DOI: 10.1111/1365-2664.13906

RESEARCH ARTICLE

Parasite spillover to native hosts from more tolerant, supershedding invasive hosts: Implications for management

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Funding information

US National Institutes of Health, Grant/ Award Number: R01TW010286-01; NSF, Grant/Award Number: DEB-2017785 and IOS 1754868

Handling Editor: Haimish McCallum

Abstract

- Introduced hosts are capable of introducing parasite species and altering the abundance of parasites that are already present in native hosts, but few studies have compared the tolerances of native and invasive hosts to introduced parasites or identified the traits of introduced hosts that make them supershedders of nonnative parasites.
- 2. Here, we compare the effects of a nematode *Aplectana hamatospicula* that is native to Cuba but appears to be introduced to Florida on the native Floridian treefrog, *Hyla femoralis*, and on the Cuban treefrog (CTF), *Osteopilus septentrionalis*. We were particularly interested in CTFs because their introduction to Florida has led to reported declines of native treefrogs.
- 3. In the laboratory, infection with *A. hamatospicula* caused a greater loss in body mass of *H. femoralis* than CTFs despite *H. femoralis* shedding fewer total worms in their faeces than CTFs. Field collections of CTFs, *H. femoralis*, and another native Floridian treefrog, *H. squirella* (Squirrel treefrog) from Tampa, FL also showed that CTFs shed more larval worms in their faeces than both native frogs when controlling for body size. Hence, the non-native CTF is a supershedder of this non-native parasite that is spilling over to less tolerant native treefrogs.
- 4. Any conservation intervention to reduce the effects of CTFs on native treefrogs would benefit from knowing the traits that contribute to the invasive host being a supershedder of this parasite. Hence, we conducted necropsies on 330 CTFs to determine how host sex and body size affect the abundance of *A. hamatospicula*, and two other common parasites in this species (acuariid nematodes and trematode metacercariae).
- 5. There was a significant linear increase in *A. hamatospicula* and encysted acuariids with CTF body size, but there was no detectable relationship between host body size and the intensity of metacercariae. Female CTFs were bigger, lived longer and, on average, had more *A. hamatospicula* than male CTFs.
- 6. Synthesis and applications. These results of the study suggest that there is parasite spillover from the invasive Cuban treefrog (CTF) to native treefrogs in Florida.

Additionally, at least some of the adverse effects of CTFs on native treefrogs could be caused by the introduction and amplification of this introduced parasite, and female and larger CTFs seem to be amplifying these infections more than males and smaller CTFs, respectively, suggesting that management could benefit from targeting these individuals.

KEYWORDS

age-intensity relationship, amphibian, disease, enemy release, helminth

1 | INTRODUCTION

Introduced hosts can disrupt host-parasite relationships of native species by either reducing or amplifying disease risk (Kelly et al., 2009; Miura et al., 2006; Peeler et al., 2011; Telfer & Bown, 2012; Young et al., 2017). For example, 'parasite spillover' can occur if introduced hosts introduce new parasites to native host species (Power & Mitchell, 2004). This can be particularly problematic to native hosts if they lack sufficient defences against these new infections or if introduced hosts amplify existing parasites (Archdeacon et al., 2010; Bar-David et al., 2006; Hershberger et al., 2010; Samuel et al., 2011). For example, in Europe native aquatic wildlife species, ranging from invertebrates, such as crayfish and mollusk species, to aquatic vertebrates, such as fishes and amphibians, have experienced population declines because of parasite spillover from introduced hosts (Peeler et al., 2011; Tompkins et al., 2003).

In contrast to amplifying infections for native hosts, introduced hosts can also reduce disease risk for native hosts. For example, introduced hosts may acquire but not transmit native parasites, thereby effectively removing them from the environment and decreasing the risk to natives by functioning as a parasite sink, a phenomenon which is often referred to as the dilution effect (Civitello et al., 2015; Lymbery et al., 2014; Paterson et al., 2011; Poulin et al., 2011; Rohr et al., 2020). Gendron and Marcogliese (2016) found that an introduced goby, *Neogobius melanostomus*, could potentially decrease an acanthocephalan, *Neoechinorhynchus tenellus*, because the parasite infects the goby but then dies prematurely in contrast to the native species of fishes that facilitate life-cycle completion (as paratenic hosts).

Whether an introduced host amplifies or dilutes disease risk for native hosts depends on the defences of introduced hosts relative to native hosts and the evolutionary history between the parasite and the hosts. Hosts can defend themselves against parasitism through resistance, (reducing parasite abundance) or tolerance (reducing damage caused by parasites at a given abundance; Raberg et al., 2009; Rohr et al., 2010). Hence, the degree of resistance and tolerance of introduced hosts relative to native hosts will help determine whether introduced hosts amplify or dilute disease risk in native hosts. Additionally, it is well documented that parasites often evolve to better infect local hosts (Lively, 2010; Lively & Dybdahl, 2000). Hosts can counter this evolution with either resistance or tolerance mechanisms, but because resistance mechanisms are detrimental to the parasite, resistance creates an evolutionary arms race that slower-evolving hosts struggle to win (Best et al., 2008). Thus, hostparasite coevolution is believed to eventually promote tolerance (Best et al., 2008).

