

THESIS

TRAMPLING BY CATTLE NEGATIVELY IMPACTS INVASIVE YELLOW-FLAG IRIS
(*IRIS PSEUDACORUS*) UNDER FLOODED CONDITIONS

Submitted by

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ABSTRACT

TRAMPLING BY CATTLE NEGATIVELY IMPACTS INVASIVE YELLOW-FLAG IRIS (*IRIS PSEUDACORUS*) UNDER FLOODED CONDITIONS

Yellow-Flag Iris (*Iris pseudacorus* L.) is a non-native, invasive wetland plant in North America that disrupts riparian ecosystem processes. Due to its physiological and morphological characteristics, *I. pseudacorus* has the capacity to exclude native vegetation and form extensive monocultures in both lotic and lentic wetland systems. Methods commonly used to manage *I. pseudacorus* infestations include manual and mechanical treatments for small populations and chemical treatment for larger populations. While these management techniques are often effective, options can be restricted by the biotic and abiotic conditions of a given site. For example, there are situations where chemical treatments near waterways (i.e. close to irrigation water diversions) may be prohibited due to label restrictions. The objective of this research was to evaluate the effectiveness of cattle trampling for reducing *I. pseudacorus* prevalence in riparian habitats. A field study was established on a ranch in northwest Nebraska to evaluate cattle trampling effects on *I. pseudacorus* density and height after two consecutive years of treatment. In a complementary greenhouse study, the effects of inundation and two different timings of simulated trampling on *I. pseudacorus* density, height, and rhizome stress (as measured by soluble sugar concentration) were also evaluated. No statistical differences in soluble sugar concentrations were observed among treatments; however, these data suggest that cattle trampling will reduce *I. pseudacorus* density and height at both timings, but trampling plus inundation was the most effective treatment combination.

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Introduction

Rangeland riparian and wetland systems play a critical role in supporting ecosystem functions in what are largely arid and semiarid landscapes. These areas rely heavily on the maintenance of appropriate hydrological and geomorphic characteristics required to support desirable flora and fauna (Goodwin et al. 1997; Silverman et al. 2019). While highly resilient, riparian and wetland systems are susceptible to disruption by stressors, such as invasive species. Exotic invaders can displace native flora, form monotypic stands, and alter the morphological characteristics (Wohl 2006) of infested waterways (Spaak 2016). Invasive species may also impact agricultural operations by clogging irrigation infrastructure, altering the timing and dependability of water available for irrigation, and reducing desirable forage production (King County DNRP 2007).

Yellow-flag iris (*Iris pseudacorus* L.) is a perennial, emergent aquatic species that produces long, blade-like leaves, showy flowers, and dense rhizomatous root systems (Stone 2009; Sutherland 1990; Tu 2003). The aesthetic appeal of the flowers has prompted *I. pseudacorus* to be planted as an ornamental outside of its native range. While other iris species, such as *Iris germanica*, are often used in an ornamental setting with no adverse effect, *I. pseudacorus* can become an aggressive invader in natural (i.e. ponds, rivers, lakes, marshes) and agricultural setting (i.e. irrigation diversions) when it escapes cultivation (Alpert et al. 2000; Tu 2003). Currently, *I. pseudacorus* is found in 48 of 50 states in the US and has been listed as a noxious weed in Montana and Washington; a designated weed quarantine species in Oregon; prohibited in Massachusetts and New Hampshire; and banned in Connecticut (King County DNRP 2007; USDA-APHIS 2013).

I. pseudacorus poses a threat to native ecosystems due to its high ecological amplitude and ability to outcompete native vegetation for resources, primarily space. (Pathikonda et al. 2008; Thomas 1980). This advantage can be mostly attributed to the robustness of the rhizomatous mats interwoven between neighboring *I. pseudacorus* shoots, vigorous asexual reproduction via rhizomes, and prolific sexual reproduction (Pennsylvania DCNR; Sutherland 1990; Tarasoff et al. 2016; Weber 2003). The rhizomatous mats and high fecundity allow *I. pseudacorus* to monopolize available space, while simultaneously increasing the probability of range expansion from the introduction site (Gaskin et al. 2016; Sutherland 1990).

Others have suggested that *I. pseudacorus*' competitive advantage may be the result of large quantities of storage carbohydrates (i.e. fructans) in the rhizomes that play a major role in growth, maintenance, and reproduction (Hanhijärvi and Fagerstedt 1994, 1995; Lambers et al. 2008; Tarasoff et al. 2016). Storage carbohydrates are multifunctional and can act as buffers against abiotic and biotic stress. Hydrolysis of these molecules provides an energy source when photosynthesis is slowed or stopped by maintaining and potentially increasing the concentration of photosynthetic byproducts (i.e. soluble sugars such as sucrose, fructose, and glucose). The concentration of these sugars are highly variable, both temporally and spatially throughout a plant, and are often adjusted in response to environmental cues, whole-plant carbon balance, and stress (Pozo et al. 2019). It can therefore be hypothesized that injury to photosynthetically active tissue could lead to increased stress and hydrolysis of storage carbohydrates, which, in turn, would increase the concentrations of soluble sugars in the near-term.

