

WIDESPREAD RANAVIRUS AND PERKINSEA INFECTIONS IN CUBAN TREEFROGS (*OSTEOPILUS SEPTENTRIONALIS*) INVADING NEW ORLEANS, USA

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Abstract.—Invasive species can negatively impact ecosystems in numerous ways, including vectoring pathogenic organisms. In amphibians, a lineage globally threatened by multiple pathogens, this spread of disease via invasive species could contribute to declines in native populations. The Cuban Treefrog (*Osteopilus septentrionalis*) is invasive in the southeastern USA. To assess whether *O. septentrionalis* is a potential reservoir host for the pathogens *Batrachochytrium dendrobatidis* (Bd; Amphibian Chytrid Fungus), Ranavirus (Rv), and Perkinsea (Pr), we sampled 82 individuals from a recently invaded site in New Orleans, Louisiana, USA. We used quantitative PCR to assess prevalence and intensity of Bd, Rv, and Pr in mouthparts and tail clips from 22 larvae and in toe clips from 60 metamorphosed frogs. We compared infection prevalence and intensity across host characteristics, including Fulton's Body Condition, sex, and life stage. None of the individuals were infected with Bd, 72% were infected with Rv, and 44% were infected with Pr. Twenty-three individuals (28%) were co-infected with Rv and Pr, but co-infection did not significantly predict the prevalence or intensity of either Rv or Pr. Although we did not observe any disease signs, Pr infections were significantly associated with lower body condition, suggesting sub-lethal fitness costs. Our study establishes that invasive *O. septentrionalis* in New Orleans are infected with two pathogens of global concern for amphibians. Understanding host-pathogen dynamics in *O. septentrionalis* in Louisiana is a critical step towards understanding how this invasive species could threaten amphibian biodiversity in the region by transmitting infectious pathogens.

Key Words.—amphibian Perkinsea; *Batrachochytrium dendrobatidis*; disease ecology; emerging infectious disease; invasive host

INTRODUCTION

Infectious diseases have negatively influenced numerous amphibian taxa and are a major focus of conservation (Daszak et al. 2003; Fisher et al. 2012; Scheele et al. 2019). Host factors, such as immune responses, can significantly drive amphibian disease dynamics at individual, population, and species levels (e.g., Mutschmann et al. 2000; Price et al. 2014; Scheele et al. 2019). Thus, an invasive congeneric species entering a novel amphibian community can alter disease dynamics by introducing or changing the pathogen prevalence in susceptible local species (Telfer and Bown 2012; Dunn and Hatcher 2015). Depending on the pathogen susceptibility of an invasive amphibian, species can be categorized as either reservoir or dilution hosts. A reservoir host spreads pathogens to susceptible hosts and therefore increases pathogen prevalence (Gog et al. 2002; Haydon et al. 2002; de Castro and Bolker 2005; Brannelly et al. 2017). In particular, when

invasive species have high pathogen tolerance this will often increase the overall community prevalence of the pathogen and drive declines in susceptible native hosts (Telfer and Bown 2012; Scheele et al. 2017a). By contrast, dilution hosts decrease pathogen prevalence because they are resistant to infection and serve as a pathogen sink by decreasing the probability of transmission to other individuals in the community (LoGiudice et al. 2003; Keesing et al. 2006; Kopp and Jokela 2007; Johnson et al. 2008; Searle et al. 2011). In some cases, dilution hosts can even eliminate the presence of the pathogen within a community (Kopp and Jokela 2007; Young et al. 2017). As the number of established invasive amphibian populations continues to increase globally (Scheele et al. 2019), we need to improve our understanding of how these species impact overall amphibian disease dynamics.

Amphibians as a group are declining globally as a direct result of emerging infectious diseases along with other factors (Halliday 1998; Daszak et al. 2003;

Scheele et al. 2019). The Amphibian Chytrid Fungus *Batrachochytrium dendrobatidis* (Bd; Berger et al. 1998; Longcore et al. 1999), viral genus Ranavirus (Rv; Daszak et al. 2009; Campbell et al. 2018), and as-yet undescribed protist amphibian Perkinsea (Pr; Landsberg et al. 2013; Isidoro-Ayza et al. 2017) infect amphibians worldwide and have contributed to disease outbreaks, population extirpations, and species extinctions. The pathogen Bd causes infections that can result in the disease chytridiomycosis, which disrupts vital functions of amphibian skin such as respiration and water balance, and has been implicated in large-scale amphibian die-offs (Mutschmann et al. 2000; Campbell et al. 2012; Scheele et al. 2017b; Scheele et al. 2019). Ranaviruses represent a genus of Iridoviruses that infect fish, amphibians, and reptiles and can cause hemorrhagic disease in these taxa (Speare and Smith 1992; Green et al. 2002; Daszak et al. 2009; Miller et al. 2011; Waltzek et al. 2014) and has resulted in significant declines in a wide range of amphibian species globally (Price et al. 2014; Campbell et al. 2018). Perkinsea is a poorly understood protist parasite that has been associated with mortality events in amphibian populations (Chambouvet et al. 2015; Atkinson 2016; Isidoro-Ayza et al. 2017). Ranavirus and Perkinsea primarily infect liver tissues, particularly in larval anurans (Miller et al. 2011; Karwacki et al. 2018; Isidoro-Ayza et al. 2019). By contrast, Bd infects keratinized tissue in the mouthparts of larvae and the epidermis of adult amphibians (Marantelli et al. 2004).

