0.001 g) to body condition at the time of death, 60 d after the start of the 96-h exposure.

Behavior was measured during the 96-h exposure by recording morning feeding activity with a camcorder (Vixia HFS21; Canon) at 24, 48, and 96 h of exposure. Eight minutes of video were analyzed starting 1 min after the researchers left the room. The total seconds each individual frog spent in the center circle (11.5 cm diameter) of the float was summed for the duration of time frogs spent on the float per tank. The number of crickets that remained in each aquarium after 20 min of undisturbed feeding was also recorded for both the morning and evening feedings throughout the 96-h test and two-month grow-out interval.

Secondary sex characteristics were recorded 44 d after the 96-h exposure and again with primary sex characteristics during liver extraction after the frogs were killed at the end of grow-out. Gonads were visually inspected for gross anomalies, and discrepancies from secondary sex characteristics were recorded.

Statistical analysis

Data were summarized and graphed in Excel (2010; Microsoft) and explored and analyzed in SPSS (PASW, version 18; SPSS). Data were evaluated for departures from normal using Shapiro-Wilk tests [18]. Differences among tanks within treatments and among treatments at the start of the experiment were tested using one-way analysis of variance (ANOVA) on initial length, weight, and body condition for each frog ($n = 3$ per tank, 15 per treatment). Differences in liver condition index among treatments at the end of the experiment were tested using one-way ANOVA [18].

Data were assessed to maximize the strength of the relationship between mass and SVL such that the most reliable estimate of b_{SMA} for calculating body condition was obtained, following Peig and Green [17]. Although use of only control individuals reduced the sample size for the SMA regression used to determine b_{SMA}, the results from the data assessment (not presented) were consistent with Peig and Green's [17] finding that data from reference individuals were more appropriate for estimating b_{SMA} in toxicological studies. Therefore, only data from the control group were used to estimate body condition as scaled mass index for each sample date.

Because individual frogs were not identified over time, tank means were used to evaluate changes in body condition and behavior among treatments across time using linear mixed models (LMMs). Frogs were measured at five different times during the experiment, but the sample days were not set at equal intervals. Therefore, the LMM for body condition using sample day as a repeated measure included an unstructured covariance. The cumulative number of crickets consumed per tank was also compared among treatments using LMM. Sample day was included as a random variable because we did not have control over the number of crickets that were consumed on a given day, only the number offered. Also, because the frogs could not be sexed when originally distributed in the tanks, the number of males in each tank was included as a random variable during LMM exploration for the number of crickets consumed. For all models, tank was included as subject, sample day as a covariate with treatment as the factor, and the fixed effects of treatment, sample day, and the interaction of treatment and sample day. Models were estimated using maximum likelihood estimates, and models with random factors were tested for significant differences from the simple model with no random factors using the difference between -2 log likelihood estimates as the χ^2 statistic.

RESULTS

At the start of the experiment, no differences existed among tanks within treatments in frog mass, SVL, and body condition as scaled mass index (Table 1). No differences were found among tanks within treatments, so all frogs within a treatment were pooled to test for differences among treatments. At the start of the experiment, no differences existed among treatments in body mass ($f_{0.05,4,14} = 0.2$, $p = 0.835$), SVL ($f_{0.05,4,14} < 0.1$, $p = 0.966$), and body condition ($f_{0.05,4,14} = 1.5$, $p = 0.237$). No mortalities occurred during the course of the present study, and no overt effects on behavior or general health were observed.

Target concentrations of imazapyr were achieved. Values were 92 to 108% of the low (4.4–5.2 ppm) and 91 to 97% of the high (8.8–9.4 ppm) nominal concentrations. Concentration did not change with time between water exchanges for either the low (4.6–5.0 ppm) or the high (9.5–9.7 ppm) treatment. Recoveries were 96 to 104% at 24 h. No imazapyr was detected in tanks with frogs exposed to high treatments 24 h after placement in clean water at the start of the grow-out.