Given this background, host-parasite coevolutionary theory suggests three main, potential outcomes when a shared parasite is introduced with an introduced host. First, the introduced shared parasite should have higher abundance in the introduced host than the native host because of the coevolutionary history (naïve host syndrome; Mastitsky et al., 2010), and thus, because of this lower resistance (or greater infectivity), the introduced host can amplify disease risk for the native host. The second outcome is that coevolutionary history selects for tolerance rather than resistance of the introduced host to the shared introduced parasite, resulting in the introduced host amplifying disease risk for native hosts. Finally, if the introduced host is able to evolve more resistance to the shared parasite than native hosts, then the introduced host could dilute disease risk for the native hosts. Coevolutionary theory has separate hypothesized outcomes for when introduced hosts become suitable hosts for parasites already common to native host species (Hudson et al., 2006; Kelly et al., 2009; Paterson et al., 2011; Young et al., 2017), but they are, for the most part, beyond the scope of this study. Given that many of the parasite-mediated effects of introduced species on native species are adverse to native hosts, there is an extensive interest in mitigating the effects invasive parasites play in facilitating host invasions and native host population declines (Dunn, 2009; Dunn & Hatcher, 2015; Lymbery et al., 2014; Sheath et al., 2015; Young et al., 2017). Thus, efforts to protect native host populations should focus on the first two outcomes above because they are most detrimental to these populations.

Importantly, any efforts to protect native host populations could be refined by understanding individual-level variation in infection intensity and shedding rates within introduced host populations. For example, for populations of introduced hosts that either become suitable hosts for native parasites or serve to introduce new parasites, exploring the relationship between the ages or sizes of hosts and their parasite infection intensities (age-intensity or sizeintensity relationship) could help to identify which introduced hosts pose the greatest risk to native hosts. Many age-intensity and sizeintensity relationships increase monotonically, either increasing continuously with age or size or reaching some asymptote because parasite death, whether natural or immune-induced, balances parasite colonization (Wilson et al., 2002; Figure 1). In these cases, the oldest or largest introduced hosts would have the most parasites and thus would represent the greatest risk of transmitting or amplifying native or introduced parasites in native hosts. In this case, targeting management efforts at the oldest or largest hosts would provide the greatest benefit to native host populations. In contrast, if hosts acguire the majority of a parasite species as juveniles but then are minimally re-infected as adults, we might expect to see a monotonically negative age-intensity relationship as parasites either die naturally or are killed by the immune system of the host (Figure 1). In this case, the youngest or smallest introduced hosts would have the most parasites and thus would represent the greatest risk of transmitting or amplifying native or introduced parasites in native hosts. In some cases, age-intensity relationships are convex, peaking at intermediate ages (Anderson & Gordon, 1982; Knudsen et al., 2002) because of acquired immunity or parasite-induced mortality in older hosts (Knudsen et al., 2002; Raffel et al., 2009, 2011; Figure 1). In this case, intermediate-aged introduced hosts might pose the greatest threat to native hosts.

The Cuban treefrog (CTF), Osteopilus septentrionalis, is an ideal host for evaluating the effects of parasites that are associated with spillover, spillback (when introduced hosts become suitable hosts for parasites already common to native host species and can then magnify the abundances of native parasites; Hudson et al., 2006; Kelly et al., 2009; Paterson et al., 2011; Young



FIGURE 1 Mean (\pm 1 *SE*) proportional mass change between week 1 and week 6 for *Aplectana hamatospicula*-infected and control individuals (*N* = 60 surviving frogs). Native frogs are more adversely impacted on average because all treatment groups gained mass except for infected native Pinewoods treefrog, *Hyla femoralis* when controlling for host initial mass. See Table S1 for statistical summary

et al., 2017) or dilution, and also for addressing size-intensity relationships. First, it is a highly invasive amphibian species in Florida that is extremely abundant and easily captured (Campbell et al., 2010; Johnson, 2010), and is already negatively impacting native treefrogs at least partly because of its large body size, broad dietary niche and extended reproductive period (Meshaka, 1996, 2001; Roznik et al., 2020). Additionally, CTFs have acquired parasites from native frogs and may be introducing parasites to native frogs (Ortega et al., 2015). Moreover, CTFs have an aquatic and terrestrial life stage, which allows us to address how their parasite loads change as a function of age for infections primarily acquired early in life as aquatic tadpoles or later in life as predominantly terrestrial juveniles and adults. Finally, although the CTF is a wellestablished invasive species throughout nearly the entire state of Florida, and native treefrog populations have been shown to increase in areas where CTFs have been removed (Rice et al., 2011). no studies have directly measured the effects of any introduced parasites of the CTF on native treefrogs.

Our first objective was to determine whether an introduced parasite of the CTF could effectively infect the native Pinewoods treefrog, Hyla femoralis (Ortega et al., 2015). To address this objective, we exposed both laboratory-reared CTFs and H. femoralis to Aplectana hamatospicula, a nematode that has likely been introduced by CTFs, and determined infection success and growth rates of each frog species. We hypothesized that A. hamatospicula would not be as infective to H. femoralis, a novel host, as CTFs, a host with which it has coevolved (naïve host syndrome; Mastitsky et al., 2010). Furthermore, we hypothesized that any H. femoralis that do become infected might experience greater pathology and lower growth rates because of this lack of coevolution with A. hamatospicula (Lymbery et al., 2014; Mastitsky et al., 2010; Vilcinskas, 2015). In a series of studies, Pizzatto and colleagues demonstrated that non-native nematodes infected native Australian amphibians in the laboratory but did not infect native amphibians in the wild (Pizzatto et al., 2012; Pizzatto & Shine, 2011a, 2011b). Thus, we then collected adult CTFs and native Floridian treefrogs to test for spillover and supershedding of this parasite in the field.

