Vol. 118: 113–127, 2016
doi: 10.3354/dao02965

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published February 25

Disease dynamics of red-spotted newts and their anuran prey in a montane pond community

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ABSTRACT: Long-term monitoring of amphibians is needed to clarify population-level effects of ranaviruses (Rv) and the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*). We investigated disease dynamics of co-occurring amphibian species and potential demographic consequences of Rv and *Bd* infections at a montane site in the Southern Appalachians, Georgia, USA. Our 3-yr study was unique in combining disease surveillance with intensive population monitoring at a site where both pathogens are present. We detected sub-clinical *Bd* infections in larval and adult red-spotted newts *Notophthalmus viridescens viridescens*, but found no effect of *Bd* on body condition of adult newts. *Bd* infections also occurred in larvae of 5 anuran species that bred in our fishless study pond, and we detected co-infections with *Bd* and Rv in adult newts and larval green frogs *Lithobates clamitans*. However, all mortality and clinical signs in adult newts and larval anurans were most consistent with ranaviral disease, including a die-off of larval wood frogs *Lithobates sylvaticus* in small fish ponds located near our main study pond. During 2 yr of drift fence monitoring, we documented high juvenile production in newts, green frogs and American bullfrogs *L. catesbeianus*, but saw no evidence of juvenile recruitment in wood frogs. Larvae of this susceptible species may have suffered high mortality in the presence of both Rv and predators. Our findings were generally consistent with results of Rv-exposure experiments and support the purported role of red-spotted newts, green frogs, and American bullfrogs as common reservoirs for *Bd* and/or Rv in permanent and semi-permanent wetlands.

KEY WORDS: *Batrachochytrium dendrobatidis* · Ranavirus · *Notophthalmus* · *Hyla* · *Lithobates* · Mortality · Co-infection

INTRODUCTION

Extensive surveys of pathogen presence in pond-breeding amphibians indicate *Batrachochytrium dendrobatidis* (*Bd*; Phylum Chytridiomycota) and ranaviruses (Family *Iridoviridae*) are widespread in North America and infect multiple families of amphibians (Miller et al. 2011, Olson et al. 2013). The aquatic zoospores of *Bd* colonize and infect keratinized skin of amphibians, which results in localized, typically sub-lethal, infections in the mouthparts of tadpoles and more severe, sometimes lethal infection

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in post-metamorphic amphibians (Voyles et al. 2009, Kilpatrick et al. 2010). Horizontal transmission of ranavirus (Rv) occurs in aquatic environments via direct and indirect mechanisms (Brunner et al. 2007, Duffus et al. 2008). Although both pathogens have been associated with numerous mortality events, in the southeastern USA ranaviral disease (particularly frog virus 3 [FV3] infection) has been linked to more outbreaks involving mass mortality (Green et al. 2002, Gray et al. 2009).

Experimentally, there are interspecific differences in susceptibility to *Bd* and differences in virulence among *Bd* strains (Kilpatrick et al. 2010, Rosenblum et al. 2010, Gervasi et al. 2013). Likewise, susceptibility to Rv differs greatly among species and among different developmental stages within a species (Hoverman et al. 2010, Haislip et al. 2011, Warne et al. 2011). Virulence and mortality rates depend on the Rv isolate, dose, and the host's genetic background (Brunner et al. 2005, Hoverman et al. 2011). Many field surveys have detected *Bd* or Rv infections in hosts lacking any gross external signs of disease (i.e. sub-clinical infections), which suggests some species or life stages may act as asymptomatic carriers (Brunner et al. 2004, Daszak et al. 2004, Rothermel et al. 2008, Miller et al. 2011). Importantly, the presence of multiple reservoirs may allow these pathogens to persist despite extirpation of susceptible hosts or the periodic drying of aquatic habitats.

One species that may serve as a reservoir for both *Bd* and Rv is the eastern newt *Notophthalmus viridescens* (Raffel et al. 2010, Strauss & Smith 2013). This species is long-lived and exhibits variable life histories throughout its range (Petranka 1998). Most populations have a juvenile eft stage that facilitates dispersal and colonization of widely separated ponds (Gill 1978). If aquatic larvae become infected and carry sub-clinical infections through metamorphosis, the terrestrial stage could be a vector for pathogen introduction to other wetlands and adjacent terrestrial habitats (Richter et al. 2013). Previous studies have documented sub-clinical infection of adult eastern newts in the wild with *Bd* (Rothermel et al. 2008, Groner & Relyea 2010, Raffel et al. 2010) and Rv (Duffus et al. 2008, Glenney et al. 2010, Todd-Thompson 2010, Richter et al. 2013). Chytrid-associated mortality events involving small numbers of *N. viridescens* have been reported from sites in southwestern Virginia (Rothermel et al. 2008) and north-central Alabama (Bakkegard & Pessier 2010). However, few newt species have been screened for more than one pathogen (but see Bovero et al. 2008, Glenney et al. 2010, Richter et al. 2013) and pathogen prevalence among different life stages has not been examined.

Permanent and semi-permanent aquatic habitats are used for breeding by species that are aquatic as adults, such as newts, or have long larval periods, such as American bullfrogs *Lithobates catesbeianus* and green frogs *L. clamitans*. Newts or other salamanders are often the dominant predators in lentic freshwater habitats (Morin 1983, Davic & Welsh 2004). In fishless ponds, the eastern newt may act as a keystone predator, exerting top-down effects on the outcome of competitive interactions among larval anurans (Morin 1983, Petranka 1998). However, larval green frogs and larval bullfrogs also prey on eggs and larvae of other anurans, including wood frogs *L. sylvaticus* (Boone et al. 2004, Jennette 2010). Wood frogs are highly susceptible to Rv infection, based on exposure trials (Hoverman et al. 2011, Warne et al. 2011) and documented mortality events (Dodd 2004, Petranka et al. 2007, Brunner et al. 2011). Thus, juvenile recruitment by wood frogs and other susceptible species, such as Cope's gray treefrog *Hyla chrysoscelis* (Hoverman et al. 2010), is likely to be severely reduced in the presence of both Rv and predators (Gray et al. 2009).

Studies combining population monitoring and disease surveillance are needed to clarify these dynamics in complex natural communities (Hoverman et al. 2012). Toward that end, we intensively monitored pond-breeding amphibians at a site in the Southern Appalachians, USA, where preliminary pathogen screening in 2005–2006 revealed *Bd* infection of multiple species (Rothermel et al. 2008). Our detection of Rv during the first year of intensive monitoring (2008) offered an opportunity to investigate potential demographic consequences of these emerging diseases in a community where both pathogens were present. Although we sought to determine prevalence of both pathogens in multiple species and life stages of co-occurring predator and prey species, we did not have sufficient resources to thoroughly screen all species present at the site. Therefore, we prioritized sampling of aquatic life stages of the 5 most abundant species in their shared breeding habitat.

MATERIALS AND METHODS

Study site

Our study was conducted at the Charles H. Wharton Conservation Center, a 52-ha research and

education facility in the Tallulah River valley of the southern Blue Ridge Mountains (Towns County, Georgia, USA; 34.991° N, 83.557° W). The river floodplain (elevation 762 m) was logged more than 45 yr ago and has since been maintained as an open meadow (Fig. 1). A series of 13 small, hydrologically connected ponds, originally created to rear trout, are fed by water diverted from the river. Less than 50 m away (approximately 100 m from the river), a previous landowner excavated 2 larger ponds and diverted seepage flow to make them hold water year-round. We chose one of these larger (0.04 ha) ponds as the focal study pond, because it is the only pond on the property that lacks fish and is used for breeding by Cope's gray treefrogs, spring peepers *Pseudacris crucifer*, green frogs, American bullfrogs, pickerel frogs *L. palustris*, wood frogs, three-lined salamanders *Eurycea guttolineata*, red salamanders *Pseudotriton ruber*, and red-spotted newts *N. viridescens viridescens*. In spring 2008, we installed a Tidbit V2 temperature logger (UTBI-001, Onset Computer Corp.) in our study pond and a HOBO Pro V2 temperature logger (U23-001, Onset Computer Corp.) in the adjacent open field (for details and resulting data, see Fig. S1 in the Supplement at www.int-res.com/articles/suppl/d118p113_supp.pdf).

