INDIVIDUAL AND COMBINED EFFECTS OF NATURAL ENEMIES ON AMPHIBIAN COMMUNITIES

by

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ABSTRACT

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Natural enemy ecology strives to integrate the fields of disease ecology and community ecology to forge a broader understanding of how pathogens and predators structure communities. To advance this field, we need a greater emphasis on: 1) quantifying pathogen-mediated effects on community structure and comparing these effects to those observed with predators and 2) determining the interactive effects of combined natural enemies on communities. I conducted a mesocosm experiment designed to assess the individual and combined effects of predators (dragonfly larvae and adult water bugs) and a pathogen (ranavirus) on a larval amphibian community. Additionally, I conducted laboratory experiments to assess whether ranavirus exposure increases the vulnerability of tadpoles to predation. In my laboratory experiments, I found that virus exposure increased predation rates with dragonflies, but not water bugs. For tadpoles in the dragonfly treatments, the probability of survival for virus-exposed tadpoles was 66-77% lower compared to unexposed tadpoles. This data suggests that predators may selectively remove infected individuals from the population, which can enhance the magnitude of the healthy herds effect. I found that the risk level of the predators largely explained effects on the community. For instance, high-risk dragonflies reduce overall survival to 30% whereas low-risk water bugs only reduced survival to 67%. Additionally, I found that virus reduce survival to 62%, which was comparable to effect of the low-risk predator. Interestingly, all three natural enemies influenced community structure (i.e. species relative abundance) in unique ways. These results demonstrate that pathogens can have effects similar to predators on communities, and that natural enemy identity is important when considering impacts on community structure. When predators were combined with the virus, I found that mortality was relatively unchanged from the predator-only treatments suggesting less than additive effects of combined natural enemies. This result was driven by the healthy herds effect; the presence of dragonflies reduced overall infection prevalence in the community to 7% compared to 30% in the virus-only treatment. This effect was observed

in the water bug treatments, to a lesser degree, suggesting that predator risk or efficiency contributes the magnitude of the effect. Collectively, my work demonstrates the importance of examining the individual and combined effects of natural enemies on ecological communities.

CHAPTER 1. VIRUS EXPOSURE INCREASES THE SUSCEPTIBILITY OF TADPOLES TO PREDATION

1.1 Introduction

Predation and disease are fundamental processes in ecological communities (Holt and Dobson 2006, Morin 2011). Because predators and pathogens frequently co-occur in nature, these natural enemies may directly and indirectly interact to influence food web dynamics (Duffy et al. 2005, Johnson et al. 2006, Cáceres et al. 2009). In particular, predation can play an important role in disease dynamics through the healthy herds effect. Predators can reduce pathogen transmission by reducing host density or inducing changes in host behaviors that reduce host contract rates (Packer et al. 2003, Lafferty 2004). For instance, removal of predatory lobsters has been shown to increase disease incidence in sea urchins, due to density effects (Lafferty 2004). In addition to the healthy herd effect, disease can indirectly influence predator-prey interactions via pathology from infections. For example, host pathology associated with infections, including sickness behaviors, could potentially increase the vulnerability of hosts to predation. Pathogen-induced vulnerability to predation is often observed in systems with trophic transmission; for instance, acanthocephalan parasites induce behavior changes in their hosts, amphipods, which increases vulnerability to fish predation (Bakker et al. 1997). Because these parasites typically have complex life cycles, trophic transmission and predation serve to propagate disease. For pathogens without trophic transmission, increased vulnerability to predation can function to reduce disease risk. Moreover, predators can select for infected individuals, and this selection has the effect of both decreasing host density and reducing infection in the system (Hudson et al. 1992, Duffy 2007). Thus, predators can play a significant role in maintaining healthier herds and lowering disease risk in certain systems. While it is recognized that non-trophically transmitted pathogens can influence the vulnerability of hosts to predation, relatively few systems have been examined outside of the context of trophic transmission (Hudson et al. 1992, Murray et al. 1997, Johnson et al. 2006, Duffy 2007).

Amphibians provide an ideal study system for addressing the interactive effects of pathogens and predators because they are likely to encounter multiple natural enemies. Amphibian larvae encounter a diverse predator community and a rich body of literature has documented predator-prey interactions in this system (Relyea 2003). For instance, activity levels are correlated with predation rates; more active individuals and species are more susceptible to predation than

those that are less active (Lawler 1989, Skelly 1994, Relyea 2001a). Amphibians also are threatened by several pathogens capable of causing massive mortality events (Daszak et al. 1999). In particular, viruses in the genus *Ranavirus* (Family Iridoviridae) have been associated with epizootic events across the globe (Duffus et al. 2015). Ranavirus transmission can occur through direct contact, necrophagy, and exposure to shed virions in the environment. Ranaviruses tend to be more virulent in larval amphibians, yet there is substantial variation in disease outcomes among species (Hoverman et al. 2011, Gray and Chinchar 2015). Importantly, pathology associated with ranavirus includes erratic swimming, lethargy, loss of equilibrium, edema, and hemorrhaging (Gray et al. 2009). Given the pathology associated with infections, there is the potential for the virus to enhance the susceptibility of hosts to predation.

While there is considerable research effort towards understanding the epidemiology of ranaviruses, few studies have addressed their effects on species interactions and communities (Gray et al. 2009). In particular, only a single study has examined the influence of ranavirus exposure on predation rates (Parris et al. 2004). In this study, salamander larvae infected with ranavirus (*Ambystoma tigrinum* virus, ATV) had lower mortality rates with dragonfly larvae compared to uninfected larvae, contrary to classic predictions (Parris et al. 2004). However, there was no difference in the activity level of infected and uninfected larvae in the presence of caged dragonfly larvae. Haislip et al. (2011) also documented that virus exposure had only limited effects on behavioral responses to caged predators in four species of larval anurans. Because virus exposure appears to have limited effects on anti-predator behavioral responses, differences in predation rates could be related to pathology associated with infection. For instance, larval anurans are known to exhibit lethargy as well as bursts of erratic activity when infected (Gray et al. 2009). Lethargy could lower predation rates with visually-oriented predators by reducing encounter rates, which would be consistent with the findings of Parris et al. (2004). Alternatively, lethargy could impair the ability of larvae to escape predators once encountered. Moreover, bursts of erratic activity could enhance predation rates by increasing detectability. To explore these potential outcomes, there is a need to examine additional amphibian species and predators to develop generalities.

To assess how virus exposure influences predation rates, I conducted short-term microcosm experiments with tadpoles of four amphibian species and two predators, dragonfly larvae (*Anax junius*) and adult water bugs (*Belostoma flumineum*). *Anax* are considered high-risk

predators because of their high capture efficiency and short handing times (Relyea 2001b). In contrast, *Belostoma* are low-risk predators because of their poor capture ability and long handling times. These differences in risk translate into differences in predation rates between the two predators, and different effects on prey traits; the magnitude of which are correlated with risk level (Relyea 2001a). I hypothesized that tadpoles exposed to virus would have higher mortality rates with predators compared to unexposed tadpoles because of the pathology associated with infection (i.e. lethargy and erratic movements). Additionally, I expected these effects to be stronger for the high-risk predator compared to the low-risk predator because of their differences in foraging efficiency.

1.2 Methods

1.2.1 Species collection and maintenance

My focal species were Spring Peepers (*Pseudacris crucifer*), Gray Treefrogs (*Hyla versicolor*), American Toads (*Anaxyrus americanus*), and Northern Leopard Frogs (*Lithobates pipiens*). I selected these species because they commonly co-occur in wetlands, represent a range of predator avoidance strategies, and vary in their susceptibility to ranavirus (Relyea 2001a, Hoverman et al. 2011, Wuerthner et al. 2017). These species were collected from ponds surrounding the Purdue Wildlife Area (PWA), West Lafayette, IN, USA. I collected Spring Peepers (n = 26 pairs) and Gray Treefrogs (n= 28 pairs) in amplexus during breeding activity and placed each pair into a 15-L tub filled with 2 L of UV-irradiated, filtered well water to oviposit overnight in the laboratory. The pairs were released the next morning. I maintained the hatchlings in the lab until they were free-swimming, at which point they were transferred to 100-L outdoor culture pools filled with 70 L of aged well water. I collected partial American Toad (n = 15) and Northern Leopard Frog (n = 18) egg masses the morning after breeding activity and placed them into outdoor 100-L culture pools filled with 70 L aged well water. I fed tadpoles Tetramin (for early stages; Tetra, Virginia, USA) or rabbit chow (Purina, Missouri, USA) *ad libitum* until the experiments began. Animal husbandry procedures followed and approved according to Purdue University PACUC protocol #13020008231.

My focal predators were larval green darner dragonflies (*Anax junius*) and adult water bugs (*Belostoma flumineum*). These predators were selected because they differ in the threat they pose

to tadpoles (Relyea 2001a). The predators were collected from ponds surrounding the PWA and housed individually in 1-L cups filled with 0.8 L UV-irradiated, filtered well water until the experiment. I fed each predator one tadpole every other day. I alternated the species identity of the feeder tadpole for each feeding to ensure predators had exposure to all four species.

1.2.2 Virus Culture

I used a ranavirus strain isolated from an infected green frog (*Rana* (*Lithobates*) *clamitans*) found at the PWA. Previous research has found that this virus strain is capable of infecting each of my focal species (Pochini and Hoverman 2017, Wuerthner et al. 2017). I cultured the virus on fathead minnow cells and Eagle's minimum essential media containing 5% fetal bovine serum (MEM) to a titer of 1.68×10^6 PFU mL^{-1}. The virus was stored at -80°C until used in the experiments.

1.2.3 Experimental Setup

My experiment was designed to examine the effect of virus exposure on predation rates for each amphibian species. The experimental design consisted of six treatments: a virus- and predator-free control (Control), virus exposure only (Virus), one lethal *Anax* (*Anax*), one lethal *Belostoma* (*Belostoma*), virus-exposed tadpoles with one *Anax* (Virus x *Anax*), and virus-exposed tadpoles with one *Belostoma* (Virus x *Belostoma*). These six treatments were replicated 4 times for 24 experimental units per species (96 total experimental units). The experimental units were 15-L tubs, filled with 7 L of aged well water. The tubs were housed on racks in a covered area shaded from sunlight outside of the laboratory, spatially blocked (n = 8 replicates per block) by shelf height (n = 3 shelf heights) with experimental units randomly assigned to treatment within each block. I added 4.5 g of oak leaves (*Quercus* spp.) to each tub to provide structure and refuge. I allowed 4 days for the leaves to settle to the bottom of the tubs.

Before adding tadpoles to the experimental units, I initiated the virus exposure. I began by randomly selecting 240 individuals per species and separating them into groups of 40, which I placed into 2-L tubs. These tubs were filled with 1 L UV-irradiated, filtered well water. Four days before the experiment began, half of these tubs (n = 3 per species) were exposed to 5.95 mL of virus in Eagles MEM (original titer: 1.68×10^6 PFU mL^{-1}) to achieve a final concentration of 10^4 PFU mL^{-1}. This dosage has been found to be sufficient for initiating infection in these species

(Hoverman et al. 2010). The remaining tubs served as controls and were exposed to an equivalent volume of sterile MEM. After two days of exposure, the tadpoles were placed into their respective experimental units (n = 10 tadpoles per unit). I allowed the tadpoles to acclimate to their experimental units for two additional days before being exposed to their predator treatments. This four day exposure period was chosen because tadpoles are likely to exhibit signs of infection but not virus-induced mortality (Hoverman et al. 2010).

The predators were added to the appropriate tubs one day after the tadpoles were added. To acclimate the tadpoles to the predator's presence, the predators were initially caged in 1-L cups covered with mesh screen and fed 1 conspecific tadpole. Caged predators release cues that are used by tadpoles in the formation of inducible defenses against predators (Petranka et al. 1987, Relyea 2001a). After 24 hours, each predator had eaten and was released into its experimental unit. At the same time, a subsample of tadpoles, exposed only to virus, were euthanized, weighed, staged, and dissected for qPCR analysis of infection. Initial masses (means ± 1 SE) of American Toads, Northern Leopard Frogs, Spring Peepers, and Gray Treefrogs were 0.036 ± 0.005g, 0.108 ± 0.011g, 0.105 ± 0.014g, 0.035 ± 0.003g, respectively. Additionally, initial average Gosner stages were 30, 25, 32, and 26, respectively.

The tubs were checked every 24 hours for mortality and prey consumption. This was accomplished by visually inspecting the contents of each tub and gently lifting the tub to look on the bottom and under the leaves. The number of tadpoles seen was recorded, as were the number of dead tadpoles found in each tub. In predator treatments, only *Belostoma* treatments had tadpoles seen dead, and there were only two individuals dead that I could not definitively attribute to predators. These were removed from the experimental unit and preserved in 70% ethanol.

The experiment ran for 8 days, and tadpoles were fed *ad libitum* throughout. At the end of the experiment, surviving tadpoles were euthanized using a 0.8 g L^{-1} concentration of MS-222 and preserved in 70% ethanol. I then staged and weighed all tadpoles and dissected those that had been exposed to virus. All tadpoles exposed to virus, except for those consumed by predators, had their liver and kidneys removed and frozen at -80°C. For each individual, the pooled kidney and liver samples was used for virus testing. DNA was extracted from these samples using DNEasy Blood and Tissue Kits (Qiagen) and stored at -80°C until qPCR analysis.

1.2.4 Ranavirus Testing

I used quantitative polymerase chain reaction (qPCR) to test for ranavirus infection in the experiment (Wuerthner et al. 2017). The reaction was carried out in 96-well plates, with 4 standards, 1 negative control, and 43 experiment samples, all run in duplicate. Each well contained 6.25 µL SsoAdvanced Universal Probes Supermix (BioRad), 2.75 µL autoclaved nanopure water, 1.0 µL of a mixture of each primer at 10 pmol mL^{-1} (rtMCP-F [50-ACA CCA CCG CCC AAA AGT AC-3'] and rtMCP-R [50-CCG TTC ATG CGG ATA ATG-3']) and a fluorescent probe (rtMCP-probe (50 - CCT CAT CGT TCT GGC CAT CAACCA-30), and 2.5 µL of DNA template or autoclaved nanopure water for a final volume of 12.5 µL. I ran qPCR reactions using a Bio-Rad real-time PCR system. The DNA standard was a synthetic double-stranded 250 bp fragment of the highly conserved *Ranavirus* major capsid protein (MCP) gene (gBlocks Gene Fragments; Integrated DNA Technologies). A standard curve was created using a log-based dilution series of 4.014×10^6 viral copies mL^{-1} to 4.014×10^3 viral copies mL^{-1}.

1.2.5 Statistical Analysis

I performed all statistical analysis using R version 3.3.2 (R Development Core 2011). To prepare the data for analysis, I combined my final survival counts with the number of tadpoles I had observed as dead and unconsumed. I constructed Cox Proportional Hazards Models to examine differences in time-to-death for tadpoles, as a proxy for predator success using the packages 'survival' and 'coxme' in R (Therneau 2013, Therneau 2015). I used survival over time as my response variable and included the presence of a predator, exposure to virus, and an interaction as predictors, and included experimental unit as a clustering factor. If my overall model showed significant differences among predator treatments for each species, I divided my dataset by predator for separate analyses and modeled survival in exposed units compared to unexposed units. Survival curves were produced using the package 'survminer' in R (Kassambara and Kosinski 2018).

1.3 Results

1.3.1 Tadpole survival

For each amphibian species, I found an overall effect of treatment on survival (Wald test ≥ 208; $P < 0.001$). Survival was generally highest in the control treatment (80 to 100%) and lowest in the *Anax* x Virus treatment (0 to 2%). To explore the specific effects of my treatments on survival, I separated the dataset by predator treatment, to examine the influence of virus exposure on tadpole survival: 1) in the absence of predators, 2) in the presence of *Anax*, and 3) in the presence of *Belostoma*.

In the absence of predators, there was no significant effect of virus exposure on survival of American Toads, Northern Leopard Frogs, or Spring Peepers ($P \geq 0.629$). However, virus exposure did decrease the survival of Gray Treefrogs ($P < 0.001$). For this species, the majority of the mortality in the virus exposure treatment occurred after 120 hours in the experiment with final survival reduced by 50% compared to the no-virus treatment.

Compared to the control treatment, *Anax* reduced tadpole survival probability by 93 to 99% in the experiments for each species, regardless of virus exposure ($P < 0.001$). Additionally, virus exposure significantly increased hazard for three of the four species in the presence of *Anax*. For Spring Peepers, Gray Treefrogs, and Northern Leopard Frogs, the addition of virus to *Anax* treatments further increased risk of death by 66-77% ($P \leq 0.009$). There was no effect of virus exposure on the probability of survival for American Toads in *Anax* treatments ($P = 0.19$).