Water quality

Water quality was similar across treatments in the mixing buckets prior to renewal of treatment water, among treatments in the tanks 24 h after renewal, and among treatments during the

Table 1. Analysis of variance parameters for frog size and body condition among tanks within treatments at the start of the experiment

	Control		Low		High	
Parameter	f[a]	p	f[a]	p	f[a]	p
SVL (mm)	1.9	0.186	0.4	0.807	1.0	0.443
Mass (g)	2.1	0.159	0.2	0.936	1.8	0.207
Body condition (SMI)	0.5	0.761	0.8	0.526	1.4	0.303

[a] Between-groups degrees of freedom = 4; total degrees of freedom = 14.
SVL = snout–vent length; SMI = scaled mass index.

Table 2. Water quality (means ± standard deviation) pooled across days from treatment mixing buckets and frog tanks

Parameter	Treatment	Temp (°C)	DO (mg/L)	pH	NH_3 (mg/L)	Conductivity (μS/cm)
Mixing buckets ($n = 3$)	Control	23.2 ± 0.3	8.2 ± 0.2	6.5 ± 0.1	<DL	80.0 ± 0.0
	Low	23.4 ± 0.2	8.1 ± 0.1	6.5 ± 0.0	<DL	80.0 ± 0.0
	High	22.6 ± 0.5	8.4 ± 0.1	6.6 ± 0.1	<DL	80.0 ± 0.0
96-h exposure ($n = 20$)	Control	23.2 ± 0.2	7.9 ± 0.1	6.8 ± 0.1	1.0 ± 0.2	90.5 ± 2.2
	Low	23.3 ± 0.2	6.9 ± 0.4	6.7 ± 0.1	<DL	90.0 ± 0.0
	High	23.2 ± 0.2	7.2 ± 0.6	6.7 ± 0.1	<DL	90.0 ± 0.0
Grow-out ($n = 90$)	Control	21.5 ± 0.7	7.5 ± 0.2	6.5 ± 0.2	1.6 ± 0.6	95.9 ± 6.5
	Low	21.4 ± 0.7	7.5 ± 0.3	6.6 ± 0.1	1.5 ± 0.6	95.1 ± 5.9
	High	21.4 ± 0.7	7.4 ± 0.4	6.6 ± 0.1	1.6 ± 0.7	95.9 ± 6.3

DO = dissolved oxygen; DL = detection limit (1 mg/L).

grow-out in clean water (Table 2). The DO in the tanks was slightly lower in high and low treatments than in controls, but it never fell below 6.0 mg/L for any treatment at any time.

Behavior

The relationship between behavior and treatment over time showed significant variance in intercepts across tanks, var = 6453.8, $\chi^2_{0.05,1} = 5.4$ (the difference between the −2 log likelihood estimates for models without and with random intercepts), $p = 0.020$. A random intercept was included in the final LMM. No significant effects of treatment ($f_{0.05,2,42.884} = 0.359$, $p = 0.701$), sample day ($f_{0.05,1,30} = 0.015$, $p = 0.903$), and the interaction of treatment × sample day ($f_{0.05,2,30} = 0.504$, $p = 0.609$) were found (Fig. 2). Also, no significant relationships existed for any treatment over time or any interactions between treatment and sample day (Table 3).

Visual inspection of graphed data of cumulative crickets consumed over time showed little difference between treatments. Differences existed when data were plotted based on the number of males in each tank (Fig. 3), and broad variation existed in the total number of crickets consumed on a daily basis (Fig. 4). The number of males in a tank contributed significantly to the variation in the cumulative crickets consumed in each treatment over time, var = 6985.6, $\chi^2_{0.05,1} = 1121.6$ (difference between −2 log likelihood for models without and with the random factor of number of males), $p < 0.0001$.