Individual hosts can be co-infected with Bd and Rv (Souza et al. 2012; Warne et al. 2016) or with Rv and Pr and these latter co-infections have led to documented mortality events in amphibian species (Cook 2008; Miller et al. 2011; Landsberg et al. 2013; Isidoro-Ayza et al. 2017). Co-infections could potentially put individuals at a higher mortality risk by adding an additional stress factor to an already compromised host (Bandilla et al. 2006; Ezenwa and Jolles 2011; Johnson and Hoverman 2012; Su et al. 2005). Thus, understanding the relationship between amphibian population declines and infection rates with these three pathogens is important for informing global biodiversity conservation strategies.

Invasive amphibian species can cause negative impacts on the communities and ecosystems they invade via changing infection dynamics and disease dynamics within native species, ultimately leading to changes in the stability of the ecosystem (Daszak et al. 2004; Lettoof et al. 2013; Soto-Azat et al. 2016; Rivera et al. 2019). For example, American Bullfrogs (*Lithobates catesbeianus*) are invasive globally and often appear to be reservoir hosts for Bd and Rv (Borzée et al. 2017; Brunner et al. 2019). In several documented cases, *L. catesbeianus* invasions have contributed to the spread of Bd and exacerbated chytridiomycosis in susceptible

native frog populations (Daszak et al. 2004; Blaustein et al. 2005; Adams et al. 2017; Urbina et al. 2018). Similarly, the African Clawed Frog (*Xenopus laevis*) can sustain high Rv pathogen loads (De Jesús Andino et al. 2012; Soto-Azat et al. 2016) and may also serve as a pathogen vector or reservoir host in areas it has invaded (Robert et al. 2007). Given the negative impact of these two invasive species on community-wide disease dynamics (Daszak et al. 2004; Soto-Azat et al. 2016; Yap et al. 2018), other invasive amphibians likely have similar harmful effects on the communities they invade.

The Cuban Treefrog (*Osteopilus septentrionalis*) is native to Cuba, the Bahamas, and the Cayman Islands (Heinicke et al. 2011). Since invading Florida, USA, in the 1920s, *O. septentrionalis* directly competed with native treefrog species for shelter and food, preying on smaller frogs including the American Green Tree Frog (*Hyla cinerea*) and causing other negative impacts to native hylid populations (Wyatt and Forsys 2004; Platenberg 2007; Heinicke et al. 2011; Andrews et al. 2015). Invasive *O. septentrionalis* populations may also serve as reservoir hosts for various pathogens. For example, one experimental study demonstrated that the endoparasite *Allodero hylae* (no common name) has a natural affinity for *O. septentrionalis* as a host, and that infected *O. septentrionalis* adults shed the parasite in high quantities into the water in which they were housed (Andrews et al. 2015). While *O. septentrionalis* individuals cleared Bd infection after sequential experimental exposure trials, suggesting acquired resistance to Bd (McMahon et al. 2014), infected *O. septentrionalis* may still spread Bd to naive frog species before infections are completely cleared by the host. Furthermore, another study documented Bd infections without disease signs in invasive *O. septentrionalis* (Rizkalla 2010), suggesting Bd amplification effects may occur in amphibian communities where *O. septentrionalis* has invaded. Finally, a recent study characterized Pr infections in invasive *O. septentrionalis*, with higher infection intensity than most other amphibian species in the community (Karwacki et al. 2018). These studies highlight the potential for invasive *O. septentrionalis* to amplify amphibian pathogens and underscore the importance of characterizing pathogen dynamics in their invasive range.

In 2016, an invasive *O. septentrionalis* population was discovered in the Audubon Zoo and adjacent Audubon Riverview Park in New Orleans, Louisiana, USA (Glorioso et al. 2018). We presume these invasive frogs first arrived in the Audubon Zoo on palm trees shipped from Florida that were planted in the elephant exhibit in March 2016. This area was not known to have an established *O. septentrionalis* population until surveys in late 2017, after which 367 individuals were removed from the Audubon Zoo and adjacent

