peptides that inhibit Bd growth have been shown to occur in ranid frogs (Rollins-Smith et al. 2002). However, *L. sphenophalus* is highly aquatic and therefore may be more exposed to Bd.

Although examination of seasonal patterns of infection is limited by our low sample sizes and haphazard sampling design, of the two species (*A. crepitanus* and *L. sphenophalus*) that were sampled during both spring (May) and summer (July), *L. sphenophalus* sampled in May (N = 8) were all Bd-positive whereas those sampled in July (N = 5) were all Bd-negative. Seasonal variation in infection level has been reported previously, and may be due to the more unfavorable conditions (decreased humidity, increased temperature) for Bd or the potential for individual amphibians to increase their body temperature to clear themselves of the infection during the summer (Berger et al. 2004; Ribas et al. 2009; Richards-Zawacki 2010).

When all individuals were pooled for analysis by habitat type, there tended to be a difference in infection rate among animals captured in different habitats (lake, wetland, creek: χ² test of independence; χ² = 0.49, p = 0.058). Amphibians captured in wetlands showed the highest infection rate (49.1%). Wetland habitat was used for breeding by all anurans within the community, likely increasing the chance for both Bd occurrence and transmission (Kriger and Hero 2007). Although our sample sizes were relatively low, restricting comparisons among habitats within species, the species with the greatest sample in multiple habitats, *A. crepitanus*, showed no difference in infection level between habitats (χ² test of independence; χ² = 4.91, p = 0.18).

Our data represent a baseline starting point for understanding Bd infection levels in this area. Increased sampling and both seasonal and annual monitoring is needed to better understand patterns of infection.

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**Detection of Ranavirus in Ohio, USA**

Of the many proximal factors that potentially contribute alone or in combination to the global concern about amphibian population declines (e.g., Stuart et al. 2004), habitat conversion (e.g., Homan et al. 2004) and infectious diseases (e.g., Daszak et al. 2003; Miller et al. 2011) are dominant concerns. To examine the differential patterns of habitat use for adult amphibians, the amphibian community of Taylor-Ochs Pond, Ohio, USA (40.0538°N, 82.3105°W) has been extensively studied since Spring 2005 (Homan et al. 2008, 2010). This 0.1-ha temporary pond supports breeding populations of *Ambystoma maculatum* (Spotted Salamanders), *Lithobates sylvaticus* (Wood Frogs), and *Anaxyrus americanus* (American Toads). As part of our long-term study, we have surveyed the pond by dip-netting weekly each summer since 2005. Prior to the summer of 2012, the only mass mortality of larval amphibians witnessed was clearly associated with early drying of the pond. However, in late May 2012, a time in which the pond had a suitably high water level, routine dip-netting of Taylor-Ochs Pond revealed evidence of mass mortality among larval amphibians. Larval individuals of four species, *A. maculatum*, *L. sylvaticus*, *A. americanus*, and, uncharacteristically, *Lithobates clamitans* (Green Frogs), were present in the pond at that time. Although we did not attempt to formally

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**LITERATURE CITED**


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quantify the magnitude of mortality in the pond, we estimate that hundreds of *A. maculatum*, *L. clamitans*, and *L. sylvaticus* showed signs of illness in the field; only *A. americanus* seemed unaffected.

We suspected ranavirus might have been associated with and contributed to the high mortality based on the signs of individuals, which included hemorrhaging, swelling, erythema, and lethargy (Miller et al. 2011). To determine whether ranavirus was present, on 24 May 2012 we haphazardly collected five each of *A. maculatum* and *A. americanus* larvae, and seven each of *L. clamitans* and *L. sylvaticus* larvae from Taylor-Ochs Pond using dip-nets. Each animal was classified as either moribund (presence of any gross signs consistent with ranavirus infection, as described above) or apparently well (no signs present) at the time of capture. Each animal was bagged individually at the pond, euthanized using MS-222 (2.5 g/L buffered to a pH of 7), frozen individually at −80°C, and screened for ranavirus as described below.

Additionally, on 29 and 30 May 2012, we haphazardly collected 49 apparently healthy *A. americanus* larvae from Taylor-Ochs Pond with dip-nets and brought them back to the laboratory. Given their apparent health in the field, we were interested in carefully monitoring individuals over time to document whether they experienced mortality and whether or not that mortality was associated with ranavirus infection. Larvae were housed individually in 500-mL plastic cups with approximately 300 mL of water. Water, either aged tap water or a blend of aged tap and Taylor-Ochs Pond water, was changed every three days. We varied the water source because we originally intended to look for effects of continued exposure to pond water on the mortality and infection status of the larvae; however, the infection rates were the same among the treatments, so we pooled the data to look only at infection and survival. Larvae were fed Tetra fish food every three days *ad libitum*, and were kept in the laboratory at room temperature (21–24°C) in a natural light:dark cycle. Larvae were kept for a period of 21 days. If an animal metamorphosed before the end of that period, it was euthanized and frozen as described above. If a pre-metamorphic animal died before the end of the study, it was bagged individually and frozen at −80°C. All surviving animals at day 21 were euthanized and frozen as above. At the completion of the study, all animals were tested for ranavirus infection, as described below.