The variation over sample day did not follow a consistent pattern, but the number of crickets consumed per day rose and fell consistently among the treatments (Fig. 4). Sample day also contributed significant variation to the cumulative crickets consumed in each treatment over time, var = 10.1, $\chi^2_{0.05,1} = 3330.05$ (difference between −2 log likelihood for models without and with the random factor of sample day), $p < 0.0001$. The model including both the number of males and the sample day as random factors was further improved over the model without random factors ($var_{males} = 87.6$, $var_{sample\ day} = 9.8$, $\chi^2_{0.05,2} = 3446.2$, $p < 0.0001$) and was a significantly better model fit than the model with males only ($\chi^2_{0.05,1} = 2324.5$, $p < 0.0001$) and the model with sample day only ($\chi^2_{0.05,1} = 116.1$, $p < 0.0001$).

In the final model for the cumulative number of crickets consumed over time, the only significant fixed factors were the intercept ($f_{0.05,1,24.8} = 25.592$, $p < 0.0001$) and sample day ($f_{0.05,1,15.0} = 382.204$, $p < 0.0001$); significant effects of neither treatment ($f_{0.05,2,34.0} = 2.291$, $p = 0.117$) nor treatment × sample day interaction ($f_{0.05,2,15.0} = 0.019$, $p = 0.982$) were found. Significant linear relationships existed between treatment and sample day for control and low treatments but not for high, and no significant linear effects of the interactions between individual treatments and sample day were found (Table 4).

Body condition

The body condition LMM using sample day as a repeated measure was a significant improvement over the simple model

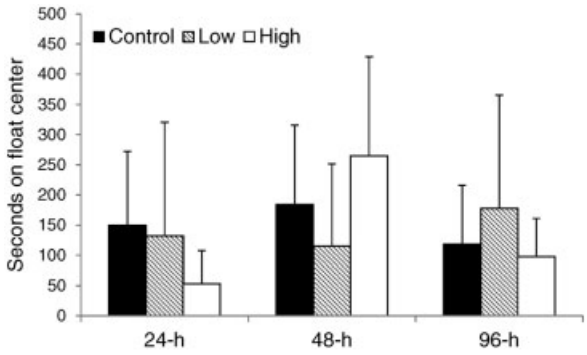

Fig. 2. Time frogs spent in the center ring of the float/feeding platform during 8 min of the morning feeding at three times during the 96-h exposure. No significant effects of treatment, sample day, and the interaction of treatment × sample day were observed. Bars = 1 standard deviation.

Table 3. Estimates of linear mixed model fixed effects on behavior as the sum of seconds that frogs were on the center of the float

Parameter	Estimate	Standard error	*df*	*t*	*p*	95% confidence interval
Intercept	182.6	68.2	42.9	2.677	0.010	(45.1, 320.1)
Sample day	−13.6	22	30	−0.619	0.540	(−58.3, 31.2)
High	−45.6	96.4	42.884	−0.473	0.639	(−240.1, 148.9)
Low	−81.5	96.4	42.884	−0.845	0.403	(−276, 113)
Control[a]	0	0	—	—	—	—
High × sample day	14.3	31	30	0.461	0.648	(−49, 77.6)
Low × sample day	31.1	31	30	1.003	0.324	(−32.2, 94.4)
Control × sample day[a]	0	0	—	—	—	—

[a] Control parameters are set to 0 because they are redundant.