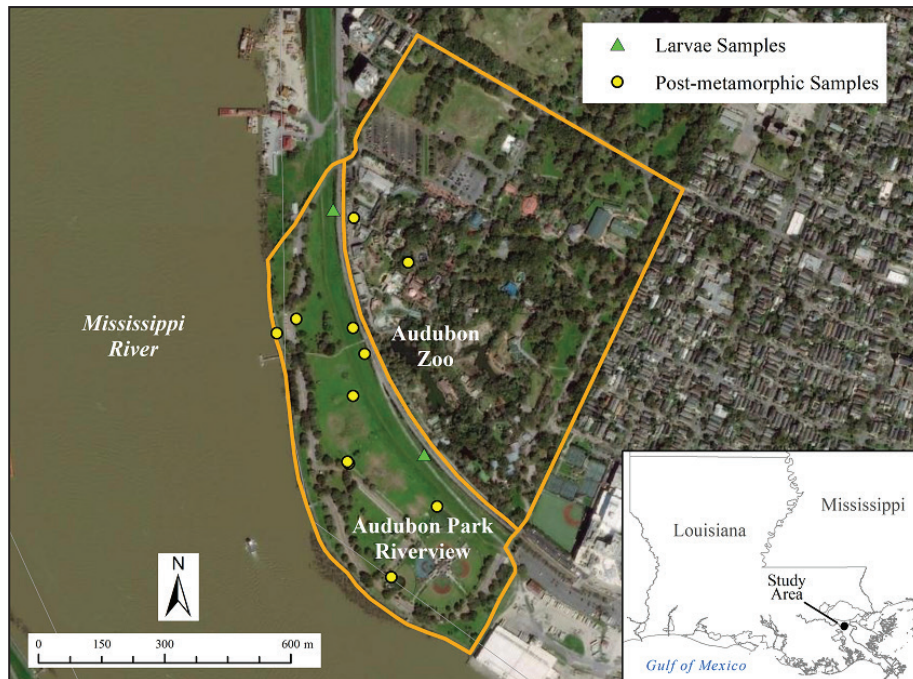


FIGURE 1. Collection locations of *Osteopilus septentrionalis* (Cuban Treefrog) sampled in New Orleans, Louisiana, USA, in September, October, and November 2017, including two locations where we collected larvae (green triangles), and 10 where we collected post-metamorphic individuals (yellow circles). (from Esri ArcGIS Desktop 10.7 Worldwide Imagery Basemap).

Audubon Riverview Park (Glorioso et al. 2018). At least two native treefrogs (*H. cinerea* and *H. squirella*, the Squirrel Tree Frog) were present on the premises prior to the invasion of *O. septentrionalis* (Glorioso et al. 2018). This large invasion raises concerns for the native amphibian community because *O. septentrionalis* may contribute to the spread of disease. Thus, we characterized the presence and infection intensity of the amphibian pathogens Bd, Rv, and Pr in this invasive population of *O. septentrionalis* using individuals removed from the site in 2017.

In this study, we tested whether infection prevalence and intensity of the three pathogens varied across life stage, month of sample collection, and sex, as well as the body condition of infected versus uninfected individuals. Given that *O. septentrionalis* are often infected with these three pathogens in other locations, we expected that the frogs in Louisiana would also be infected with at least some of these pathogens. Furthermore, we expected to find prevalence and intensity differences across life stages, months, and body condition based on previous studies of pathogen infections in *O. septentrionalis*. Our study highlights the importance of pathogen surveillance as a component of amphibian conservation efforts.

MATERIALS AND METHODS

Tissue collection.—We removed 367 *O. septentrionalis* individuals by hand during visual

encounter surveys from Audubon Zoo and adjacent Audubon Riverview Park in New Orleans, Louisiana, USA, from September through November 2017 (Fig. 1; Glorioso et al. 2018; Glorioso and Waddle 2018). Of those 367, we randomly selected 15 metamorphosed individuals per sampling date ($n = 60$; Table 1) for disease analysis. For every individual captured and removed, we measured body mass (g) and snout-to-vent length (SVL; cm), and recorded the capture location. We used a topical application of 20% benzocaine to euthanize post-metamorphic *O. septentrionalis* and collected toe clips in the laboratory before preservation. We sterilized scissors after each toe clip by flaming or alcohol immersion. On 23 October 2017 we also collected and euthanized larvae from two drying puddles using a dipnet in Audubon Riverview Park (Fig. 1; Glorioso et al. 2018). We dissected a randomly selected subset of these larvae ($n = 22$), removing the mouthparts to test for Bd, taking tail clips to test for Rv and Pr, and storing each tissue separately for each individual. Though liver tissue would have been ideal for Rv and Pr detection, we obtained only tail and toe clips from this sampling for genetic analysis, and we know these tissues slightly under-estimate Rv and Pr prevalence (Gray et al. 2012; Karwacki et al. 2018). We preserved all tissues in 99% molecular grade ethanol and stored in a -20°C freezer prior to DNA extractions. We determined age class in metamorphosed frogs via SVL, classifying any individual with $\text{SVL} < 3.9$ cm as a juvenile (Blair 1958). Finally, we attempted to confirm

the sex of all individuals by opening the body cavity and directly observing the gonads. When we were unable to determine the sex of individuals, we classified them as unknown sex.

Pathogen quantification.—We extracted DNA from all preserved tissues using a Qiagen DNeasy Blood and Tissue kit (Qiagen Corporation, Germantown, Maryland, USA) according to the protocol of the manufacturer. We bleached all surfaces prior to DNA extractions and only opened one sample tube at a time during all extraction steps, minimizing the potential for contamination. We used three established qPCR protocols to determine the prevalence and infection intensity (in genomic equivalents) of Pr (Karwacki et al. 2018), Bd (Boyle et al. 2004), and Rv (Allender et al. 2013). We ran qPCR reactions in duplicate for all samples and performed said reactions in volumes of 25 μ L, consisting of the following: 2 μ L of forward primer (10 μ M), 2 μ L of reverse primer (10 μ M), 3 μ L of molecular grade water, 5 μ L of qPCR Taqman probe (1 μ M) and 8 μ L of qPCR Supermix (Bio-Rad, Hercules, California, USA) and 5 μ L of template DNA. We used serially diluted gBlock gene fragments (Integrated DNA Technologies, Coralville, Iowa, USA) comprising the qPCR target region of the genome of each pathogen as standards in each reaction and ran them in duplicate on each plate. Pr gBlock standard dilutions ranged from 1×10^9 to 1 genomic equivalents (GE), Bd from 1×10^7 to 1 GE, and Rv from 5.29×10^9 to 5.29×10^1 GE. We ran negative controls consisting of 5 μ L molecular grade water in place of template DNA in duplicate for each plate. We retained all plates retained in our analyses with a minimum r^2 value of 0.90. We ran third replicate runs for any sample replicates that deviated by more than two threshold cycles (the cycle at which the DNA amplification first exceeds background levels). We calculated mean GE across replicates, and for samples run in triplicate, we took the mean GE of the two most similar replicates.