If this non-native parasite of the non-native CTF is indeed detrimental to native treefrogs, then any conservation intervention to reduce the effects of the CTF on native treefrogs would benefit from knowing the traits of the invasive host that produce supershedders of this parasite (Martin et al., 2010, 2019). Hence, our second objective was to offer insights into the traits of CTFs that might predict their potential for parasite spillover and amplification by quantifying the relationship between the abundance of their parasitic infections (native and introduced) and the size of both male and female CTFs. To address this second objective, we determined the best-fitting model of parasite abundance as a function of size for both sexes of CTFs. These analyses were conducted on the most common parasites recovered from CTFs: A. hamatospicula (gut nematode; likely introduced from Cuba), acuariids (encysted nematodes in gut wall; native to FL) and trematode metacercariae (encysted in mesenteries and body cavity; native to FL).

2 | MATERIALS AND METHODS

2.1 | Study system and hypotheses

2.1.1 | Life cycles

For A. hamatospicula, amphibians serve as the only host in the nematode's direct life cycle (Knutie et al., 2017; Roznik et al., 2020). Gravid, adult female worms live in the intestinal tract of the CTF and release juveniles or eggs (viviparous or ovoviviparous) in the faeces of infected frogs. These juveniles penetrate the skin of the same or another frog to complete the life cycle (Knutie et al., 2017; Roznik et al., 2020). We know of only one study that has documented A. hamatospicula in a native frog species in the United States. Vhora and Bolek (2013) provided evidence of A. hamatospicula infecting a native Microhylidae (Gastrophryne olivacea) in Oklahoma, where prevalence and mean intensity were 85% and 33.1 worms, respectively (see Supplement for documentation of all the field evidence of A. hamatospicula infecting wild amphibians in the Caribbean and Americas, see also Walton, 1940 for a suspect positive in the United States). Otherwise, this parasite is only documented in CTFs in the United States and in various amphibian species in Cuba. Hence, CTFs have the potential to introduce a new parasite to treefrogs native to Florida.

For acuariids, the CTF is a paratenic host. Acuariids are nematodes that require a bird as a final host with arthropods serving as intermediate hosts, but sometimes they will infect paratenic hosts, such as fishes or amphibians that have ingested the infected arthropod (White et al., 2016). Thus, CTFs acquire larval acuariids in the gastrointestinal tract when consuming another infected host, but the parasite does not develop in amphibians. The degree to which CTFs acquire acuariids and are consumed by bird definitive hosts relative to native frogs will dictate whether CTFs amplify or dilute these infections for native hosts.

Finally, CTFs are intermediate hosts for trematode metacercariae. Metacercariae are primarily acquired during the aquatic stage, where the tadpoles are exposed to cercariae, a larval swimming stage of the trematode life cycle. Cercariae typically infect amphibians by penetrating their skin or entering their cloaca. Metacercariae are common in both CTFs and native frog species.

2.1.2 | Size-intensity hypotheses

Based on host traits, we can make general predictions about how size and sex of CTFs should affect the parasite abundance of each of the three focal parasite species. First, we hypothesized that older, larger frogs would have the largest abundance of both *A. hamatospicula* and acuariids because the predominantly terrestrial CTFs have accumulated exposure to these predominantly terrestrial parasites throughout their life span, which can be 2–5 years (Meshaka, 2001; Roznik et al., 2020). Furthermore, CTFs are sexually dimorphic, with females often being much larger than males (Meshaka, 2001; Roznik et al., 2020). Thus, females have more surface area to contact larval A. *hamatospicula* and need to eat more than males to maintain their larger body size, increasing their likelihood of consuming acuariids. Consequently, we expect larger females to have the highest abundance and pose the biggest risk of transmitting *A. hamatospicula* and acuariids to natives.

Unlike A. *hamatospicula* and acuariids, trematode metacercariae are mostly obtained when hosts are young and exclusively aquatic. Thus, CTFs should have predominantly decreasing exposure to metacercariae with age/size post-metamorphosis. For female CTFs, we hypothesize that the encysted metacercariae would either die naturally or be cleared as the host ages (LaFonte & Johnson, 2013), producing a negative linear age-intensity relationship. Relative to females, males spend more time in the water during the breeding season in an effort to maximize mating opportunities (Wells, 2007). Hence, males should acquire more of these aquatic infections as adults than females and should therefore have a less negative sizeintensity relationship for this parasite.

2.2 | Experimental infections

2.2.1 | Host and parasite collections

Cuban treefrogs, Osteopilus septentrionalis, and Pinewoods treefrogs, Hyla femoralis, were collected as tadpoles in July of 2015 from Flatwoods Conservation Park in Tampa, FL (28°07'01.08"N 82°18'11.15"W). Pinewoods treefrogs were selected as our focal native treefrog because they are the most abundant native treefrog in the park and adults regularly inhabit the same polyvinyl chloride (PVC) collecting pipes as CTFs. Tadpoles were separated by species and reared outdoors in the shade in plastic boxes $(33 \text{ cm} \times 203 \text{ cm} \times 15 \text{ cm})$ containing roughly 8 L of artificial spring water with approximately 10 tadpoles per box. Tadpoles were fed ad libitum on a spirulina-agar diet. After metamorphosis, the juvenile frogs were housed in the laboratory (22°C, 12 hr light-dark photoperiod) in individual plastic containers along with a wet, unbleached paper towel for bedding. All frogs received a new, clean container with bedding on a weekly basis. These juvenile frogs were fed ad libitum with vitamin-dusted crickets until all frogs reached a minimum snout-vent length (SVL) of 20 mm. To ensure that none of the juvenile frogs had been previously infected as tadpoles with nematodes in the wild, two weekly faecal checks for juvenile nematodes were conducted prior to experimental exposure. Faecal checks were performed by rinsing individual frog containers with approximately 2 ml of 0.7% saline water and then observing this rinse water and any faeces under a dissecting microscope to check for the presence of juvenile nematodes.