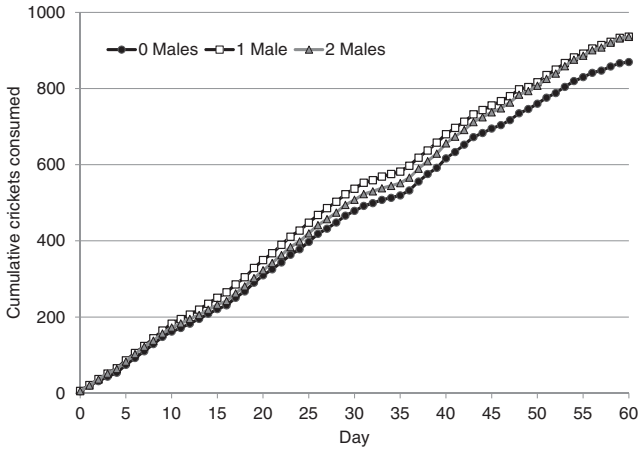

Fig. 3. Cumulative number of crickets consumed per day by the number of males in a tank. Black circles indicate that none of the three frogs in a tank was male, white squares indicate that one of the three frogs in a tank was a male, gray triangles indicate that two of the three frogs in a tank were male. The number of males in a tank contributed significantly to the variation in crickets consumed over time.

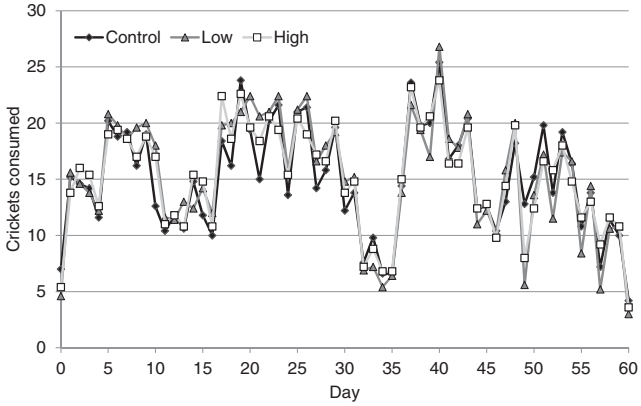

Fig. 4. Mean crickets consumed per day by treatment. Sample day contributed significantly to the variation in crickets consumed over time, but treatment did not.

($\chi^2_{0.05,14} = 52.1$, $p < 0.0001$). Sample day was the only significant predictor for changes in body condition over time ($f_{0.05,1,15} = 2935.8$, $p < 0.0001$). No significant effects of treatment ($f_{0.05,2,15} = 3.354$, $p = 0.063$) or the treatment × sample day interaction ($f_{0.05,2,15} = 1.115$, $p = 0.354$) were found. Significant linear relationships existed between treatment and sample day for the control and high treatments but not for low, and no significant effects of the treatment × sample day interactions existed on the linear relationship of body condition over time (Fig. 5 and Table 5).

Liver condition

No differences existed in liver condition among tanks within treatments (ANOVA, control: mean 0.098 ± 0.009 SD, $f_{0.05,4,14} = 0.6$, $p = 0.641$; low: mean 0.098 ± 0.007 SD, $f_{0.05,4,14} = 2.5$, $p = 0.112$; high: mean 0.096 ± 0.010 SD, $f_{0.05,4,14} = 1.6$, $p = 0.256$) and among treatments with frogs pooled across tanks ($f_{0.05,2,44} = 0.1$, $p = 0.864$). Because frogs within tanks may violate assumptions of independence, liver condition was also compared among treatments using the tank mean values. No differences existed in tank mean liver condition among treatments ($f_{0.05,2,14} = 0.1$, $p = 0.898$).

Gonads

No gross anomalies were observed in gonads. Primary and secondary sexual characteristics were consistent for all but one frog. One male frog in the high treatment had well-developed nuptial pads and underdeveloped testes compared with the relative primary and secondary sexual characteristics of all other frogs examined.

DISCUSSION

No acute or latent effects of imazapyr tank mixes on OSF juveniles were observed in the present study. We evaluated several different end points to establish confidence in our assessment that no differences existed between individuals exposed to imazapyr tank mixes and controls. A disconnection was observed between the external and internal states of sexual characteristic development of one male frog relative to other frogs. This was unlikely to be treatment-related because only one individual expressed the condition. It is also unlikely to be ecologically important. Presumably, gonad development would catch up with the secondary sexual characteristic development by the approximate time of the breeding season, four months later.