Statistical analyses.—We conducted all statistical analysis in R (R Development Core Team 2015) using the packages ggplot2 (Wickham 2016), rcompanion (Mangiafico 2016), gridextra (Baptiste and Antonov 2017), and bbmle (Bolker and License 2009) with an alpha value of 0.05 as our significance threshold. Prior to running any analysis, we tested and confirmed that the data fit the assumptions of the statistical analysis used. We tested whether pathogen prevalence differed significantly between month of capture, life stage, and sex using Chi-square tests followed by post hoc Fisher's Exact Tests where more than two categories were present (Shan and Gerstenberger 2017) for Rv and Pr. We also tested whether Fulton's Body Condition (K), measured

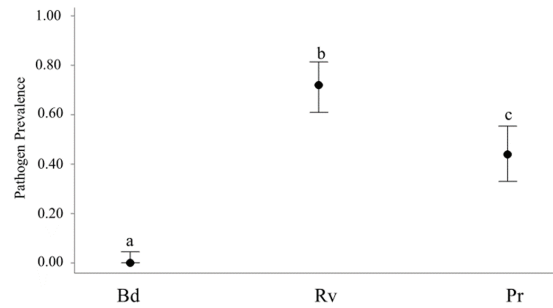


FIGURE 2. Overall prevalence ($\pm$ 95% Clopper-Pearson binomial confidence intervals) of Perkinsea (Pr), Ranavirus (Rv), and *Batrachochytrium dendrobatidis* (Bd) in *Osteopilus septentrionalis* (Cuban Treefrog) individuals sampled in New Orleans, Louisiana, USA, in 2017. Significant differences among groups are indicated by different letters above confidence intervals as determined via Chi-square with Fisher exact tests.

as the ratio of body length (SVL) to mass (Băncilă et al. 2010), differed significantly by infection status (infected versus uninfected) in metamorphosed frogs using a One-way ANOVA with Tukey HSD post-hoc tests where more than two categories were present for Rv and Pr. Finally, we tested whether pathogen infection intensity (in log GE) differed significantly between sampling month in metamorphosed frogs, life stage, sex, K, and whether the animal had a single infection or multiple infections using One-way ANOVAs with Tukey HSD post-hoc tests where more than two categories were present.

RESULTS

We analyzed 82 invasive *O. septentrionalis* individuals that we sampled in September, October, and November of 2017 (Table 1; all raw data available in Dryad, doi:10.5061/dryad.7wm37pvs8). We definitively determined the sex of 57 of the 60 metamorphosed frogs, which consisted of 34 males and 23 females (Table 2). No individuals were infected with Bd (95% CI: 0–4%), 59 individuals were infected with Rv (72%; CI: 61–81%), and 36 individuals were infected with Pr (44%; CI: 33–55%; Table 2; Fig. 2).

TABLE 1. *Osteopilus septentrionalis* (Cuban Treefrog) samples collected for this study in New Orleans, Louisiana, USA, in 2017. Average monthly temperature was obtained via the WorldClim database (Fick and Hijmans 2017). Abbreviations are NAS = number of adults sampled, NJS = number of juveniles sampled, NLS = number of larvae sampled, and AMT = average monthly temperature.

Sampling date	NAS	NJS	NLS	AMT
18 September 2017	15	0	0	26.1° C
28 September 2017	8	7	0	26.1° C
23 October 2017	12	3	22	21.4° C
13 November 2017	10	5	0	16.7° C

TABLE 2. *Perkinsea* (Pr), *Ranavirus* (Rv), and *Batrachochytrium dendrobatidis* (Bd) prevalence across sex and life stage in *Osteopilus septentrionalis* (Cuban Treefrog) sampled in New Orleans, Louisiana, USA, in 2017.

Group	Total	Bd		Rv		Pr	
		Number Positive	Proportion infected	Number Positive	Proportion infected	Number Positive	Proportion infected
Male	34	0	0.00	28	0.82	19	0.56
Female	23	0	0.00	20	0.87	9	0.39
Unknown Sex	25	0	0.00	11	0.44	8	0.32
Adult	45	0	0.00	36	0.80	24	0.53
Juvenile	15	0	0.00	15	1.00	6	0.40
Larvae	22	0	0.00	8	0.36	6	0.27

Among infected frogs, mean Rv infection intensity was 38,692,557 GE (range: 1,143 to 374,221,683 GE) and mean Pr infection intensity was 6,786,477 GE (range: 392,370 to 22,230,475 GE). Fulton's Body Condition (K) did not differ significantly in Rv infected versus uninfected individuals ($F_{1,58} = 2.30$, $P = 0.134$). By contrast, Pr infected individuals had significantly lower body condition compared to uninfected individuals ($F_{1,58} = 8.29$, $P = 0.005$; Fig. 3).