Techniques employed to manage *I. pseudacorus* range from physical removal to herbicide treatments. Because *I. pseudacorus* infestations are often large by the time managers attempt to address the issue, chemical treatments are the predominant management strategy, with

glyphosate and imazapyr being the most commonly used herbicides (King County DNRP 2007; Jacobs et al. 2011; Pennsylvania DCNR; Simon 2008; Spaak 2016). Fall imazapyr treatments alone and in combination with glyphosate controlled *I. pseudacorus* better than glyphosate alone (Simon, 2008). It is important to note though that while imazapyr is often considered the most effective herbicide for *I. pseudacorus* management, label restrictions limit its use near irrigation diversion due to the herbicide's residual soil activity (Anonymous 2011). The non-selective nature of imazapyr and glyphosate and restrictions around flowing water used for irrigation, illustrates the importance of identifying alternative treatment options for managing *I. pseudacorus* in wetlands and riparian areas close to irrigation diversions.

The idea to use trampling as a management technique arose because of the observation of *I. pseudacorus* mortality along paths used by researchers. Subsequent research found that human trampling reduced *I. pseudacorus* density and height by 75% and 58%, respectively (Spaak 2016). The research presented here builds on these previous findings with the overall goal of evaluating the effectiveness of cattle trampling as a novel technique for *I. pseudacorus* management. The objectives of this project were to: 1) determine the impact that two years of cattle trampling would have on *I. pseudacorus* density and height, and 2) determine the impact that inundation and timing of simulated trampling would have on the density and height *I. pseudacorus*, and soluble sugar concentration in its rhizomes.

Materials and Methods

*Field Study of Cattle Trampling *I. pseudacorus**

Site Description. A field study was conducted in Sioux County, Nebraska (42.25°N, 103.43°W), roughly 60 km north of Mitchell, Nebraska, just outside the eastern border of Agate Fossil Beds National Monument (AGFO) on a working cattle ranch. The site has an elevation of 1,372 meters above sea level, a mean annual precipitation between 36 cm and 41 cm, and a mean annual temperature between 7 and 9 °C (USDA-NRCS 1998). The vegetation was dominated by *I. pseudacorus*; however, there were several patches of common spike rush (*Eleocharis palustris* L.) and sedge (*Carex* L.). The soils were primarily Bigwinder fine sandy loam (Coarse-loamy, mixed, superactive, calcareous, mesic Aeric Fluvaquents) with several areas being classified as part of the Las Animas-Lisco complex (USDA-NRCS 2013). Due to the soil properties, and the proximity to the Niobrara River, the area is prone to frequent flooding during the spring and early summer months and covered in ice throughout the winter from roughly November through February (USDA-NRCS 2013). The Niobrara River at this location has an average flow of 0.4 m³ s⁻¹ [1958-1991; the USGS stream gauge was reactivated in February, 2014 (Spaak 2016)].

It should be noted that previous treatment at this site included a 2016 glyphosate application (Rodeo®). There was little impact to this infestation from the treatment, as evidenced by the uniformly robust *I. pseudacorus* population present at the beginning of this study in summer, 2017. The roughly 1.5-hectare stand is located in a meadow and bounded by the Niobrara River to the south, an irrigation ditch used by the ranch to the north, a perimeter of trees to the east, and a fence line to the west.

Experimental Design. This field study began in June 2017, lasted until June 2019, and was designed to focus on the potential impacts of cattle trampling on the density and height of established *I. pseudacorus* plants (mature individuals) in the interior of an existing infestation. The study design consisted of non-trampled plots inside constructed exclosures and trampled plots outside the exclosures (Figure 1). Seven, 7.5 m², circular exclosures were built and randomly located in the study area. Each exclosure consisted of two, welded-wire cattle panels and five t-posts. Sample units in trampled plots were paired with sample units in non-trampled plots to ensure initial plant compositions in trampled and non-trampled plots were similar (Figure 1). Sampling in trampled plots occurred between 1.5 and 4-m away from exclosures to prevent confounding effects from human trampling that occurred directly adjacent to exclosures during construction and subsequent data collection.

Treatments. The ranch where the field experiment was conducted runs a cow/calf operation. During the first year of the study in 2017, 140 cow/calf pairs were present in the roughly 145 acre pasture between late June and late July. In August 2017, 10 bulls were also present in the pasture; however, they appeared to congregate outside the *I. pseudacorus* infestation. In 2018, 140 cow/calf pairs were present in the pasture from June 9 until June 25 and another ten days in early September. In order to encourage cattle use in the study site, six salt blocks were placed in the meadow among the plots after water levels declined during both years of the study.

Data Collection. All data were collected from four, 0.5-m² quadrats (subsamples) in each trampled and non-trampled plot (Figure 1). Initial density and height measurements for all plots were taken in June 2017 prior to cattle turnout. For both trampled and non-trampled plots, shoot

density was recorded inside each of the 0.5-m² quadrats. Shoots were identified by following leaves to their base to ensure individual plant counts as opposed to just counting leaves. For both trampled and non-trampled plots, height was measured by selecting a live, standing leaf that appeared to represent the average leaf height inside each 0.5-m² subsample. The selected leaf was then held straight up at full height and measured from the soil to the leaf tip. Density and height measurements were taken again in June 2018 to quantify year 1 trampling impacts. The final data collection occurred in June 2019 to quantify additional year 2 trampling impacts.

Additional data were collected in September 2017 and September 2018 to describe the impacts of cattle in trampled plots. These data included height, visual estimates of percent trampled, and percent grazed. Percent trampled and grazed were visual estimates of *I. pseudacorus* in each 0.5-m² quadrat that showed signs of trampling (broken/ bent shoots) or grazing (chewed leaves or those that had been bitten or torn off). The presence or absence of seedpods and flowers in each quadrat was also documented during field visits.

