Vol. 121: 97–104, 2016
doi: 10.3354/dao03046

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published September 26

Seasonal infection rates of *Batrachochytrium dendrobatidis* in populations of northern green frog *Lithobates clamitans melanota* tadpoles

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ABSTRACT: Few studies have documented seasonal variation of *Batrachochytrium dendrobatidis* (*Bd*) infection rates in larval amphibians. We identified 4 natural populations of northern green frogs *Lithobates clamitans melanota* in Pennsylvania (USA) that contained *Bd*-infected tadpoles during post-wintering collections in May and June, after hibernating tadpoles had overwintered in wetlands. However, we failed to detect infected tadpoles at those wetlands when pre-wintering collections were made in late July through early September. We observed 2 cohorts of tadpoles that appeared to lack *Bd*-infected individuals in pre-wintering collections, yet contained *Bd*-infected individuals the following spring. We also observed 4 cohorts of pre-wintering tadpoles that were *Bd*-free, even though post-wintering tadpoles collected earlier in the year were infected with *Bd*. Our results suggest that tadpoles either reduce *Bd* infections during the summer months, and/or infections proliferate sometime prior to (or shortly after) tadpoles emerge from hibernation. It is unlikely that pre-wintering tadpoles were too small to detect *Bd* zoospores because (1) there was no correlation between *Bd* zoospore levels and tadpole size or stage, and (2) size was not a significant predictor of infection status. These results suggest that, while sampling larvae can be an effective means of collecting large sample sizes, investigators in our Mid-Atlantic region should conduct sampling by early summer to maximize the chances of detecting *Bd*. Further research is warranted to determine whether wetland topography and warm, shallow microhabitats within wetlands contribute to a population's ability to drastically reduce *Bd* prevalence prior to overwintering at ponds.

KEY WORDS: *Batrachochytrium dendrobatidis* · Chytrid fungus · Temporal · Amphibian · Disease prevalence

INTRODUCTION

Chytridiomycosis is an emergent infectious disease caused by the chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) that has resulted in population declines of hundreds of amphibian species (Daszak et al. 2003, Wake & Vredenburg 2008, Forzán et al. 2010). *Bd* has a global distribution, and has been detected in 48% of sites, 68% of countries, and 42% of species where samples have been tested (Olson et al. 2013). *Bd* infects keratinized areas in the outer layers of the epidermis in adult amphibians, while attaching mainly to mouthparts of anuran larvae (Berger et al. 2005, Searle et al. 2013). Chytridiomycosis results in excessive keratinization (toughening) of the skin of adults, thus disrupting electrolyte transport and ultimately causing death via cardiac arrest (Voyles et al. 2009). Larval amphibians do not suc-

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cumb to chytridiomycosis because they have little or no keratin in their skin (Marantelli et al. 2004). *Bd* has a thallus stage that grows inside skin cells, and thalli develop into zoosporangia that produce flagellated zoospores. Zoospores are the motile, waterborne stage that can disperse to other organisms or re-infect the same individual (Berger et al. 2005). Amphibians that harbor *Bd* without developing chytridiomycosis, such as bullfrogs *Lithobates catesbeianus* and green frogs *L. clamitans* (Greenspan et al. 2012), could be important reservoirs of *Bd* because they have relatively high infection intensities (Forzán et al. 2010), and their tadpoles have extended exposure to aquatic *Bd* zoospores because they overwinter in ponds for 1 or 2 yr. *Bd* has been detected on non-amphibian species such as Canada geese *Branta canadensis* and domestic geese *Anser anser domesticus* (Garmyn et al. 2012), as well as crayfish (*Procambarus* spp. and *Orconectes* spp.; McMahon et al. 2013, Brannelly et al. 2015). The ability for non-amphibian species to act as effective vectors, however, remains speculative.