Field methods and sample collection for disease testing

We dip-netted the study pond periodically from February 2008 to August 2010, with more frequent sampling during spring months. We also regularly inspected all ponds at the site for evidence of amphibian die-offs. Sick or dead amphibians were sent (fresh or frozen) to The University of Georgia's Veterinary Diagnostic Investigational Laboratory (VDIL) for examination (see below). For routine screening, we attempted to capture at least 20 individuals of each focal species (i.e. red-spotted newt, green frog, American bullfrog, wood frog, Cope's gray treefrog) during each dip-netting session, although in practice this was only possible when a particular life stage was present and relatively abundant. When possible, we relied on nonlethal techniques, such as swabbing (for *Bd*) or tail-clipping (for Rv). However, on a subset of sampling dates each year, we collected and euthanized (in MS-222 or chloretone) a random sample of focal species for detailed histopathological examination. During handling of amphibians, we followed steps to avoid cross-contaminating samples and to minimize risk of spreading infection, including changing disposable nitrile gloves between individuals and keeping individuals in separate plastic bags. We also disinfected

Fig. 1. Main study pond in the Tallulah River valley (34.991° N, 83.557° W), Towns County, Georgia, USA, in early spring (March 2008)

boots and nets with a 3–5% bleach solution before and after every sampling event.

Adult newts

We captured adult red-spotted newts in our study pond during 17 dip-netting sessions in June 2008–June 2009 and 12 trapping sessions in June 2009–September 2010. We deployed 10 double-ended minnow traps during each 2- to 4-d trapping session and checked traps approximately every 12 h. Each captured newt was placed in a separate plastic bag with water from the pond and typically processed within 2 h.

In June 2009, we started marking each new individual by clipping unique combinations of 2–5 toes. Each newt was also photographed and assigned a spot identification code based on the number of red spots on each side of the dorsum and head. In 2010, we discontinued toe-clipping, because by then we were confident we could identify individuals from photographs. We swabbed each newt upon its first capture and upon recapture in a different trapping session (10–12 sweeps of skin on venter and 5 sweeps per hind limb). Toe clips and skin swabs were frozen for *Bd* testing (see 'Molecular diagnostics'). We also collected a tail clip from each newly captured newt and preserved the tissue in 70% ethanol for Rv testing (Gray et al. 2012). Lastly, we recorded snout–vent length (SVL), weight (to the nearest 0.1 g), and sex of every individual.

Larval newts and efts

We captured larval newts with dip-nets and placed each larva in a separate plastic bag with pond water. Larvae were euthanized in chloretone on the day of capture and preserved whole in separate vials containing 70% ethanol. Recently metamorphosed efts and immigrating adult-sized newts (some with vestiges of red-orange coloration suggesting they might be maturing efts) were intercepted by a drift fence and captured in pitfall traps installed in June 2009. The drift fence was made of silt fencing (0.9 m high) buried 20–30 cm below ground. Fifteen pairs of pitfall traps (7.6 l) were opened and checked at least once daily in 2009 (17 June–11 September) and 2010 (6 March–12 September). During the intervening fall and winter, we closed the traps and cut gaps in the fence to allow animals to move freely in and out of the pond.

Upon first capture, efts were measured (SVL) and given a cohort mark by injecting visual implant elastomer (VIE; Northwest Marine Technology) at the base of the tail. We collected skin-swab samples from the first 30 efts leaving the pond in July 2009 and all maturing efts entering the pond in August–September 2009. We thoroughly swabbed the ventral skin, hind limbs, and hind feet and preserved swabs in vials containing 70% ethanol. Newts were then released on the opposite side of the drift fence to resume migrating.

Larval and juvenile anurans

Because *Bd* is localized in the keratinized mouthparts of tadpoles, we swabbed only the mouthparts of large tadpoles. Swab samples were individually preserved in vials containing 70% ethanol. Larvae that were too small to swab (<35 mm total length) were euthanized and preserved whole in 70% ethanol. Upon capture at the drift fence, recently metamorphosed ranids were measured (SVL) and cohort-marked on the hind limbs using VIE, then released on the opposite side of the fence. We collected skin-swab samples from a subset of juvenile bullfrogs (n = 30) and juvenile green frogs (n = 31) in June–August 2009.

Histopathology

Necropsies were performed on amphibians submitted to VDIL for detailed examination. Whereas *Bd* only infects the skin, Rv infects multiple cell types and causes necrosis in the liver, spleen, and kidney of severely affected amphibians (Miller et al. 2011). Animals were sectioned longitudinally such that all organs, digits, and mouthparts could be viewed. The tissues were routinely processed, embedded in paraffin, sectioned at 3–5 µm, placed on glass slides, stained with hematoxylin and eosin, and viewed with light microscopy. Any changes from normal were documented and described.

Molecular diagnostics

Bd testing

In 2008, all skin-swab and tissue samples were tested at the Southeastern Cooperative Wildlife Disease Study (SCWDS; Athens, Georgia, USA) using

polymerase chain reaction (PCR)-based assays. Genomic DNA was extracted from each skin-swab sample using the DNeasy Blood and Tissue Kit (Qiagen) following the manufacturer's protocol and amplification was conducted with primers Bd1a and Bd2a as described by Annis et al. (2004), except we amplified for 40 cycles. The positive control was *Bd* DNA obtained from a Georgia amphibian and confirmed to be *Bd* by multi-locus sequence typing. For the negative controls, we used molecular-grade water extracted with each set of samples and run as an additional PCR sample. Each sample was run in triplicate and a sample was considered positive if *Bd* was detected in at least 2 replicates.

In 2009 and 2010, SCWDS continued testing swab samples for *Bd*, but most tissue samples were tested by VDIL. The VDIL ran samples using both conventional and quantitative real-time PCR (qPCR), following the protocols reported by Annis et al. (2004) and Boyle et al. (2004), respectively. The DNA was extracted from tissue samples using a commercially available kit (Qiagen). For qPCR assays, the genomic DNA was measured in each sample and a set amount used for PCR. The assay was processed for 40 cycles. Based on standard curve results and given the possibility of fluorescence artifacts (i.e. non-specific amplification of background nucleic acids) represented by higher threshold cycle (CT) values (Caraguel et al. 2011), CT values >35 were not counted as positive. The PCR assays were run with 2 positive and 2 negative controls. Positive controls included cultured *Bd* and tissue from a positive amphibian (i.e. *Bd*-infected according to histology). Negative controls included DNA-grade water and tissue from a negative amphibian (i.e. not infected according to PCR and histology).

Rv testing

Both conventional PCR and qPCR techniques were employed by VDIL to test for Rv. Genomic DNA was extracted from tissues (i.e. tail clips from adult newts or the liver of larval ranids) using the DNeasy Blood and Tissue Kit (Qiagen). Conventional PCR was performed using the protocol and primer sets (MCP4 and MCP5) found in Mao et al. (1996, 1997) and targeting an approximately 450 bp region of the major capsid protein gene. The PCR products were resolved via electrophoresis on a 1.0% agarose gel. Each PCR run included 2 negative controls (water and tissue from a Rv-negative tadpole) and 2 positive controls (cultured Rv and tissue from a Rv-positive tadpole). For qPCR the extracted DNA was assayed according to Picco et al. (2007). The assay was processed for 40 cycles. Based on our standard curve and for reasons noted above (Caraguel et al. 2011), samples having CT values >35 were not counted as positive. As a cost-saving measure in 2010, we relied mainly on conventional PCR to test for Rv, and only retrospectively ran samples with qPCR if they were suspect according to conventional PCR. Samples that were suspect (i.e. displayed a possible faint band) for Rv by conventional PCR but negative by qPCR were considered negative.

Interpretation of diagnostic results

For a subset of tissue samples tested with both conventional and qPCR for *Bd*, there was 100% agreement between methods (Table S1 in the Supplement). Results of conventional PCR on swabs versus conventional PCR on tissues were slightly less concordant (84% agreement; Table S1). Others have similarly noted differences in sensitivity of PCR depending on sample type (Hyatt et al. 2007, Burrowes et al. 2011). In cases of disagreement, we classified the individual as positive if PCR detected *Bd* in either the swab or tissue. However, to avoid biasing estimates of seasonal *Bd* prevalence for adult newts (and because toe clips were only collected from a subset of newts), we relied solely on the one sample type tested consistently throughout the study, i.e. conventional PCR on skin swabs.

For Rv testing of newt tissues, results of conventional and qPCR did not agree in 52% of cases (Table S2 in the Supplement). As above, we classified individuals as positive if either method detected Rv, but because >80% of samples were tested with only one method, our data may not be reliable for comparisons of Rv prevalence over time. Most tissue samples of larval anurans (68%) were tested for Rv using both conventional and qPCR and there was 92% agreement between methods (Table S3 in the Supplement).