Greenhouse Experiment: Investigating Impacts of Timing of Trampling and Inundation

Collection and Growth of Experimental Plants. *I. pseudacorus* plants for the greenhouse portion of this research were collected in late-April 2018 at green-up from the aforementioned meadow in Sioux County, NE (42.25°N, 103.43°W). Collection involved identifying groups of roughly ten *I. pseudacorus* shoots, digging up the shoots, roots, rhizomes, and soil (i.e. plug), and placing them in a pot to be transported back to the Plant Growth Facilities at Colorado State University for planting. Two days following field collection, each plug was placed into an individual, 11-liter pot. The size of each plug was ~28 cm in diameter, and final planted samples included rhizomes, soil from the study site, Pro-Mix BX potting soil, and roughly 10 *I. pseudacorus*

shoots. Greenhouse conditions remained constant throughout the study with temperatures between 21 and 24 °C and 16 hours light and 8 hours dark.

Treatments. The study aimed to investigate the interaction between timing of simulated cattle trampling and inundation on *I. pseudacorus* shoot density and height, and soluble sugar concentrations in the rhizomes. As a result, the study was designed to be a 3 by 2 factorial with a total of six treatment groups (Trampling: early trample, late trample, non-trampled; Inundation: inundated and non-inundated). Each treatment group consisted of 9 replicate pots with 10 iris shoots (subsamples) per pot. Pots were randomly assigned to one of the six possible treatments. The simulated trampling treatment was a modification of the approach used by Spaak (2016) and consisted of applying roughly 176 kPa of pressure to each individual shoot as close to the crown as possible to mimic trampling by cattle (Higgins et al. 2017). Trampling events took place during the growing season at two different times prior to flowering in order to simulate an early trampling event as well as a late trampling event. Early simulated trampling occurred one week after *I. pseudacorus* plugs were placed in the greenhouse when shoots were roughly 15 cm tall, and late simulated trampling occurred four weeks later.

In order to maintain the inundation groups and adequate water levels for non-inundated treatments, pots were placed in 11-liter buckets. The water levels of inundated groups were held constant at 2.5 cm below the bucket rim (roughly 5-7 cm above the crowns of *I. pseudacorus*). Water levels in the non-inundated groups were held at 25 cm from the bottom of the bucket, leaving the soil surface and crowns of the plants exposed to air but providing adequate moisture to maintain growth.

Data Collection: Density and Height. Density and height data were collected at the start of the study (May 2018), prior to each trample treatment, and at the conclusion of the study (August 2018). Density was measured by counting the shoot number per pot. Shoots were identified by following individual leaves to their base. Height was measured by making a visual estimate of an average sized leaf in each pot. The leaf was then held straight up at full height and measured from the soil surface to the leaf tip.

Rhizome Sample Preparation. Rhizome samples for soluble sugar quantification were collected at the beginning of the study for non-trampled *I. pseudacorus* (May 2018), immediately prior to all simulated trampling events (May 2018 for early trampled and June 2018 for late trampled), and at the conclusion of the study (August 2018). Following the rhizome harvest from each pot, samples were microwaved for 90 seconds to stop enzymatic activity and then placed in a drying oven at 55 °C for 72 hours. Samples were then ground to pass through a 40-mesh (425 micron) screen and placed in cold storage at -3 °C until sugar extraction and quantification (R O'Connor, personal communication).

Soluble Sugar Extraction. An ethanol extraction following methods outlined in Landhäusser et al. (2018) was used in which 30 mg of ground plant material was placed in a 2 mL screwcap tube and hydrated with 1.5 mL of 80% ethanol. Tubes were shaken to suspend solids and then heated in a 90 °C water bath for 10 minutes. After cooling to room temperature, tubes were placed in a microcentrifuge at 13,000g for 1 minute. Following the centrifugation, 200µL of the supernatant was transferred into a new 2 mL screwcap via a micropipette and stored at -20 °C until the soluble sugar assay occurred.

Soluble Sugar (Glucose, Fructose, Sucrose) Assay. The stored supernatant was removed from the -20 °C freezer and placed in a 60 °C oven overnight to remove ethanol. After heating, 1.0 mL of deionized water (DI H₂O) was added to each sample. Screwcap tubes were vortexed for 5 seconds, heated in a 90 °C water bath for 5 minutes, and vortexed for another 5 seconds. This sequence was repeated a second time and then tubes were allowed to cool to room temperature and placed in a microcentrifuge at 13,000g for 1 minute.

Following centrifugation, 96-well plates with sugar standards (glucose: 0 to 0.5 mg mL⁻¹; sucrose and fructose: 0.5 mg mL⁻¹), samples in triplicate (20 µL each), and blanks were prepared. One hundred µL of hexokinase-glucose 6-phosphate dehydrogenase solution (GHK, Sigma G3293; Appendix A) was added to each well, including standards and blanks, for the glucose assay. Water plates were also prepared which included the standards (glucose: 0 to 0.5 mg mL⁻¹; sucrose and fructose: 0.5 mg mL⁻¹), samples in triplicate, and blanks. For water plates, 100 µL of DI H₂O was added to each well in lieu of GHK. Once the GHK and DI H₂O were added, plates were covered and incubated at room temperature for 60 minutes until absorbance readings were taken.

Following the initial absorbance reading, which was used to quantify glucose, 10 µL of phosphoglucose isomerase solution (Sigma P538; Appendix A) was added to each well for the fructose assay. Once added, plates were covered and incubated at room temperature for 60 minutes until absorbances were read. In order to quantify sucrose, an additional 10 µL of invertase solution (Sigma I9274; Appendix A) was added to each well from the fructose assay. Plates were then incubated a final time at room temperature for 60 minutes until absorbances were measured. All absorbance values were read at 340 nm on a Model UV2600 (Shimadzu

Scientific Columbia, MD 21046) spectrophotometer. Details on solution preparation and quantification equations can be found in Appendix A of this thesis.