Seasonal variation in *Bd* infections has been documented in the adult stages of several species of amphibian. In many populations of adult anurans, *Bd* prevalence is highest during cooler seasons of the year (Retallick et al. 2004, Woodhams & Alford 2005, Savage et al. 2011, Phillott et al. 2013, Korfel & Hetherington 2014, Bletz et al. 2015), and this pattern holds true for infection intensity (Whitfield et al. 2012) and mortality events (Berger et al. 2004). This pattern might be explained by temperature-dependent life history characteristics of *Bd*. Woodhams et al. (2008) found that *Bd* zoosporangia produced more zoospores at cooler temperatures (7–10°C, compared to 17–25°C), and both Woodhams et al. (2008) and Piotrowski et al. (2004) found that zoospores survived twice as long at cooler temperatures. In addition, hibernation during cooler temperatures can suppress amphibian immune systems (reviewed by Rollins-Smith & Woodhams 2011), and warmer temperatures have been shown to up-regulate the expression of genes responsible for producing antimicrobial peptides in *Bd*-infected frogs (Ribas et al. 2009).

In tadpoles of the midwife toad *Alytes obstetricans*, *Bd* prevalence and infection loads (mean zoospore copies ind.$^{-1}$) were lowest during the warmest moths of the year (Fernández-Beaskoetxea et al. 2015). By tracking *Bd* infections among tadpoles in 6 cattle troughs in Spain, those authors also found that *Bd* loads displayed a negative relationship with temperature. Furthermore, they found that short-term temperature trends (2–5 d) were more predictive of *Bd* loads than longer-term trends (10–30 d). Smith et al. (2007), however, failed to detect seasonal variation in *Bd* prevalence among *Amietia* (*Strongylopus*) *hymenopus* and *Hadromophryne* (*Heleophryne*) *natalensis* tadpole populations in South Africa, but they found that older (later-stage) tadpoles were more likely to be infected with *Bd*. We suspect that seasonal variation in prevalence could have been masked by the increased likelihood of infection among later-stage tadpoles. If, during all sampling periods, their tadpole populations included individuals from a wide range of developmental stages, they would be unlikely to detect seasonal variation in prevalence. This type of demographic structure could be expected because *A. hymenopus* is able to breed year-round (Minter et al. 2004), and *H. natalensis* remains in the tadpole stage for nearly 2 yr (Wager 1986, Minter et al. 2004). Thus, both species would likely have populations with multiple tadpole cohorts living sympatrically.

It is important to determine whether anuran larvae show seasonal variation in *Bd* prevalence because larvae are important life stages to sample in disease monitoring and inventory initiatives. Retallick et al. (2006) suggested that tadpoles can be easier to sample than adult anurans because they are often easier to capture, more conspicuous, and remain active at wetlands for a longer period of time. In our study, we examined how *Bd* prevalence changed in green frog tadpoles pre- and post-wintering. In 4 different populations, we collected mouth swab samples from tadpoles and used quantitative PCR (qPCR) to compare *Bd* prevalence and intensity during 2 different sampling periods. The first sampling period was in spring/early summer from individuals that had emerged from overwintering in ponds, while the second was in the late summer/early fall from tadpoles which had never overwintered in ponds (Table 1).

MATERIALS AND METHODS

Field collections

In 2013 and 2014, northern green frog *Lithobates clamitans melanota* tadpoles were collected from 4 populations located in human-created, permanently-flooded wetlands in Pennsylvania, USA. Emergent vegetation extended less than 3 m from each wetland's flooded perimeter, and the flooded surface areas of wetlands were smallest at Penn State University (PSU) Altoona (0.04 ha, Blair County), largest at Huntingdon (1.35 ha, Huntingdon County), and 0.33 ha and 0.47 ha, respectively, for Mowry (Blair

Table 1. Collection dates, sample sizes, and *Batrachochytrium dendrobatidis* (*Bd*) prevalence (Prev., in %) among pre- and post-wintering tadpole cohorts. PSU: Penn State University; nd: *Bd* not detected

Site	2012 cohort			2013 cohort						2014 cohort		
	Post-wintering			Pre-wintering			Post-wintering			Pre-wintering		
	Prev.	n	Date	Prev.	n	Date	Prev.	n	Date	Prev.	n	Date
PSU Altoona	8.9	45	23 May 2013	nd	15	1 Aug 2013	73.3	60	14 May 2014	nd	38	19 Sep 2014
Mowry		—			—		21.7	60	27 May 2014	nd	30	1 Oct 2014
Titusville		—		nd	60	24 Jul 2013	26.7	30	26 Jun 2014		—	
Huntingdon		—			—		95.0	60	19 May 2014	nd	45	26 Sep 2014

County) and Titusville (Crawford County). Perennial, herbaceous vegetation dominated the landscape within the 100 m surrounding each wetland, although 40% of Titusville was bordered by deciduous forest land cover (Fry et al. 2011).