Two weeks before nematode exposures, adult CTFs were collected from Flatwoods Wilderness Park, euthanized with tricane methanesulfonate (MS-222), and necropsied to obtain adult *A. hamatospicula*. Once the gravid, adult female *A. hamatospicula* nematodes were isolated from the CTF intestinal tract, the worms were then individually transferred to petri dishes ($3.53 \text{ cm} \times 1 \text{ cm}$) along with approximately 2 ml 0.7% saline water where they shed juvenile worms. Juvenile worms were allowed to grow for approximately 2 weeks to ensure that they had reached their infective, skinpenetrating stage before exposing them to the juvenile frogs.

2.2.2 | Experimental design

For the nematode exposures, each frog was first placed in a single petri dish (3.53 cm \times 1 cm) that was capped and then sealed with parafilm (n = 20 worm-exposed frogs and n = 20 sham-exposed frogs per species). Next, 20 juvenile A. hamatospicula were counted with a dissecting microscope and then transferred to each worm-exposed frog (along with approximately 1 ml of 0.7% saline water) through one large, premade hole in the petri dish lid. For sham-exposed frogs, approximately 1 ml of the same nematode water solution (but without nematodes) was pipetted through the hole into the petri dish. Each large hole was then sealed with tape to reduce evaporation of the water, but a smaller, premade hole in the lid was left uncovered to allow for airflow. These petri dishes containing the frogs were then placed in a dark cabinet to minimize stress to the frog and left for 24 hr. After the 24-hr exposure time, frogs were removed from the petri dishes and placed back into their original individual plastic housing containers. The remaining juvenile A. hamatospicula nematodes in each petri dish that were unable to successfully penetrate the frog were counted with a dissecting microscope. All frogs were kept at the same 22°C, 12 hr light-dark photoperiod throughout the remainder of the experiment.

Before exposure to A. hamatospicula and each week thereafter, frog snout-vent length (estimate of frog length; SVL; to the nearest 0.1 mm) and mass (to the nearest 0.001 g) were recorded once per week for 6 weeks. Additionally, successful infections of established adult A. hamatospicula nematodes in the gut were confirmed by performing faecal checks as described above. To isolate the effect of A. hamatospicula on frog growth, each frog was fed a restricted diet of three vitamin-dusted crickets 2 days per week to ensure that frogs could not compensate for resources lost to parasitism by consuming crickets ad libitum.

2.3 | Test of spillover and supershedding in the field

We requested permission to collect adult CTFs and native treefrogs from Flatwoods Conservation Park to necropsy them for *A. hamatospicula* infections. Unfortunately, because CTFs have caused declines in native treefrogs since they arrived at the park, park managers did not allow us to euthanize any native treefrogs. We did receive permission to collect adult Pinewoods (*H. femoralis*) and Squirrel treefrogs (*H. squirella*), as well as adult CTFs, for the purposes of getting faecal samples from them in the laboratory, after which we returned them to their collection locations. We collected 52 CTFs from six wetlands and collected 30 adult Pinewoods and 30 adult Squirrel treefrogs, each from three wetlands of the six from which we sampled CTFs. These frogs were immediately brought back to the laboratory. Their mass and SVL were obtained and then they were placed individually into 1-L plastic containers and fed crickets ad libitum until they defecated. Fresh faecal samples were placed under a dissecting microscope and the number of live nematode larvae were recorded. Obtaining fresh faecal samples allowed us to discriminate between ovoviviparous and oviparous nematode species, the former of which will have moving larvae in a fresh faecal sample, whereas the latter will only have ova.

Although it is not possible to morphologically identify larval nematodes to species and we could not molecularly identify A. *hamatopsicula* because it is not in Genbank, it is likely that most larval nematodes that we observed in faecal samples are A. *hamatospicula*. Of the 330 CTFs we necropsied from this park, we counted 4,100 adult ovoviviparous parasitic nematodes; of these, 4,096 or 99.9% were A. *hamatospicula*, whereas 4 or 0.1% were *Rhabdias* sp. Thus, by examining faecal samples soon after they were deposited, it seems likely that we are predominantly estimating numbers of A. *hamatospicula* larvae. However, we cannot be certain that the percentages of ovoviviparous worms that are A. *hamatospicula* are similar in CTFs and native treefrogs.