Compared to the control treatment, *Belostoma* reduced survival probability of Gray Treefrogs, Northern Leopard Frogs and American Toads by 76 to 97% ($P \leq 0.005$). However, there was no evidence that virus exposure further influenced the probability of survival for any of the amphibian species with *Belostoma* ($P \geq 0.13$).

1.3.2 Infection prevalence

I measured a random subsample of exposed tadpoles from each species to determine initial infection prevalence at the start of the experiments (n=10 per species). For American Toads, Northern Leopard Frogs, Spring Peepers, and Gray Treefrogs, I found 30, 0, 10, and 80% infection prevalence, respectively. In addition, I tested all animals from the no-predator, virus exposure treatment for infection at the end of the experiment. I found 38, 0, 26, and 80% infection for American Toads, Northern Leopard Frogs, Spring Peepers, and Gray Treefrogs, respectively

(Table 1-1). Similar patterns in infection prevalence across species were observed in the survivors from the *Belostoma* treatments (Table 1-1). However, too few tadpoles were recovered alive from the *Anax* treatments to assess infection patterns.

1.4 Discussion

My results demonstrate that virus exposure can increase predation rates on larval anurans; however, the magnitude of this effect was dependent on both predator and prey identity. For three of the four amphibian species, individuals exposed to virus had 66-77% higher predation rates by *Anax* compared to unexposed individuals. In contrast, there were no differences in predation rates between exposed and unexposed individuals with *Belostoma*. These differences between predators could be related to differences in their capture efficiency and handling times; *Anax* are more efficient at capturing prey and have shorter handling times compared to *Belostoma* (Relyea 2001). Thus, *Anax* appear to be better able to take advantage of any virus-induced change in the vulnerability of tadpoles. Although I only examined two predator species, these results suggest that the magnitude of pathogen-induced vulnerability to predation is dependent on the risk posed by the predator.

Differences in prey species identity also influenced the effect of virus on predator-prey interactions in the *Anax* treatments. Virus exposure had the largest effect on predation rates of Spring Peepers, moderate effects on Gray Treefrogs and Northern Leopard Frogs, and no effect on American Toads. These species-level differences could be related to baseline activity levels of the species and how virus exposure influences behavior. For instance, Spring Peepers generally have low activity levels while American Toads are highly active (Morin 1986, Skelly 1994, Relyea 2001b). Moreover, high prey activity levels generally influence predation rates such that highly active species will experience higher predation rates than less active species (Lawler 1989). If virus exposure increases activity or erratic movements of prey, I would expect the effects on predation rates to be greater for prey that are generally less active. Although I did not measure behavior in my experiment to reduce disturbance and impacts on predation, this may suggest that behavior could contribute to the effects in my results. Alternatively, virus exposure could elicit chemical distress cues that signal vulnerability to predators. For example, red grouse infected with nematode parasites are easier to detect with scent by predators (Hudson et al. 1992). If such cues exist in my system, my data suggests that *Anax* are more responsive to them compared to

Belostoma. Before generalizations can be drawn regarding types of predators that may be capable of exploiting the vulnerability induced by virus, more research should be conducted regarding both the role of predator identity and the mechanism by which this vulnerability is conveyed.

It should be noted that I did not detect infection in Northern Leopard Frogs, regardless of exposure to virus via the same protocol as other species. The techniques used in this study have been used in other studies to elicit infection in Northern Leopard Frogs (Hoverman et al. 2011), which suggests that methodology played a minor role in this finding. Despite this lack of infection, Northern Leopard Frogs experienced 66% higher probability of predation after exposure to virus. One possibility is that I did successfully infect Leopard Frogs, but that the infection was not detectable or was cleared. Thus, individuals with resistance or tolerance to ranavirus might still experience adverse effects. Another possibility is that simply exposure to virus initiates an energetic shift towards immunity, altering prey traits as early as this initial exposure, and not requiring successful infection.

My results are counter to the findings of Parris et al. (2004). They found that ATV reduced predation rates by *Anax* on Tiger Salamanders. The contrasting results of these two studies could be driven by differences in the virus or amphibian species used in the experiments. For example, ATV and FV3 could influence amphibian behavior differently. It is also possible that salamanders respond differently to virus exposure than anurans. Because I did not examine behavior, I am unable to directly assess this possibility. However, Parris et al. (2004) did not detect difference in the activity level of infected and uninfected larvae in the presence of caged *Anax* suggesting that behavior was not the driving mechanism of their predation results. Given the limited number of studies in this area, more research examining different predators, amphibians, and virus isolates is needed to develop generalities.

Many species encounter multiple co-occurring natural enemies within their communities (Borer et al. 2007, Hatcher and Dunn 2011). Importantly, natural enemies can influence each other via their interactions with hosts or prey within the community (Hatcher et al. 2006). A recent study documented the healthy herds effects in amphibians such that free-ranging *Anax* reduced ranavirus prevalence in an amphibian assemblage by 83% compared to predator-free treatments (Gallagher et al. in review). While this result appeared to be mediated by predator driven reductions in host density and transmission, the results of the current study demonstrate that virus exposure could also enhance the vulnerability of prey to predation. For host populations, higher consumption rates

of virus-exposed individuals by predators could reduce pathogen transmission within natural systems. However, an additional question that must be addressed is whether predators selectively consume infected over uninfected prey. While selective predation is not a requirement for the healthy herds effect to occur, it does strengthen the magnitude of the effect (Packer et al. 2003). Collectively, these results suggest that a combination of density-mediated and trait-mediated effects may contribute to the healthy herds effect in amphibian communities. In light of my study, future work should focus on the role of predator density and identity, as well as understanding how community composition influences these dynamics.

1.5 Tables and Figures

Table 1-1. Total number of survivors, total number infected, and overall infection prevalence in the three predator treatments within the virus treatment for each species.

	No Predator			***Belostoma***			***Anax***		
Species	N	Infected	Prevalence	N	Infected	Prevalence	N	Infected	Prevalence
Spring Peeper	39	10	26%	29	12	41%	3	0	0%
Gray Treefrog	25	20	80%	12	9	75%	0	N/A	N/A
Northern Leopard Frog	37	0	0%	17	1	6%	1	0	0%
American Toad	34	13	38%	10	5	50%	0	N/A	N/A

N/A = Not Applicable, no survivors from these treatments.

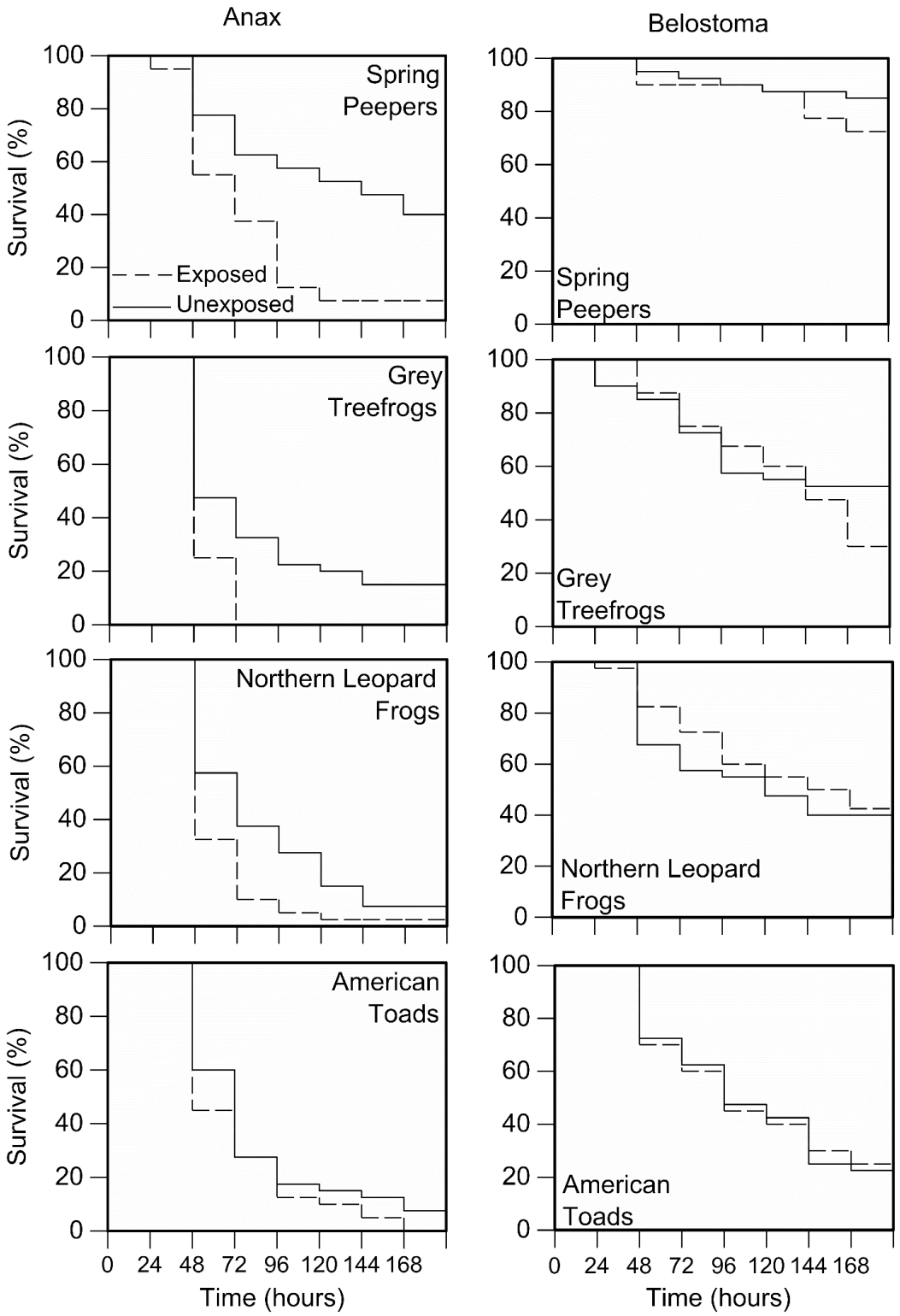

Figure 1-1. Survival curves showing the effects of exposure to ranavirus on survival over time for tadpoles in the presence of larval dragonflies (left) and adult water bugs (right). Solid lines indicate unexposed tadpoles and dashed lines indicate exposed tadpoles.

CHAPTER 2. INDIVIDUAL AND COMBINED EFFECTS OF NATURAL ENEMIES ON THE STRUCTURE OF LARVAL AMPHIBIAN COMMUNITIES

2.1 Introduction

For decades, ecologists have examined the factors that generate and influence community structure (species richness, composition, relative abundance; Morin 2011). Predators, in particular, play an important role in structuring many communities. As keystone species, predators can enhance species diversity by reducing the abundance of competitively dominant prey, thus favoring inactive or well-defended prey (Paine 1966, Morin 1983, Wellborn et al. 1996). There is also evidence that pathogens have parallel effects on community structure. For instance, pathogens were able to reverse the outcome of competition and promote coexistence in flour beetles (*Tribolium*) and fruit flies (*Drosophila*) due to disproportionate effects on the competitively dominant host species (Park 1948, Jaenike 1995). Additionally, disease outbreaks and major epidemics can dramatically alter community structure (Dobson and Hudson 1986). Despite the recognition that predators and pathogens can influence community structure, there have been few attempts to directly compare the effects of different natural enemies on the structure of a focal community. Such research is critical to advance natural enemy ecology, which strives to unify predator-prey and host-pathogen interactions under a single framework within community ecology (Raffel et al. 2008).

Research suggests a common mechanism underlying consumer-mediated effects on community structure – that species vary in their vulnerability to natural enemies. In predator-prey systems, factors such as activity level, habitat use, body size, and morphology can influence vulnerability to predation (Turner and Mittelbach 1990, Skelly 1994, Relyea 2001a). Likewise, species-level variation in immunity, habitat selection, and activity levels affect susceptibility to pathogens (Hoverman et al. 2011, Johnson and Hoverman 2014). Using information on the relative vulnerability of species to natural enemies, we should be able to predict their influence on community structure. More specifically, we would expect the community structure of prey communities to shift to more predator-tolerant taxa following the introduction of predators while the introduction of pathogens should shift community structure to more disease-tolerant taxa.

An additional question to emerge from this direction of inquiry is the degree of similarity between communities influenced by different natural enemies. If the traits that increase vulnerability to predation also tend to increase exposure or susceptibility to pathogens (e.g., high activity level), we might expect natural enemies to have similar influences on community structure. Alternatively, if the defensive mechanisms differ according to the natural enemy, community structures could differ dramatically. To date, no studies have directly contrasted the influence of different natural enemies on community structure. To advance the field of natural enemy ecology, we need a greater emphasis on quantifying pathogen-mediated effects on community structure and comparing these effects to those observed with predators.

An important consideration in natural enemy ecology is that the effects of natural enemies on community structure will depend on the characteristics of the enemies. There is substantial research demonstrating that predators vary in the risk they pose to prey (Sih 1987, Lima and Dill 1990, Relyea 2001b). This variation is generated by differences among predators in their abundance within the community and their ability to capture, handle, and consume prey, which collectively determine predation rates. Due to differences in predation risk and overall predation rates, high-risk predators generally have greater effects on community structure compared to low-risk predators (Van Buskirk 1988, Relyea 2003). Similarly, pathogens vary in their virulence to host species (Schmid-Hempel 2011). Consequently, we might expect parallel effects on community structure; highly virulent pathogens should have greater effects on community structure than less virulent pathogens. To advance our understanding of how natural enemies influence community structure, we need studies that incorporate multiple natural enemies to assess how variation in risk or virulence influences the magnitude of community-level effects.

Many species are found in communities in which they are exposed to multiple natural enemies simultaneously (Hatcher and Dunn 2011). Natural enemies can potentially influence each other directly or indirectly via their interactions with victim species. In predator-prey systems, emergent multiple predator effects have been demonstrated; risk reduction can occur if predator-predator interactions alter consumption rates of prey while risk enhancement can occur if prey have conflicting responses to predators (Sih et al. 1998). Similarly, there is evidence that pathogens influence predator-prey interactions. A classic example is seen in systems with trophically transmitted pathogens (Hatcher and Dunn 2011). In this case, infections increase the vulnerability of hosts to predation resulting in transmission of the pathogen to the next host in the life cycle.

While less frequently examined, there is also evidence that predators can influence host-pathogen interactions (Raffel et al. 2008). For example, research found that dragonfly larvae reduced the prevalence of ranavirus infections in an amphibian assemblage by reducing tadpole density and thereby transmission rates (i.e. healthy herds effect; Gallagher et al. in review). While this research collectively demonstrates the reciprocal effects of predators and pathogens on interactions with their respective victims, there have been few attempts to extend this research to communities with multiple victims to assess emergent effects of combined natural enemies on community structure (Veddeler et al. 2010, Philpott et al. 2012, Stephens et al. 2013).

Larval amphibians are an excellent model system for the study of natural enemy ecology. Amphibian larvae encounter a diverse predator community and a rich body of literature has documented predator-prey interactions in this system (Relyea 2003). There is substantial research demonstrating that amphibian predators vary in the risk they pose to prey (Relyea 2003). This variation is generated by differences among predators in their abundance within the community and their ability to capture, handle, and consume prey, which collectively determine predation rates. For instance, my two focal predators exist at opposing ends of the predation risk gradient; *Anax* are considered high-risk predators because of their high capture efficiency and short handing times, while *Belostoma* are low-risk predators because of their poor capture ability and long handling times (Relyea 2001b). These differences in risk translate into differences in predation rates between the two predators.

In addition to predators, amphibian larvae encounter a broad diversity of pathogens. Ranaviruses are broadly distributed pathogens of ectothermic vertebrates (Chinchar 2002). In amphibians, infections have been detected in a broad diversity of species across the globe (Duffus et al. 2015). Moreover, epizootics have been reported in numerous species and are characterized by rapid onset and high mortality in larvae (Duffus et al. 2015). Transmission of ranaviruses is horizontal, and can occur by exposure to infected water or soil, or via ingestion of infected individuals (Harp and Petranka 2006, Brunner et al. 2007). Ranaviral disease is characterized by systemic hemorrhage and tissue necrosis, ultimately resulting in organ failure (usually of the liver or kidneys), which can occur within one week of exposure (Gray et al. 2009, Miller et al. 2011). Research over the last decade has characterized the relative vulnerability of amphibian species to ranavirus infection and disease outcomes (Hoverman et al. 2011). However, no studies have

examined the impacts of ranaviruses on the structure of amphibian communities (Hoverman et al. 2011).