In late June, after the confirmation of ranavirus in Taylor-Ochs Pond (see below), we sampled one pond directly upstream of Taylor-Ochs (Griesse Pond) and four ponds downstream of Taylor-Ochs Pond (Gurvis, Olde Minnow, Spring Peeper, and Wood Duck Ponds, Fig. 1). Griesse Pond is a permanent pond that directly feeds Taylor-Ochs Pond via a single stream channel. It has a surface area of ~1.5 ha, and is 200 m upstream of Taylor-Ochs Pond. Gurvis Pond is a permanent pond that directly feeds Taylor-Ochs Pond via a single stream channel. It has a surface area of ~1.5 ha, and is 200 m upstream of Taylor-Ochs Pond. Olde Minnow Pond is a permanent pond with an area of ~0.1 ha and is 684 m downstream of Taylor-Ochs Pond; Spring Peeper Pond is a temporary pond with an area of ~0.1 ha and is 783 m downstream of Taylor-Ochs Pond; and Wood Duck Pond is a temporary pond with an area of ~0.1 ha and is 783 m downstream of Taylor-Ochs Pond. Given their apparent health in the field, we were interested in carefully monitoring individuals over time to document whether they experienced mortality and whether or not that mortality was associated with ranavirus infection. Larvae were housed individually in 500-mL plastic cups with approximately 300 mL of water. Water, either aged tap water or a blend of aged tap and Taylor-Ochs Pond water, was changed every three days. We varied the water source because we originally intended to look for effects of continued exposure to pond water on the mortality and infection status of the larvae; however, the infection rates were the same among the treatments, so we pooled the data to look only at infection and survival. Larvae were fed Tetra fish food every three days *ad libitum*, and were kept in the laboratory at room temperature (21–24°C) in a natural light:dark cycle. Larvae were kept for a period of 21 days. If an animal metamorphosed before the end of that period, it was euthanized and frozen as described above. If a pre-metamorphic animal died before the end of the study, it was bagged individually and frozen at −80°C. All surviving animals at day 21 were euthanized and frozen as above. At the completion of the study, all animals were tested for ranavirus infection, as described below.

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downstream; Wood Duck Pond is a semi-permanent pond with an area of ~0.2 ha and is 806 m downstream of Taylor-Ochs pond (Fig. 1). Surface areas of ponds and distances between ponds were determined using ImageJ software and satellite photos (Schneider et al. 2012). To avoid potential cross-contamination among ponds, equipment was disinfected with a 10% bleach solution between ponds. In all cases, larvae in these ponds were sampled haphazardly (see Table 1 for sample sizes). We could not find any amphibian larvae in either Gurvis Pond or Wood Duck Pond, but one recent A. americanus metamorph was collected from the shore of Wood Duck Pond. All animals were euthanized and frozen as described above and tested for ranavirus. Individuals were kept isolated from one another throughout all procedures.

To screen for ranavirus, we extracted DNA from a combination of tissues from each animal (liver, interrenal glands, and the upper intestine, dissected with sterilized forceps and scissors) using the Qiagen DNeasy Blood and Tissue kit following the manufacturer’s instructions (QIAGEN Inc, Valencia, California, USA). We ground up the entirety of very small individuals in 2-μL screw cap tubes filled with a small volume of 1.0-mm diameter silicon-carbide sharp particles (BioSpec Products, Bartlesville, Oklahoma, USA), and then topped up with tissue lysis buffer. These individuals were then homogenized for 45 s using a Mini-BeadBeater-16 (BioSpec Products, Bartlesville, Oklahoma) and DNA was extracted from a 200-μL aliquot of the homogenate. The concentration of extracted DNA was measured using a NanoDrop-2000 (Thermo-Scientific) and, if necessary, diluted to approximately 20ng DNA/μL with elution buffer.

Extracted DNA from each sample was screened for ranavirus in triplicate 20-μL reactions on 96-well plates on a StepOnePlus (Applied Biosystems, Foster City, California, USA) with 5 μL of DNA template (~100ng) using a Taqman realtime polymerase chain reaction (qPCR) with primers and probe that amplify a 70-bp region within the major capsid protein of all known ranaviruses (Brunner and Collins 2009). The ~500 pb product was sequenced with an ABI 3730 (Applied Biosystems, Foster City, California, USA) and compared with published ranavirus sequences on GenBank using a BLAST search.