Data Analysis

Field Experiment. A repeated-measures analysis of variance (ANOVA) was used to determine trampling effects on *I. pseudacorus* density and height. Factors considered in the model were treatment (trampled and non-trampled), year, and interactions as fixed effects; year as the repeated measure; and plot as a random factor. A post hoc pairwise comparison using Tukey's Honestly Significant Difference was performed, and all main effects, interactions, and pairwise comparisons were tested at a significance level of $\alpha = 0.05$. All data were analyzed utilizing the R and R Studio software ('lmerTest' package; R Core Team 2017).

Greenhouse Experiment. Data were found not to be normally distributed via the Shapiro-Wilk test and were analyzed in R Studio using a non-parametric Kruskal-Wallis test to determine the interaction between timing of trampling and inundation on *I. pseudacorus* density, height, and soluble sugars. The density, height, and total sugars of pre-treatment samples were not statistically different, confirming that treatment groups were initially similar, so analysis of post-treatment data were deemed an appropriate way to assess treatment effects. Individual Kruskal-Wallis analyses for each sugar fraction (i.e. glucose, fructose, and sucrose) were also performed in order to understand whether treatments had differential effects on specific osmolytes. A post hoc Wilcoxon Rank-Sum test was performed to determine differences among treatment groups. All analyses were tested at a significance level of $\alpha = 0.05$.

Results and Discussion

Field Experiment

The dynamics of *I. pseudacorus* densities sampled between 2017 and 2019 differed between trampled and non-trampled plots ($P=0.0038$). *I. pseudacorus* density increased steadily in non-trampled plots over the course of the study (2017-2019). Following one year of trampling, there was a significant increase in plant density between 2017 and 2018, which could be due to a compensatory growth response (Figure 2A). In 2018, after one year of trampling in the trampled plots, *I. pseudacorus* density did not differ from the non-trampled plots. It is not until 2019, after two years of trampling, that a significant within year difference was present and trampling appeared to negatively impact *I. pseudacorus* density.

I. pseudacorus plant heights sampled between 2017 and 2019 also differed between trampled and non-trampled plots ($P=0.00093$). Plant heights remained fairly consistent throughout the study in non-trampled plots and were not statistically different across years (Figure 2B). Plant height in trampled plots steadily decreased with time and by 2019, after two years of trampling, leaf height was lower than at the start of the study in 2017. Significant within year differences between trampled and untrampled plots were also observed following both the first and second years of treatment.

In addition to density and height, visual estimates of percent trampling and grazing impact were recorded in each trampled plot following cattle removal (Table 1). Percent impact was measured using a 0.5-m² frame and included two categories: percent grazed, and percent trampled. These impacts were not mutually exclusive, so overall impact could be greater than 100%. In September 2017, a higher percentage of plants were grazed than were trampled;

however, in September 2018 estimated grazing impact was slightly lower than the estimate of trampling impacts. Overall estimated impact appeared slightly greater in 2017 than in 2018.

These data support the hypothesis that cattle trampling would reduce *I. pseudacorus* density and height. Despite the significant decrease observed in plant height following years one and two of trampling, significant differences in density only occurred after the second year.

Physical management treatments for this species, such as cutting or mowing, applied early in the growing season seem to have the greatest impact (Simon 2008; Spaak 2016). This could be due to the limited time for plants to replenish carbohydrates that are heavily utilized earlier in developmental stages (Whitehead 1971). While cattle were placed in the meadow in late June in 2017, it was not until mid- to late-July (following placement of salt blocks) that most of the trampling impacts occurred. In 2018, cattle were placed in the meadow in early June, and the highest concentration of cattle occurred in early July. This slight change in timing of trampling could have contributed to the more pronounced decrease in density observed in 2019.

The difference in timing of trampling may also have led to differences in the impacts observed between 2017 and 2018. While cattle do not generally consume *I. pseudacorus* because it contains glycosides that irritate their digestive system, the iris toxins consumed by the cattle in this study could have been diluted by forage they consumed outside the infested area. This could have contributed to greater than expected iris consumption (Damhoureyeh and Hartnett 1997). In September of 2018, the average impact from trampling appeared to be greater than grazing. This shift in impact type may have led to differential responses in growth following trampling and could be related to the shift in timing.

The difference in timing of trampling between 2017 and 2018 also resulted in differences in inundation of the meadow during treatment. Inundation levels during trampling differed

between years. In 2017, trampling occurred during dry conditions and 2018 trampling partly occurred while the meadow was still inundated. The impact to aboveground biomass from trampling, as well as the reduced ability for gas exchange, could have led to a reduction in available resources required to produce leaves of the same height and density in trampled plots following the 2018 treatment. This is supported by other research where aggressive aboveground biomass removal of *I. pseudacorus* and subsequent reductions in regrowth has been tied to differences in carbohydrate utilization in conjunction with saturated conditions (Tarasoff et al. 2016).