Rv and Pr co-infected 23 individuals (28%). *Ranavirus* prevalence ($X^2 = 1.41$, $df = 1$, $P = 0.234$ and Pr prevalence ($X^2 = 1.41$, $df = 1$, $P = 0.234$) did not differ significantly between singly versus co-infected individuals. Likewise, *Ranavirus* infection intensity ($F_{1,80} = 2.89$, $P = 0.094$) and Pr infection intensity ($F_{1,80} = 1.52$, $P = 0.227$) did not differ significantly between singly versus co-infected individuals. By contrast, K differed significantly between co-infected and singly infected individuals ($F_{2,57} = 4.59$, $P = 0.014$; Fig. 3),

with co-infected individuals exhibiting significantly lower K than frogs only infected with Rv ($F_{1,57} = 4.59$, $P = 0.024$).

Ranavirus prevalence in post-metamorphic individuals significantly differed across sampling month ($X^2 = 6.66$, $df = 2$, $P = 0.035$; Fig. 4), but individuals sampled in November did not have significantly higher prevalence than those sampled in October ($X^2 = 6.66$, $df = 2$, $P = 0.073$) or September ($X^2 = 6.66$, $df = 2$, $P = 0.236$). *Ranavirus* prevalence also varied significantly across life stage ($X^2 = 21.10$, $df = 2$, $P < 0.001$; Fig. 5), with lower prevalence in larvae compared to adults or juveniles ($X^2 = 21.10$, $df = 2$, $P = 0.001$). *Ranavirus* infection intensity also varied significantly by life stage ($F_{2,79} = 86.78$, $P < 0.001$; Fig. 5), but with significantly lower infection intensities in larvae compared to adults or juveniles ($F_{1,80} = 86.78$, $P < 0.001$). *Ranavirus* infection intensity did not vary across sampling months ($F_{2,57} = 0.48$, $P = 0.624$), and neither Rv prevalence (X^2

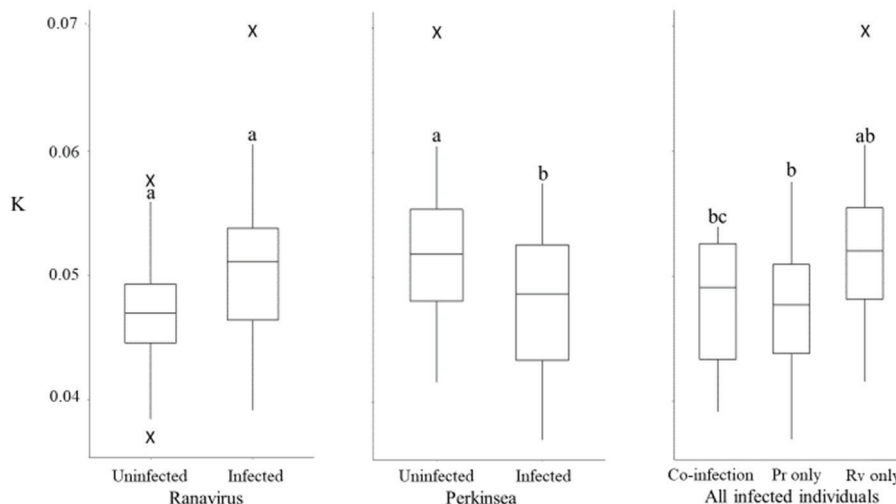


FIGURE 3. Body condition for *Osteopilus septentrionalis* (Cuban Treefrog) individuals infected with *Ranavirus*, *Perkinsea*, and both, sampled in New Orleans, Louisiana, USA, in 2017. Body condition (K) is depicted as boxplots representing median (horizontal line within box), interquartile range (outer box), range exclusive of outliers (vertical line), and outliers ($> 1.5 \times$ interquartile range; black X) for *Ranavirus* (Rv, left panel) and *Perkinsea* (Pr, center panel) infected compared to uninfected, and body condition (K) for co-infection (right panel) compared to single infection. Significant differences among groups are indicated by different letters above vertical lines using ANOVA with Tukey HSD post-hoc test where more than two categories were present.