My objectives were to address the individual and combined effects of natural enemies on the structure of larval amphibian communities. My experimental design consisted of each natural enemy (*Anax*, *Belostoma*, and ranavirus) at low and high densities. Additionally, I included treatments that crossed the presence of ranavirus with either *Anax* or *Belostoma*. I made several predictions using previous research in this system (Table 2-1). Based on the risk posed by each natural enemy, I predicted overall amphibian abundance to be lowest with *Anax*, intermediate with ranavirus, and highest with *Belostoma*. I also predicted that community structure would diverge among the natural enemy communities because of differences in the relative vulnerability of amphibians to each natural enemy. With increases in the density of each natural enemy, I predicted the magnitude of effects to increase. Given previous research demonstrating that predators reduce infection prevalence in amphibians (Gallagher et al. in review), I predicted less than additive effects of combined natural enemies on community structure.

2.2 Materials and Methods

2.2.1 Focal species

My focal amphibians were Spring Peepers (*Pseudacris crucifer*), Gray Treefrogs (*Hyla versicolor*), American Toads (*Anaxyrus americanus*), Northern Leopard Frogs (*Rana* (*Lithobates) pipiens*), and Wood Frogs (*R. sylvatica*). I selected these species because they commonly co-occur in wetlands (Werner et al. 2007), represent a range of predator avoidance strategies, and vary in their susceptibility to pathogens and predators (Relyea 2001a, Hoverman et al. 2011). Wood frogs served as my source of infections in the experiment because of their high susceptibility to ranavirus (see *Experimental design and setup*, Haislip et al. 2011, Hoverman et al. 2011). All the species except Wood Frogs were collected from ponds surrounding the Purdue Wildlife Area (PWA), West Lafayette, IN, USA. I collected Spring Peepers and Gray Treefrogs in amplexus (n= 28 and 26 pairs, respectively) and placed each pair into a 15-L tub filled with UV-irradiated, filtered well water to oviposit overnight in the laboratory. I maintained the hatchlings in the lab until they were free-swimming, at which point they were transferred to covered 100-L outdoor culture pools filled with 70 L of aged well water. I collected partial American Toad (n = 15) and Northern Leopard

Frog (n = 18) egg masses the morning after breeding activity and placed them into outdoor culture pools. I collected partial Wood Frog egg masses (n = 10) from a forested wetland near Nashville, Indiana, USA and placed them into outdoor pools. I fed tadpoles Tetramin (for early stages; Tetra, Virginia, USA) or rabbit chow (Purina, Missouri, USA) *ad libitum* until the experiment began.

My focal predators were larval green darner dragonflies (*Anax junius*) and adult water bugs (*Belostoma flumineum*). These predators were selected because they differ in the threat they pose to tadpoles (Relyea 2001b). The larval *Anax* are voracious predators of larval anurans, with an average handling time of ~2 min; whereas *Belostoma* are inefficient tadpole predators, with a handling time ranging from 17-65 minutes (Relyea 2001b). The predators were collected from ponds surrounding the PWA and housed individually in 1-L cups filled with 0.8 L UV-irradiated, filtered well water until the experiment. I fed each predator one tadpole every other day alternating the species identity of the feeder tadpole each feeding, to ensure predators had exposure to all five species.

I used a ranavirus strain isolated from an infected green frog (*R. clamitans*) found at the PWA. Previous research has found that this virus strain is capable of infecting each of my focal species (Pochini and Hoverman 2017, Wuerthner et al. 2017). I cultured the virus on fathead minnow cells and Eagle's minimum essential media (MEM) containing 5% fetal bovine serum to a titer of 1.68×10^6 PFU mL^{-1}. The virus was stored at -80°C until used in the experiment.

2.2.2 Experimental design and setup

In May 2017, I began a mesocosm experiment to examine the community-level effects of natural enemies on amphibians. My experimental design consisted of a control (no natural enemies), each natural enemy (*Anax*, *Belostoma*, and Virus) at low and high densities, and two predator-pathogen combinations (Low *Anax* + Low Virus and Low *Belostoma* + Low Virus). I replicated each of the nine treatments eight times for a total of 72 experimental units. My experimental units were 1200-L cattle tanks (Rubbermaid, Georgia, USA) filled with 700 L of aged well water and covered with 70% shade cloth lids. I arranged the tanks in an 8 x 9 grid and randomly assigned one replicate of each treatment to a row within the grid. To each tank, I added 200 g of dried oak (*Quercus* spp.) leaves for cover and 30 g of rabbit chow as an initial source of nutrients. I also added 1.3 L of pond water, which had been passed through a 250-micron sieve to exclude predators, to introduce algae into the tanks. Two days later, I inoculated each unit with

concentrated zooplankton, which was hand-sorted and passed through a 1 mm filter to exclude tadpole predators. I added 10 clay tiles (7.6 cm x 15.2 cm) facing south against the inside of the units to monitor periphyton growth throughout the experiment. I allowed the zooplankton and algal communities in the mesocosms to establish for three weeks before initiating the experiment.

On 17 May, I randomly selected and added tadpoles of American Toads, Gray Treefrogs, Northern Leopard Frogs, and Spring Peepers to each tank (n = 20 per species; Appendix Table 2-1). After adding the tadpoles to the tanks, I implemented the predator treatments. For the *Anax* treatments, I used one individual for the low-density treatment and two for the high-density treatment. For the *Belostoma* treatments, I used two individuals for the low-density treatment and four for the high-density treatment. Because *Belostoma* are relatively inefficient predators compared to *Anax* (Relyea 2001a), I used these numbers to increase predation rates and enhance risk levels. For the first day of the experiment, all predators were placed into individual cages (plastic cups with window screen secured to the bottom) and fed 1 Gray Treefrog tadpole. This approach allowed the tadpoles in the experimental units to acclimate to the predators prior to their release.

For the virus treatments, I added either 5 (50% of individuals exposed) or 10 (100% of individuals exposed) Wood Frog tadpoles that were previously exposed to ranavirus for the low- and high-density treatments, respectively. Each control tank received 10 unexposed Wood Frog tadpoles. I generated exposed and unexposed Wood Frogs in the laboratory five days before I introduced them into the experimental units using the methodology of Wuerthner et al. (2017). I began by moving tadpoles inside and placing them into 15-L tubs filled with 1 L of UV-irradiated, aged well water. I added 50 tadpoles to each of 15 tubs (Appendix Table B-1). I allowed the tadpoles to acclimate to laboratory conditions (12:12 day:night cycle at 21°C) for 1 d before virus exposure. For tubs assigned the virus exposure, I added 0.595 µL of virus stock (1.68×10^6 plaque-forming units (PFU) mL^{-1}) to achieve a concentration of 10^3 PFU mL^{-1}. The control tubs received a sham exposure to 0.595 µL of sterile virus growth media. After 1 d of exposure, I increased the water level to 7 L and maintained the tadpoles until added to the experimental units. At this time, I randomly selected infected or uninfected tadpoles for addition to the appropriate treatments. I also set aside a sample of 20 individuals per exposure treatment

to monitor handling mortality. All individuals survived for 24 hr. This approach simulates natural ranavirus transmission within the experimental units (Wuerthner et al. 2017).

On day 10, I destructively sampled three of the eight replicates from each treatment to assess treatment effects on the tadpole assemblage before expected virus-driven mortality. I removed all tadpoles from each tank, euthanized them in MS-222, and preserved them in 70% ethanol. Later, I measured the mass and Gosner stage of each tadpole; and dissected the liver and kidneys of individuals from virus treatments (Gosner 1960). On day 26, it was determined that many tadpoles were nearing metamorphosis, and the experiment was ended. I took down the remaining units using the same protocol as on day 10.

2.2.3 Tadpole Activity

I measured tadpole behavior throughout the experiment using visual scan sampling (Relyea and Hoverman 2003). Every three days at ~11:00h, beginning on experimental day 1, I walked the perimeter of each tank and recorded the number of individuals seen and the number of individuals moving. Moving was defined as any behavior that resulted in forward motion (tail-wagging for feeding was excluded). For each observation, I calculated percent activity as the number of tadpoles moving divided by the number of tadpoles seen multiplied by 100. I observed each tank 10 times on each observation day, and averaged the observations for my response variable. Because I was unable to reliably differentiate between species in the mesocosms, this activity measure is a composite measure across all species.

2.2.4 Periphyton Measurement

Prior to each sampling day, I precombusted grade A/E (47mm) glass fiber filters in a muffle furnace at 550°C. Every three days starting on day 3, I removed one clay tile from each mesocosm and placed it into a sealed plastic bag. I filled the bags with 1 L of reverse osmosis water and scrubbed the tiles with a rough-bristle toothbrush. I collected 100-mL subsamples, which I passed through the grade A/E glass fiber filters using Büchner funnels. After drying, at 70°C for 24 hours, I weighed the filter and used the difference in weight to calculate total dry mass. Then, I combusted each filter at 550°C to attain ash-free dry mass, and the difference in these weights was used to determine organic mass (EPA 1992).

2.2.5 Ranavirus Infection Determination

I used quantitative polymerase chain reaction (qPCR) to test for ranavirus infection in the experiment (Wuerthner et al. 2017). The reaction occurred in 96-well plates, with 4 standards, 1 negative control, and 43 experimental samples, all run in duplicate. Into each well, I added 6.25 µL SsoAdvanced Universal Probes Supermix (BioRad), 2.75 µL autoclaved nanopure water, 1.0 µL of a mixture of each primer at 10 pmol mL^{-1} (rtMCP-F [50-ACA CCA CCG CCC AAA AGT AC-3'] and rtMCP-R [50-CCG TTC ATG ATG CGG ATA ATG-3']) and a fluorescent probe (rtMCP-probe (50 - CCT CAT CGT TCT GGC CAT CAACCA-30), and 2.5 µL of DNA template or autoclaved nanopure water for a final volume of 12.5 µL. I ran qPCR reactions using a Bio-Rad real-time PCR system. The DNA standard was a synthetic double-stranded 250 bp fragment of the highly conserved *Ranavirus* major capsid protein (MCP) gene (gBlocks Gene Fragments; Integrated DNA Technologies). I created a standard curve using a log-based dilution series of 4.014×10^6 viral copies mL^{-1} to 4.014×10^3 viral copies mL^{-1}. Using this protocol, I calculated species-level and overall infection prevalence and viral load. Species-level results are presented in Appendix B (Figures B-1, B-2).

2.2.6 Statistical analyses

My response variables were tadpole community structure (i.e. Species abundance), total tadpole abundance, overall infection prevalence, viral load, tadpole behavior, and periphyton biomass. In order to understand the influence of my treatments on tadpole community structure, I used package 'vegan' in R to create Euclidean distance measures, which were analyzed using a PERMANOVA with 9,999 permutations (Oksanen et al. 2018). Euclidean dissimilarity was used rather than the Bray-Curtis method because the experiment started with a known species composition and, therefore, zeros indicate a significant absence. I conducted multiple tests with the dataset. First, I examined the influence of each individual natural enemy's presence and density on tadpole community structure relative to the control by setting up isolated distance measures between the control and the density treatments (High and Low) for each enemy, and testing for an effect of the natural enemy and the density of the enemy. Then, I tested for differences among natural enemies within each of the density treatments, using distance measures that included only two enemies at one density (e.g. Low *Anax* and Low Virus) and testing for an effect of enemy in pairwise combinations. Lastly, I tested for interactive effects between the virus and each of the

predators, using the Control treatment, the Low Virus treatment, the Low predator treatment, and the virus-predator cross treatment and testing for the effect of predator, virus and the interaction. All PERMANOVA P-values were corrected together using the False Discovery Rate method (Benjamini and Hochberg 1995). For visualization of these differences, and to understand which amphibian species influenced shifts among treatments, I used Linear Discriminant Analysis via the 'candisc' package (Freindly and Fox 2017).

Total tadpole abundance was analyzed using a one-way ANOVA with linear contrasts. The contrasts were designed to assess: 1) the effect of each natural enemy compared to control, 2) the effect of density within each natural enemy, and 3) the effect of natural enemy within each density treatment. I conducted two-way ANOVAs to determine whether the effects of combined natural enemies (i.e. *Anax* and virus, *Belostoma* and virus) were additive or synergistic. For these analyses, I used the control, the low-density treatment for each natural enemy, and the combination treatment. Overall virus prevalence and viral load were analyzed using Kruskal-Wallis rank sum tests, with pairwise contrasts using Wilcoxon rank-sum tests. Results for species-level prevalence and viral load are reported in the Appendix B (Figures 1-2). I analyzed tadpole behavior and periphyton biomass using repeated-measures ANOVA. Although I detected a significant treatment-by-day interaction for behavior, treatment effects were largely consistent across time. Thus, for simplicity, I focused on overall treatment effects using least squares means. Results by day are presented in the Appendix (Figures B-3, B-4). Pairwise comparisons were conducted using Tukey's Honest Significant Difference test (Tukey 1949). Analysis of periphyton also contained a significant treatment-by-day interaction, however organic mass showed intense variability by day, and thus I use the final day of periphyton measurement as an endpoint. Full periphyton results can be found in Appendix B. For each species, I also examined treatment effects on mass, and developmental stage. These data are reported in Appendix B (Figures 5-8). I performed all statistical analysis using R version 3.3.2 (R Development Core 2011).

2.3 Results

2.3.1 Day 10 tadpole community structure, total abundance

Early in the experiment, I observed that the presence of natural enemies was contributing to divergence in tadpole community structure relative to the control (Table 2-2, Figure 2-1). Most

of the divergence was driven by the presence of predators rather than virus. Additionally, there was an influence of *Anax* density on tadpole community structure. Generally, I also found that tadpole community structure differed between predator and virus treatments. There was a significant interaction between *Anax* and virus, resulting in a more-than-additive effect on abundance, and a community structure significantly different than that of Low *Anax* treatments. Linear discriminant analysis shows much of the difference between *Anax* treatments and controls were mediated by reductions in leopard frogs, treefrogs, and Spring Peepers; while differences in *Belostoma* treatments were mostly driven by reductions in American Toad abundance (Figure 2-1, Table 2-3, Figure 2-2).

Total tadpole abundance was significantly influenced by treatment ($F_{8, 18} = 15.7$, $P < 0.001$; Figure 2-3). There was a significant effect of *Anax* presence and density on tadpole abundance (Table 2-4). Compared to the control, tadpole abundance was 10% and 37% lower in the Low and High *Anax* treatments, respectively. The presence of *Belostoma* reduced tadpole abundance by 16% compared to the control; however, there was no effect of *Belostoma* density. Additionally, there was no influence of virus presence or density on tadpole abundance. Comparing effects within each density treatment, I only found differences in tadpole abundance among the natural enemies within the high-density treatment. Tadpole abundance was lowest with *Anax*, intermediate with *Belostoma*, and high with virus. Lastly, I found interactive effects of *Anax* and virus on tadpole abundance and additive effects of *Belostoma* and virus (Table 2-5). The combination of *Anax* and Virus reduced tadpole abundance by 33% compared to the single low *Anax* treatment.

2.3.2 Day 26 tadpole community structure, total abundance

Overall, each natural enemy had a unique effect on tadpole community structure relative to the control and the other natural enemies (Table 2-2). However, natural enemy density was only a significant factor for *Anax*. Collectively, these results suggest that tadpole communities diverged in the presence of individual natural enemies. However, when each predator was combined with virus in the system, I found that the community structure was similar to that found with the predator alone. This suggests that community-level effects of combined natural enemies are largely driven by predators rather than the virus. Discriminant analysis shows that *Anax* communities were characterized by a reduction in treefrogs, leopard frogs, Spring Peepers, and Wood Frogs; and that

differences between Low *Anax* treatments and High *Anax* treatments were mediated by an increase in predation on American Toads (Figure 2-4, Table 2-6, Figure 2-2). *Belostoma* treatments were defined mostly by their reduction in toad abundance. Lastly, Virus treatments were characterized by a uniform reduction in abundance across species.

Total tadpole abundance was significantly influenced by treatment ($F_{8, 36} = 28.5$, $P < 0.001$; Figure 2-2, Table 2-4). I found an effect of *Anax* presence and density; abundance was 64% and 85% lower in the Low and High *Anax* treatments, respectively, compared to Control. There was a significant effect of *Belostoma* and virus presence on tadpole abundance, but not of their density. Abundance was 25% lower with *Belostoma* and 23% lower with Virus compared to the control. Comparing among natural enemies, *Anax* reduced abundance 53 to 80% compared to Virus and *Belostoma* treatments. However, there was no difference between the *Belostoma* and Virus treatments. Similar to day 10, I found interactive effects of *Anax* and virus on tadpole abundance and additive effects of *Belostoma* and virus (Table 2-5). However, in contrast to day 10, the combination of *Anax* and virus did not alter tadpole abundance relative to the Low *Anax* single natural enemy treatments, suggesting less than additive effects of combined natural enemies.