Analyses of adult newt body condition and recapture rates

We used 2-way analysis of variance (ANOVA; Type II SS) to test for effects of month, *Bd* infection status, and their interaction on body condition of adult newts in March–May 2010, the season with the highest number of captures of this life stage. As a meas-

ure of body condition, we used the scaled mass index (SMI; Peig & Green 2009), which is highly correlated with protein and fat stores in another North American salamandrid (*Taricha granulosa*; MacCracken & Stebbings 2012). Recognizing the potential for false negatives and differences in sensitivity between different diagnostic techniques (in this case, PCR on toe tissue and skin swab), we only used observations that were either negative or positive by both methods of *Bd* screening. This essentially restricted the test of *Bd* effects to newts that were truly uninfected versus those with presumably high infection intensities. To ensure independence of data, we only used the first observation for individuals captured more than once. We also excluded the few Rv-positive newts from analyses. The relatively few detections of Rv did not provide enough data for a similar analysis of Rv effects on body condition.

Although we intended to use formal capture-mark-recapture analysis to estimate recapture probabilities and survival with respect to infection status, newt recapture rates were too low to provide adequate sample sizes (i.e. survival estimates had unacceptably high standard errors and even fairly simple models had poor goodness-of-fit). In the absence of data on survival rates, one might still expect to see lower recapture rates of infected newts if *Bd* or Rv infection were causing mortality. To explore this, we determined the percentages of infected newts in July–August 2009 that were subsequently recaptured in 2010, for qualitative comparison with random samples of uninfected newts over the same time period, thus accounting for the highly unequal sample sizes owing to the low proportion of infected newts in summer 2009.

RESULTS

Prevalence and clinical signs in adult newts

Prevalence of *Bd* among adult newts ranged from 0% in August–September 2009 to 72% in February–March 2008 (Fig. 2). In all 3 yr of the study, *Bd* prevalence tended to be higher in winter through early spring then declined sharply in mid- to late summer (Fig. 2).

Of 319 newts for which we obtained at least one tail-clip sample during 2009–2010, 23 (7.2%) were positive for Rv. This is likely an underestimate of true prevalence because PCR testing of tail clips has a false negative rate of approximately 20% (Gray et al. 2012). We could not evaluate seasonal or annual variation in Rv prevalence given our heavy reliance on this less-sensitive method, lack of year-round sampling, and use of different diagnostic methods for Rv over time (Tables 1 & S2).

On 28 March 2008, we found a dead newt that was positive for both Rv and *Bd*, and we detected additional cases of co-infection every year (Table 1). A male newt collected in March 2009 had small, red lesions (i.e. hemorrhages) on its tail and was co-infected with Rv and *Bd* (Fig. 3). Microscopically, we observed necrosis and degeneration of the muscle within the hemorrhagic areas and also noted intracytoplasmic inclusion bodies in leukocytes within the same areas. The liver tissue also showed early signs of degeneration. Only one other co-infected newt exhibited gross clinical signs, a male in March 2010 with edema and reddening of the gular region.

Body condition and recapture rates of adult newts

Data for 76 adult newts (65 males and 11 females; range 43–58 mm SVL) captured in spring 2010 were used in the analysis of month and *Bd* effects on SMI. Mean (±SD) SMI was similar between infected (1.466 ± 0.186) and uninfected (1.498 ± 0.163) newts ($F_{1,70}$ = 0.092, p = 0.762), but differed significantly among months ($F_{2,70}$ = 7.361, p = 0.001). Mean SMI

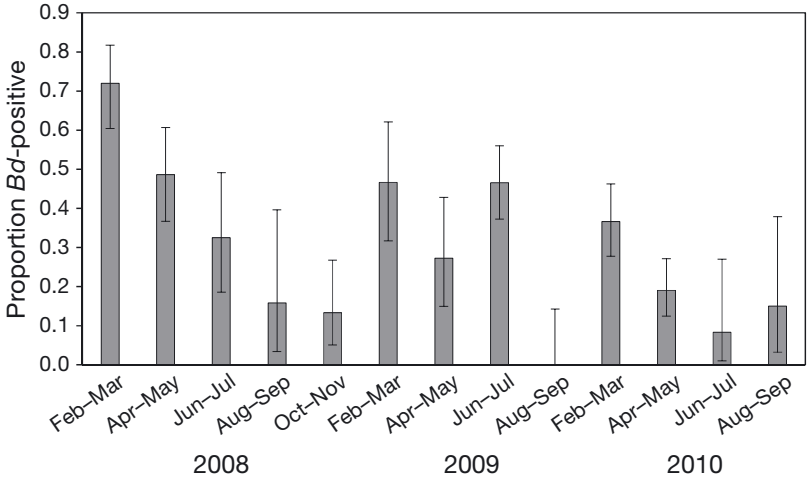

Fig. 2. Prevalence (based on conventional PCR testing of skin swabs) of *Batrachochytrium dendrobatidis* (*Bd*) in adult red-spotted newts *Notophthalmus v. viridescens* (± Clopper-Pearson 95% binomial confidence intervals; Zar 1999) in the main study pond in the Tallulah River valley, Georgia, USA. Only aquatic adults captured by dip-netting or minnow trapping are included. Sample sizes for each time period ranged from n = 19–121

Table 1. Occurrence of ranavirus (Rv) infection (and co-infection with *Bd*) in aquatic adult and larval red-spotted newts in the main study pond in the Tallulah River valley, Georgia, USA. Only adults (A) and larvae (L) captured by dip-netting or minnow trapping are included. For Rv, individuals were considered infected if tissues were positive by either conventional or qPCR (see Table S2 in the Supplement). The sample type tested for *Bd* is in parentheses

Month	Life stage	Sample type	n	No. pos.	No. co-infected with *Bd*
Mar 2008	A	Organs	1	1	1 (toe tissue)
May 2008	A	Organs	20	13	13 (toe tissue)
Oct 2008	L	Organs	1	1	—
Mar 2009	A	Organs	2	1	1 (skin swab)
May 2009	A	Organs	8	1	1 (skin swab)
Jun 2009	A	Tail clip	50	0	—
Jul 2009	A	Tail clip	58	0	—
Aug 2009	A	Tail clip	15	15	0 (skin swab)
Aug 2009	L	Organs	8	4	0 (toe tissue)
Mar 2010	A	Tail clip	111	5	4 (toe tissue)
Apr 2010	A	Tail clip	38	2	2 (toe tissue)
May 2010	A	Tail clip	47	0	—
Jun 2010	A	Tail clip	7	0	—
Jul 2010	A	Tail clip	10	1	1 (toe tissue)
Aug 2010	A	Tail clip	4	0	—
Aug 2010	L	Tail clip	30	0	—
Sep 2010	A	Tail clip	11	0	—

was low in March (1.426 ± 0.184), increased significantly in April (1.618 ± 0.108; Scheffé's test, p = 0.004), then declined again in May (1.542 ± 0.111), regardless of infection status (*Bd* × Month: $F_{2,70}$ = 0.085, p = 0.919).

Two (16.7%) of the 12 newts that were *Bd*-positive in July–August 2009 were subsequently recaptured in March–August 2010, versus 1 of 15 (6.7%) randomly selected *Bd*-negative newts. Recapture rates were similar for the 15 Rv-positive (33.3%) and 15 randomly selected Rv-negative newts (26.7%) between the same 2 time periods.

Prevalence and clinical signs in larval and post-metamorphic newts

In contrast to the high prevalence of *Bd* infection in adult newts, we detected *Bd* in only 1 of 91 larval newts (Table 2). In August 2010, we found 4 dead newt larvae while dip-netting our study pond. Three were suspect for *Bd* by PCR but no *Bd* was observed microscopically in sections of toes or skin from elsewhere on the body and all were negative for Rv. Thus, the 4 mortalities were not associated with *Bd* or Rv infection. However, Rv infection was detected by PCR in a larval newt collected in October 2008 (confirmed with histology) and 4 larval newts collected in August 2009, none of which exhibited gross clinical signs of disease (Table 1).

All skin-swab samples of emigrating efts (n = 30) were negative for *Bd*. Four recently metamorphosed efts died in pitfall traps during summer 2009. All 4 were negative for both *Bd* and Rv based on qPCR, so they may have simply died from heat stress. None of the maturing efts (n = 46) entering the pond in fall 2009 were infected with *Bd* according to skin swab-conventional PCR assays. Prevalence of *Bd* was low in adult newts captured entering the pond (4.3% infected; n = 23) in late March to early June 2010.