Greenhouse Experiment

Density and Height. Density and height of *I. pseudacorus* were both affected by trampling and the responses of inundated plants to trampling differed from non-inundated plants ($P < 0.0001$). Both density and height of inundated individuals were significantly reduced by early and late trampling. Densities for the inundated/early trample group and inundated/late trample group were also significantly lower than all treatment groups that were non-inundated (Figure 3A). Height of the inundated/early trample plants was significantly lower than the inundated/non-trampled plants and all three treatment groups that were non-inundated, while height of the inundated/late trample only differed from the non-inundated/non-trampled and inundated/non-trampled plants (Figure 3B). Further, both density and height of the early and late trampled plants under inundated conditions were statistically similar to each other. Results indicate clear interactive effects of trampling and inundation but no impact of either trampling or inundation alone. Interestingly, time of tampling was not a critical factor under either inundated or non-inundated conditions. These results provide additional insight into the findings from the field

study; it may not have been the timing of trampling alone that caused differences between 2017 and 2018 but, rather, the change in level of inundation at the time of trampling.

Similar findings suggesting *I. pseudacorus* aboveground biomass stress due to inundation have been reported in other studies. Thomas 1980 used elevation as a proxy for inundation length. A positive relationship between biomass and elevation was observed; elevation was found to explain 47% of biomass variation. Of all factors investigated (i.e. light, vegetation structure, soil color, and presence of soil hardpan), length of inundation periods was asserted as being the most limiting factor for *I. pseudacorus* growth (Thomas 1980). In another study, cutting *I. pseudacorus* leaves while plants were continuously inundated resulted in no regrowth one-year post treatment (Tarasoff et al 2016); however, shorter periods of inundation did not significantly impact plants one-year post treatments.

Soluble Sugars. There were no statistical differences among total soluble sugar concentrations, within individual sugar fractions (i.e. glucose, fructose, sucrose), or when comparing between pre- and post-treatment (Figure 4). Although not statistically significant, there are several apparent trends that may provide additional insight into the density and height results presented above.

In all 6 treatment groups, sucrose accounts for the greatest percentage of total soluble sugar content in *I. pseudacorus* rhizomes, followed closely by fructose and then glucose. The timing of simulated trampling had no noticeable impact on density and height of *I. pseudacorus* following treatment, regardless of inundation; there were indications of differences (although not statistically significant) in total free sugar concentrations between the early and late trampled treatment groups with and without inundation. Plants that were non-inundated produced a spike

in total soluble sugar concentration in the late trampled group that may indicate a lag effect of stress. Early trampled samples had two months of recovery time post-treatment prior to rhizome harvest, while the late trampled samples had only one month for recovery. Although regrowth potential was similar, the elevated concentrations of soluble sugars (i.e. sucrose and fructose) indicate elevated levels of stress in non-inundated/late trample plants compared to inundated/early trample individuals.

In contrast, when inundated, it is the early trampled group that had the highest (numerical) concentration of soluble sugars. This again could be a lag effect indicating stress, but an inverse relationship to what was discussed for the non-inundated groups; inundated/early trample individuals would have been stressed for an additional month compared to the inundated/late trample individuals. This may have been caused by the complete lack of aboveground biomass regrowth of the inundated/early trample plants following treatment. Without the production of new leaf material, the rhizomes would have remained in an anoxic state and continued with anaerobic respiration. While anoxic environments often lead to decreases in metabolic activity and often dormancy, there is evidence that *I. pseudacorus* does not downregulate its metabolic activity under anoxic stress and continues with glycolysis and ethanol fermentation (Tarasoff et al. 2016; Hanhijärvi and Fagerstedt 1994).

The lack of downregulation of metabolic activity could also account for the apparent increase in fructose levels, which would have accumulated following hydrolysis of the primary storage carbohydrate fructans as a means of mitigating stress (Hanhijärvi and Fagerstedt 1994). It is unknown whether fructan levels in the rhizomes following treatment could be sufficient to support regrowth the following growing season; however, the continued presence of, and often increase in free sugars suggests there was at least enough stored carbon at the conclusion of the

study for continued plant function. Stored carbon in the inundated/ early trample treatment group may have continued to be depleted over time and lead to eventual cell death (Tarasoff et al. 2016). This is supported by the cell death of rhizomatous tissue quantified by Tarasoff et al. (2016) one-year post treatment following complete inundation. In order to obtain a more holistic picture of carbon starvation as a potential mechanism driving decreased growth capacity of *I. pseudacorus* under prolonged inundation, continued research into the concentrations of fructans and starch are required.

Management Implications

The aquatic, invasive species yellow-flag iris (*Iris pseudacorus*) causes considerable damage to riparian and wetland ecosystems through exclusion of native species and alteration of the hydrological and geomorphic characteristics of these systems. Although these ecosystems comprise a small proportion of total landmass in rangelands, riparian areas and wetlands provide critical habitat and ecosystem functions. The most commonly reported management technique for yellow-flag iris is chemical treatment with herbicides such as glyphosate and imazapyr, with imazapyr often showing greater efficacy. While these herbicides can be effective at reducing yellow-flag iris prevalence, restrictions on use of imazapyr close to irrigation diversions and the relatively low efficacy of glyphosate suggest the need to identify other options for *I. pseudacorus* management.

The results presented in this research suggest that cattle trampling when coupled with inundation could be an effective option for land managers working to reduce yellow-flag iris abundance. Results also suggest that timing of trampling should be matched with the occurrence of standing water (inundation) rather than *I. pseudacorus* phenology early in the growing season. Simulated trampling at two different time points prior to flowering resulted in no differences in density or height of plants, however inundation coupled with trampling resulted in drastic reductions of both variables. Attractants, such as salt blocks, may be necessary to draw cattle into *I. pseudacorus* infestations.

Total concentrations of soluble sugars present in the rhizomes of iris were also assessed. No effect of treatment was observed, however elevated sugar concentrations in the rhizomes of inundated and trampled iris support the need for continued research into potential carbon starvation.