2.3.3 Infection prevalence and viral load

On day 10, infection prevalence was significantly different among virus treatments (Chi-square = 8.7, P = 0.033; Figure 2-6). While pairwise comparisons could not distinguish differences among treatments, prevalence trended higher in the Virus x *Belostoma* treatment compared to the other treatments. Viral load did not differ among treatments on day 10 (Chi-Square = 5.2, P = 0.157; Figure 2-6).

On day 26, infection prevalence was significantly affected by virus treatments (Chi-square = 14.5, P = 0.002; Figure 2-7). Prevalence in the Virus x *Anax* treatment was 79 and 65% lower compared to the Low and High Virus treatments, respectively ($P < 0.018$). Prevalence in the Virus + *Belostoma* treatment was 61% lower compared to the Low Virus treatments. Viral load was also affected by virus treatments (Chi-square = 13.6, P = 0.004), with Virus + *Anax* treatments reducing viral load by 83 and 80% when compared to the Low and High Virus treatments ($P < 0.005$; Figure 2-7).

2.3.4 Tadpole behavior

There was a significant effect of treatment ($F_{8, 216} = 14.8$, $P < 0.001$), day ($F_{6, 216} = 13.3$, $P < 0.001$), and the interaction ($F_{48, 216} = 2.5$, $P < 0.001$) on behavior (Table 2-7, Figure 2-8). Because treatment effects were relatively similar across time, I focused on the overall treatment effects for simplicity (see Appendix for results graphed by day). Behavioral effects were largely driven by *Anax*. The Low and High *Anax* treatments reduced tadpole activity by 61 to 94% compared to controls. Additionally, activity was 84 to 86% lower in the high *Anax* treatment compared to the low *Anax* and Virus + *Anax* treatments.

2.3.5 Periphyton biomass

There was a significant Day x Treatment interaction on periphyton biomass (Table 2-7). There were no clear trends in periphyton biomass across days; moreover, on the final day of the experiment, there were no differences across treatments ($F_{8, 36} = 1.3$, $P = 0.282$; Figure 9).

2.4 Discussion

While predation and disease are core concepts in ecology, there have been relatively few attempts to compare and contrast their effects on a focal community. My results demonstrate that each natural enemy had a unique influence on the structure of my amphibian community. These effects were largely predictable based on prior knowledge of species vulnerability to natural enemies. In the presence of *Anax*, overall tadpole abundance was dramatically reduced, and community structure shifted to dominance by toads and Spring Peepers. Toads are generally avoided by *Anax* because of the toxins present in their skin while Spring Peepers are generally less susceptible to predators than other amphibian species because of their low level of baseline activity (Formanowics and Brodie Jr. 1982, Skelly 1994). With *Belostoma*, overall tadpole abundance was moderately reduced and community structure shifted to dominance by treefrogs and leopard frogs. These species appeared to be able to grow large enough to reach a size refuge from predation (Appendix Figure 2-7). Toads were preferred by *Belostoma* despite the presence of toxins. Because the toxins are largely sequestered in the skin, *Belostoma* can bypass them with their proboscis. Similar to *Belostoma*, ranavirus had moderate effects on overall tadpole abundance. Shifts in community structure were less pronounced with ranavirus compared to predators; Spring Peepers and leopard frogs tended to dominate the communities. In a mesocosm experiment

containing the same amphibian species, Wuerthner et al. (2017) documented lower infection prevalence and higher survival of Spring Peepers and leopard frogs compared to toads and treefrogs. Given similar effects of *Belostoma* and virus on total tadpole abundance, my results suggest that ranavirus could be characterized as a low-risk predator along the predation risk gradient. However, as found with different predator species, ranavirus can have unique effects on community structure driven by species differences in vulnerability. Collectively, these results demonstrate that the impact of natural enemies on victim communities varies based on the identity of the natural enemy but are largely predictable based on knowledge of the relative susceptibility of individual species to each natural enemy.

My temporal sampling also provided evidence that shifts in community structure occurred relatively quickly within the experimental units. Within 10 days, patterns in overall abundance and community structure were emerging in the predator treatments. However, such patterns were not detected in the virus treatments. These differences in pace are likely explained by the inherent characteristics of predation and disease. Unlike predators, a successful 'attack' by a pathogen does not necessarily result in immediate mortality of the host. Even in cases where pathogens are virulent, the time between initial infection and mortality will be longer than a typical predator attack. Consequently, predators are expected to have more immediate effects on victim density than pathogens. However, as infection builds in a system and disease progression occurs, pathogen-mediated mortality is likely to increase. In my system, it required at least 26 days before pathogen-mediated effects were observed. Given the longer temporal dynamics of pathogen transmission within this system, future research that tracks community responses over longer time frames would be valuable to determine if the magnitude of pathogen-mediated effects on the community increase.

I included treatments with each natural enemy at two densities to understand the influence enemy density plays in the structure of amphibian communities. I predicted that increasing density of natural enemies would increase the magnitude of the effects on communities. While *Anax* density did significantly influence tadpole communities, there were no density effects for the other natural enemies. The differences in *Anax* density treatments was largely driven by the reduction of toads in the high-density treatment. In the Low *Anax* treatment, the predator may be able to avoid toads because of the availability of other prey. However, high predator densities and limited prey availability in the High *Anax* treatment could force individuals to consume less preferred prey

items. In the *Belostoma* treatments, high predator densities could have resulted in predator-predator interference and a reduction in per capita predation rates compared to the low-density treatments. I recovered 9/10 *Belostoma* from Low treatments, but only recovered 14/20 *Belostoma* from High treatments. Cannibalistic behavior has been shown in *Belostoma* in high densities or with low food (Flosi 1980). In the virus treatments, the addition of more virus-exposed Wood Frogs did not significantly affect the system. This would imply that ranavirus is not strictly a density-dependent pathogen, and supports the idea that transmission depends on individual-level heterogeneity (Brunner et al. 2017).

When amphibian communities were exposed to predators and pathogens simultaneously, I found that community structure mirrored the patterns found with the predator alone. Thus, the effects of combined natural enemies on community structure were less-than additive, which was consistent with my prediction. This result can be explained by the healthy herds effect in which predators indirectly reduce virus transmission. Indeed, I found that both predators reduced overall infection prevalence by 61-75%. Moreover, in the *Anax* treatments, Spring Peepers and Gray Treefrogs were free of infections suggesting that predation can function to exclude the pathogen from some species and potentially the community. Collectively, these data demonstrate that the combination of predators and pathogens in this system leads to risk reduction, which parallels work in predator-predator systems (Sih et al. 1998).

The healthy herd effect in my system could have been driven by several mechanisms. It could have been mediated by reductions in tadpole abundance or changes in behavior that reduce contract rates and, consequently, transmission rates. In a recent study, Gallagher et al. (in review) demonstrated that predation-mediated reductions in tadpole abundance were more important than reductions in tadpole activity induced by predators in explaining lower ranavirus prevalence in this system. Additionally, predators could consume infected individuals before they have the opportunity to transmit the infection. In a laboratory experiment, I demonstrated that virus exposed individuals are predated by *Anax* faster than unexposed tadpoles (Chapter 1). However, the same pattern was not observed with *Belostoma*. Whether predators selectively remove infected individuals over uninfected individuals has not been assessed in this system. Collectively, this data suggests that multiple mechanisms likely contribute to the healthy herd effect in this system.

Although I observed the healthy herds effects with both predators, the magnitude was greater for *Anax* than *Belostoma* suggesting that predator identity and predator riskiness are

important in this process. This is the first study to document predator-specific differences in the healthy herds effect. A possible mechanism underlying these differences could be predation rates. Based on my day-10 timepoint, *Anax* reduced overall tadpole abundance faster than *Belostoma*. It is possible that the more-dramatic reduction in tadpole density in *Anax* treatments simply translates to less transmission in these treatments. It is curious that at Day 10, Virus x *Anax* treatments experienced such a dramatic increase in predation, compared to Low *Anax*, while *Belostoma* treatments do not show the same trend. Given evidence that *Anax* are more efficient predators with virus-exposed tadpoles than unexposed (Chapter 1), it could be that given the choice *Anax* select infected tadpoles, explaining this increase in consumption at day 10 and the differences in virus reduction between *Anax* and *Belostoma*.

I also examined whether the presence of natural enemies influenced trophic cascades in the system. However, there was limited evidence of trophic cascades despite dramatic reductions in tadpole abundance and activity in some treatments. Although not significant, there was a general trend for higher periphyton abundance in the presence of *Anax*. Previous research with *Anax* and other predators has demonstrated strong trophic cascades in this system. However, much of this work has focused on relatively species-poor communities. In more diverse communities, other species could compensate for the reduction in overall tadpole abundance. For instance, leopard frogs, which are dominant competitors in this system, were more abundant in the *Anax* treatments and were twice as massive compared to conspecifics in the other treatments (Appendix Figure 2-7). Thus, this species could have compensated for reductions in the abundance of the other species, thereby reducing the potential for trophic cascades on periphyton to be observed.

In this study, I found evidence that different natural enemies influence the structure of communities in unique ways. This shift can occur rapidly, in the case of predators, but may take more time for pathogens due to differences in the timescale of this interaction. Additionally, the importance of enemy density varies based on the identity of the enemy, with less-threatening enemies posing no additional threat at higher densities. In combination, predators dominate the effects of virus on this tadpole community, and both predators reduced virus prevalence, with the magnitude of this effect dependent on the relative threat of the predator. These results collectively imply that natural enemy identity is important in determining the interactions between enemies and their communities; and importantly, that predator identity is an important factor in the healthy herds effect. Future efforts should focus on determining the effects of additional enemies,

including alternate predators and pathogens as well as determining the effects of changing the base community.

2.5 Tables and Figures

Table 2-1. Relative susceptibility of my five focal amphibian species to my natural enemies: larval dragonflies (*Anax*), adult water bugs (*Belostoma*), and ranavirus. Information collected from Relyea 2001b, Haislip et al. 2011, Hoverman et al. 2011, Wuerthner et al. 2017, Haislip et al. 2012.

Species	*Anax*	*Belostoma*	*Ranavirus*
Spring Peeper	Low	Low	Low
Gray Treefrog	Intermediate	Intermediate	Intermediate
Northern Leopard Frog	Intermediate	Intermediate	Low
American Toad	Low	Intermediate	Intermediate
Wood Frog	High	High	High

Table 2-2. Results of PERMANOVA analysis on species abundances for both sampling points. P-values have been adjusted using the False Discovery Rate method.

		Day 10			Day 26		
Treatment	**Effect**	df	F	P	df	F	P
Anax Treatments	**Enemy**	1, 8	7.3	**0.031**	1, 14	100.1	**<0.001**
	Density	1, 8	7.4	**0.036**	1, 14	9.6	**0.010**
Belostoma Treatments	**Enemy**	1, 8	10.3	**0.022**	1, 14	26.4	**<0.001**
	Density	1, 8	1.1	0.365	1, 14	3.3	0.067
Virus Treatments	**Enemy**	1, 8	2.8	0.124	1, 12	11.7	**<0.001**
	Density	1, 8	0.7	0.591	1, 12	2.1	0.137
Low *Anax*- Low Virus	**Enemy**	2, 8	2.3	0.104	2, 13	19.7	**<0.001**
Low *Belostoma*- Low Virus	**Enemy**	2, 8	3.9	**0.036**	2, 13	8.6	**<0.001**
Low *Anax*- Low *Belostoma*	**Enemy**	2, 8	4.7	**0.014**	2, 14	34.6	**<0.001**
High *Anax*- High Virus	**Enemy**	2, 8	8.5	**0.014**	2, 13	71.3	**<0.001**
High *Belostoma*- High Virus	**Enemy**	2, 8	9.1	**0.014**	2, 13	17.3	**<0.001**
High *Anax*- High *Belostoma*	**Enemy**	2, 8	8.5	**0.014**	2, 14	79.5	**<0.001**
Anax x Virus	**Predator**	1, 11	11.3	**0.005**	1, 18	39.0	**<0.001**
	Virus	1, 11	6.0	**0.031**	1, 18	3.6	0.062
	Predator: Virus	1, 11	3.5	0.098	1, 18	7.1	**0.012**
Belostoma x Virus	**Predator**	1, 11	11.2	**0.005**	1, 18	10.5	**<0.001**
	Virus	1, 11	2.7	0.104	1, 18	9.9	**<0.001**
	Predator: Virus	1, 11	0.3	0.815	1, 18	4.8	**0.011**

Table 2-3. Summary of linear discriminant analysis for differences in amphibian abundances across natural enemy treatments on day 10. Standardized discriminant function coefficients and correlations between scores and abundances for significant linear discriminant functions are presented.

	LD1		LD2	
Species	Coefficient	Correlation	Coefficient	Correlation
American Toad	-1.03	-0.49	0.90	0.74
Gray Treefrog	-0.45	-0.84	-0.49	-0.17
Northern Leopard Frog	0.29	-0.78	-0.18	-0.05
Spring Peeper	-1.34	-0.85	-0.13	-0.37
Wood Frog	0.13	-0.39	0.75	0.29

Table 2-4. Linear contrasts after one-way ANOVA to test for treatment effect on both sampling days. P-values have been adjusted using the False Discovery Rate method.

	Day 10 tadpole abundance		Day 26 tadpole abundance	
Test	t	P	t	P
Presence of *Anax*	5.561	**<0.001**	12.283	**<0.001**
Density of *Anax*	-5.316	**<0.001**	-2.990	**0.008**
Presence of *Belostoma*	3.737	**0.005**	4.155	**<0.001**
Density of *Belostoma*	-1.233	0.280	-1.078	0.332
Presence of Virus	1.246	0.280	3.894	**<0.001**
Density of Virus	-0.308	0.762	1.043	0.336
Low *Anax* – Low Virus	1.233	0.280	-5.249	**<0.001**
Low *Belostoma* – Low Virus	-1.695	0.1839	-9.282	**<0.001**
Low *Anax* – Low *Belostoma*	0.462	0.708	0.834	0.410
High *Anax* – High Virus	-6.241	**<0.001**	-1.286	0.275
High *Belostoma* – High Virus	-2.620	**0.035**	-6.084	**<0.001**
High *Anax* – High *Belostoma*	-3.621	**0.005**	-7.996	**<0.001**

Table 2-5. Results of two-way ANOVA for both Belostoma cross treatments and Anax cross treatments. P-values have been adjusted using the False Discovery Rate method.

			Day 10 tadpole abundance		Day 26 tadpole abundance		
Treatment	**Effect**	df	F	P	df	F	P
Anax Treatments	**Predator**	1, 8	45.3	**<0.001**	1, 16	66.7	**<0.001**
	Virus	1, 8	24.4	**0.001**	1, 16	3.3	0.089
	Predator:Virus	1, 8	12.9	**0.007**	1, 16	8.4	**0.010**
Belostoma Treatments	**Predator**	1, 8	86.0	**<0.001**	1, 16	4.7	**0.045**
	Virus	1, 8	11.9	**0.009**	1, 16	10.8	**0.005**
	Predator:Virus	1, 8	0.1	0.798	1, 16	3.6	0.076

Table 2-6. Summary of linear discriminant analysis for differences in amphibian abundances across natural enemy treatments on day 26. Standardized discriminant function coefficients and correlations between scores and abundances for significant linear discriminant functions are presented.