Prevalence and clinical signs in larval and post-metamorphic anurans

Larval green frogs and bullfrogs

Prevalence of *Bd* determined from PCR testing of mouthpart swabs or tissues ranged from zero in August 2009 to 57.1% in green frogs (April–May 2009) and 76.0% in bullfrogs (May 2010; Table 2). We also detected co-infection with *Bd* and Rv in 4 of

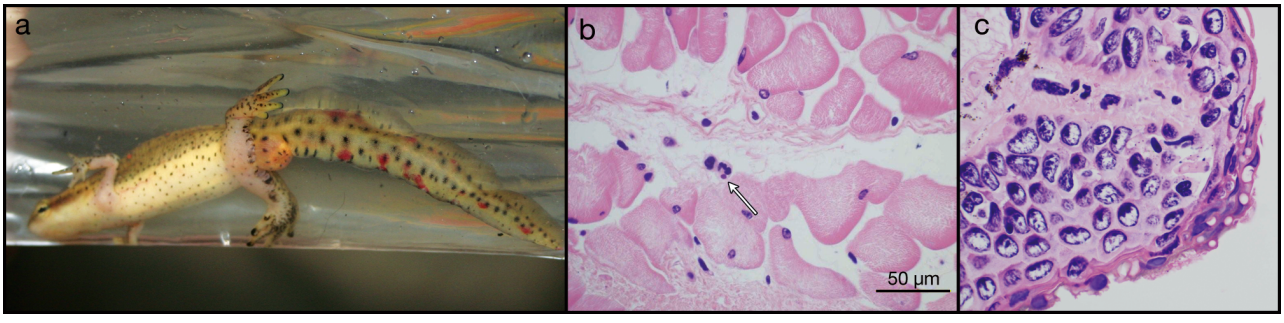

Fig. 3. Male red-spotted newt *Notophthalmus v. viridescens* collected on 26 March 2009; PCR and histology showed it to be co-infected with ranavirus and *Batrachochytrium dendrobatidis* (*Bd*). (a) Lesions on the tail. (b) Viral inclusion (white arrow) in the cytoplasm of a leukocyte in one of the tail lesions. (c) Characteristic fungal zoosporangia of *Bd* in the epidermis

Table 2. Prevalence (based on PCR results) of *Batrachochytrium dendrobatidis* in larval red-spotted newts and larval anurans in the main study pond in the Tallulah River valley, Georgia, USA. Sample sizes varied because of interspecific differences in length of larval period and relative abundance, which affected our capture success via dip-netting. We tested toes of larval newts and mouthparts (swabs or tissue) of larval anurans (for details, see Table S1 in the Supplement at www.int-res.com/articles/suppl/d118p113_supp.pdf). The number positive is in parentheses

Month	*Notophthalmus v. viridescens*		*Lithobates catesbeianus*		*Lithobates clamitans*		*Lithobates sylvaticus*		*Hyla chrysoscelis*		*Pseudacris crucifer*	
	n	% pos.	n	% pos.	n	% pos.	n	% pos.	n	% pos.	n	% pos.
Feb–Mar 2008					83 (15)	18.1	85 (2)	2.4				
Apr–May 2008			2 (2)	–	92 (34)	37.0			22 (6)	27.3	19 (2)	10.5
Jun–Jul 2008	31 (0)	0.0			47 (15)	31.9			24 (0)	0.0		
Aug–Sep 2008	22 (0)	0.0	5 (0)	–	27 (2)	7.4						
Oct–Nov 2008			45 (3)	6.7	46 (3)	6.5						
Feb–Mar 2009			23 (4)	17.4	44 (5)	11.4	60 (0)	0.0				
Apr–May 2009					42 (24)	57.1	19 (4)	21.1				
Jun–Jul 2009			48 (17)	35.4	47 (5)	10.6			33 (0)	0.0		
Aug–Sep 2009	8 (0)	0.0	24 (1)	4.2	29 (0)	0.0						
Apr–May 2010			25 (19)	76.0	41 (16)	39.0						
Jun 2010									24 (0)	0.0		
Aug 2010	30 (1)	3.3										

100 larval green frogs screened for both pathogens throughout the study. We noticed missing keratin in the mouthparts of 34 larval American bullfrogs and green frogs, which is an indication of heavy *Bd* infection in ranid larvae (Rachowicz & Vredenburg 2004). Although *Bd* prevalence was higher in bullfrog and green frog larvae noted as having sparse or missing keratin (68%) than in larvae with normal-looking mouthparts (21%), the majority of infected larvae (84%) did not have obvious mouthpart deformities.

In October 2008, we found 2 dead and 5 moribund green frog larvae in our study pond. The affected larvae were negative for *Bd* according to PCR. Histologically there was no evidence of infection by Perkinsea-like alveolates, which are protistan parasites that have been implicated in mortality of larval ranids at other sites in Georgia (Davis et al. 2007) and Florida (Landsberg et al. 2013). Histopathological findings were consistent with Rv and all 7 larvae were PCR-positive for Rv (Table 3). Two of 20 green frog larvae collected in November also exhibited signs consistent with ranaviral disease (i.e. swelling of the body and legs, hemorrhages or erythema of the legs and ventrum; Miller et al. 2011), but both were PCR-negative for Rv.

We detected additional mortality of larval green frogs in our study pond in 2009–2010, including 1 larva found dead in May 2009, 2 in August 2009, and 1 in March 2010. We also found 1 dead green frog larva in a nearby fish pond in June 2009. Four of the 5 dead larvae were PCR-positive for Rv and the larva collected in March 2010 was also PCR-positive for *Bd*. The heart muscle of 1 Rv-positive larva had areas

Table 3. Occurrence of ranavirus (Rv) infection in larval anurans at our study site in the Tallulah River valley, Towns Co., Georgia, USA. Small sample sizes and nonrandom collections on many dates precluded estimation of prevalence. Infection status was based on PCR testing of organs (liver and kidney; see Table S3 in the Supplement) and larvae were from our main study pond unless noted otherwise. **Boldface** indicates at least one specimen on that date exhibited gross clinical signs consistent with Rv infection. Full genus names in Table 1

Month	*L. catesbeianus*		*L. clamitans*		*L. sylvaticus*		*H. chrysoscelis*	
	n	No. pos.	n	No. pos.	n	No. pos.	n	No. pos.
Mar 2008					30	5		
May 2008			13	2				
Oct 2008			**7**	7				
Nov 2008			**2**	0				
Feb 2009	**3**	0	**1**	0				
Mar 2009			1	0	20	1		
Apr 2009					10	4		
May 2009			**12**	1	**38**[a]	20		
Jul 2009							**9**	8
Aug 2009	20	0	**22**	2				
Mar 2010			**1**	1				
Apr 2010			**21**	2				
May 2010	**24**	0	**20**	2				
Jun 2010							24	0

[a]In May 2009, we collected larval wood frogs from 2 smaller fish ponds during a die-off

of degeneration, necrosis, and few inflammatory cell infiltrates. The epithelial cells lining the intestines were occasionally necrotic or vacuolated, and infiltrated by few inflammatory cells.

Despite consistent detection of Rv in larval green frogs, we did not detect Rv in larval American bullfrogs inhabiting the same pond (Table 3). Some larval bullfrogs in February 2009 and May 2010 exhibited possible signs of Rv (i.e. erythema or swelling), but all were PCR-negative (Table 3, Table S3).

Juvenile green frogs and bullfrogs

Despite high prevalence of *Bd* in larvae during April-May 2009 (Table 2), only 1 of 31 emigrating juvenile green frogs was positive for *Bd* via skin swab-conventional PCR assays. One juvenile green frog that died in a minnow trap in August 2009 was positive for both Rv and *Bd*. Three juvenile green frogs were found dead and bloated in September 2009, but they were not tested for Rv (due to funding limitations); all were PCR-negative for *Bd*. All 30 juvenile American bullfrogs (2 emigrating and 28 immigrating into the study pond) were negative for *Bd* via skin swab-conventional PCR assays.

Larval wood frogs

Numbers of wood frog egg masses in our study pond declined over the 3 yr of observation, from ≥100 in February 2008, to 80–100 in February 2009 and only about 38 in March 2010, a year in which breeding was likely delayed due to late winter storms. In 2008, there were abundant wood frog larvae on 28 March, but none were observed on 18 April. Two of 25 larvae collected on 3 March 2008 were PCR-positive for *Bd* but overall prevalence during this season was low (Table 2). Of the 30 larvae collected on 28 March, 5 were PCR-positive for Rv (Table 3) and all were negative for *Bd* by PCR and histology.

In 2009, wood frog larvae were still present in mid-April, but we did not find any on 12 May. Using PCR, we detected Rv in larvae in late March and mid-April (Table 3). However, there were no significant histologic abnormalities in larvae from the April sample. We did not detect *Bd* in whole larvae collected on 16 March or 26 March (n = 60), but 4 of 19 larvae collected on 16 April were PCR-positive for *Bd* (Table 2). In March 2010, we noted very few wood frog larvae during visual surveys and did not catch any when we dip-netted in mid-April.