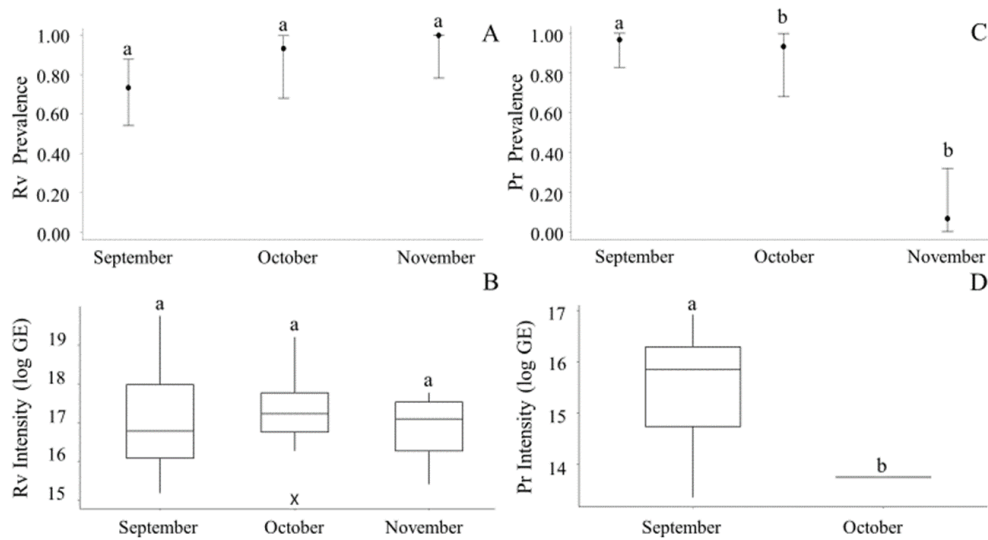


FIGURE 4. Prevalence and infection intensity by month for the pathogens, Ranavirus (Rv; A and B) and Perkinsea (Pr; C and D), in post-metamorphic *Osteopilus septentrionalis* (Cuban Treefrog) sampled in New Orleans, Louisiana, USA (n = 60), in 2017. Panels A and C depict Pathogen prevalence ($\pm$ 95% Clopper-Pearson binomial confidence intervals) and panels B and D depict infection intensity in log genomic equivalents (GE); boxplot symbology as in Fig. 3. Significant differences among groups are indicated by different letters above confidence intervals or vertical lines via Chi-square with Fisher exact test for prevalence and ANOVA with Tukey HSD post-hoc test for infection intensity.

= 0.79, df = 2, $P = 0.683$) nor infection intensity varied between males and females ($F_{2,57} = 0.88$, $P = 0.422$).

Perkinsea prevalence in post-metamorphic individuals also varied significantly based on sampling month ($X^2 = 52.40$, df = 2, $P < 0.001$; Fig. 4), but dynamics were different than for Rv. Specifically, individuals collected in September had significantly higher Pr prevalence than individuals caught in October or November ($X^2 = 52.40$, df = 2, $P < 0.001$). Similarly,

Pr infection intensity varied significantly across sampling month ($F_{1,58} = 16.36$, $P < 0.001$; Fig. 4), with significantly higher infection intensity in individuals collected during September than October ($F_{1,58} = 16.36$, $P < 0.001$). Perkinsea prevalence did not vary with life stage ($X^2 = 4.19$, df = 2, $P = 0.123$), but Pr infection intensity varied significantly by life stage ($F_{2,79} = 6.08$, $P = 0.005$; Fig. 5). Larvae had significantly lower Pr infection intensity compared to adults and juveniles

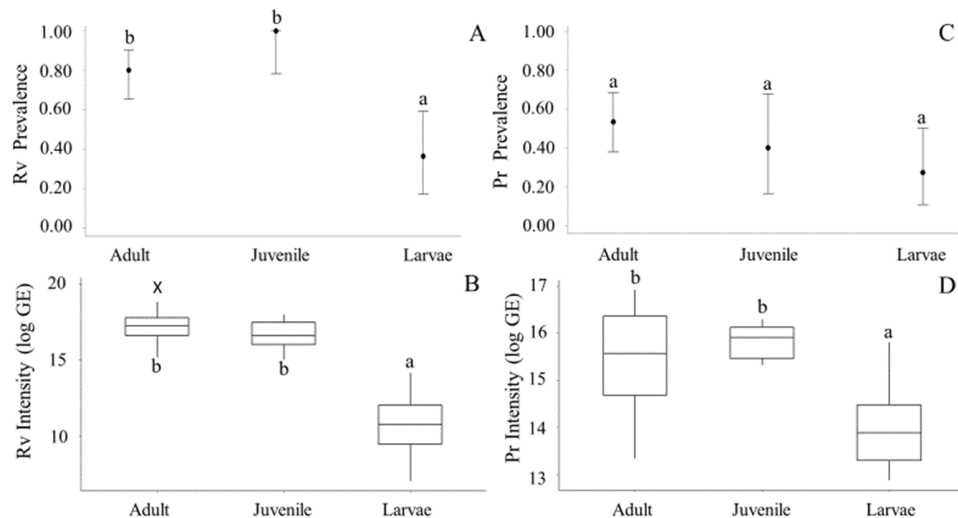


FIGURE 5. Prevalence and infection intensity by lifestage for the pathogens, Ranavirus (Rv; A and B) and Perkinsea (Pr; C and D), in *Osteopilus septentrionalis* (Cuban Treefrog) sampled in New Orleans, Louisiana, USA (n = 82), in 2017. Panels A and C depict Pathogen prevalence ($\pm$ 95% Clopper-Pearson binomial confidence intervals) and panels B and D depict infection intensity in log genomic equivalents (GE); boxplot symbology as in Fig. 3. Significant differences among groups are indicated by different letters above confidence intervals or vertical lines via Chi-square with Fisher exact test for prevalence and ANOVA with Tukey HSD post-hoc test for infection intensity.

($F_{1,80} = 6.08$, $P < 0.010$). Neither Pr prevalence ($X^2 = 1.89$, $df = 2$, $P = 0.388$) nor infection intensity varied between males and females ($F_{2,57} = 0.29$, $P = 0.751$).

DISCUSSION

In our study, we found high Rv and Pr prevalence and infection intensity but no Bd infections in the invasive *O. septentrionalis* population in New Orleans, Louisiana. Because we measured Rv and Pr from toe and tail clips, which harbor lower pathogen burdens than liver tissue (Gray et al. 2012; Karwacki et al. 2018), our estimates may represent an underestimate of true pathogen prevalence and intensity. Perkinsea (Isidoro-Ayza et al. 2017) has been documented previously in Louisiana so it is unsurprising that *O. septentrionalis* in New Orleans are infected. While these animals could have been infected prior to their arrival in New Orleans, at least some of the individuals sampled in our study likely obtained the infections after invasion given that we sampled infected tadpoles from clutches deposited within the study area in Louisiana. We found that co-infection with Rv and Pr in these frogs was common (occurring in 28% of sampled animals), consistent with the literature (Landsberg et al. 2013; Karwacki et al. 2018). Previous studies have also reported that Rv and Pr co-infections have contributed to large anuran mortality events (Green et al. 2002; Landsberg et al. 2013), suggesting that infection with one pathogen could increase susceptibility to another. Co-infection did not significantly affect the prevalence or intensity of either pathogen in the *O. septentrionalis* individuals we analyzed, however. Furthermore, co-infected individuals had significantly lower K compared to Rv-only infected frogs, but not compared to Pr-only infections, suggesting that Pr affects body condition independent of other pathogens. Thus, we find no evidence of co-infections producing different responses than single infections. This finding highlights that co-infection needs further investigation in amphibian hosts, particularly in an experimental context, in order to understand how pathogen interactions with each other and the host immune system may influence disease severity.