While trampling was the primary technique investigated in this research, these findings can be applied more broadly and suggest that other management techniques beyond trampling should focus on applying the most damage to aboveground leaf material while water levels at infested sites are high and result in inundation of rhizomes and plant crowns.

Tables

Table 1. Estimated impacts of cattle on *Iris pseudacorus*. Impacts were quantified as percent of *Iris pseudacorus* trampled or grazed in a 0.5-m² frame.

Impact Type	September 2017	September 2018
Trampled	47%	55%
Grazed	66%	43%
Overall Estimated Impact	113%	98%

Figures

A)



B)

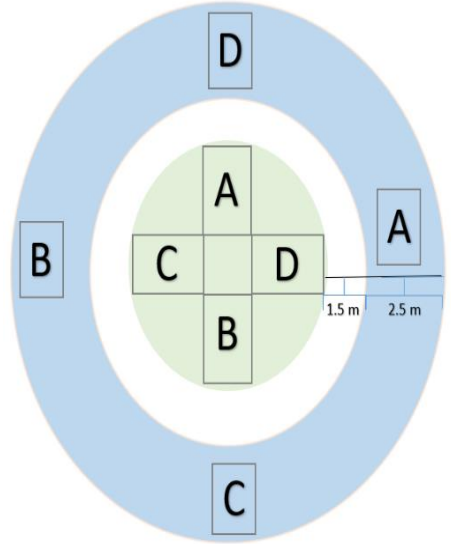


Figure 1. Field study plot layout A) photo showing the structure of exclosures used for untrampled plots, and B) diagram detailing untrampled plots inside exclosure (green circle) and paired trampled plot (blue circle). The sampled 0.5-m² quadrats are represented by the rectangles labeled a-d (subsamples).

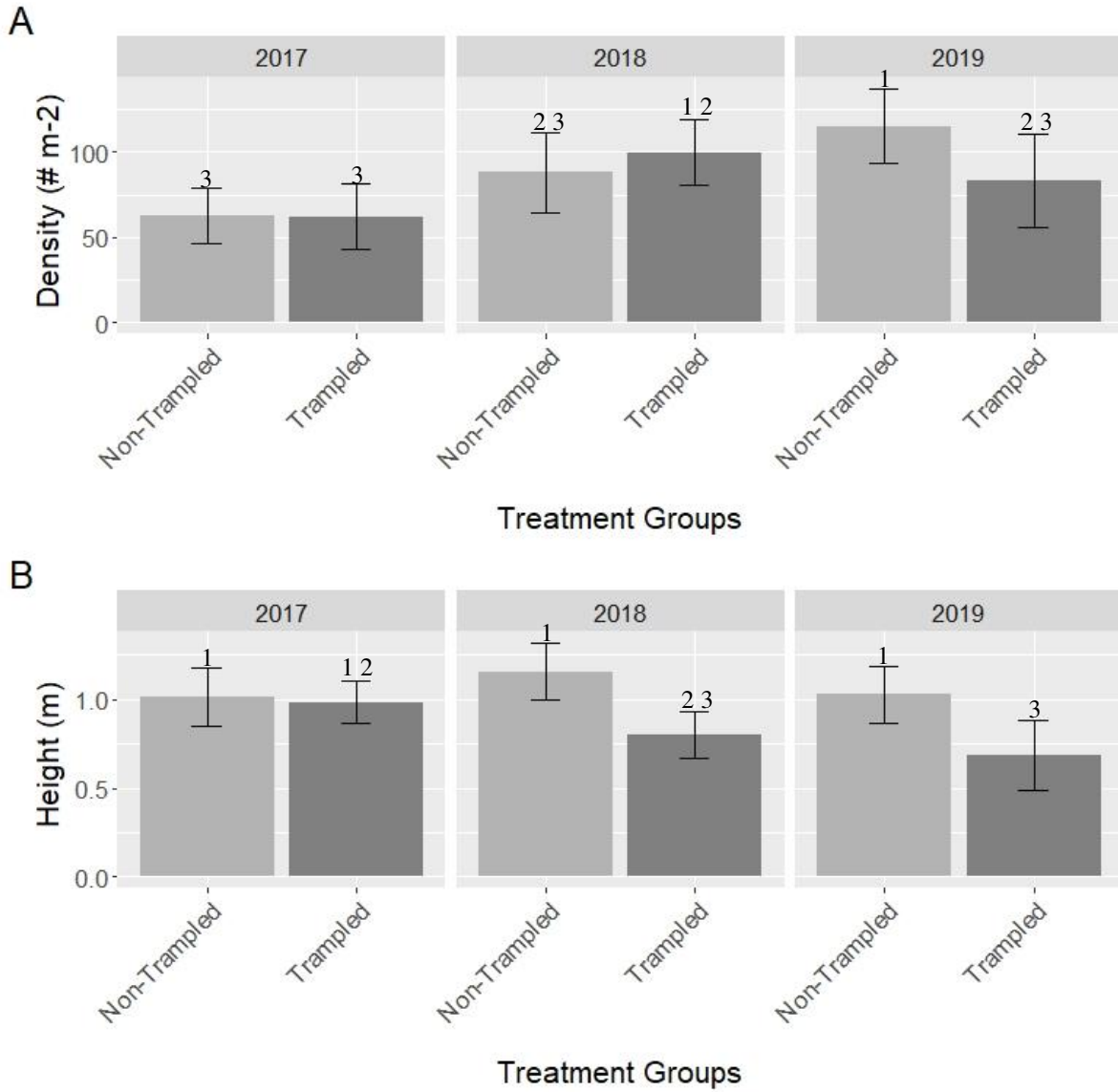


Figure 2. Yellow flag iris (*Iris pseudacorus*) A) density (# m⁻²; mean + se) and B) height (m; mean + se) as affected by cattle trampling in wetlands along the Niobrara River, Nebraska, USA. Means with a number in common are not different (Tukey's HSD; $\alpha=0.05$; $n=7$).