We witnessed a die-off of wood frog larvae in nearby ponds in 2009. On 12 February 2009, we noted a total of 22 wood frog egg masses in several small fish ponds located 45 m west of our study pond. Wood frog larvae were still present in these ponds in early May, and neither *Bd* nor Rv was detected by PCR in a sample of 15 larvae collected on 12 May (B. Rothermel, 'Georgia amphibian disease dynamics'; data available at https://mantle.io/grrs/studies). However, a die-off of late-stage larvae occurred between 28 May and 13 June. In total we observed 117 dead and 27 sick wood frog larvae. The majority exhibited edema and erythema of the mouthparts and cloacal area. We did not see any metamorphosed wood frogs in or near these ponds, suggesting there was complete mortality. More than half of the wood frog larvae collected from the fish ponds during the die-off were infected with Rv based on PCR (Table 3). Several specimens exhibited changes in the kidney and liver tissues consistent with ranaviral disease, including degeneration of hepatocytes and renal tubular epithelium, as well as inclusion bodies within occasional renal tubular epithelial cells. *Bd* was not detected histologically or by PCR.

Larval hylids

We found low prevalence of *Bd* infection in larval Cope's gray treefrogs and spring peepers, with the only detections occurring in April–May 2008 (Table 2). We did not screen spring peepers for Rv, but 8 of 9 Cope's gray treefrogs in summer 2009 were PCR-positive for Rv, including 1 individual exhibiting erythema of the hind limbs (Table 3).

Juvenile recruitment

Hundreds of recently metamorphosed efts emigrated from the pond in 2009 and 2010 (Table 4). We undoubtedly missed capturing even more efts because we had to close the traps in early September, before all larvae completed metamorphosis. Large numbers of juvenile green frogs emigrated in both years (Table 4). American bullfrog recruitment increased substantially between 2009 and 2010 (Table 4). Based on the large size of larval bullfrogs and green frogs caught in early spring, and the emergence of metamorphs as early as April, we think most larval bullfrogs and some larval green frogs overwintered in the pond and metamorphosed the following spring–summer. Notably, we never captured or saw juvenile

Table 4. Numbers of juvenile (i.e. recently metamorphosed) amphibians that emigrated from the main study pond in June–September 2009 and March–September 2010, determined from captures in pitfall traps on the inside of the drift fence encircling the pond. Recaptures of marked American bullfrogs *L. catesbeianus*, green frogs *L. clamitans*, and red-spotted newts *N. v. viridescens* were excluded from the totals

Species	2009	2010
Notophthalmus v. viridescens	884	392
Lithobates clamitans	335	280
Lithobates catesbeianus	18	274
Eurycea guttolineata	11	13
Hyla chrysoscelis	16	5
Pseudacris crucifer	3	0
Lithobates palustris	0	1
Pseudotriton ruber	0	1
Lithobates sylvaticus	0	0

wood frogs at any ponds at our study site. We captured some juvenile Cope's gray treefrogs in both years (Table 4), despite detection of Rv in larval treefrogs in July 2009 (Table 3). Pickerel frogs, spring peepers, three-lined salamanders, and red salamanders also bred in the study pond, but we caught relatively few metamorphs of these species (Table 4; see data in B. Rothermel, 'Georgia amphibian disease dynamics' at https://mantle.io/grrs/studies for results of our limited pathogen screening of these species). The ability of juvenile hylids and three-lined salamanders to cling to vertical surfaces reduced trapping efficiency and likely contributed to the low number of captures of these species.

DISCUSSION

At our montane study site in the Southern Appalachians, USA, we observed patterns of *Bd* and Rv prevalence consistent with results from previous field surveys and experimental exposure of native amphibian species to Rv. We detected sub-clinical *Bd* infections in larval and adult red-spotted newts and in larvae of 5 anuran species that bred in our study pond in 2008–2010, including 3 ranids and 2 hylids. However, all cases of mortality and clinical signs in adult newts and larval anurans appeared to result from ranaviral disease, including a mass die-off of wood frog larvae in smaller fish ponds located near our study pond. We strongly suspect the lack of wood frog recruitment in our study pond was a consequence of poor larval survival in the presence of Rv and multiple potential predators (e.g. newts, larval bullfrogs and green frogs, larval aeshnids; Walters 1975, Brodie & Formanowicz 1983, Boone et al. 2004, Jennette 2010, Pitt et al. 2011). Our results support the purported role of red-spotted newts, green frogs, and American bullfrogs as common reservoirs for *Bd* and/or Rv in permanent and semi-permanent wetlands (Daszak et al. 2004, Duffus et al. 2008, Gray et al. 2009, Hoverman et al. 2012).

Overall patterns of *Bd* prevalence

Seasonal patterns of *Bd* prevalence at our study site were generally similar to patterns observed previously in this and other temperate regions (Kriger & Hero 2006, Rothermel et al. 2008), although we did not see the rise in prevalence in autumn observed at higher-latitude sites in North America (Ouellet et al. 2005, Lenker et al. 2014). Prevalence of *Bd* among adult red-spotted newts, larval green frogs, and larval American bullfrogs declined sharply in mid- to late summer every year (Fig. 2, Table 2). In July–August, the temperature of our study pond sometimes exceeded 30°C (Fig. S1), a temperature lethal to *Bd* (Woodhams et al. 2003, Piotrowski et al. 2004). However, many abiotic and biotic factors besides temperature may influence infection prevalence via effects on *Bd* and the host species.

The lack of mortality attributable to *Bd* at our study site and rarity of *Bd*-associated mortality throughout the southeastern USA (Daszak et al. 2005, Rothermel et al. 2008) could be related to temperature-mediated host defenses and thermal limits to *Bd* growth in this relatively warm climate. Interestingly, we detected *Bd* in only 1 of 91 larval newts (Table 2). *Bd* may be unable to colonize the skin of larval newts if, like most other urodeles, their epidermal tissues do not become keratinized until metamorphosis (Venesky et al. 2010). Larval and metamorphic newts may also be protected from infection by prevailing high temperatures suppressing *Bd* growth during the latter part of their larval period (Fig. S1). We did not detect *Bd* in skin-swab samples of emigrating juveniles or immigrating maturing efts. Likewise, despite seasonally high prevalence of *Bd* infections in larval green frogs and bullfrogs (Table 2), skin-swab samples of 30 recently metamorphosed bullfrogs and 30 of 31 recently metamorphosed green frogs were negative for *Bd*. Warm terrestrial microhabitats are readily available at our study site in the southern Blue Ridge Mountains, where daily maximum air temperatures are typically >25°C in summer (Fig. S1). Thus, daytime temperatures during the post-metamorphic

period may be warm enough to slow *Bd* growth and allow juvenile amphibians to maintain infection intensities below lethal levels, if not clear infection altogether (Woodhams et al. 2003, Daskin et al. 2011, Raffel et al. 2015).

Co-infection and effects of *Bd* and Rv in newts

Whereas several species of Eurasian salamandrids have experienced mortality from ranaviruses (Balseiro et al. 2010, Kik et al. 2011, Stöhr et al. 2013), most Rv infections in *N. viridescens* have been subclinical (Miller et al. 2011, Richter et al. 2013). One adult newt found dead at the edge of our study pond was co-infected with *Bd* and Rv. The few clinical signs in this and other co-infected newts were consistent with ranaviral disease, however, the majority of co-infected individuals exhibited no gross signs of disease. To our knowledge, our observations represent the first documented cases and description of co-infection in a wild-caught salamandrid (Fig. 3). With the exception of 2 sampling events in May 2008 and August 2009, there appeared to be low overall prevalence of Rv in newts (Table 1).

We did not obtain sufficient recaptures of marked adult newts to permit a formal survival analysis. Our limited data on the proportions of newts that were infected with *Bd* or Rv in August 2009 and recaptured in 2010 suggest infected and uninfected newts had similar recapture rates over this interval. We also found no evidence of sub-lethal effects of either *Bd* or Rv on adult newts. The highly skewed sex ratio of newts captured in minnow traps during spring 2010 was likely a result of mate-seeking males being attracted to conspecifics already caught in the traps (Rohr et al. 2004), as we have no reason to suspect differential effects of either pathogen on males and females. Newts infected with *Bd* did not exhibit reduced body condition, based on the lack of significant difference in SMI between *Bd*-positive and *Bd*-negative newts during spring 2010. This was a good year to test for potential effects of *Bd* on adult newts, because relatively cold temperatures persisted into March (daily minimum temperatures in the study pond ranged 5.1–10.8°C in March 2010 versus 9.2–20.7°C in March 2009 and 6.8–12.4°C in March 2008; Fig. S1). Certain aspects of immune function in red-spotted newts are suppressed at colder temperatures (Raffel et al. 2006, 2015).