The absence of Bd in our sampled *O. septentrionalis* could arise from a variety of factors. *Batrachochytrium dendrobatidis* occurs at low prevalence in most species sampled in the southeastern USA (Bielby et al. 2015; Horner et al. 2017) as well as in *O. septentrionalis* sampled in Florida (Rizkalla 2010), and in our dataset we have 95% confidence that Bd infection is 0–4%, suggesting the invasive *O. septentrionalis* population may truly be free of the pathogen. *Batrachochytrium dendrobatidis* infections are seasonally variable (Savage et al. 2011), and Bd grows optimally at cooler temperatures (Piotrowski et al. 2004) that are typical

during the fall months when we sampled in New Orleans. Thus, seasonality is likely not causing us to miss Bd infections. Additionally, most other species of native treefrogs exhibit few infections and do not exhibit negative impacts from infections (Brannelly et al. 2012); however, some native hylids in southern Louisiana show persistent Bd infection in recent years, with increasing prevalence in cooler months (Sonn et al. 2019). While this study did not detect Bd infected *O. septentrionalis*, we recommend follow-up pathogen studies in this invasive population using larger sample sizes collected during cooler months.

Among the two pathogens detected, we only found evidence of negative fitness consequences for frogs infected with Pr. Specifically, Pr infections were significantly associated with decreased Fulton's Body Condition. This finding is consistent with previous studies documenting that Pr infections are associated with mortality (Green et al. 2002; Isidoro-Ayza et al. 2017, 2019) and that *O. septentrionalis* tend to harbor higher Pr infection intensities than other species in the same amphibian communities (Karwacki et al. 2018). Pr infection may be amplified in local amphibian communities where *O. septentrionalis* occurs, and this is particularly concerning in regions such as New Orleans, because many of the Pr die-offs in native species have been reported from the southeastern U.S. (Davis et al. 2007; Landsberg et al. 2013), suggesting high pathogenicity, host susceptibility, or both. The lack of a significant difference in Fulton's Body Condition among Rv infected individuals suggests *O. septentrionalis* may exhibit some level of tolerance to Rv, which could lead to amplification of this pathogen as has been documented in other species (Brunner et al. 2019). Alternatively, the Rv strain in Louisiana may be less virulent than other strains present in different locations (Schock et al. 2009). Because we do not have pathogen data from the native amphibian community prior to the arrival of *O. septentrionalis*, we are unable to resolve whether the pathogen dynamics we recovered are typical of the area versus unique to *O. septentrionalis*, or if they represent novel pathogen introductions. This highlights the importance of conducting amphibian pathogen surveillance whenever possible to generate a baseline for understanding future pathogen and/or host introductions.

Environmental factors can have an indirect role in disease dynamics (Carey and Alexander 2003) and variation in infection prevalence and intensity across sampling months has previously been documented for both Rv (Green et al. 2002; Hall et al. 2018) and Pr (Karwacki et al. 2018). Likewise, we found that Rv and Pr prevalence varied significantly across sampling months in post-metamorphic individuals; however, we found a trend for lower Rv prevalence in October

compared to November, suggesting an increase in infections during cooler months, whereas spikes in Rv prevalence and disease outbreaks are commonly associated with the warmest months of the year (Green et al. 2002; Gahl and Calhoun 2008). Higher infection prevalence in cooler months could be explained by the thermal mismatch hypothesis (Cohen et al. 2017) given that *O. septentrionalis* is not cold tolerant and may not clear infections efficiently during cooler months (Haggerty and Crisman 2015). We also found higher Pr prevalence and intensity in September compared to October or November, which contradicts the only other study of Pr infection dynamics across sampling months that found prevalence and intensity to be highest in the coolest months of January, February, and March (Karwacki et al. 2018). While we were not able to test for significance between months, relatively small differences in temperature have been well documented to cause difference in infection outcome (Gahl and Calhoun 2008). One plausible explanation for the unique temporal pathogen dynamics we observed is that our study was primarily conducted during the drier part of the year in New Orleans, which could have limited the spread of Rv and Pr in the cooler and drier months because anurans prefer moist environments (Pitt et al. 2017) and Pr spores primarily spread through water and Rv has limited transmission in water (Cook 2008; Miller et al. 2011).