2.4 | Age-intensity relationships for CTFs and A. hamatospicula, acuariid and metacercariae

2.4.1 | Host and parasite collections

During the summers of 2005-2008, 330 CTFs were collected from PVC pipes encircling 18 wetlands within the Flatwoods Wilderness Park in northeastern Hillsborough County, Florida (28°07'01.08"N, 82°18'11.15"W). Within this park, the plant community is mainly a second-growth pine flatwoods forest matrix with numerous borrow pits, hardwood swamps, freshwater marshes and cypress domes (Campbell et al., 2010; Guzy et al., 2006). PVC pipes (diameter: 3.8 cm, length: 1 m) were placed equidistant at all wetlands so that the number of pipes at each wetland was proportional to its perimeter. All 18 wetlands were checked an equal number of times throughout the year and the pipes in all 18 wetlands were always checked within 1 week of one another. After removing frogs from the PVC pipes, the date, wetland and pipe location were recorded, and each frog was placed in a plastic bag, euthanized with MS-222 and kept frozen until necropsied for parasite quantification. Frog SVL and wet weight were recorded. For frogs ≥42 mm in SVL, sex was determined by the presence/absence of nuptial pads and evidence of mature reproductive organs. All frogs ≤41 mm were considered juveniles due to a lack of discernible reproductive organs. The body cavity was opened by a longitudinal incision from vent to throat and all internal body organs and mesenteries were examined for A. hamatospicula, acuariids and trematode metacercariae. Parasites were counted and preserved in 70% ethanol. Identification and confirmation of parasites were provided by Charles Bursey at Pennsylvania State University's Shenango Campus and by Dr. Omar M. Amin at Parasitology Center, Inc. (PCI) in Scottsdale, Arizona.

2.5 | Statistical analyses

2.5.1 | Experimental infections

All statistical analyses were conducted with R statistical software (R Development Core Team, 2016). To test pre-infection resistance, the proportion of larvae that successfully penetrated each frog was the binomial response variable, species (CTF versus H. femoralis) was the independent variable, and initial frog size was the covariate. To test for species variation in post-infection resistance, total worms shed by each frog was the negative binomial response variable, species (CTF versus H. femoralis) and week of the experiment were the crossed independent variables, initial frog mass was the covariate, and individual frogs were treated as random intercepts. To assess the consequences of infection on native and invasive hosts, we used a linear model to determine the effect of treatment (control versus infected), species (CTF versus H. femoralis) and their interaction on proportional mass change while controlling for the initial mass of each individual. To test the hypothesis that the native and invasive species differ in their tolerance of this non-native parasite, we used a linear model to quantify how proportional host mass change between week 1 and week 6 was affected by the interactions among log total worms shed per infected individual (a proxy for intensity of infection), host species and initial mass. Thus, our test of tolerance uses the reaction norm approach recommended by several researchers (Raberg et al., 2009; Rohr et al., 2010). Initial mass of the frogs was included in all models because bigger frogs might be able to support larger worms that could then shed more larvae. We used the ANOVA function in the CAR PACKAGE IN R to generate probability values via log-likelihood ratio tests.

2.5.2 | Test of spillover and supershedding in the field

We conducted a mixed-effects generalized linear model with number of larval worms in a faecal sample as the zero-inflated negative binomial response variable (because it had a lower AICc than a negative binomial, zero-inflated Poisson and Poisson models), species of treefrog host as the categorical independent variable, host mass as the continuous covariate and wetland as the random effect. We used the ANOVA function in the CAR package in R to generate probability values via log-likelihood ratio tests.

2.5.3 | Age-intensity relationships for CTFs and *A. hamatospicula*, acuariid and metacercariae

To address our second objective of quantifying the relationship between the intensity of parasitic infections (response variable) and host size (SVL), controlling for year of collection for both male and female CTFs (explanatory variables treated as fixed effects), we used model selection to compare among several plausible models (Table S4). To best account for the overdispersion of parasite counts in the hosts, our models included the comparison of negative binomial and Poisson error distributions. To facilitate identifying nonlinear relationships that might exist between predictors and parasite intensity, we considered generalized additive models (GAMs) using the gam function the MGCV package of R (with the default smoothing spline; Zuur et al., 2009). With the MGCV package, a term called the effect degrees of freedom (edf) was used to assess how linear or nonlinear the models were. These values range between 0 and infinity, and lower values indicate that the relationship is more linear. Finally, because CTFs were captured across multiple wetlands, we also considered models that treated wetland as a random effect (generalized additive mixed models, GAMMs). CTF infection intensity was modelled independently for each parasite (A. hamatospicula, metacercariae and acuariids) and separately for each sex of the CTF because of the considerable size differences between the sexes. Juvenile frogs were used to model both the male and female populations because juveniles are not sexually dimorphic and because, in their absence, we lose important content on the accumulation of parasites early in life.

To determine the best fitting body size-intensity model for each parasite (A. *hamatospicula*, metacercariae and acuariid) and each host sex, we compared among models by comparing their AIC values (Zuur et al., 2009). AIC considers a trade-off between the goodness of fit and complexity of a statistical model. Model assumptions were confirmed by visually inspecting plots of the fitted values versus the residuals (Zuur et al., 2009).

3 | RESULTS

3.1 | Experimental infections

The average (\pm SE) initial mass for CTFs and *H. femoralis* was 0.709 \pm 0.040 g and 0.547 \pm 0.038 g, respectively. Although there was a positive relationships between body size and the proportion of *A. hamatospicula* larvae that successfully penetrated frogs ($\chi^2 = 4.44$, p = 0.035, slope coefficient \pm SE: 0.0951/mm \pm 0.0453), there was no significant difference in the proportion of larvae that penetrated CTFs and *H. femoralis* (marginal means, upper 95% CI, lower 95% CI: 0.556, 0.495, 0.615; 0.513, 0.453, 0.573, respectively) when controlling for body size ($\chi^2 = 0.72$, p = 0.396). A total of 13 CTFs (65%) and 9 *H. femoralis* (45%) released larval worms in their faeces and thus became infected with *A. hamatospicula*, consistent with CTFs being the more competent host; however, these prevalence were not significantly different ($\chi^2 = 1.62$, p = 0.20). Given that 9 *H. femoralis* were infected, *A. hamatospicula* can indeed spillover from CTF to the native *H. femoralis* under laboratory settings.