	LD1		LD2		LD3	
Species	Coefficient	Correlation	Coefficient	Correlation	Coefficient	Correlation
American Toad	-0.05	0.21	0.91	0.87	-0.38	-0.37
Gray Treefrog	0.37	0.94	-0.36	-0.19	-0.23	-0.03
Northern Leopard Frog	0.42	0.94	-0.33	-0.09	-0.29	-0.10
Spring Peeper	0.59	0.87	0.09	0.14	-0.30	0.26
Wood Frog	0.38	0.87	0.51	0.20	0.89	0.44

Table 2-7. Results of repeated measures ANOVA on both periphyton organic mass and behavior, using percent activity as a proxy for behavioral changes

Response Variable	**Effect**	**df**	**F**	**P**
Behavior	Treatment	8, 36	13.6	**<0.001**
	Day	6, 216	16.7	**<0.001**
	Treatment:Day	48, 216	3.1	**<0.001**
Periphyton	Treatment	8, 251	1.6	0.157
	Day	7, 251	8.3	**<0.001**
	Treatment:Day	56, 251	1.9	**<0.001**

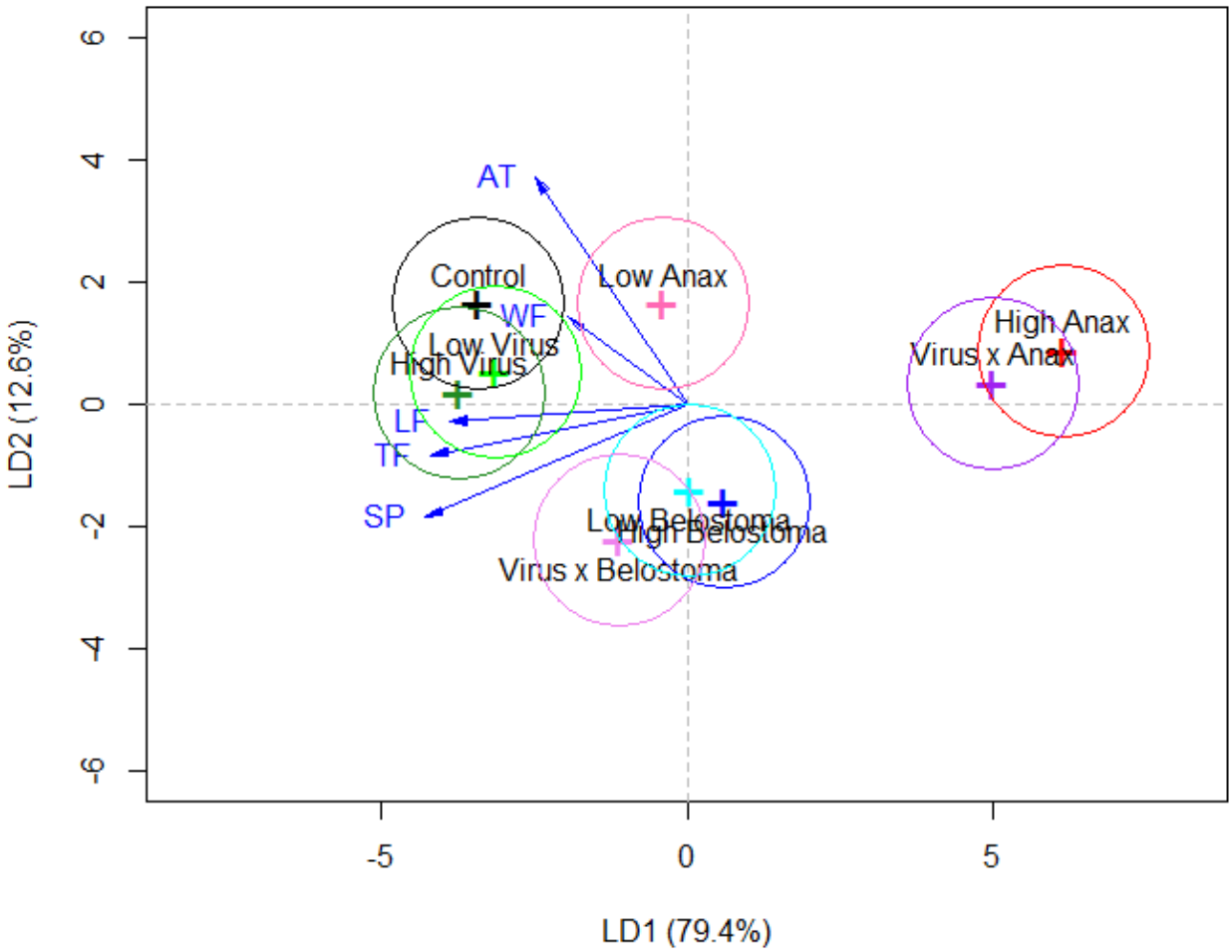

Figure 2-1. Plot of day 10 treatment centroids on axes described by linear discriminants 1 and 2. Centroids represent average community composition across three replicates.

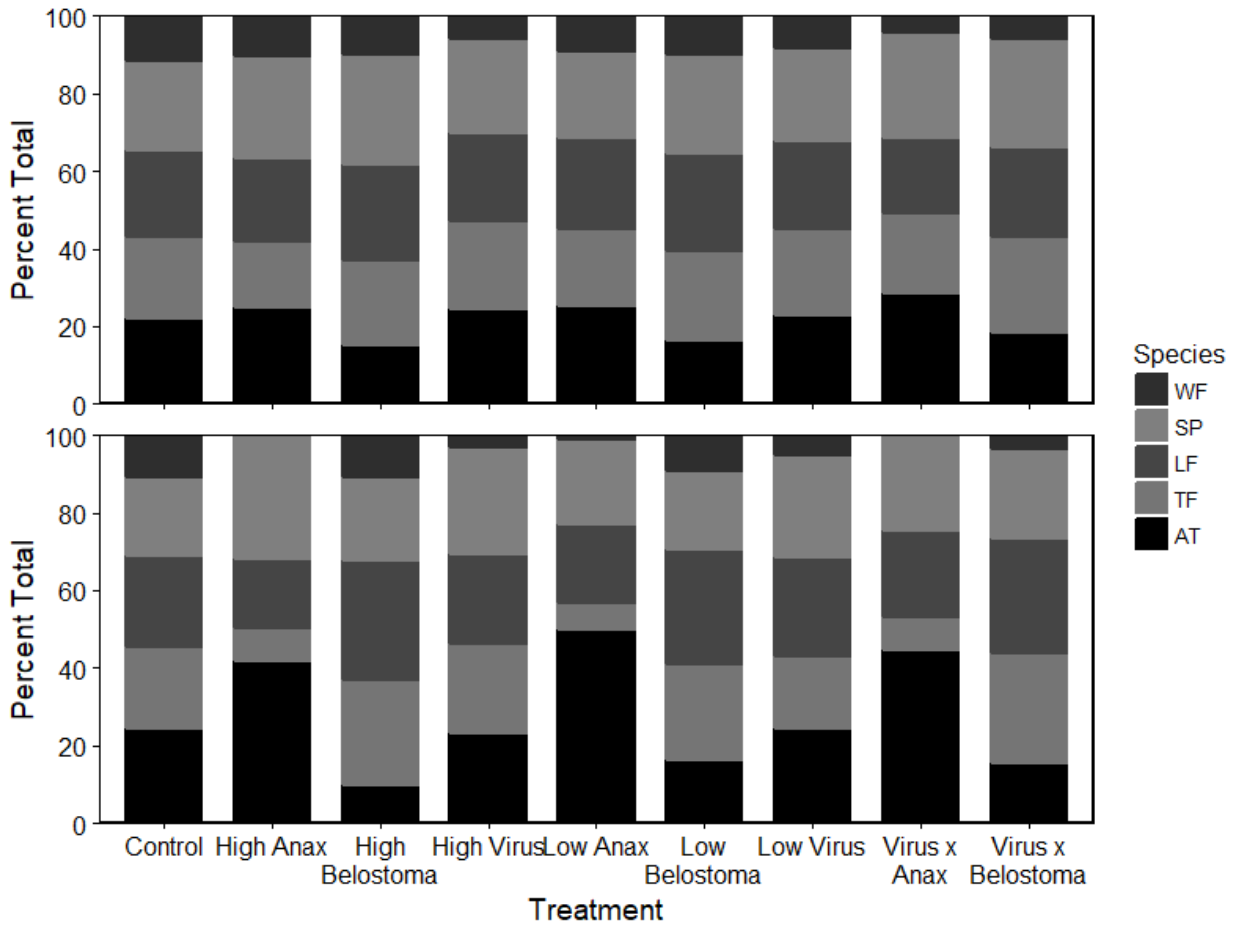

Figure 2-2. Relative abundance of the 5 focal species of anurans on Day 10 (top) and Day 26 (bottom), separated by treatment. Values are means among treatments.

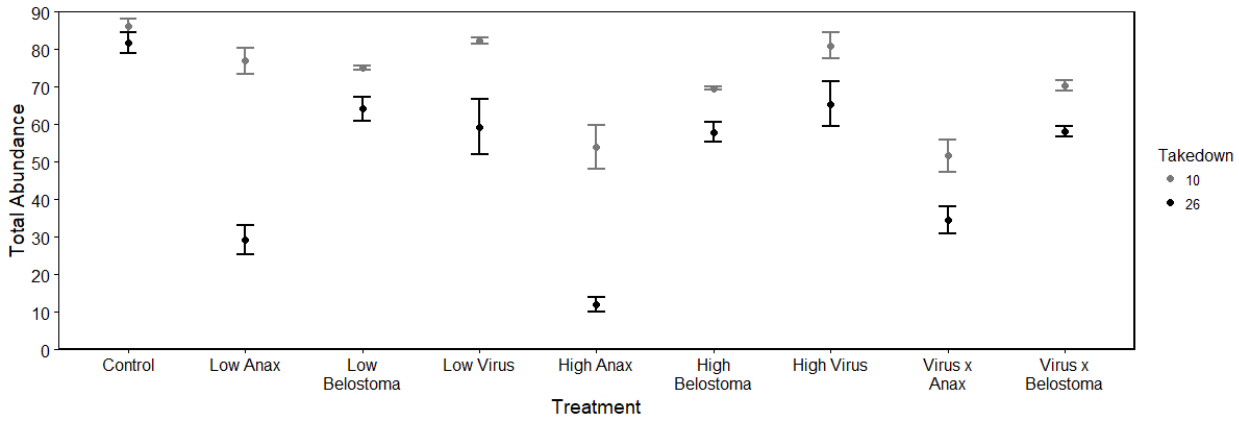

Figure 2-3. Total abundance across treatment for both Day 10 and Day 26. Data are means ± 1 SE.

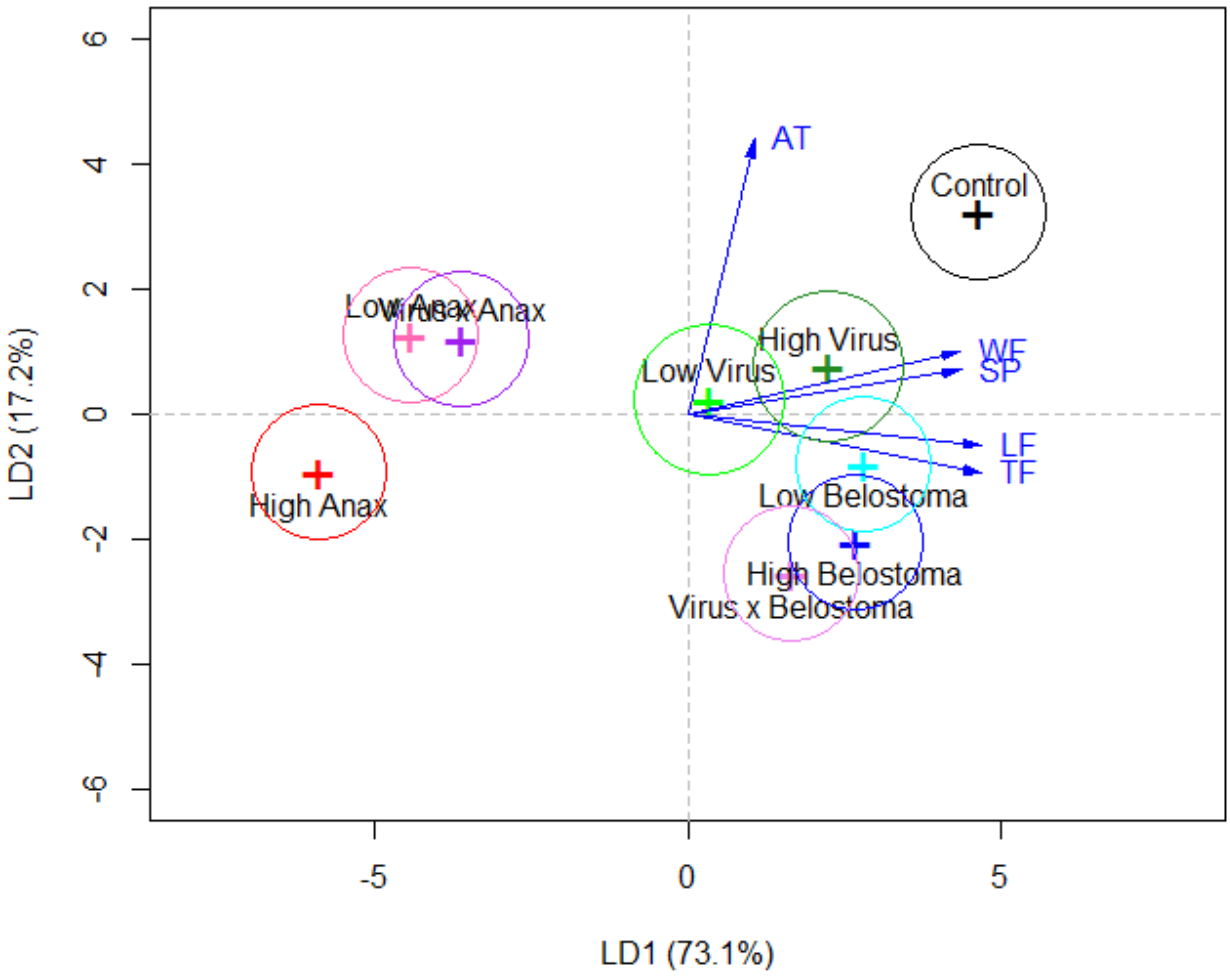

Figure 2-4. Plot of day 26 treatment centroids on axes described by linear discriminants 1 and 2. Centroids represent average community composition across five replicates.

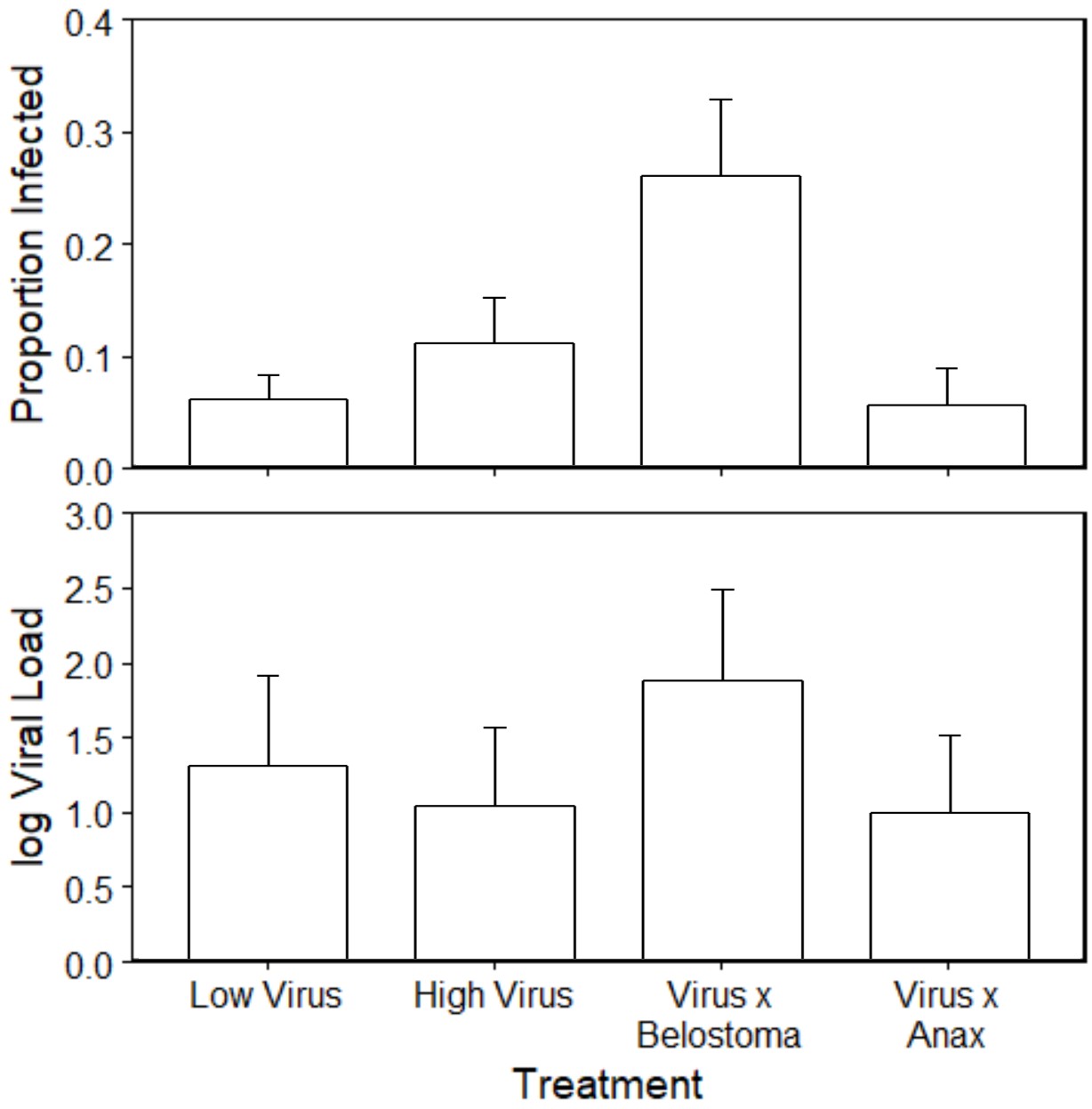

Figure 2-5. Average infection prevalence and log viral load among all species in each virus-exposed treatment on day 10. Data are means ± 1 SE.