Another surprising finding in our study was that larvae exhibited significantly lower Rv prevalence and intensity compared to juvenile and adult metamorphosed frogs. This is in contrast to numerous other studies documenting that Rv infections primarily occur in larval populations (Green et al. 2002; Greer et al. 2005; Haislip et al. 2011), and while some evidence exists for impacts from Rv infections in metamorphic frogs (Sauer et al. 2018), our data suggest that more attention should be paid to Rv infections in future studies of Rv in metamorphosed frogs. Given the smaller number of larvae relative to adults in this study, the different tissues analyzed, and the single sampling date when larvae were collected, further data are needed to draw conclusions about life stage differences in Rv infection dynamics. Similarly, we did not find any difference in Pr prevalence or intensity across life stages, which was an unexpected finding as previous studies have identified that Pr primarily infects larvae (Karwacki et al. 2018) or recently metamorphosed frogs (Davis et al. 2007; Landsberg et al. 2013). Again, our results should be interpreted with caution due to limited sampling events, different tissue types, and small sample sizes. Nonetheless, our study adds to the small body of literature confirming Pr is not only found in larvae and juveniles but also in adult frogs (Landsberg et al. 2013; Karwacki et al. 2018).

Based on our finding of high pathogen prevalence and intensity, we propose that invasive *O. septentrionalis* is a reservoir host for both Rv and Pr in New Orleans. The presence of numerous *O. septentrionalis* infected with one or multiple pathogens present a conservation concern because *O. septentrionalis* are potentially facilitating the spread of pathogens to susceptible species across the amphibian communities they invade. Furthermore, the likely origin of these *O. septentrionalis* on a shipment of palm trees highlights the many transportation routes that can facilitate spread of invasive treefrogs and their pathogens (e.g., Hartigan et al. 2012; Plenderleith et al. 2015). Our study also adds to the growing body of literature detecting cryptic, temporally variable infections causing sub-lethal effects in amphibian hosts (e.g., Horner et al. 2017; Karwacki et al. 2018), underscoring the need to better understand the relationship between climate, pathogen exposure, amphibian disease, and amphibian population declines. Our study also provides a baseline for tracking the relationship between novel *O. septentrionalis* introductions and pathogen spread in the local amphibian community. Further monitoring of Pr and Rv infection rates and intensity in *O. septentrionalis* and native amphibian populations in the study area would characterize the disease dynamics in the local amphibian community and help determine if *O. septentrionalis* is acting as a pathogen reservoir.

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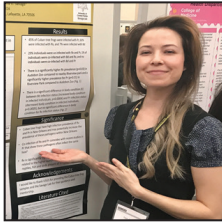
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Herpetological Conservation and Biology



NET GALT is a fourth-year Biology student working on her second Bachelor's degree in Psychology at the University of Central Florida, Orlando, Florida, USA. She has an appreciation for amphibian disease ecology, and a commitment to working as a Lab Research Assistant and in collecting samples during fieldwork. (Photographed by Sarah Ramirez).



MATTHEW S. ATKINSON is currently a Ph.D. candidate at the University of Central Florida Orlando, Florida, USA. He received his M.S. in Biology at Western Carolina University, Cullowhee, North Carolina, USA, and a B.S. in Wildlife Management at the University of Georgia, Athens, Georgia, USA. His primary research interests include amphibian disease, with a particular focus on the protist parasite *Perkinsea*, immunogenetics, reptile and amphibian conservation biology, and invasion ecology. (Photographed by Dustin Balschi).



BRAD M. GLORIOSO is an Ecologist with the U.S. Geological Survey at the Wetland and Aquatic Research Center in Lafayette, Louisiana, USA. He earned his B.S. from Southeastern Louisiana University, Hammond, Louisiana, USA, and his M.S. from Middle Tennessee State University, Murfreesboro, Tennessee, USA, where his thesis focused on population ecology and feeding activity in Stinkpots (*Sternotherus odoratus*). While freshwater turtles remain a passion and research area, much of his current work focuses on amphibians as part of the U.S. Geological Survey Amphibian Research and Monitoring Initiative (ARMI). (Photographed by C.J. Hillard).



HARDIN WADDLE is a Research Ecologist at the U.S. Geological Survey Wetland and Aquatic Research Center, Hammond, Louisiana, USA, and regional Principal Investigator for the U.S. Geological Survey Amphibian Research and Monitoring Initiative (ARMI). He holds a B.S. degree from Auburn University, Auburn, Alabama, USA, a M.S. degree from Florida International University, Miami, Florida, USA, and a Ph.D. from the University of Florida, Gainesville, Florida, USA. His research focuses on population ecology of amphibians and reptiles. (Photographed by Brad Gloriosos).



MELANIE LITTON is the Assistant Curator of Herpetology at Audubon Zoo, New Orleans, Louisiana, USA, where she has been since 2006. Prior to working at Audubon, she was the senior Aquarist and Herpetologist at the National Aquarium in Washington, D.C., USA, and a keeper in the Smithsonian National Zoological Park Department of Herpetology. Melanie has been actively involved in conservation efforts for the critically endangered Jamaican Iguana (*Cyclura collei*) since 2014 and is the World Association for Zoos and Aquariums (WAZA) international studbook keeper for the critically endangered Chinese Alligator (*Alligator sinense*). Among her many interests, she has an affinity for crocodilians and monitor lizards. (Photographed by Tandora Grant).



ANNA SAVAGE is an Associate Professor in the Biology Department at the University of Central Florida, Orlando, Florida, USA. She received her Ph.D. in Ecology and Evolutionary Biology at Cornell University, Ithaca, New York, USA. Her research program investigates the genetic and ecological drivers of emerging infectious diseases in amphibians and reptiles, with a focus on the amphibian fungal disease chytridiomycosis. (Photographed by Renee Ciulla).