There was a significant effect of treatment on change in proportional mass overall ($F_{1,55} = 3.83$, p = 0.028, Table S1); both the control and infected CTFs and the control *H. femoralis* tended to gain mass throughout the experiment, but the infected *H. femoralis* lost mass (Figure 1). Infected *H. femoralis* lost mass despite being more resistant as they shed less than half the number of worms throughout the experiment (mean \pm SE: 77.12 \pm 31.21) as CTFs (mean \pm SE: 171.5 \pm 19.74; df = 20, t = 2.686, p = 0.001; Table S2, Figure 2). Although all very small *H. femoralis* and small CTFs (<0.65 g) had low tolerance to *A. hamatospicula* infections (i.e. negative slopes between worms abundance and host mass change), larger CTFs (>0.70 g) were significantly more tolerant of infections than similar-sized *H. femoralis* (Species × worms shed × initial mass: $F_{1,14} = 12.07$, p = 0.004; Table S3, Figure 3). In fact, these larger CTFs showed no evidence of mass loss with increasing *A. hamatospicula* infections, whereas the similar-sized *H. femoralis* did (Figure 3). The transition from a negative to a neutral slope in mass change occurred between 0.6 and 0.7 g, suggesting that CTFs >0.7 g might be the most tolerant treefrogs.

3.2 | Test of spillover and supershedding in the field

There was a significant effect of treefrog host species on the number of ovoviviparous nematode larvae in faecal samples (Species: $\chi_2^2 = 10.73$, p = 0.005) when controlling for host mass (Mass: $\chi_1^2 = 2.04$, p = 0.154). CTFs released more than two times as many ovoviviparous nematode larvae as Pinewoods treefrogs ($\chi_1^2 = 3.48$, p = 0.03) and more than three times as many as Squirrel treefrogs ($\chi_1^2 = 11.16$, p < 0.001; Figure 4).



FIGURE 2 Mean (±1 SE) weekly faecal Aplectana hamatospicula worm counts for infected Cuban and Pinewoods treefrogs. Once A. hamatospicula began producing eggs/larvae, Cuban treefrogs consistently shed more worms each week (when controlling for host initial mass) with the exception of week 3, suggesting that Cuban treefrogs are less resistant or more competent hosts than Pinewoods treefrogs. The peak in shedding for the Pinewoods treefrogs in week 3 was associated with one individual that shed >200 larvae in that week, but shed fewer and fewer larvae in each subsequent week and eventually shed no larvae by the end of the experiment. Asterisks represent a significant difference in worms shed between Cuban and Pinewoods treefrogs. See Table S2 for statistical summary

3.3 | Size-intensity relationships for CTFs and A. *hamatospicula*, acuariid and metacercariae

Of the 330 CTFs necropsied for this study, 50% were infected with A. hamatospicula (mean intensity \pm SE: 24.82 \pm 5.2, maximum: 150), 36% were infected with metacercariae (mean intensity \pm SE: 5.20 ± 0.71 , maximum: 52) and 32% were infected with acuariid larvae (mean intensity \pm SE: 2.49 \pm 0.07, maximum: 105). For all parasite species and each host sex, a negative binomial error distribution fit the size-intensity data better than Poisson, zero-inflated Poisson or zero-inflated negative binomial distributions (Tables S4 and S5). For both males and females (Figure 5), plots of the best fitting models against the partial residuals showed that the relationships for all parasite intensities and host size were monotonic as the GAM edfs were all close to one (Table S6). Host size was a significant positive predictor for both A. hamatospicula and acuariid intensities in both males (Figure 5a,b) and females (Figure 5d,e), but there was no significant relationship between metacercarial intensity and size for males (Figure 5c) or females (Table S6; Figure 5f). The slope between A. hamatospicula and CTF body size increases sharply above ~60 mm for female CTFs and above ~40 mm for male CTFs (Figure 5), but because females had significantly more A. hamatospicula than males (p < 0.05; Figure 5), targeting the removal of females above 60 mm would be most beneficial at reducing risk to native frogs.

4 | DISCUSSION

Several studies show that introduced parasites can have negative effects on native populations (Archdeacon et al., 2010; Bar-David et al., 2006; Hershberger et al., 2010; Samuel et al., 2011). Although the CTF is a well-established invasive species throughout nearly the entire state of Florida, and native treefrog populations have been shown to increase in areas where CTFs have been removed (Rice et al., 2011), no studies have directly measured the effects of any introduced parasites of the CTF on native treefrogs. Here we show that large infected CTFs do not experience a significant loss in body mass when infected with A. hamatospicula nematodes, yet they shed more juvenile worms than the native H. femoralis, which is significantly less tolerant of these infections than CTFs. Moreover, fieldcollected CTFs shed more live ovoviviparous nematode larvae than native H. femoralis and H. squirella collected from the field. These laboratory and field patterns suggest that CTFs are a non-native amphibian host that is a supershedder of a non-native nematode parasite that is spilling over to less tolerant native treefrogs.