We found that all 24 specimens initially collected from Taylor-Ochs Pond were infected with ranavirus (Fig. 2). We successfully isolated a Ranavirus from two moribund frogs. The sequence of the MCP gene of these viruses was >99% identical to the type ranavirus frog virus 3 (FV3). To examine the patterns of log_{10} viral quantities among the four amphibian species in Taylor-Ochs Pond, we ran a two-factor ANOVA to look for effects of species, condition, and any interaction between the two (JMP 10.0, 2012). Excluding A. americanus, none of which were moribund, moribund tadpoles had a much higher titer than those that were apparently well (F_{0,9} = 17.5, P < 0.001). Although sample sizes were small, L. clamitans had a significantly higher mean virus titer than A. maculatum, A. americanus, and L. sylvaticus among apparently well individuals (F_{1,5} = 13.5, P = 0.003; Tukey HSD, P < 0.05), whereas there was no significant difference in virus titers among moribund individuals of different species (F_{2,10} = 1.8, P = 0.22, Fig. 2).

Among the 49 A. americanus larvae that were maintained in the laboratory, ranavirus DNA was detected in eight. We generated Kaplan-Meier Survival Curves and used log-rank tests (JMP 10.0, 2012) to look for differences in survivorship between infected and uninfected animals, as well as ANOVA to look for differences in the log_{10} viral titers among those that died or survived. Infected individuals had significantly lower survivorship than uninfected individuals (x^2 = 15.2, P < 0.0001; Fig. 3). Three of the
eight infected larvae survived to the end of the 21-day experiment; two of these survivors metamorphosed during the study. Among the larvae that were infected, there was no difference in virus titer between those that died during the study (mean ± SD = 3.67 ± 3.45 log_{10} pfu) and those that survived (mean ± SD = 0.90 ± 0.53 log_{10} pfu; t = -1.76, P = 0.22), nor was there a difference in virus titer between the surviving metamorphs (mean ± SD = 1.0 ± 0.66 log_{10} pfu) and the others (mean ± SD = 3.16 ± 3.34 log_{10} pfu; t = -0.85, P = 0.43). However, our statistical power was low for both tests (Power = 0.21 and 0.11, respectively).

In our upstream and downstream sampling, we did not detect ranavirus in the four *A. maculatum* larvae found in Griesse Pond, upstream of Taylor-Ochs, but we did find ranavirus downstream, in *L. catesbeianus* from Olde Minnow Pond and in *A. maculatum* and *L. clamitans*, but not *Hyla versicolor*, from Spring Peeper Pond (Table 1). Given our small sample sizes it is not possible to exclude ranavirus occurrence from any site or make meaningful comparisons in prevalence among ponds or species.

In the summer of 2012, Taylor-Ochs Pond in Granville, Ohio experienced a mass die-off event concurrent with the confirmed presence of an FV3-like ranavirus. To our knowledge, this is the first published report of ranavirus in Ohio although ranviruses have been confirmed in other nearby states, including Illinois and Wisconsin (J. Sekowska, pers. comm.), New York (Brunner et al. 2011), Pennsylvania (Glenney et al. 2010), and Tennessee (Gray et al. 2009; Green et al. 2002). While we cannot be sure that ranavirus caused the observed mortality event, the prevalence of infection among *A. maculatum, L. sylvaticus*, and *L. clamitans*, each of which experienced high levels of mortality, as well as the positive relationship between infection and mortality in the laboratory-housed *A. americanus*, suggest that ranavirus likely played a role. In addition to finding ranavirus in our focal pond, we also confirmed its presence in two downstream ponds. Across these three ponds, we identified ranavirus infection among *A. maculatum, L. catesbeianus, L. clamitans, L. sylvaticus*, and *A. americanus*, all of which have had documented ranavirus infections previously (as reviewed by Miller et al. 2011). Whether *H. versicolor* was infected and we simply did not have the sample size to detect it remains unknown, although ranavirus infection in this species has been documented elsewhere (Duffus et al. 2008).

Among the larvae initially sampled from Taylor-Ochs Pond, ranavirus titers were higher in moribund animals than in apparently healthy animals, which is consistent with the finding of Brunner et al. (2005) that higher viral doses are associated with more rapid expression of symptoms. Interestingly, apparently well *L. clamitans*, a species not found in Taylor-Ochs in the preceding seven years, had viral titers that were higher than those of the other species. We hypothesize that a large spring rain event that occurred in 2012 brought the *L. clamitans* larvae to Taylor-Ochs from the upstream Griesse Pond. Although we cannot confirm that *L. clamitans* larvae were the source of the Taylor-Ochs infection, their unusual presence in the community as well as their relatively high viral loads are consistent with this hypothesis. We plan to continue monitoring Taylor-Ochs Pond, as well as the upstream and downstream ponds to document further occurrence and potential impacts of continuing ranavirus presence. Understanding the role of potential co-infection of *Batrachochytrium dendrobatidis*, or another pathogen, in larval health is a secondary objective for future monitoring.

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**Literature Cited**