Treatment effect on body condition approached a significant difference ($p = 0.063$). Due to the randomized distribution of frogs, mean body condition values at the start of the experiment were ranked control > low > high. At the end of the 96-h exposure period, mean body condition values had converged such that body condition improved while the frogs were in the tank mix. That may be an effect of feeding during the exposure. Prior to testing, frogs were reared in large cattle tanks with many other individuals. The effects of competition during rearing may have resulted in lower body conditions that subsequently improved after frogs were placed in lower densities in

Table 4. Estimates of linear mixed model fixed effects on cumulative number of crickets consumed over time

Parameter	Estimate	Standard error	*df*	*t*	*p*	95% confidence interval
Intercept	7.3	2.6	553.9	2.786	0.006	(2.2, 12.5)
Sample day	15.6	1.4	15	11.143	<0.001	(12.6, 18.6)
High	6.6	6.6	16.9	0.998	0.333	(−7.3, 20.4)
Low	9.1	4.4	293.1	2.078	0.039	(0.5, 17.8)
Control[a]	0.0	0.0	—	—	—	—
High × sample day	0.2	2.0	15	0.115	0.91	(−4.0, 4.5)
Low × sample day	0.4	2.0	15	0.192	0.85	(−3.8, 4.6)
Control × sample day[a]	0.0	0.0	—	—	—	—

[a] Control parameters are set to 0 because they are redundant.

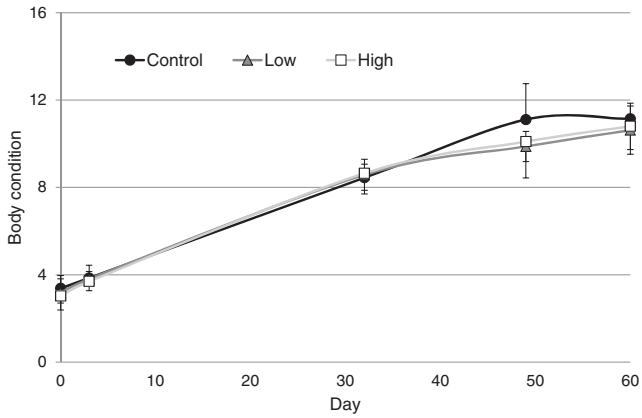

Fig. 5. Mean body condition per treatment on five sample days. No significant effects of treatment and the interaction of treatment × sample day were observed. Bars = 1 standard deviation.

the laboratory. It should also be noted that frogs did not restrict foraging to the floats. Crickets that had jumped into the water were also consumed, so frogs in treatment tanks were also ingesting the tank mix, thereby effectively receiving the tank mix through two routes of exposure: absorption through skin and ingestion.

The frogs in this experiment were fed and had no exposure to stressors that can occur in their normal habitats. We did not make any determination of imazapyr effects on animals under stressful conditions. In this preliminary experiment, we were primarily concerned with evaluating acute and latent effects of the tank mix itself. In wild populations, several natural stressors can impact the health and survival of individuals. The effects of predation, reduced water levels as ponds and wetlands dry during the summer, and/or high population densities can increase individual stress levels and potentially reduce fitness and survival. The presence of predators alone can increase nonconsumptive mortality in some tadpole species, presumably due to increased stress [19,20]. The toxicity of pesticides may also be altered in the presence of predators. Tadpoles in experimental mesocosm communities with a newt (*Notophthalmus viridescens*) predator experienced higher mortality when a glyphosate product with a toxic surfactant (Roundup with polyethoxylated tallow amine) was added [19,20].

Pond-drying may also affect individual fitness and survival. Typically, pond-drying has been evaluated in larval amphibians, showing that many species have developmental plasticity that allows them to adapt by metamorphosing sooner. This comes at a potential cost to survival later, due to often smaller sizes post-metamorphosis [21]. Introducing a pesticide to amphibian communities experiencing the effects of pond-drying may alter the norm of reaction (as described in Newman [21]) to the natural stressor.