Aplectana hamatospicula is likely an introduced parasite of the CTF (Ortega et al., 2015), and their shared evolutionary history might explain why infected CTFs shed more worms and experience less pathology (weight loss) than native treefrogs. When hosts are investing in costly immune functions (e.g. inflammation) to resist pathogens, the host may have fewer resources to devote towards growth and/ or reproduction (Kutzer & Armitage, 2016; Rigby et al., 2002; Sears et al., 2011). Consequently, it could be beneficial for hosts to mount



FIGURE 3 Proportional mass change for infected Cuban and Pinewood treefrogs as a function of the number of *Aplectana hamatospicula* worms shed and host initial mass. For small and large treefrogs, the model was conditioned at a mass of 0.63 g and 0.70 g, respectively. All small Pinewood and Cuban treefrogs (<0.64 g) had low tolerance to *A. hamatospicula* infections, represented by the negative slopes between worm abundance and host mass change, but larger Cuban treefrogs (\geq 0.70 g) were significantly more tolerant of *A. hamatospicula* infections (neutral slope) than similar-sized Pinewoods treefrogs (negative slope). Grey bands are 95% confidence bands. See Table S3 for statistical summary

less of this type of resistance response if, for example, increased tolerance (i.e. less demand of energetic resources) towards the infection improves host fitness in the presence of this parasite (Kutzer & Armitage, 2016; Rohr et al., 2010). Hosts that do not share an evolutionary history with a pathogen, such as native treefrogs and A. *hamatospicula*, might suffer increased pathology due to this lack of evolved tolerance (Lymbery et al., 2014; Mastitsky et al., 2010; Rohr et al., 2010; Vilcinskas, 2015). In this case, it is likely that *H. femoralis* may have invested in resistance to *A. hamatospicula*, and the use of resources to fuel such an investment may explain why infected *H. femoralis* were unable to invest in growth (McDade et al., 2016; Tschirren & Richner, 2006).

In addition to showing no significant decline in mass of large CTFs, our data also show that the CTFs are capable of shedding significantly more worms, and thus likely harbour a greater number of adult nematodes or larger adult nematodes in the intestinal tract



FIGURE 4 Mean predicted (±95% confidence intervals) ovoviviparous nematode larvae in the faeces of field-collected Cuban, Pinewoods and Squirrel treefrogs. Species that do not share lower case letters shed significantly different numbers of larvae in their faeces. Cuban treefrogs shed more ovoviviparous nematode larvae, which are likely *Aplectana hamatospicula*, than the two native treefrogs

than native treefrogs. Thus, CTFs have the potential to amplify these worms in the environment relative to *H. femoralis*. Given that *A. hamatospicula* is one of the most abundant nematodes in the CTF at our study site (Ortega et al., 2015; Roznik et al., 2020) and *H. femoralis* and *H. squirella* were infected at this same site, it is possible that the negative effects of *A. hamatospicula* are being experienced by several species of native treefrogs. This contributes to the growing body of literature that shows that populations of native hosts can be altered by introduced parasites (Archdeacon et al., 2010; Bar-David et al., 2006; Hershberger et al., 2010; Peeler et al., 2011; Samuel et al., 2011; Young et al., 2017).

In addition to exploring the potential effect that introduced parasites might have on native populations, it is also beneficial to understand how host traits influence the spread or acquisition of parasites, regardless of whether they are introduced or not (Ezenwa et al., 2016; Izhar & Ben-Ami, 2015; Lloyd-Smith et al., 2005; Martin et al., 2019, 2010; Viljoen et al., 2011). Deciphering how traits influence parasite dynamics can be particularly useful for exploring how a species may be contributing to parasite spillover, spillback and dilution effects (Raffel et al., 2010; Rohr et al., 2015; Sears et al., 2015; Venesky et al., 2014). By understanding these processes, we can better predict how CTFs directly or indirectly change parasite abundance within native treefrogs. Here, we show that older/larger CTFs continue to gain *A. hamatospicula* and acuariids, but that there is no relationship between metacercariae loads and CTF age/size.

As CTFs get older and larger, they harbour more A. *hamatospicula*. This larger host size increases surface area for worms to penetrate, FIGURE 5 Plots of partial residuals and 95% confidence bands for the GAM bestfit models of parasite intensity against host snout-vent length (proxy for age) for male Cuban treefrogs infected with (a) Aplectana hamatospicula (model I), (b) acuariids (model II) and (c) metacercariae (model I) and for female Cuban treefrogs infected with (d) A. hamatospicula (model I), (e) acuariids (model II) and(f) metacercariae (model I). Snout-vent length is a significant predictor of parasite intensity for A. hamatospicula (Males: $\chi^2 = 43.38$, p < 0.001; Females: $\chi^2 = 44.01, p < 0.001$) and acuariids (Males: $\chi^2 = 18.16$, p < 0.001; Females: $\chi^2 = 13.22, p < 0.001$). See Tables S4–S6 for statistical summary



as demonstrated by the positive association between the proportion of worms successfully penetrating and frog body size, and provides more gut resources to support larger A. hamatospicula abundances within the intestinal tract because these nematodes feed on digested food. This might explain the greater shedding rates of CTFs than H. femoralis. Older hosts might also harbour more parasites due to accumulated exposures (cumulative hypothesis; Hawlena et al., 2005). Therefore, older CTFs should have a greater number of A. hamatospicula than younger CTFs, which is exactly what our data show. Because juvenile A. hamatospicula exit the CTF in the faeces, larger CTFs can increase the abundance of A. hamatospicula in the wild that can infect native frogs. Given that female CTFs are on average bigger than male CTFs and there is a positive size-intensity relationship for A. hamatospicula, female and older CTFs seem to be amplifying these infections more than males and younger CTFs, especially given that the tolerance of CTFs to A. hamatospicula infections increases with body size (Figure 3).

