Herpetological Review, 2023, 54(2), 204–207.

No Detection of Ranaviruses nor Chytrids Among Salamanders and Newts in Algonquin Provincial Park, Ontario, Canada

In the midst of the current amphibian extinction crisis, pathogen surveillance is crucial to limit disease-driven population declines (DiRenzo and Grant 2019). Ranaviruses (RV) and chytrid fungi being the primary pathogens associated with mass-mortality events (Blaustein et al. 2012; Cunningham 2018), the regular assessment of their distribution is essential to inform amphibian conservation management and promptly respond to introduction events (Mörner et al. 2002; Weldon et al. 2013; Grear et al. 2021). For example, although the salamander chytrid fungus *Batrachochytrium salamandrivorans* (*Bsal*) is currently geographically restricted to Asia and Europe (Martel et al. 2014; Castro Monzon et al. 2022), it is intensively screened for in North-America (Gray et al. 2015, 2023; Waddle et al. 2020; Koo et al. 2021; Castro Monzon et al. 2022; Crawshaw et al. 2022; North American *Bsal* Task Force 2022) as international trade and suitable climatic conditions for the fungus put this region at high risk of a *Bsal* invasion (Yap et al. 2017; Gray et al. 2023). Even for widely distributed pathogens such as RV and the chytrid fungus *B. dendrobatidis* (*Bd*), which are present on all continents where amphibian hosts occur (Fisher et al. 2009; Duffus et al. 2015), regular surveillance is important to further our understanding of disease dynamics and to reassess extinction risk for susceptible populations (Langwig et al. 2015).

Here, we participate in these monitoring efforts by screening Canadian caudates for RV, *Bd* and *Bsal* at two locations within Algonquin Provincial Park, Ontario, Canada: Bat Lake (BL), situated on a public trail and Lost Ray Lake (LL), a remote site within the Algonquin Wildlife Research Station area. Conducting pathogen surveillance in caudates rather than anuran models allows simultaneously testing for the presence of RV, *Bd* and *Bsal*, since this latter fungus is lethal to salamander hosts (Martel et al. 2014; Gray et al. 2023). Previous pathogen surveillance efforts conducted at BL in 2005 had revealed a prevalence of 6% of RV and no *Bd* in Green Frogs (*Lithobates clamitans*; St-Amour et al. 2008) but had not investigated the presence of *Bsal* among salamanders since this fungus was undescribed until 2013 (Martel et al. 2013). Considering that *Bd* was already present in Ontario at the time of this initial sampling (Ouellet et al. 2005), including 48-km away from our study sites where it was associated with a die-off of American Bullfrog (*Lithobates catesbeianus*) tadpoles (Charbonneau et al. 2005), and that the park is visited by over a million people yearly (Ontario Parks 2022), it is plausible that *Bd* may have spread into BL since the survey of St-Amour et al. (2008). Furthermore, although a more recent screening conducted as part of microbiota-focused research found Spotted Salamander (*Ambystoma maculatum*) larvae free of pathogens in both BL and LL (Fieschi-Méric et al. 2023), amphibian larvae have very little keratinized epidermis, which may limit the colonizable area for fungi (Keitzer et al. 2011; Van Rooij et al. 2015); therefore, screening adult life stages, with more keratinized tissue, should have a greater likelihood of detecting chytrids (Brunelli et al. 2015).

We herein conducted field surveillance for RV, *Bd* and *Bsal* among native caudate hosts from BL and LL at various life-history stages susceptible to RV and/or to chytrids: recently metamorphosed Eastern Newts (*Notophthalmus viridescens*; Fig. 1A), adult Spotted Salamanders (Fig. 1B) and adult Blue-spotted Salamanders (*Ambystoma laterale;* Fig. 1C). Sub-clinical infections with RV and *Bd* have been reported in natural populations of Eastern Newts (Rothermel et al. 2016), whereas infection trials showed that *Bsal* can be particularly deadly to juvenile stages of that species (Carter et al. 2021). Spotted Salamanders can be infected with *Bd* (Lenker et al. 2014) and with RV without apparent signs of disease (Duffus et al. 2008), and previous research suggests they may be resistant to *Bsal* (Martel et al. 2014; DiRenzo et al. 2021). Blue-spotted Salamanders can be infected with RV and *Bd* (Standish et al. 2018) but their susceptibility to *Bsal* is unknown.

Between May and August 2019, we screened 152 different individuals (Table 1) in BL (45.58611°N, 78.51861°W; 400 m elev.) and LL (45.59028°N, 78.53694°W; 417 m elev.). Terrestrial adults were caught during their reproductive migration towards their breeding lake, along a drift-fence already in place at BL (Moldowan et al. 2019). Aquatic adults were captured in their reproductive phase with minnow-traps. Individuals sampled during their terrestrial migration were maintained in mesocosms for the purpose of another study, and as a consequence were not recaptured during the aquatic phase. Recently metamorphosed juvenile Eastern Newts were caught during their migration away from LL, under logs found along the shore. Each individual was held with a new pair of nitrile gloves and was skin-swabbed (MW100 rayon tipped dry swabs, MWE) with 10 strikes back-and-forth on the ventrum, five on each side of the tail, five on each side of the back, and five rolls on each hand and foot. All swabs were preserved dry, on ice during sampling in the field, at -25°C upon arrival in the Algonquin Wildlife Research Station facilities, and at -80°C after being transported to our laboratory. All the equipment was disinfected with a 3% solution of Virkon®S (DuPont, Wilmington, DE, USA) after each use as a biosecurity measure (Olson et al. 2021). DNA was extracted from

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A) PHOTO BY M. DENÔEL; B, C) PHOTO BY L. FIESCHI-MÉRIC

FIG. 1. Study species at the sites and life-stage tested in this disease screening: A) a juvenile Eastern Newt at Lost Ray Lake; B) an adult Spotted Salamander at Bat Lake; C) an adult Blue-spotted Salamander at Bat Lake. Both sites are within Algonquin Provincial Park, Ontario, Canada.