Late winter storms in February 2010 also delayed arrival of anuran eggs and larvae, an important food resource for newts in the spring (Petranka 1998). We believe nearly all adults in our population overwintered in the pond, based on lack of emigrants in late summer and few captures of immigrating adults in March–April (in contrast to migratory populations studied by Gill 1978 and Grayson & Wilbur 2009). Seasonal variation in food availability could potentially explain the large increase in newt SMI between March and April followed by a decline in May. Adults presumably emerge from winter in poor body condition and food may remain scarce until temperatures warm enough for wood frogs and spring peepers to breed. Anuran eggs and larvae provide an energy-rich food source that allows predatory newts to attain higher body condition (Sztatecsny et al. 2013). By May, newts in our pond may have depleted larval anuran populations and surviving tadpoles either metamorphosed or reached a size where they could evade predation, resulting in reduced food resources and lower SMI. Additional research would be needed to confirm these mechanisms for the seasonal pattern we observed, as we could find no studies of correlations among body condition, fat stores, and food availability for this species. The energetic expenditures of courtship and oviposition, as well as intraspecific density, would also have to be taken into account.

Red-spotted newts bred successfully in both years and juvenile production was relatively high (Table 4) compared to mountain ponds in Virginia studied by Gill (1978); only 1 of 5 ponds produced >200 juveniles during the 3 yr of his study. Juvenile production in this species is strongly affected by intraspecific density (Harris 1987) and can vary by orders of magnitude among years, even within the same pond (Hurlbert 1970, Gill 1978). In our study pond, the few cases of mortality in larval newts were unrelated to *Bd* or Rv and we saw no clinical signs of disease in early life stages. The successful recruitment combined with low incidence of clinical signs and disease-related mortality implies that neither *Bd* nor Rv had severe population-level effects during our study period.

Prevalence and effects of Rv in anurans

We detected Rv in larval green frogs (spring, summer, and fall), larval wood frogs (spring), and larval Cope's gray treefrogs (summer; Table 3). Anuran species that have rapid larval development and use short-hydroperiod wetlands for breeding tend to be more susceptible to ranaviral infection (Hoverman et al. 2011). Larval wood frogs, in particular, are highly

susceptible to ranaviruses, especially when exposed during late developmental stages (Haislip et al. 2011, Warne et al. 2011). Of 19 amphibian species experimentally exposed to ranaviruses, wood frogs exhibited the highest infection prevalence and mortality rates (Haislip et al. 2011, Hoverman et al. 2011). Consistent with results from exposure trials, wood frogs were the only species to experience mass mortality at our study site. In late May 2009, we confirmed Rv to be associated with a die-off of larval wood frogs in 3 small, hydrologically connected fish ponds. The die-off progressed rapidly, with apparently 100% mortality occurring within 17 d. In general, die-offs due to ranaviruses are characterized by sudden onset, range in duration from only 5 d to several weeks, and often result in >90% mortality of the affected species (Green et al. 2002, Petranka et al. 2007, Russell et al. 2011).

Although we never found dead wood frog larvae in our main study pond, we suspect their annual disappearance and lack of recruitment were partly attributable to ranaviral disease. Consumption by red-spotted newts and other predators also likely contributed to declining abundance of wood frog larvae every spring. The minimum larval period in wood frogs is 45 d (Dodd 2004). In late March 2008, wood frog larvae were still undeveloped (Gosner stage ≤25) and they could not have reached metamorphosis by mid-April, when we noted their absence from the pond. Similarly, though wood frog larvae were still present in mid-April 2009, they lacked visible limb buds. We detected Rv in larval wood frogs in early spring 2009 and 2010 (Table 3) and never captured juveniles at drift fences surrounding the pond. In contrast, we captured some juvenile Cope's gray treefrogs in both years, despite the moderate susceptibility of larvae to Rv (Haislip et al. 2011, Hoverman et al. 2011). Unfortunately, we could not evaluate impacts of Rv on hylids given our limited testing and the ineffectiveness of pitfall traps for capturing hylids. Dead wood frog or hylid larvae would have been hard to detect in our study pond because it was much deeper and more turbid than the fish ponds. Ranaviral disease may also go undetected because Rv-affected larvae may be consumed via intraspecific cannibalism, necrophagy, or predation (Gray et al. 2009, Green et al. 2010).

Despite extensive temporal overlap within the same pond, larval green frogs apparently had higher Rv infection prevalence than American bullfrogs. Green frogs seem to have experienced low levels of mortality throughout our study period, judging by occurrence of sub-clinical and clinical Rv infections in spring, summer, and fall (Table 3). However, we never saw mass mortality and hundreds of juvenile green frogs emigrated from the pond in both 2009 and 2010 (Table 4). We did not detect Rv or find any severely affected larval bullfrogs in the study pond, though our sample size for PCR testing was relatively low (<50; Table 3). We detected Rv infection in 1 larval bullfrog from a fish pond (B. Rothermel, 'Georgia amphibian disease dynamics' available at https://mantle.io/grrs/studies) and others have documented sub-clinical infections (Gray et al. 2007) and mortality (Hoverman et al. 2012) in wild bullfrogs. Exposure trials suggested that green frog larvae may be more susceptible to FV3-like ranavirus than bullfrog larvae (Haislip et al. 2011, Hoverman et al. 2011). Overall, for the 4 anuran species we monitored, there seems to be good concordance between results of laboratory exposure trials and ranaviral disease dynamics in this natural system.

Implications for disease dynamics and population persistence

The scenario we observed may allow for density-independent transmission of Rv, which could set the stage for local extirpation of some host species (Miller et al. 2011). In communities with multiple alternative hosts, a highly susceptible species such as the wood frog has high probability of acquiring infection via interspecies transmission, regardless of intraspecific density. Furthermore, any benefits to larval wood frogs of red-spotted newts reducing densities of competitively dominant species (i.e. newts acting as keystone predators; Morin 1983) may be outweighed by the effects of a virulent pathogen. Mesocosm experiments would be particularly useful for investigating such interactions and generating predictions of disease dynamics in complex natural communities, especially if experiments included both pathogens (*Bd* and Rv) and varied the composition of the amphibian community.

Research is also needed to clarify pathogen prevalence and disease effects in terrestrial life stages. Because we did not monitor the newt population year-round, we are uncertain if all adults overwintered in the pond, or whether some individuals overwintered in terrestrial habitats, as in some populations of this species (Gill 1978, Grayson & Wilbur 2009). The 23 unmarked adults that immigrated in March–June 2010 may have been migratory adults that overwintered on land, adult dispersers from other ponds, or first-time breeders. Migratory and non-migratory in-

dividuals might have different infection rates. Additionally, the stresses imposed by migration could make migratory individuals more susceptible to *Bd* or Rv, imposing a fitness cost on the migratory phenotype. Such questions could be addressed with capture-mark-recapture and multi-state modeling (see for example Murray et al. 2009) and would help clarify the potential for migratory adult newts to act as reservoirs (Brunner et al. 2004) or vectors (Richter et al. 2013). Adult anurans could also serve as reservoirs, but our screening was limited to one cohort of adult wood frogs (n = 53) entering the pond in March 2010. We detected *Bd* and Rv in toe tissue (prevalence <4% for both; B. Rothermel, 'Georgia amphibian disease dynamics' available at https://mantle.io/grrs/studies), which suggests future studies should undertake more extensive monitoring of adult life stages.

To date, wood frogs have not been extirpated from our study site, as evidenced by continued deposition of eggs in various ponds in 2011–2016 (B. Rothermel, C. Jenkins & J. Jensen unpubl. data). Predator- and/or disease-free ponds in the surrounding area may be acting as sources to sustain a metapopulation of this species. Such aquatic habitats appear to be a limited resource in the Upper Tallulah River valley, where most lentic habitats are man-made fish ponds. In this and similar landscapes, a potential management strategy is to create shallow ponds or partially fill existing ponds so they dry completely every year, thereby reducing habitat for reservoir hosts and facilitating wood frog persistence. However, occasional outbreaks of ranaviral disease would still be likely and would be hard to predict, considering research to date has not yet identified combinations of landscape variables and within-pond stressors that trigger mortality events (Gahl & Calhoun 2008, Haislip et al. 2012, Reeve et al. 2013). Finally, the lack of severe *Bd* and Rv effects on most species in our study should not be construed as cause to relax biosecurity protocols. The introduction of novel, highly virulent strains or species — especially the recently described salamander pathogen *Batrachochytrium salamandrivorans* — into the Southern Appalachians could have devastating consequences for native amphibian communities (Martel et al. 2014, Price et al. 2014).