The green frog is among the largest anurans in North America, and the species is distributed throughout the entire eastern coast from southern Canada through central Florida (Conant & Collins 1998). Its breeding season in the US Mid-Atlantic region (where our study occurred) is late spring through early summer, typically lasting from May through July (Lee 1973). Breeding occurs in permanent (and occasionally semi-permanent) ponds/wetlands throughout the range because tadpoles typically overwinter in ponds. In the US Mid-Atlantic region, the emergence of metamorphosed individuals occurs in June and July (Ryan 1953, Whitaker 1961, Wells 1976), although Ting (1951) showed that captive green frog tadpoles can complete metamorphosis without overwintering, and Martof (1956) reported a cohort of green frogs in the northern US (Michigan) that completed metamorphosis within 70 to 85 d.

Populations of tadpoles were sampled during 2 different times of the year: once after tadpoles had overwintered, and once prior to tadpoles overwintering. Post-wintering collections were made between May and June during the active egg-laying season, while pre-wintering collections were made in late July, August, and September after the majority of tadpoles from the previous year's cohort were expected to have metamorphosed (Table 1). The number of tadpoles collected at each site ranged from 15 to 60 individuals for each sampling event, with a mean (±SD) of 37.6 (± 16.8) tadpoles for pre-wintering samples and 51.0 (± 13.4) tadpoles for post-wintering samples. In a state-wide survey of 24 populations of green frog tadpoles throughout Pennsylvania using the same methodology as in this study, the mean prevalence of *Bd* was approximately 23% (J.T. Julian unpubl.). Therefore, we estimated that in a sample size of 30 tadpoles we would fail to detect *Bd* only 0.04% of the time ($p = [1.00 - 0.23]^{30}$), and recommendations by Skerratt et al. (2008) suggested that a sample size of 13 would be sufficient to detect *Bd* with 95% confidence given our estimated prevalence. Once collected, tadpoles were transported to laboratories at the campus of Penn State Altoona in a 5 gallon (~18.9 l) bucket filled with pond water. In the laboratory, tadpoles were euthanized with a lethal dose of MS-222, their Gosner stage and snout to vent lengths were recorded, and their mouthparts were swabbed with a sterile cotton swab with 5 rotations to collect *Bd* zoospores. Swabs were placed into 1.5 ml polypropylene centrifuge tubes and stored in a −16°C freezer until taken to the US Fish and Wildlife Service's Northeast Fisheries center in Lamar, PA, for DNA extraction and quantitative polymerase chain reaction (qPCR) analysis.

Tissue digestion, DNA extraction, and qPCR

DNA was extracted from cotton swabs following the Tissue DNA Protocol outlined in the Omega Mag-Bind® Tissue DNA KFA 96 kit (M6329-02) for DNA extraction using magnetic bead technology. Swab samples were placed in a mixture of tissue lysis buffer and Proteinase K, and incubated at 56°C on a shaker for 12 to 18 h. For each DNA extraction plate, we added a negative control sample (a sterile swab tip), and a previous *Bd*-positive sample (a swab sample from a different wetland that was *Bd*-positive). The following day, DNA was extracted into 96-well plates using a Life Technologies KingFisher™ magnetic bead extraction machine. qPCR was conducted using protocols and primer sets developed by Annis et al. (2004). The qPCR reactions were run at a total volume of 30 µl, consisting of 5 µl of extracted DNA template, primers 5.85 Chytr and ITS1-3, DNA probe Chytr MGB2, Taqman Universal Master Mix (Life Technologies), and distilled water. A plasmid culture obtained from Dr. J. E. Longcore (University of Maine) was used to create the 4 known *Bd* concentrations in order to generate a standard curve to esti-

mate the number of *Bd* zoospore equivalents per sample, and each sample was run in duplicate to estimate and average number of zoospore equivalents. In total, 8 internal positive controls (Applied Biosystems 4308323, TaqMan® Exogenous Internal Positive Control [VIC™-Probe]) were used per 96-sample qPCR run to test for potential PCR inhibition. In addition to 1 previous positive control, 1 negative non-template control of water was used.