TABLE 1. Number of individuals (N) of each life stage and species sampled in this survey. The sites (Site; BL = Bat Lake; LL = Lost Ray Lake) and the date of capture (Date) are also indicated. Both sites are within Algonquin Provincial Park, Ontario, Canada.

Species	Life stage	N	Date	Site
Ambystoma maculatum	Adult, terrestrial	30	May 2019	BL
	Adult, aquatic	30		
Ambystoma laterale	Adult, terrestrial	30	May 2019	BL
	Adult, aquatic	22		
Notophthalmus viridescens	Juvenile	40	Aug 2019	LL

the swabs using the DNeasy PowerSoil Pro kit (QIAGEN) and was amplified through quantitative PCR (qPCR) performed on an Mx3005P Real-Time PCR System (Agilent Technologies, USA). Simplex assays were carried out for each pathogen, following protocols to detect the MCP gene of ranaviruses (Leung et al. 2017), the ITS1 rRNA gene of *Bd* (Kriger et al. 2006) and the 5.8S rRNA gene of *Bsal* (Blooi et al. 2013). Because we expected low prevalence of chytrids, samples were pooled by five for *Bd* and for *Bsal* detection (Sabino-Pinto et al. 2019). Five concentrations (1, 10, 10^3, 10^5 and 10^{10} genomic equivalents per 1µL) of gBlocks (IDT) synthetic standards designed as in Standish et al. (2018) and three negative controls were included in each reaction plate to obtain the calibration curve. Samples were tested in duplicate reactions, randomly assigned to different plates. A reaction was considered positive if it provided an amplification signal above the cycle threshold (CT). In the event that results from duplicates were inconsistent, a third reaction was run and concluded from (Sabino-Pinto et al. 2018). The prevalence of each pathogen at each site was estimated using the 'rule of three', which states that if none of *N* individuals is positive to a pathogen, true prevalence is at most 3/*N* with 95% confidence (Hanley and Lippman-Hand 1983; Skerratt et al. 2008).

We did not detect RV, *Bd* nor *Bsal* in any of the 152 samples tested, indicating that the prevalence of these pathogens was below 7.5% in juvenile Eastern Newts from Lost Ray Lake, below 6% in adult Blue-spotted Salamanders from Bat Lake and below 5% in adult Spotted Salamanders from Bat Lake with 95% confidence at the time of our study. For 10 samples, one of the qPCR duplicates provided an amplification signal above the cycle threshold (four *Bd* and six RV reactions), so a triplicate reaction was run. Each of these third reactions was negative and the pathogen concentrations initially detected in the putative positive reactions were very low (below 4 genomic equivalents per 1µL DNA extract), so we concluded that these samples were negative. These results suggest that RV and chytrids are either absent from the sample sites or present at an extremely low prevalence.

While encouraging, our findings should be conservatively interpreted. First, species-specific life history traits can influence susceptibility to infection (Daszak et al. 2003; Hoverman et al. 2011) and thus alter pathogen detectability; in this study, terrestrial salamanders returning to BL may have been less likely to harbour pathogens because diseased individuals had a lower probability to survive overwintering (Rumschlag and Boone 2018; Wetsch et al. 2022). Moreover, given the phenology of our pathogens of interest, a larger sampling time-window would have maximized the probability to come across periods of highest occurrence of RV or chytrids; indeed, RV are associated with rapid and brief epidemics (Brunner et al. 2015) and the detectability of chytrid fungi is often lowered in summer months because their growth is temperature-dependent (Savage et al. 2011; Sapsford et al. 2013). Since virions may persist in other herpetofaunal, osteichthyan or even environmental reservoirs (Gray et al. 2009), it is possible that RV did not completely disappear from BL since its detection in 2005 (St-Amour et al. 2008). In fact, while Green Frogs were present in both of our study sites and showed no apparent signs of disease, they should be tested for RV as a follow up to that previous study (St-Amour et al. 2008). Moreover, this previous screening was based on liver samples, a more sensitive method than our non-invasive skin swabbing, especially when infection intensity is low (Gray et al. 2012). Further screenings during different seasons, in other host species and with different sample types are therefore necessary to disentangle potential seasonal variations in pathogen abundance and detectability from actual changes in pathogen distribution at BL. Conversely, given its remoteness, it is likely that RV and chytrids have not spread to the LL site yet. Nevertheless, regular disease screenings and most particularly *Bsal* surveillance are important efforts to continue for the conservation of North-American salamanders amidst the current fungal panzootic that currently threatens amphibians (Waddle et al. 2020; Crawshaw et al. 2022).

Acknowledgments.—We thank Noah Loiselle and César Doublet for their assistance in the field, and more generally all scientists from the Algonquin Wildlife Research Station for their support. We are grateful to Jake Kerby for his guidance on qPCR protocols and thank researchers from the Living With Lakes Center for access to their qPCR machine. At the time of the study, LFM was a Ph.D. student

funded by the ReNewZoo program through an NSERC Create Grant. MD is a Research Director at F.R.S.-FNRS (Fonds de la Recherche Scientifique). Our protocol was conducted with the approval of the Ontario Ministry of Natural Resources (license #1093589) and of Laurentian University's Animal Care Committee (protocol #2019-02-01).

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