For many juvenile frogs, pond-drying may not have the physiological impact it has on tadpoles. However, for a more aquatic species like the OSF [6], pond-drying may concentrate individuals into remaining wet habitats, potentially making them more vulnerable to inter- and intraspecies predation [22]. Although we did not observe any treatment-related effects on growth or behavior of OSF juveniles exposed to imazapyr that might indicate a higher vulnerability to predation, we did not test for responses in natural conditions of pond-drying, concentration of individuals and/or increased competition, or presence of predators (e.g., Boone and James [23]). Furthermore, we did not test any other native species that might be present at the time of herbicide application. Other species present may be predators of OSF juveniles [22]. The disruption of predator–prey dynamics has occurred with exposure of either the predator or the prey to other contaminants (e.g., carbaryl, an acetylcholinesterase-inhibiting insecticide [24]).

A paucity of data exists for direct effects of imazapyr tank mixes on any of the fauna in native wetland communities. This creates uncertainty for land managers who desire to implement its use for habitat restoration in wetlands. In several herbicide studies, the surfactant in the terrestrial formulation is more toxic than the active ingredient [25–27]. Although these data are compelling evidence to support concerns associated with the use of more toxic tank mixes in terrestrial habitats where amphibians occur, they are more difficult to interpret in relation to aquatic herbicide tank mixes used in wetland restoration. In Washington, surfactants are regulated such that only those that have low aquatic toxicity are allowed for use in wetlands. Therefore, using an active ingredient with an approved surfactant in Washington should provide some level of protection against direct toxic effects to fauna. For amphibians, indirect effects may be more important [28].

In amphibian habitats, stressors often occur simultaneously with multiple predator species, the effects of pond-drying, and high amphibian densities. Many potential predators of OSFs were observed in OSF habitats during the weed-management season, and many of them co-occurred in time and location (A. Yahnke, unpublished data). In this case, the indirect effects of herbicide treatments may be of concern in that habitat alteration may increase vulnerability to predation through the removal of plants that provide cover or change food-web dynamics. Future research should focus on ecological interactions with herbicide treatments.

Other aspects of herbicide treatment were outside the scope of the present study but may be important to consider in evaluating herbicides for use in aquatic systems. We did not

Table 5. Estimates of linear mixed model fixed effects on body condition over time

Parameter	Estimate	Standard error	t[a]	p	95% confidence interval
Intercept	3.6	0.1	45.743	<0.001	(3.5, 3.8)
Sample day	0.1	0.004	32.21	<0.001	(0.1, 0.1)
High	−0.3	0.1	−2.585	0.021	(−0.5, −0.1)
Low	−0.2	0.1	−1.432	0.173	(−0.4, 0.1)
Control[b]	0.0	0.0	—	—	—
High × sample day	−0.003	0.01	−0.500	0.625	(−0.02, 0.01)
Low × sample day	−0.01	0.01	−1.469	0.163	(−0.02, 0.004)
Control × sample day[b]	0.0	0.0	—	—	—

[a] All parameters analyzed with 15 degrees of freedom.
[b] Control parameters are set to 0 because they are redundant.

evaluate latent effects on the reproductive ability or resistance to desiccation of exposed individuals [29]. Nor did we investigate effects on a histological or molecular level that may reveal symptoms not visible in our gross examination. More in-depth analyses may be required to identify effects such as those that have raised concern with other herbicides, including effects on endocrine [14] or immune system [30] functions.

Evidence that minimal harm to species targeted for protection will come from habitat management is important in conservation work. The peer-reviewed literature provides little information about the toxicity of imazapyr to amphibians. Documenting the potential for minimal harm is essential to maximizing the effectiveness of habitat-restoration tools such as aquatic herbicides. Thus, studies that show no effects of an aquatic herbicide to nontarget organisms are a critical contribution to the toxicological literature. Imazapyr is an important and effective tool in conservation and habitat restoration to manage invasive plants such as reed canarygrass that degrade critical wetland habitat.

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