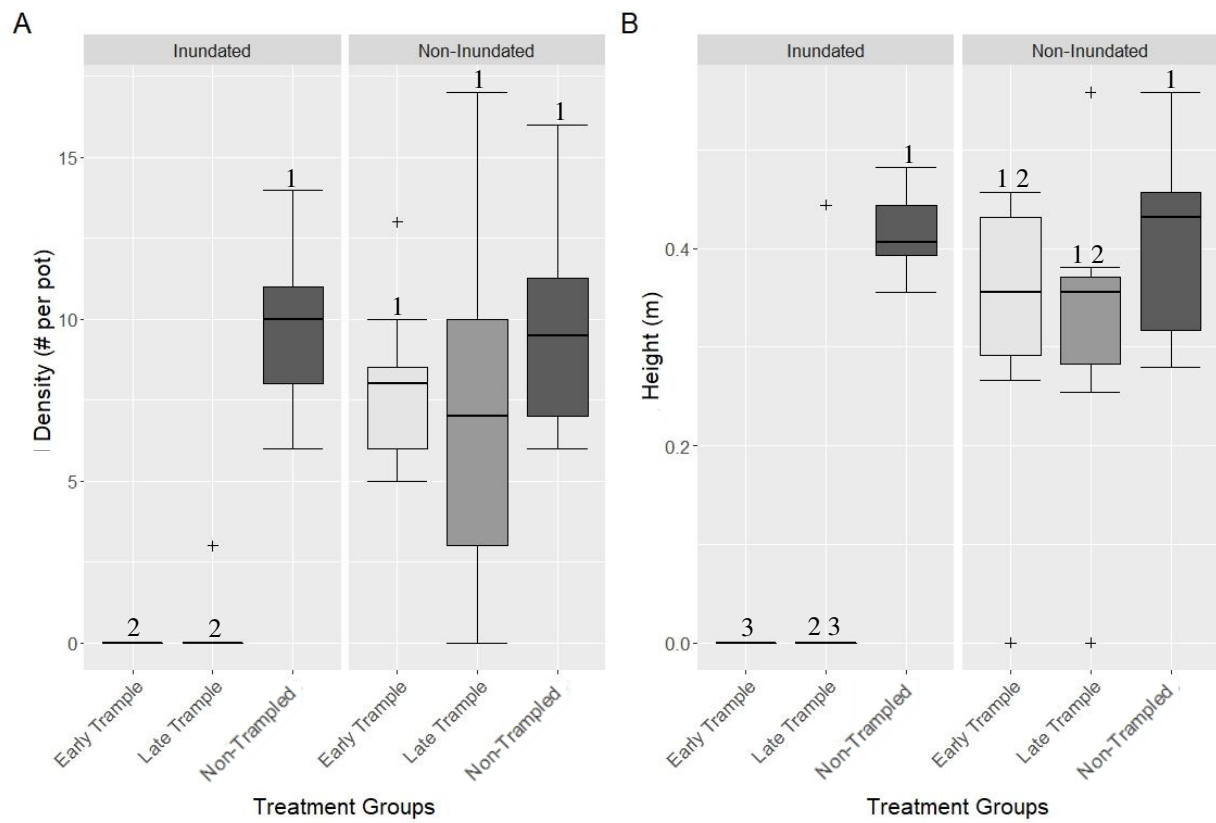


Figure 3. Yellow-flag iris (*Iris pseudacorus*) density (panel A; # per pot) and height (panel B; m) as affected by simulated cattle trampling and inundation. Results reflect differences in medians (bold horizontal lines). Treatment groups with a number in common are not different (Wilcoxon Rank Sum Test; $\alpha=0.05$).