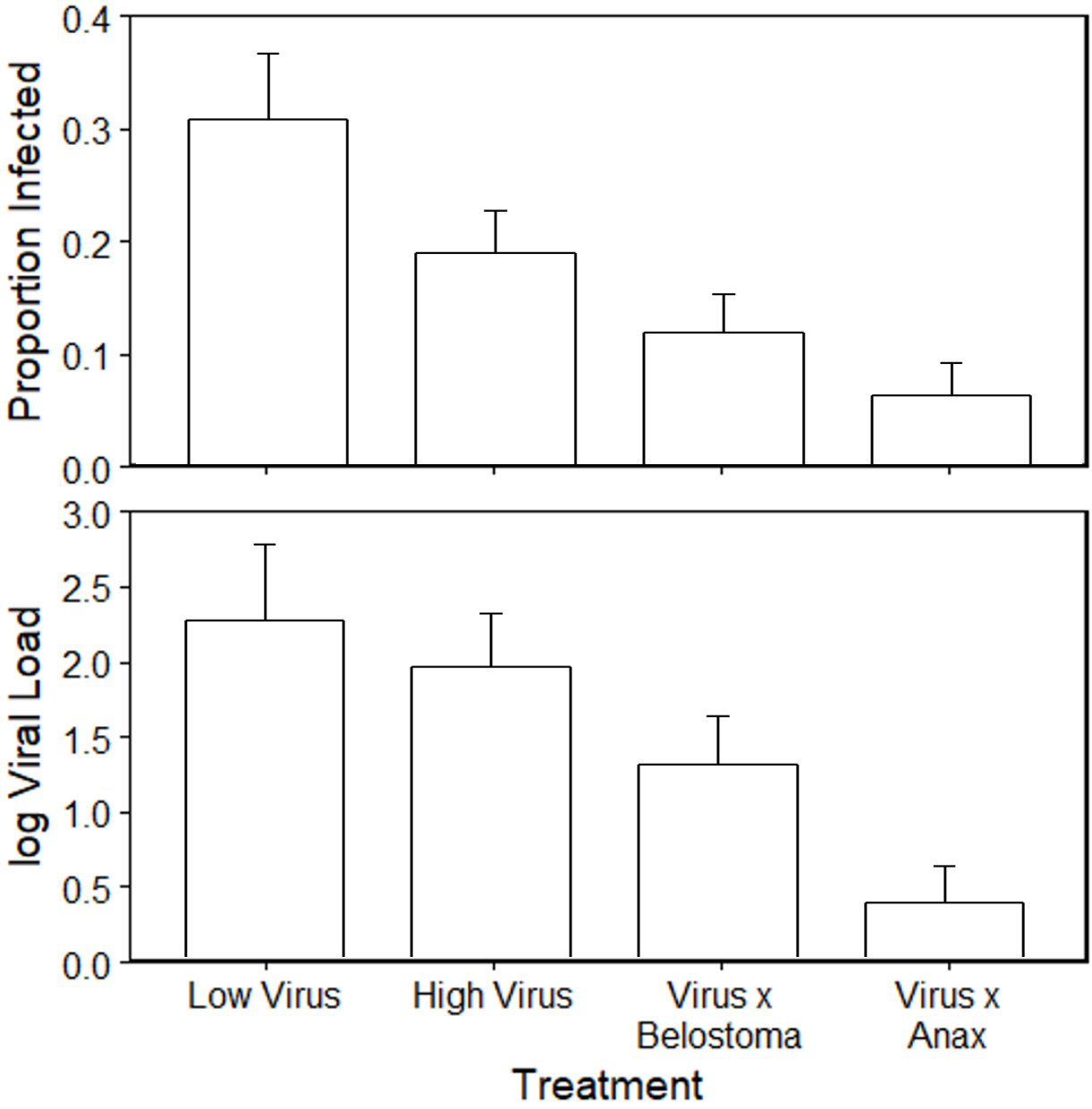

Figure 2-6. Average infection prevalence and log viral load among all species in each virus-exposed treatment on day 26. Data are means ± 1 SE.

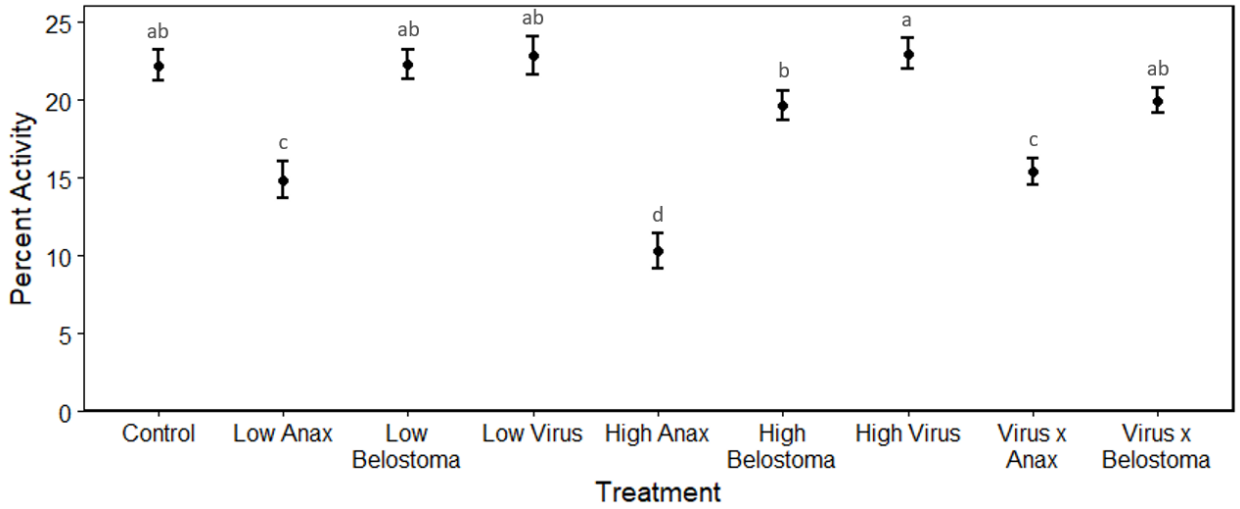

Figure 2-7. Average percent activity within each treatment. Data are least-square means from repeated measures ANOVA ± 1 SE.

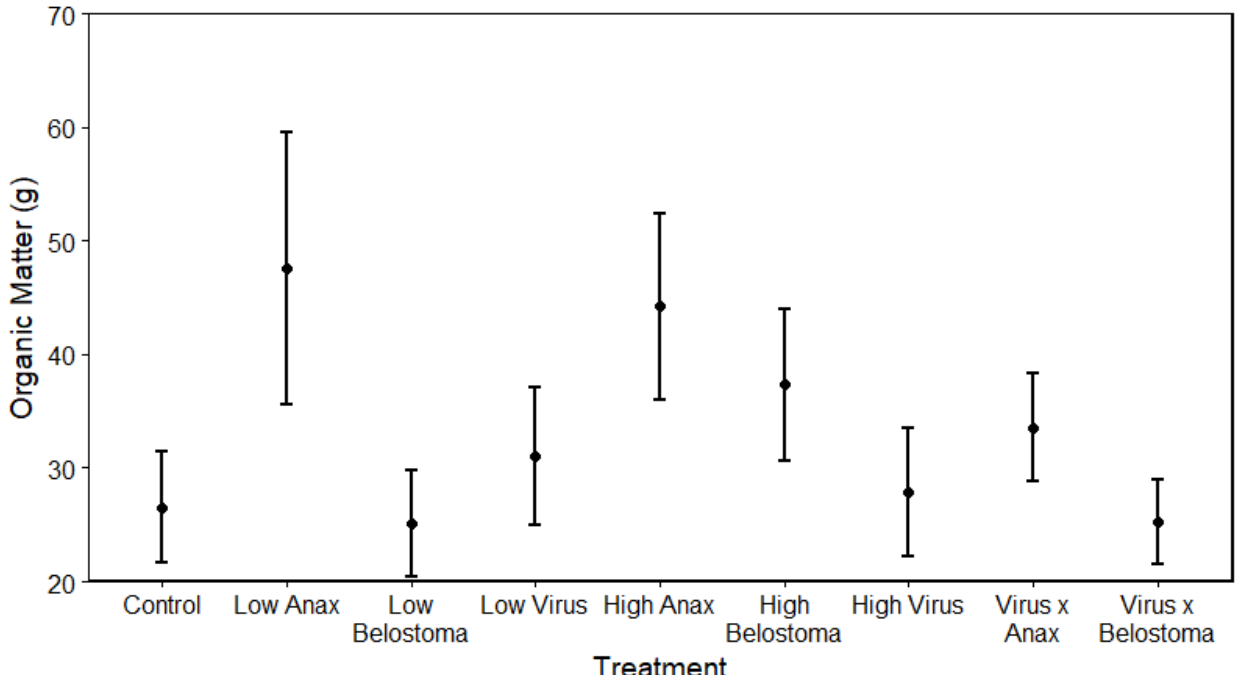

Figure 2-8. Periphyton organic matter in grams on experimental day 24, the last day of periphyton measurement. Data are means ± 1 SE.

CHAPTER 3. CONCLUSIONS

Uniting predators and pathogens under a common framework is inherently difficult because these natural enemies have a number of differences. In particular, they differ in the time scale of interactions with their victims and the number of victims attacked. Despite these differences, predation and disease are ultimately consumer strategies that are linked via their influence on victim mortality rates. This similarity allows for the integration of pathogen and predator ecology under a common framework to compare their effects on ecological communities. My experiments utilize this framework to understand the individual and combined effects of ranavirus, a viral pathogen of amphibians, and predatory invertebrates on amphibian species and communities.

An important consideration in natural enemy ecology is the diversity of enemies, hosts, and prey within a system. Focusing on a single consumer-resource interaction could bias and limit our understanding of ecological processes. Thus, my experiments incorporated multiple natural enemies along with multiple victim species to assess general patterns. Importantly, species identity was a major driver in my results. For instance, my observation that baseline activity of different amphibian species was related to the strength of the pathogen-induced vulnerability to predators. Other authors have used similar strategies to test vulnerability among species to predators and pathogens in isolation, discovering species-level trends in vulnerability. In the same way, our use of community structure as an endpoint was illuminating. Looking at only univariate results, such as total abundance, important trends in community structure could be overlooked. For example, ranavirus and *Belostoma* had similar effects on the total abundance of amphibians in a community, but their community compositions were different. Incorporating multivariate measures such as community structure into ecological experimentation is an important step between laboratory experiments and natural systems and allows us to better understand the dynamics of these systems.

Placing pathogens into the same context as other natural enemies can be helpful in understanding their impacts on communities. In particular, I discovered that the effects of ranavirus on amphibian communities was similar to the low-threat predator suggesting that pathogens can be placed along the same risk gradient as predators. Broadening these experiments to include more predators and pathogens is important to our ability to assess risk level and the potential for impacts on natural systems. Likewise, altering our base amphibian community can be illuminating. In Virus x *Anax* treatments, we eliminated virus from two species, Spring Peepers

and Gray Treefrogs. It is possible that in altering community composition, we discover that we can completely exclude ranavirus in certain communities, or we find certain communities where the trends we identified do not hold true.

My findings demonstrate that the healthy herds effect occurs in amphibian systems with invertebrate predators. While the healthy herds effect has been extensively explored in theoretical studies, few studies have experimentally demonstrated the healthy herds hypothesis. Moreover, no studies have examined it within the context of communities or compared across predators to determine their potential to mediate the healthy herds effect. My results demonstrate that the magnitude of the healthy herds effect is mediated by predator risk level; a stronger healthy herds effect was observed with high-risk predators compared to low-risk predators. Future work examining the healthy herds effect in natural systems is a critical next step. In particular, field surveys that correlated predator presence and abundance with infection prevalence would help to illuminate whether the patterns observed in experimental settings are found in nature. Given the global pattern of trophic downgrading (i.e. the loss of top predators from systems), disease emergence could be driven by the loss of the healthy herds effect provided by predators.

APPENDIX A

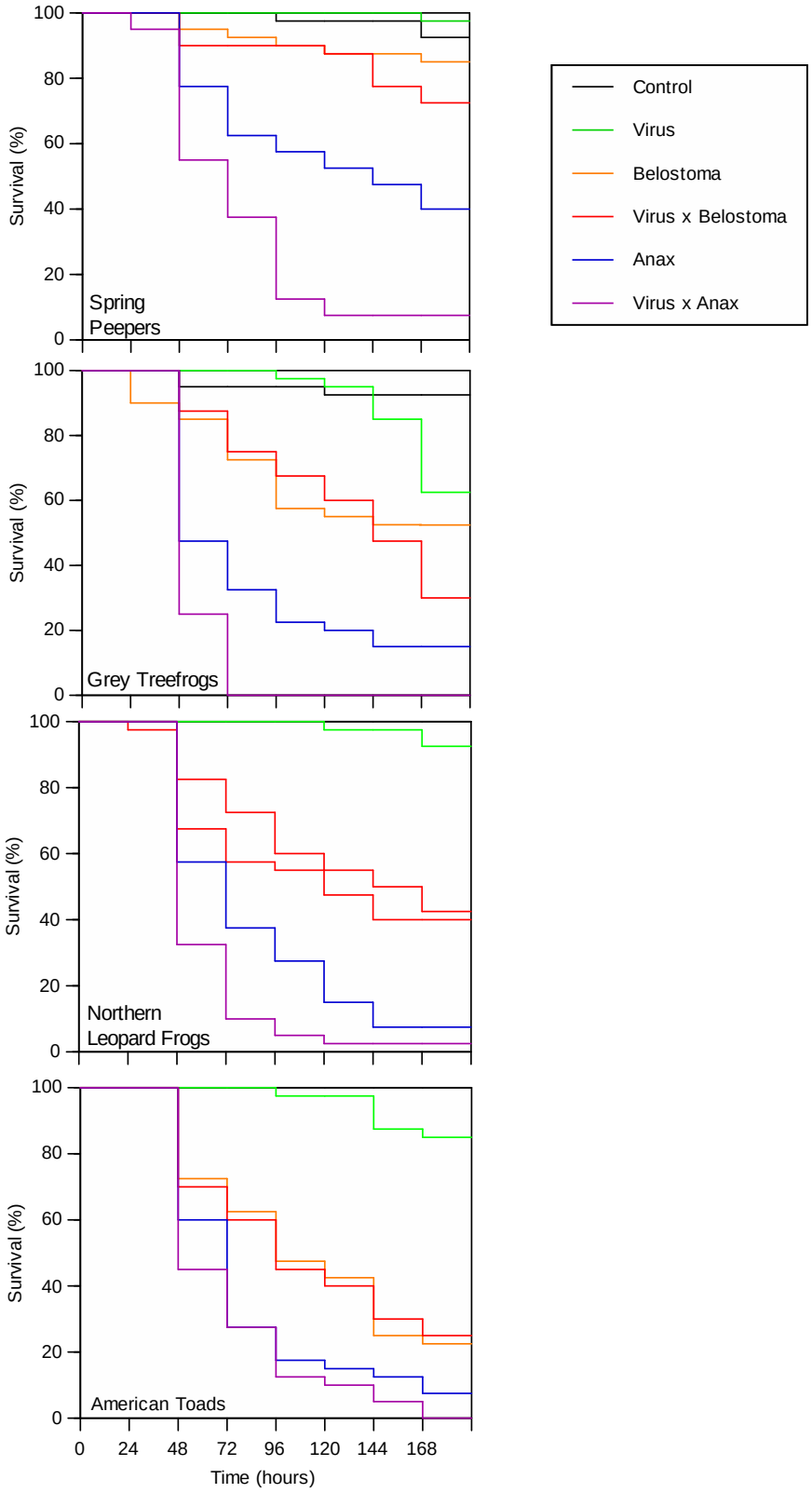

Figure A-1. Survival curves showing the effect of each treatment on each species.