The increasing amounts of acuariids in older/larger hosts are likely a result of these hosts consuming a larger quantity of arthropods that are infected with acuariids. Because the acuariids are encysted in the CTF (a paratenic host), there is no chance of the parasite being transmitted back to the native treefrogs unless the CTF is consumed by a final host (bird), whereby the acuariid can complete its life cycle and then exit in the faeces of the birds (White et al., 2016). If birds preferentially consume larger CTFs, this invasive treefrog may then contribute to the spillback effect (Hudson et al., 2006; Kelly et al., 2009; Paterson et al., 2011; Young et al., 2017) because birds may become infected with a larger quantity of acuariids, thus eventually releasing more eggs into the environment. On the other hand, if birds selectively consume smaller CTFs, then the larger CTFs may be contributing to the dilution effect because they essentially become a sink for the acuariids, which removes them from the environment.

Metacercarial loads did not significantly change with CTF size. Although CTFs are likely to be predominantly infected as tadpoles, it is possible that these infections can be cleared as frogs mature. For example, bluegill sunfish infected with Ribeiroia ondatrae metacercariae showed a decline in their infection over the course of roughly 2 months, and these declines were correlated with circulating leukocytes and neutrophils (Calhoun et al., 2015). Given that metacercariae can be cleared, we should expect a decline in metacercariae with age of the CTF, but instead, our results show that there is no difference between the adult and juvenile metacercarial infections in CTFs. These infections in the adult frogs can be explained by the adult frogs returning to water bodies to breed, at which time they can become re-infected by cercariae. Studies have also shown that fishes infected with trematodes tend to be easier prey for fish-eating birds (the final host; Gopko et al., 2017; Lafferty & Morris, 1996). Additionally, Goodman and Johnson (2011) showed that metacercarial-induced morphological changes result in decreased survivorship for frogs because of increased predation. In our case, if the metacercarial-infected CTFs are preferentially consumed by predators, then more eggs may be passed from the final host into the environment whereby native hosts may eventually encounter more cercariae in the water. In such case, the CTF could amplify parasites of the native treefrogs. This increase in cercariae by CTFs would lead to a spillback effect of native trematodes.

5 | CONCLUSIONS

In summary, our data show that parasite spillover from the invasive CTF to native Floridian treefrogs is possible because A. *hamatospicula*, a non-native nematode common to CTFs, is capable of infecting native Floridian treefrogs in the laboratory and appears to be infecting native treefrogs in the field. Moreover, the native treefrog host is less tolerant of this introduced parasite than the invasive CTF (Figure 3) and CTFs shed more of these parasites than the native hosts. Thus, at least some of the adverse effects of the CTF on native treefrogs could be caused by the introduction and amplification of this introduced parasite. This is supported by recent evidence from a parasite removal study showing that A. *hamatospicula* reduces monthly survival rates of CTFs and native treefrogs by 2.2% (Roznik et al., 2020).

A study in southern Florida found that the abundance of native treefrogs increased after CTFs were removed (Rice et al., 2011) and an unpublished mark-recapture study at the same location as our study revealed that removing only CTFs for several years increased the population growth rates of native treefrogs (T. Campbell, unpublished data). Our findings suggest that focusing on removal of large CTFs would be a beneficial management approach, because these frogs, on average, have the most of this introduced parasite and are the most tolerant of these infections,. Larger CTFs also are more likely to outcompete and prey on smaller native treefrogs and are the most fecund. Importantly, large CTFs cannot fit in small PVC pipes. Thus, using PVC pipes that are of sufficient diameter to attract the largest CTFs for removal (3.81 cm or larger), or using a mix of sizes that would attract different-sized frogs, should more effectively benefit native treefrog populations than distributing smaller PVC pipes alone.

Our findings suggest that CTFs can amplify A. *hamatospicula* infections in native treefrogs. Future work could compare A. *hamatospicula* infections in native treefrog populations in the presence versus absence of CTFs, and investigate the impact of infection resistance and tolerance in other native species. Additionally, future work should test the efficacy of our management suggestions. By investigating the mechanisms by which invasive species can lead to declines in native populations while also discerning which host traits result in alterations to relationships between native hosts and their parasites, biologists should be able to better predict how new invasions may impact native populations that are already experiencing declines.

ACKNOWLEDGEMENTS

Frog collections were authorized by Florida Fish and Wildlife Conservation Commission Conditional/Prohibited Species Special Permit ESC 06-07, ESC 06-07A, ESC 07-02 and ESC 08-04, and by yearly SWFWMD Special Use Authorization Letters. Advice for modelling techniques and troubleshooting in R were provided by Philipp Boersch-Supan and Erin Feichtinger. Funds for this project were provided by grants from the NSF (DEB-2017785, IOS 1754868) and NIH (R01TW010286-01) to J.R.R. and by NSF to N.O.

AUTHORS' CONTRIBUTIONS

N.O. and J.R. developed the ideas, conducted the statistical analyses and wrote the manuscript; N.O. conducted the laboratory experiment and the frog necropsies; W.P. helped with parasite identification and T.C. provided the Cuban treefrogs for the necropsies; The study on worm shedding rates of field-collected frogs was conducted by E.A.R., K.L.S. and N.C. All authors contributed to the editing of the manuscript.

DATA AVAILABILITY STATEMENT

Data available via the FigShare Repository https://figshare.com/artic les/dataset/Data_for_Figshare_xlsx/14479692 (Ortega et al., 2021).

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