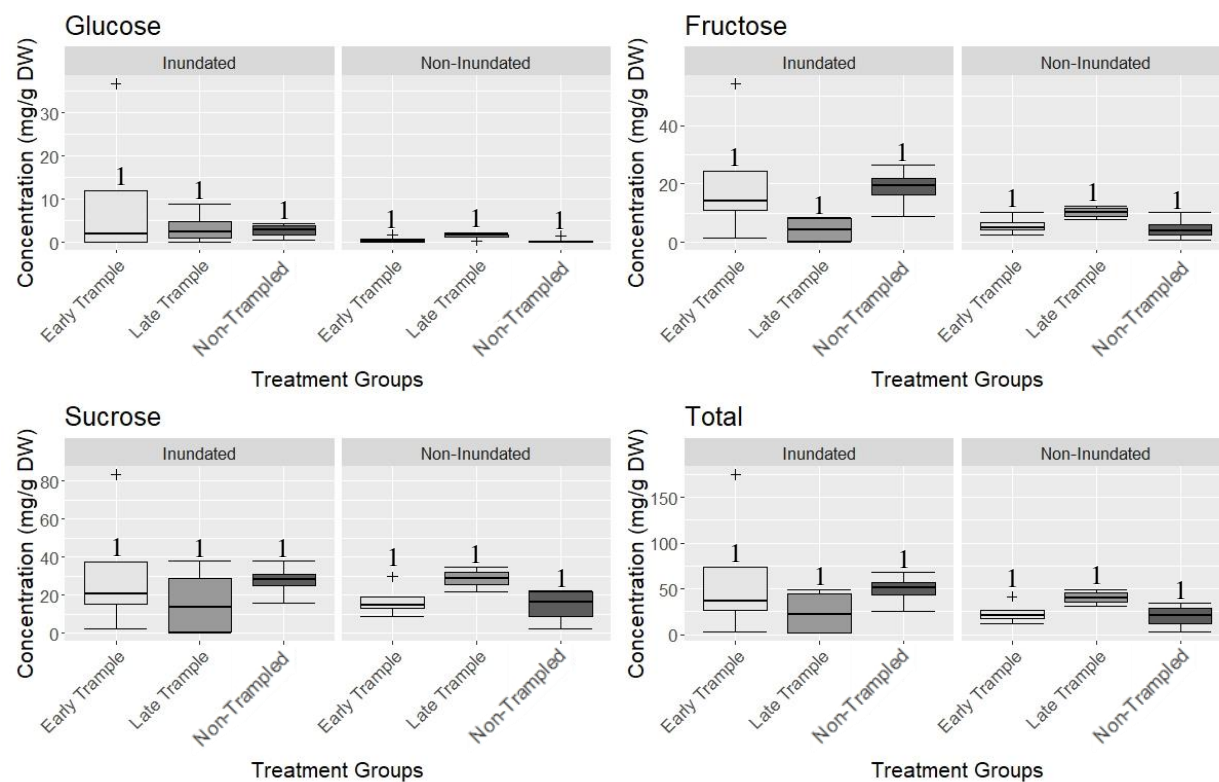


Figure 4. Yellow-flag iris (*Iris pseudacorus*) concentration of soluble sugars (mg/g dw) as affected by simulated cattle trampling and inundation. Results reflect differences in medians (bold horizontal lines), and treatment groups with a number in common are not different (Wilcoxon Rank Sum Test; $\alpha=0.05$).

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Appendix A: Solution Preparation and Quantification Equations

1. Solution Preparation

1.1 Sodium Acetate (NaOAc) Buffer Solution (25 mM, pH 4.6):

Dissolve 1.025 g of sodium acetate in 450 mL of deionized water (dH₂O). Adjust to pH 4.6 with acetic acid. Bring to a total volume of 500 mL with dH₂O.

1.2 Hexokinase-Glucose 6-Phosphate Dehydrogenase (GHK) Solution:

Add 50 mL of dH₂O to the bottle of Glucose Assay Reagent (Sigma G3293-50ML), and invert gently to dissolve.

1.3 0.2 M 4-(2-Hydroxyethyl)piperazine-1-Ethanesulfonic Acid (HEPES) Buffer (pH 7.8):

Add 4.766 g of HEPES (Sigma H3375) to 100 mL of dH₂O. Titrate the solution to pH 7.8 with 5 M NaOH. Dilute 10-fold for 0.02 M buffer.

1.4 Phosphoglucose Isomerase Solution:

Add 1.0 mL of 0.2 M HEPES (pH 7.8) to 100 U of PGI (Sigma P5381-5KU). Dilute 50 μ L of the stock solution with 2 mL of 0.2 M HEPES

1.5 Invertase Solution (60 U/mL):

Dissolve 600 U of invertase (Sigma I9274, from baker's yeast (*S. cerevisiae*)) in 10 mL of 25 mM NaOAc buffer.

1.6 Glucose Standard Solutions:

Make serial 1:2 dilutions of the 1 mg/mL stock solution (Sigma G6918) with dH₂O for standard solutions of 1000 to 62.5 μ g/mL

1.7 Fructose and Sucrose Standard Solutions (1000 μ g/mL):

Dissolve 100 mg of each sugar (Fructose: Sigma F0127; Sucrose: Sigma S0389) in 100 mL of dH₂O)

2. Concentration (mg/g DW) Quantification Calculations

The following equations were used to quantify the concentration of sugars ($\mu\text{g/g DW}$) in each sample. Variables used throughout the equations are as follows: Abs_{stand} (absorbance reading of sugar standard), Abs_{blank} (absorbance reading of blank cell), Abs_{samp} (absorbance reading of cell with iris tissue sample), V_1 (volume of sample or dH_2O in each cell [mL]), V_2 (total volume in each cell [mL]), and wt (weight of ground iris tissue used during sugar extraction [g]).

Glucose Standard Curve

$$[\text{glucose}_{standard}] = m \times (Abs_{stand} - Abs_{blank}) + b \quad [1]$$

Quantification of Glucose

$$[\text{glucose}] = \left\{ \left((Abs_{samp} - Abs_{blank}) \times m + b \right) \times \left(\frac{1}{V_1} \right) \times \left(\frac{V_2}{wt} \right) \right\} \quad [2]$$

Quantification of Fructose

$$[\text{fructose}] = \left\{ \left[\left((Abs_{samp} - Abs_{blank}) \times m + b \right) \times \left(\frac{1}{V_1} \right) \times \left(\frac{V_2}{wt} \right) \right] - [\text{glucose}] \right\} \quad [3]$$

Quantification of Sucrose

$$[\text{sucrose}] = \left\{ \left[\left((Abs_{samp} - Abs_{blank}) \times m + b \right) \times \left(\frac{1}{V_1} \right) \times \left(\frac{V_2}{wt} \right) \right] - [\text{glucose}] \right\} \times 2 \quad [4]$$