APPENDIX B

Tadpole individual-level traits

Because I expect mass and stage to vary by species during this experiment, these values are presented by species. For each species I analyzed Gosner stage and individual mass using one-way ANOVAs and Tukey post-hoc tests.

Species-level abundance data

Due to the use of Discriminant Analysis as a follow-up to PERMANOVA analysis, species-level abundance is presented here without statistics, on both day 10 and 26 of the experiment.

Table B-1. Initial mass and stage for a sample of the focal tadpoles added to experimental units.

Species	N	Mass (g)		Stage		
		Mean	SD	Median	Min	Max
Spring Peeper	20	0.125	0.035	33	29	36
Gray Treefrog	20	0.031	0.013	26	26	28
Northern Leopard Frog	20	0.099	0.039	26	26	28
American Toad	20	0.023	0.006	28	27	29
Wood Frog	20	0.096	0.046	27	26	31

Table B-2. Results of ANOVA analyses of mass data for each species, and Kruskal-Wallis tests for each species stage data.

	Mass				Stage			
	Day 10		Day 26		Day 10		Day 26	
Species	F	P	F	P	Chi^2	P	Chi^2	P
American Toad	17.2	<0.001	7.9	<0.001	33.5	<0.001	14.2	0.077
Gray Treefrog	7.0	<0.001	0.8	0.57	38.4	<0.001	11.1	0.197
Northern Leopard Frog	3.9	<0.001	5.0	<0.001	48.8	<0.001	16.1	0.040
Spring Peeper	7.2	<0.001	1.2	0.315	42.7	<0.001	9.9	0.266
Wood Frog	1.6	0.144	3.3	0.004	15.7	0.05	7.7	0.256

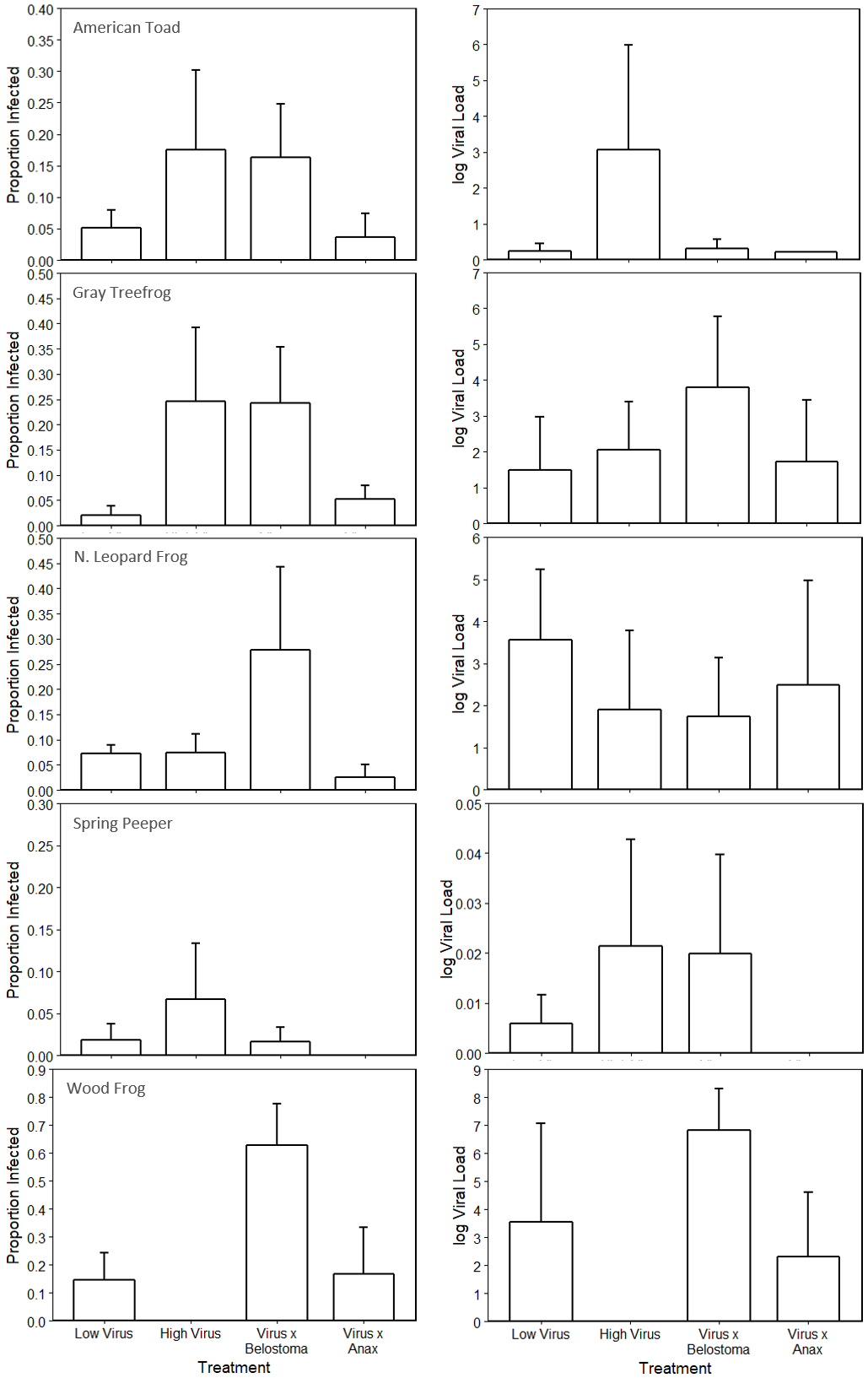

Figure B.1. Average infection prevalence and log viral load in all species in each virus-exposed treatment on day 10. Data are means ± 1 SE.

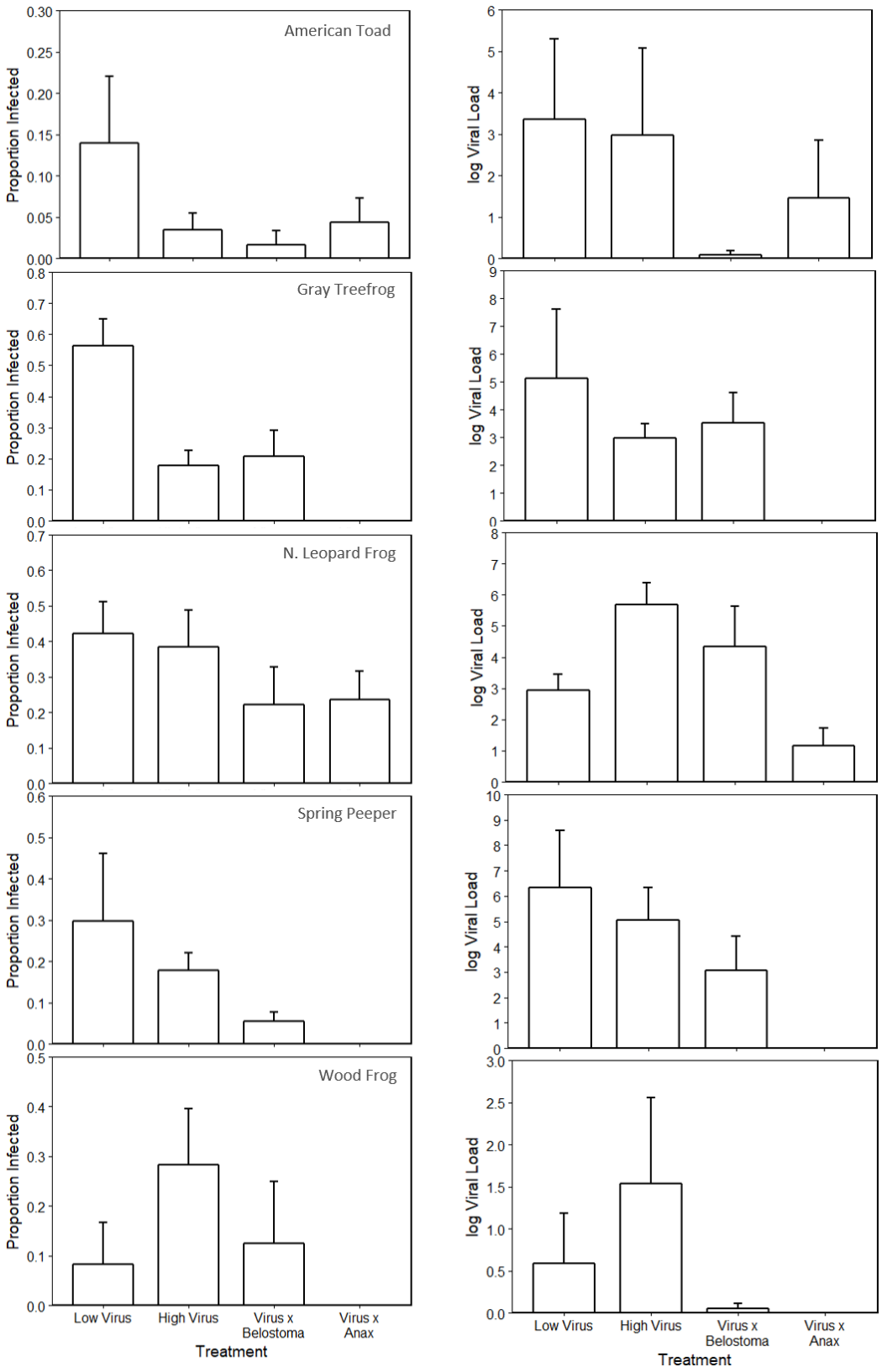

Figure B-2. Average infection prevalence and log viral load in all species in each virus-exposed treatment on day 26. Data are means ± 1 SE.

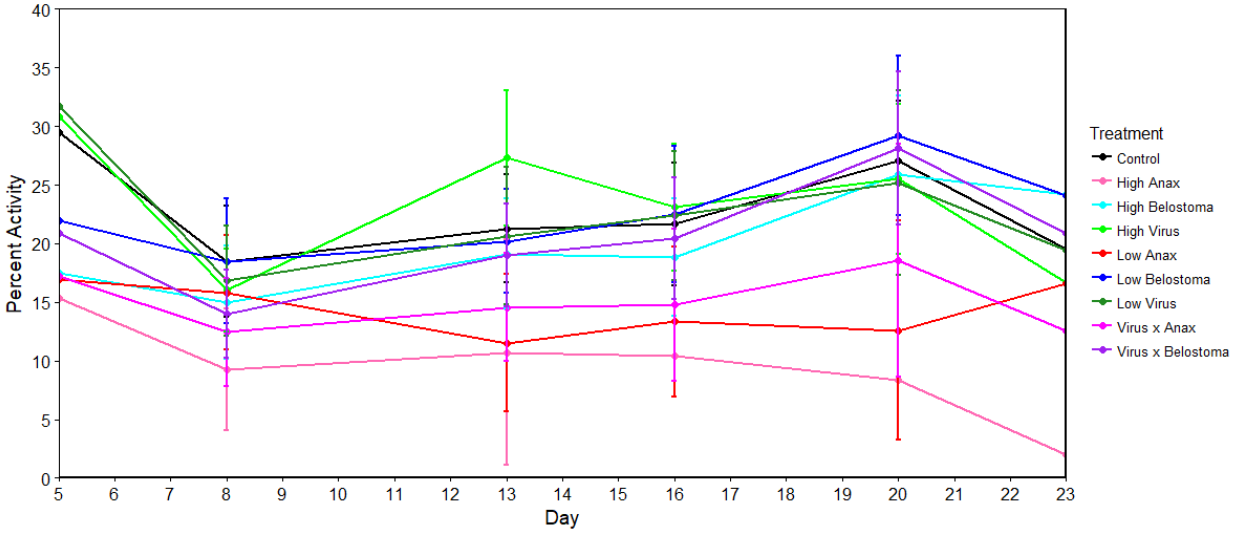

Figure B-3. Percent activity pooled among ten observations on each tank each day. Data are treatment means ± 1 SE.

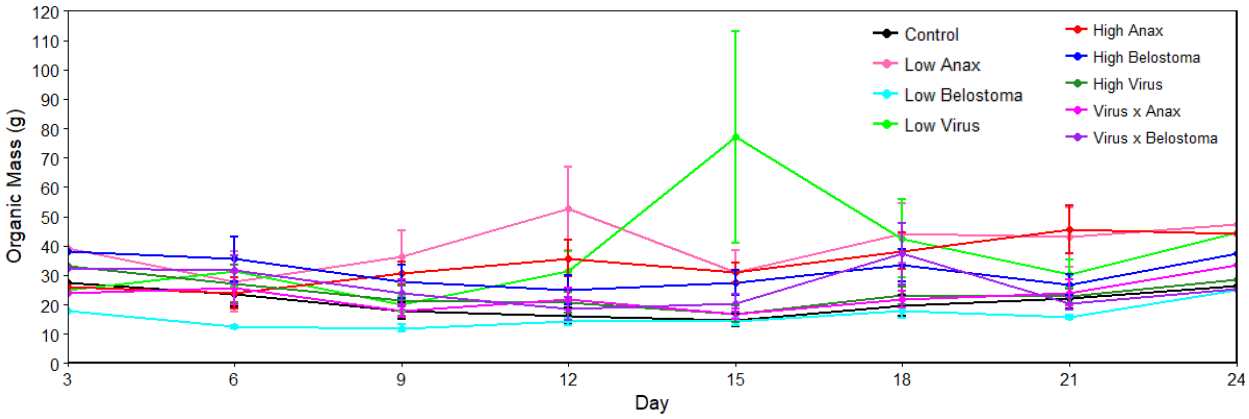

Figure B-4. Periphyton organic matter over time. Data are treatment means ± 1 SE

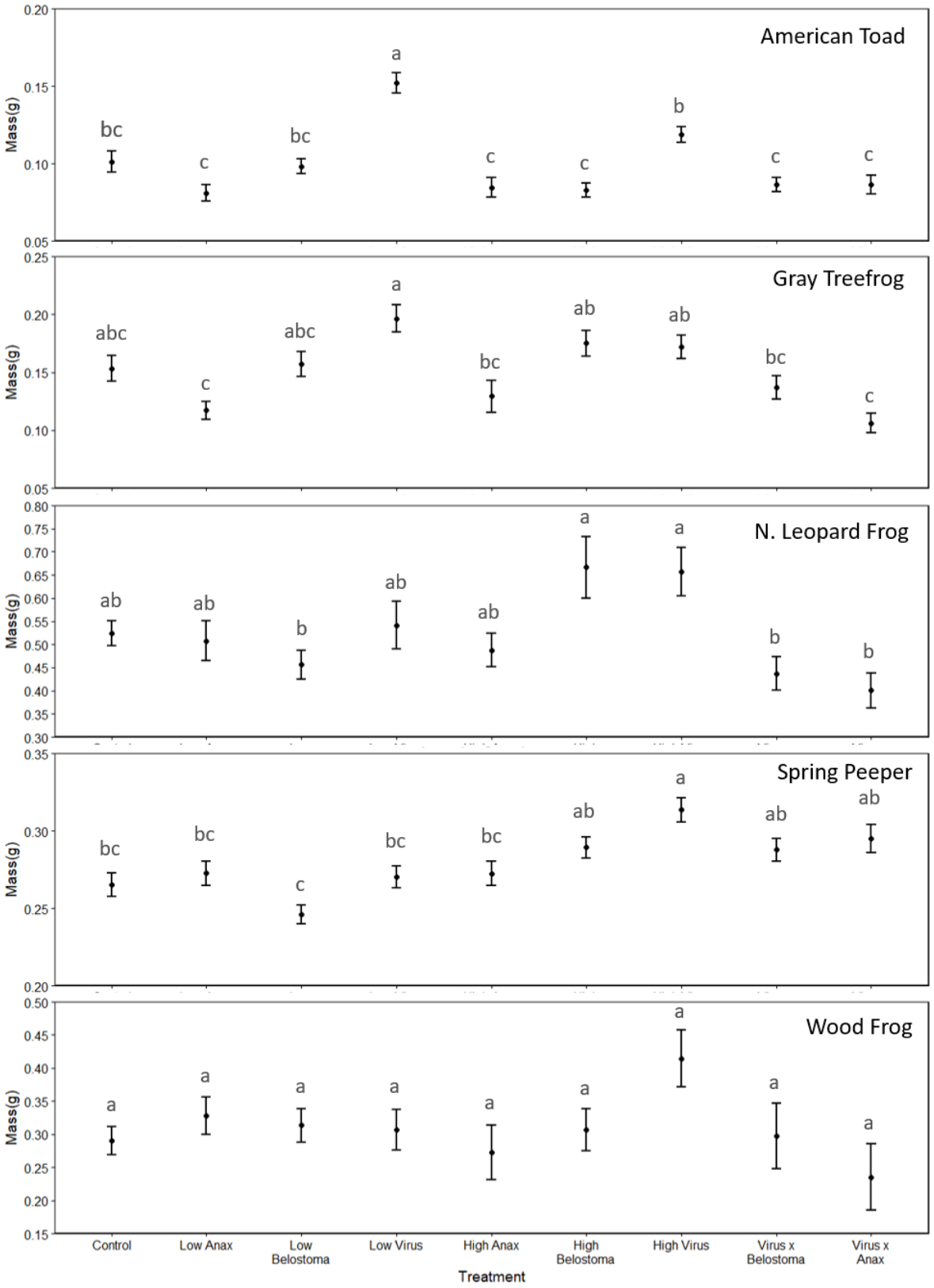

Figure B-5. Mean mass for each of my focal species on the day 10 takedown. Error bars are ± 1 SE