Acknowledgements. This research was supported by the US Fish and Wildlife Service (Georgia State Wildlife Grant T-34-R) and Nongame Wildlife Conservation Funds administered by Georgia Department of Natural Resources. We thank J. McCollum and Georgia Wildlife Federation for access to the field site and significant in-kind support. We are grateful to many people and organizations for field assistance: R. Hill (Atlanta Botanical Garden); J. Brock, D. Brothers, E. Kabay, A. McDaniel, L. Wyrwich, and A. Taylor (Zoo Atlanta); C. Jenkins and J. Bauder (The Orianne Society); and volunteers S. Caster, S. Downing, A. McGhee, M. Purdee, D. Satterfield, and K. Sorensen. We also thank L. Whittington, D. Rousey, and K. Bridges for processing samples in the laboratory and S. Pruett for assisting with data analysis. Helpful comments from J. Mendelson and 3 anonymous reviewers greatly improved the manuscript. Field research was performed under Georgia collecting permits 29-WBH-09-152 and 29-WBH-10-55. Use of animals was reviewed and approved by The University of Georgia IACUC (A2007 10-186).

LITERATURE CITED

Annis SL, Dastoor FP, Ziel H, Daszak P, Longcore JE (2004) A DNA-based assay identifies *Batrachochytrium dendrobatidis* in amphibians. J Wildl Dis 40:420–428

Bakkegard KA, Pessier AP (2010) *Batrachochytrium dendrobatidis* in adult *Notophthalmus viridescens* in north-central Alabama, USA. Herpetol Rev 41:45–47

Balseiro A, Dalton KP, del Cerro A, Márquez I, Parra F, Prieto JM, Casais R (2010) Outbreak of common midwife toad virus in alpine newts (*Mesotriton alpestris cyreni*) and common midwife toads (*Alytes obstetricans*) in Northern Spain: a comparative pathological study of an emerging ranavirus. Vet J 186:256–258

Boone MD, Little EE, Semlitsch RD (2004) Overwintered bullfrog tadpoles negatively affect salamanders and anurans in native amphibian communities. Copeia 683–690

Bovero S, Sotgiu G, Angelini C, Doglio S, Gazzaniga E, Cunningham AA, Garner TWJ (2008) Detection of chytridiomycosis caused by *Batrachochytrium dendrobatidis* in the endangered Sardinian newt (*Euproctus platycephalus*) in southern Sardinia, Italy. J Wildl Dis 44: 712–715

Boyle DG, Boyle DB, Olsen V, Morgan JAT, Hyatt AD (2004) Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using real-time Taqman PCR assay. Dis Aquat Org 60:141–148

Brodie ED Jr, Formanowicz DR Jr (1983) Prey size preference of predators: Differential vulnerability of larval anurans. Herpetologica 39:67–75

Brunner JL, Schock DM, Davidson EW, Collins JP (2004) Intraspecific reservoirs: complex life history and the persistence of a lethal ranavirus. Ecology 85:560–566

Brunner JL, Richards K, Collins JP (2005) Dose and host characteristics influence virulence of ranavirus infections. Oecologia 144:399–406

Brunner JL, Schock DM, Collins JP (2007) Transmission dynamics of the amphibian ranavirus *Ambystoma tigrinum* virus. Dis Aquat Org 77:87–95

Brunner JL, Barnett KE, Gosier CJ, McNulty SA, Rubbo MJ, Koloszvary MB (2011) Ranavirus infection in die-offs of vernal pool amphibians in New York, USA. Herpetol Rev 42:76–79

Burrowes PA, Alicea A, Longo AV, Joglar RL (2011) Toes versus swabs? Evaluation of the best tissue source for detection of *Batrachochytrium dendrobatidis* in field-caught amphibians. Herpetol Rev 42:359–362

Caraguel CGB, Stryhn H, Gagné N, Dohoo IR, Hammell KL (2011) Selection of a cutoff value for real-time polymerase chain reaction results to fit a diagnostic purpose:

analytical and epidemiologic approaches. J Vet Diagn Invest 23:2–15

Daskin JH, Alford RA, Puschendorf R (2011) Short-term exposure to warm microhabitats could explain amphibian persistence with *Batrachochytrium dendrobatidis.* PLoS ONE 6:e26215

Daszak P, Streiby A, Cunningham AA, Longcore JE, Brown CC, Porter D (2004) Experimental evidence that the bullfrog (*Rana catesbeiana*) is a potential carrier of chytridiomycosis, an emerging fungal disease of amphibians. Herpetol J 14:201–207

Daszak P, Faggioni C, Scott DE, Gibbons JW, Porter D (2005) Chytridiomycosis does not cause population declines in long-term monitored amphibian metapopulations at the Savannah River Site. Ecology 86:3232–3237

Davic RD, Welsh HH Jr (2004) On the ecological roles of salamanders. Annu Rev Ecol Evol Syst 35:405–434

Davis AK, Yabsley MJ, Keel MK, Maerz JC (2007) Discovery of a novel alveolate pathogen affecting southern leopard frogs in Georgia: description of the disease and host effects. EcoHealth 4:310–317

Dodd CK Jr (2004) The amphibians of Great Smoky Mountains National Park. University of Tennessee Press, Knoxville, TN

Duffus ALJ, Pauli BD, Wozney K, Brunetti CR, Berrill M (2008) Frog virus 3-like infections in aquatic amphibian communities. J Wildl Dis 44:109–120

Gahl MK, Calhoun AJK (2008) Landscape setting and risk of *Ranavirus* mortality events. Biol Conserv 141:2679–2689

Gervasi S, Gondhalekar C, Olson DH, Blaustein AR (2013) Host identity matters in the amphibian–*Batrachochytrium dendrobatidis* system: Fine-scale patterns of variation in responses to a multi-host pathogen. PLoS ONE 8: e54490

Gill DE (1978) The metapopulation ecology of the red-spotted newt, *Notophthalmus viridescens* (Rafinesque). Ecol Monogr 48:145–166

Glenney GW, Julian JT, Quartz WM (2010) Preliminary amphibian health survey in the Delaware Water Gap National Recreation Area. J Aquat Anim Health 22: 102–114

Gray MJ, Miller DL, Schmutzer AC, Baldwin CA (2007) *Frog virus 3* prevalence in tadpole populations at cattle-access and non-access wetlands in Tennessee, USA. Dis Aquat Org 77:97–103

Gray MJ, Miller DL, Hoverman JT (2009) Ecology and pathology of amphibian ranaviruses. Dis Aquat Org 87: 243–266

Gray MJ, Miller DL, Hoverman JT (2012) Reliability of nonlethal surveillance methods for detecting ranavirus infection. Dis Aquat Org 99:1–6

Grayson KL, Wilbur HM (2009) Sex- and context-dependent migration in a pond-breeding amphibian. Ecology 90: 306–312

Green DE, Converse KA, Schrader AK (2002) Epizootiology of sixty-four amphibian morbidity and mortality events in the USA, 1996–2001. Ann NY Acad Sci 969:323–339

Green DE, Gray MJ, Miller DL (2010) Disease monitoring and biosecurity. In: Dodd CK Jr (ed) Amphibian ecology and conservation: A handbook of techniques. Oxford University Press, New York, NY, p 481–505

Groner ML, Relyea RA (2010) *Batrachochytrium dendrobatidis* is present in northwest Pennsylvania, USA, with high prevalence in *Notophthalmus viridescens.* Herpetol Rev 41:462–465

Haislip NA, Gray MJ, Hoverman JT, Miller DL (2011) Development and disease: How susceptibility to an emerging pathogen changes through anuran development. PLoS ONE 6:e22307

Haislip NA, Hoverman JT, Miller DL, Gray MJ (2012) Natural stressors and disease risk: does the threat of predation increase amphibian susceptibility to ranavirus? Can J Zool 90:893–902

Harris RN (1987) An experimental study of population regulation in the salamander, *Notophthalmus viridescens dorsalis* (Urodela: Salamandridae). Oecologia 71: 280–285

Hoverman JT, Gray MJ, Miller DL (2010) Anuran susceptibilities to ranaviruses: role of species identity, exposure route, and a novel virus isolate. Dis Aquat Org 89:97–107

Hoverman JT, Gray MJ, Haislip NA, Miller DL (2011) Phylogeny, life history, and ecology contribute to differences in amphibian susceptibility to ranaviruses. EcoHealth 8: 301–319

Hoverman JT, Gray MJ, Miller DL, Haislip NA (2012) Widespread occurrence of ranavirus in pond-breeding amphibian populations. EcoHealth 9:36–48