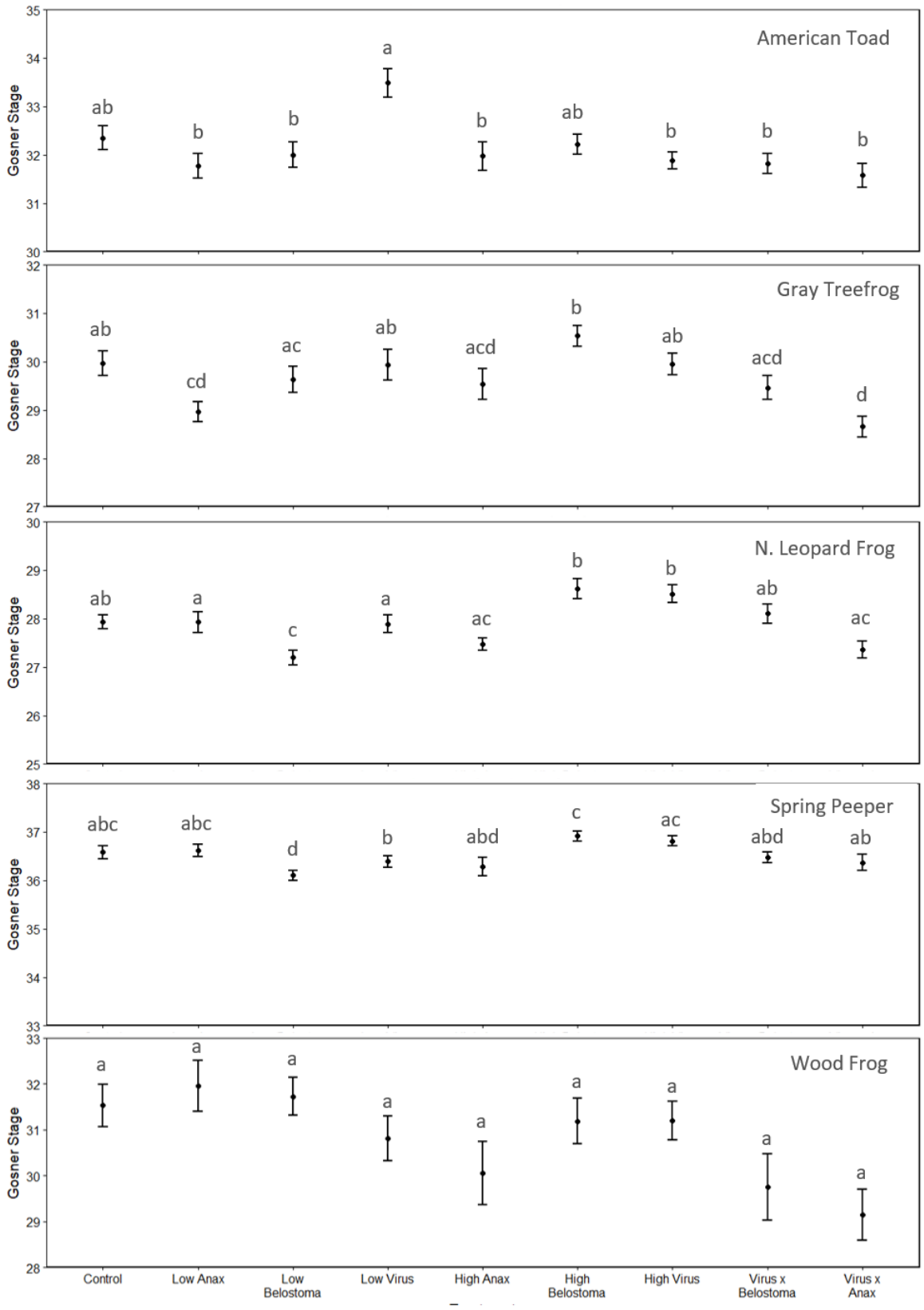

Figure B-6. Mean Gosner stage for each of my focal species on the day 10 takedown. Error bars are ± 1 SE

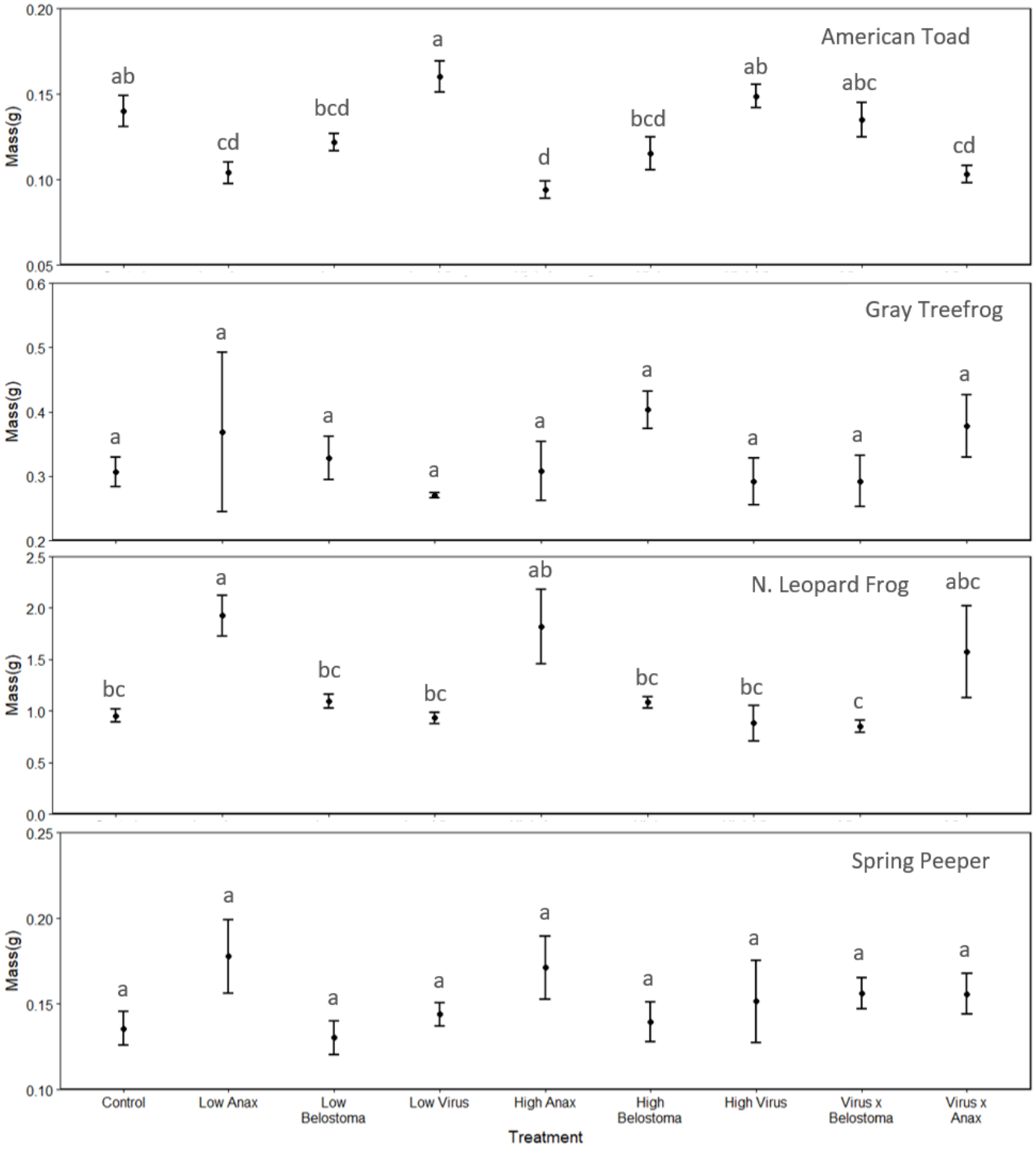

Figure B-7. Mean mass for each of my focal species on the day 26 takedown. Error bars are ± 1 SE

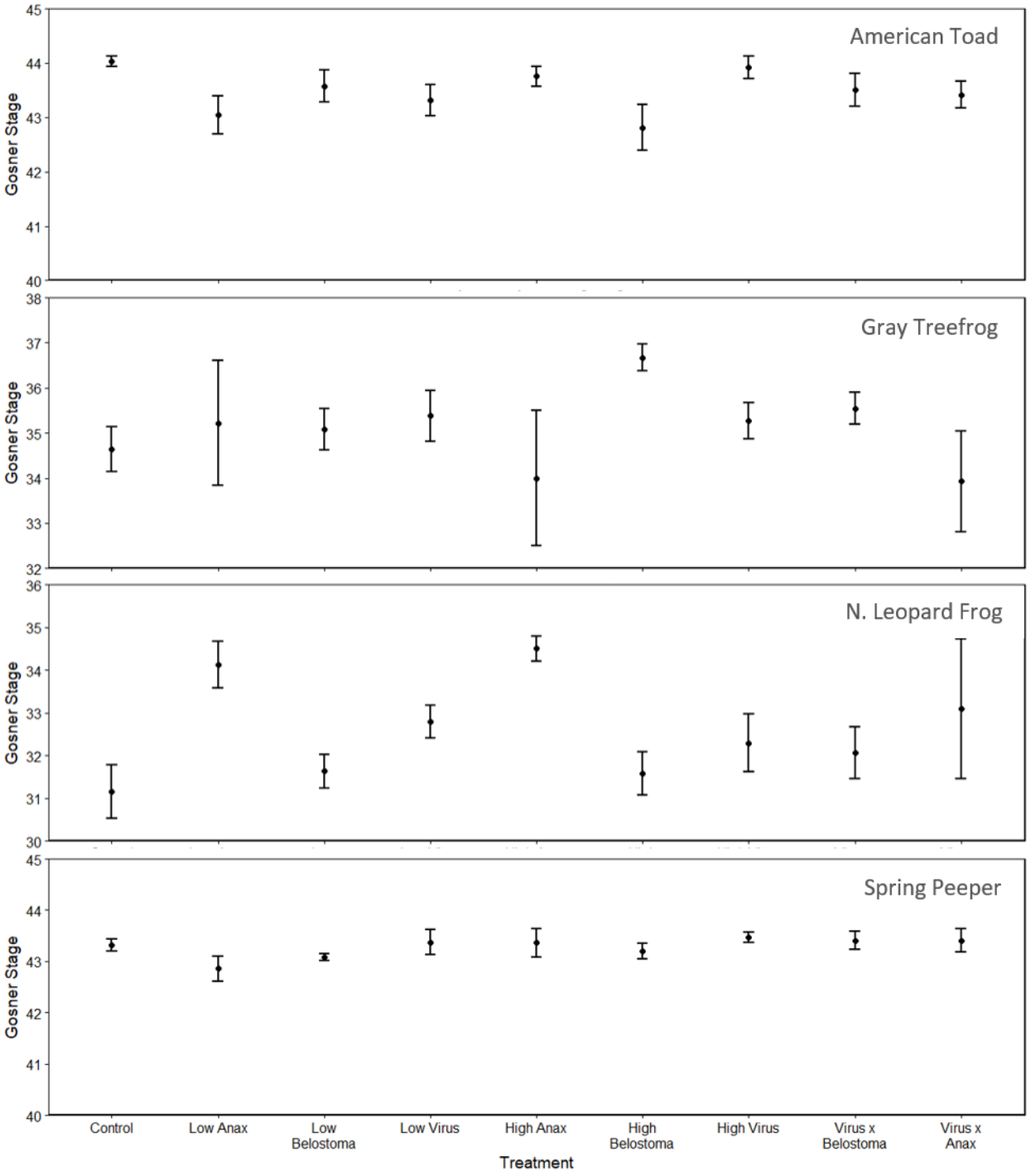

Figure B-8. Mean Gosner stage for each of my focal species on the day 26 takedown. Error bars are ± 1 SE

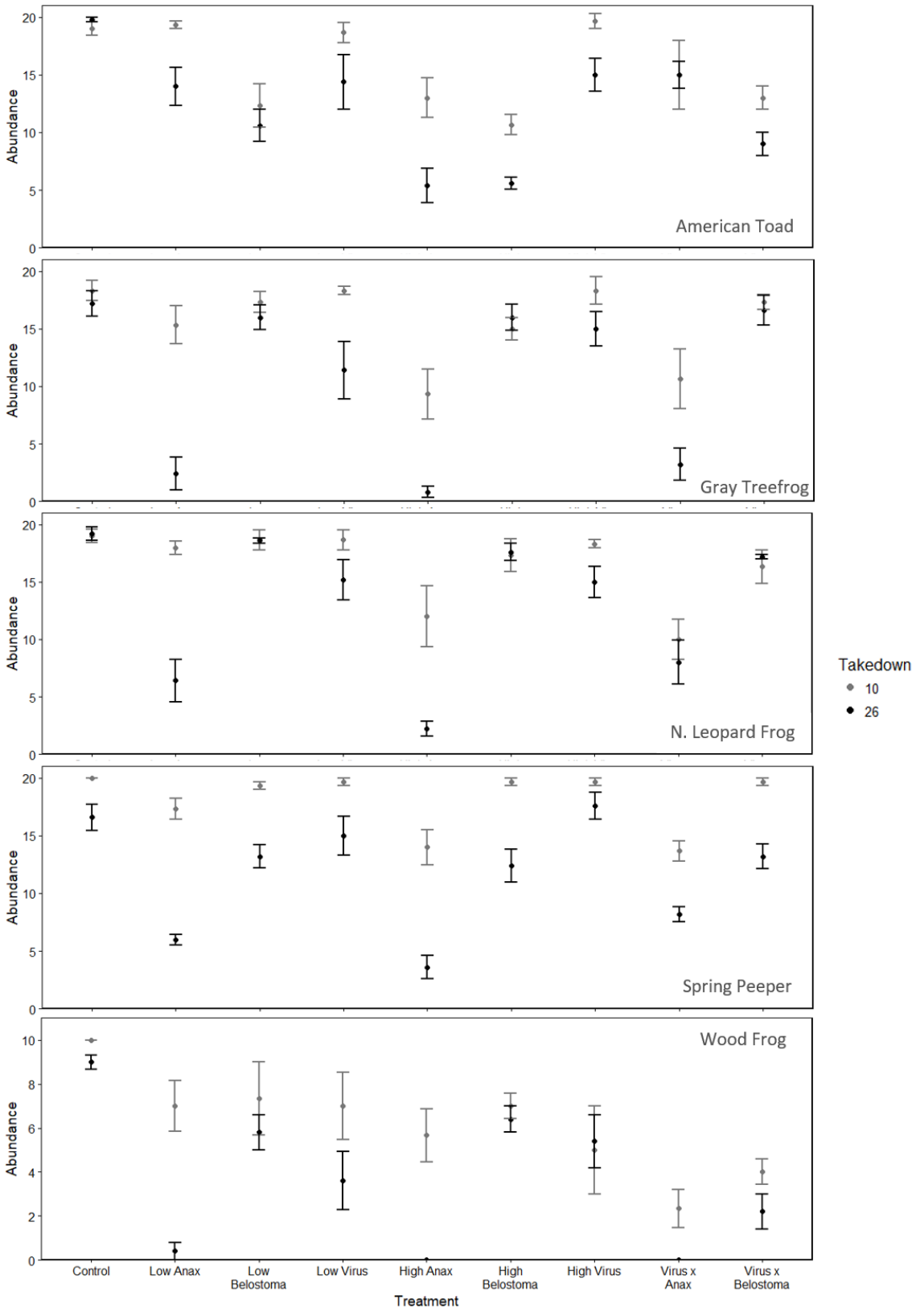

Figure B-9. Species-level abundance across treatment for both Day 10 and Day 26. Data are means ± 1 SE.

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