Hurlbert SH (1970) The post-larval migration of the red-spotted newt *Notophthalmus viridescens* (Rafinesque). Copeia 1970:515–528

Hyatt AD, Boyle DG, Olsen V, Boyle DB and others (2007) Diagnostic assays and sampling protocols for the detection of *Batrachochytrium dendrobatidis.* Dis Aquat Org 73:175–192

Jennette MA (2010) *Lithobates sylvaticus* (wood frog) egg predation. Herpetol Rev 41:476–477

Kik M, Martel A, Spitzen-van der Sluijs A, Pasmans F, Wohlsein P, Gröne A, Rijks JM (2011) Ranavirus-associated mass mortality in wild amphibians, The Netherlands, 2010: A first report. Vet J 190:284–286

Kilpatrick AM, Briggs CJ, Daszak P (2010) The ecology and impact of chytridiomycosis: an emerging disease of amphibians. Trends Ecol Evol 25:109–118

Kriger KM, Hero JM (2006) Large-scale seasonal variation in the prevalence and severity of chytridiomycosis. J Zool 271:352–359

Landsberg JH, Kiryu Y, Tabuchi M, Waltzek TB and others (2013) Co-infection by alveolate parasites and frog virus 3-like ranavirus during an amphibian larval mortality event in Florida, USA. Dis Aquat Org 105:89–99

Lenker MA, Savage AE, Becker CG, Rodriguez D, Zamudio KR (2014) *Batrachochytrium dendrobatidis* infection dynamics vary seasonally in upstate New York, USA. Dis Aquat Org 111:51–60

MacCracken JG, Stebbings JL (2012) Test of a body condition index with amphibians. J Herpetol 46:346–350

Mao J, Tham TN, Gentry GA, Aubertin A, Chinchar VG (1996) Cloning, sequence analysis, and expression of the major capsid protein of the iridovirus frog virus 3. Virology 216:431–436

Mao J, Hedrick RP, Chinchar VG (1997) Molecular characterization, sequence analysis, and taxonomic position of newly isolated fish iridoviruses. Virology 229:212–220

Martel A, Blooi M, Adriaensen C, Van Rooij P and others (2014) Recent introduction of a chytrid fungus endangers Western Palearctic salamanders. Science 346:630–631

Miller D, Gray M, Storfer A (2011) Ecopathology of ranaviruses infecting amphibians. Viruses 3:2351–2373

Morin PJ (1983) Predation, competition, and the composition of larval anuran guilds. Ecol Monogr 53:119–138

Murray KA, Skerratt LF, Speare R, McCallum H (2009) Impact and dynamics of disease in species threatened by the amphibian chytrid fungus, *Batrachochytrium dendrobatidis.* Conserv Biol 23:1242–1252

Olson DH, Aanensen DM, Ronnenberg KL, Powell CI and others (2013) Mapping the global emergence of *Batrachochytrium dendrobatidis*, the amphibian chytrid fungus. PLoS ONE 8:e56802

Ouellet M, Mikaelian I, Pauli BD, Rodrigue J, Green DM (2005) Historical evidence of widespread chytrid infection in North American amphibian populations. Conserv Biol 19:1431–1440

Peig J, Green AJ (2009) New perspectives for estimating body condition from mass/length data: the scaled mass index as an alternative method. Oikos 118:1883–1891

Petranka JW (1998) Salamanders of the United States and Canada. Smithsonian Institution Press, Washington, DC

Petranka JW, Harp EM, Holbrook CT, Hamel JA (2007) Long-term persistence of amphibian populations in a restored wetland complex. Biol Conserv 138:371–380

Picco AM, Brunner JL, Collins JP (2007) Susceptibility of the endangered California tiger salamander, *Ambystoma californiense*, to ranavirus infection. J Wildl Dis 43:286–290

Piotrowski JS, Annis SL, Longcore JE (2004) Physiology of *Batrachochytrium dendrobatidis*, a chytrid pathogen of amphibians. Mycologia 96:9–15

Pitt AL, Allard CM, Hawley JE, Baldwin RF, Brown BL (2011) *Lithobates sylvaticus* (wood frog) predation. Herpetol Rev 42:263

Price SJ, Garner TWJ, Nichols RA, Balloux F, Ayres C, de Alba AMC, Bosch J (2014) Collapse of amphibian communities due to an introduced ranavirus. Curr Biol 24: 2586–2591

Rachowicz LJ, Vredenburg VT (2004) Transmission of *Batrachochytrium dendrobatidis* within and between amphibian life stages. Dis Aquat Org 61:75–83

Raffel TR, Rohr JR, Kiesecker JM, Hudson PJ (2006) Negative effects of changing temperature on amphibian immunity under field conditions. Funct Ecol 20:819–828

Raffel TR, Michel PJ, Sites EW, Rohr JR (2010) What drives chytrid infections in newt populations? Associations with substrate, temperature, and shade. EcoHealth 7:526–536

Raffel TR, Halstead NT, McMahon TA, Davis AK, Rohr JR (2015) Temperature variability and moisture synergistically interact to exacerbate an epizootic disease. Proc R Soc B 282:20142039

Reeve BC, Crespi EJ, Whipps CM, Brunner JL (2013) Natural stressors and ranavirus susceptibility in larval wood frogs (*Rana sylvatica*). EcoHealth 10:190–200

Richter SC, Drayer AN, Strong JR, Kross CS, Miller DL, Gray MJ (2013) High prevalence of *Ranavirus* infection in permanent constructed wetlands in eastern Kentucky, USA. Herpetol Rev 44:464–466

Rohr JR, Park D, Sullivan AM, McKenna M, Propper CR, Madison DM (2004) Operational sex ratios in newts: field responses and characterization of a constituent chemical cue. Behav Ecol 16:286–293

Rosenblum EB, Fisher MC, James TY, Stajich JE, Longcore JE, Gentry LR, Poorten TJ (2010) A molecular perspective: biology of the emerging pathogen *Batrachochytrium dendrobatidis.* Dis Aquat Org 92:131–147

Rothermel BB, Walls SC, Mitchell JC, Dodd CK Jr and others (2008) Widespread occurrence of the amphibian chytrid fungus *Batrachochytrium dendrobatidis* in the southeastern USA. Dis Aquat Org 82:3–18

Russell DM, Goldberg CS, Sprague L, Waits LP, Green DE, Schuler KL, Rosenblum EB (2011) Ranavirus outbreaks in amphibian populations of northern Idaho. Herpetol Rev 42:223–225

Stöhr AC, Fleck J, Mutschmann F, Marschang RE (2013) Ranavirus infection in a group of wild-caught Lake Urmia newts *Neurergus crocatus* imported from Iraq into Germany. Dis Aquat Org 103:185–189

Strauss A, Smith KG (2013) Why does amphibian chytrid (*Batrachochytrium dendrobatidis*) not occur everywhere? An exploratory study in Missouri ponds. PLoS ONE 8:e76035

Sztatecsny M, Gallauner A, Klotz L, Baierl A, Schabetsberger R (2013) The presence of common frogs (*Rana temporaria*) increases the body condition of syntopic Alpine newts (*Ichthyosaura alpestris*) in oligotrophic high-altitude ponds: benefits of high-energy prey in a low-productivity habitat. Ann Zool Fenn 50:209–215

Todd-Thompson M (2010) Seasonality, variation in species prevalence, and localized disease for *Ranavirus* in Cades Cove (Great Smoky Mountains National Park) amphibians. Master's thesis, University of Tennessee, Knoxville, TN

Venesky MD, Parris MJ, Altig R (2010) Pathogenicity of *Batrachochytrium dendrobatidis* in larval ambystomatid salamanders. Herpetol Conserv Biol 5:174–182 http://www.herpconbio.org/Volume_5/Issue_2/Venesky_etal_2010.pdf

Voyles J, Young S, Berger L, Campbell C and others (2009) Pathogenesis of chytridiomycosis, a cause of catastrophic amphibian declines. Science 326:582–585

Walters B (1975) Studies of interspecific predation within an amphibian community. J Herpetol 9:267–279

Warne RW, Crespi EJ, Brunner JL (2011) Escape from the pond: Stress and developmental responses to ranavirus infection in wood frog tadpoles. Funct Ecol 25:139–146

Woodhams DC, Alford RA, Marantelli G (2003) Emerging disease of amphibians cured by elevated body temperature. Dis Aquat Org 55:65–67

Zar JH (1999) Biostatistical analysis, 4th edn. Prentice Hall, Upper Saddle River, NJ

Editorial responsibility: Louise Rollins-Smith, Nashville, Tennessee, USA

Submitted: May 28, 2015; Accepted: January 6, 2016
Proofs received from author(s): February 5, 2016