Comparisons

We estimated the prevalence of *Bd* (proportion of infected individuals) for populations of larvae from 5 pre-wintering samples (collected late July to October) and 5 post-wintering samples (collected May to June, Table 1). In 2 populations, we estimated prevalence among tadpoles hatched in 2014 (i.e. Cohort 2104) for both a pre-wintering sample and a post-wintering sample. In 3 populations, we were able to make comparisons between the pre-wintering prevalence of a cohort to the post-wintering prevalence of the prior year's cohort. For each individual, we measured the presence or absence of *Bd* and the zoospore equivalents. We used Spearman rank-correlation analysis to determine whether *Bd* intensity was associated with tadpole stage or snout to vent length (SVL), and we used Kruskal-Wallis tests to compare size and stage between *Bd* infected versus non-infected individuals.

We constructed a binary logistic regression model for all tadpoles to determine whether tadpole size (SVL in mm) or cohort (a combination of breeding population and collection year) could predict whether an individual tadpole was infected with *Bd* (*Bd* detected = 1; *Bd* not detected = 0). While the main goal of this analysis was to determine whether we were less likely to detect *Bd* in smaller individuals, cohort was an important factor to include in this model because conditions that influence disease dynamics would likely differ between populations and result in different baseline infection rates. Furthermore, this model allowed us to calculate odds ratios between cohorts to estimate how much more likely an individual from one cohort was to be infected than an individual from another cohort.

RESULTS

In 6 comparisons between post- and pre-wintering cohorts, we failed to detect *Bd* in populations prior to overwintering in ponds, even though each population tested positive for *Bd* after tadpoles emerged from winter hibernation (Table 1). We observed this despite *Bd* prevalence that exceeded 70% in 2 post-wintering cohorts and testing 188 pre-wintering individuals (Table 1). Out of all post-wintering tadpoles sampled (n = 252), 50.0% were infected with *Bd*, and infected individuals averaged 3426 ± 530 (SE) zoospore equivalents. Among post-wintering tadpoles, those infected with *Bd* tended to be smaller than uninfected tadpoles (mean SVL = 20.0 ± 0.6 mm compared to 24.9 ± 0.8 mm), and they were from earlier Gosner stages (mean = 30.1 ± 0.4 compared to 33.6 ± 0.4). However, these differences were insignificant when we made comparisons of *Bd*-infected (*Bd*+) vs. uninfected (*Bd*−) tadpoles within each population (Fig. 1).

The intensity of *Bd* infections in post-wintering tadpoles did not appear to be related to tadpole size or developmental stage, nor did these factors appear to predict whether a tadpole was infected with *Bd.* Within each population that we sampled, we failed to detect significant correlations between *Bd* intensity and tadpole stage or tadpole size ($p > 0.178$ and $p > 0.121$ among all sites, respectively; see Table A1 in the Appendix). Within each population, the size and stage of *Bd*+ individuals was similar to the size and stage of individuals that were *Bd*−, thus Kruskal-Wallis tests failed to find significant differences in size or developmental stage ($p > 0.590$ and $p > 0.432$ among all sites, respectively; Table A1).

Our binary logistic regression model explained 38.4% (deviance R^2) of the variation in infection status, with the probability of *Bd* infection significantly differing between cohorts, but not between tadpoles of different size. Logistic regression estimated that tadpoles from the Huntingdon cohort were at least 6.0 times more likely to be *Bd*+ than any other cohort, and tadpoles from the PSU Altoona 2013 cohort were at least 6.3 times more likely to be *Bd*+ than tadpoles from the remaining cohorts (Table 2). After considering the cohort to which a tadpole belonged, tadpole size was not found to be a significant predictor of its *Bd* infection status ($\beta_{Size} = -0.0201 \pm 0.0227$, $p = 0.380$).

DISCUSSION

We detected *Bd* in tadpoles during the months that followed an overwintering period, and failed to detect *Bd* in tadpoles that had yet to overwinter. This suggests that either prevalence decreased to the

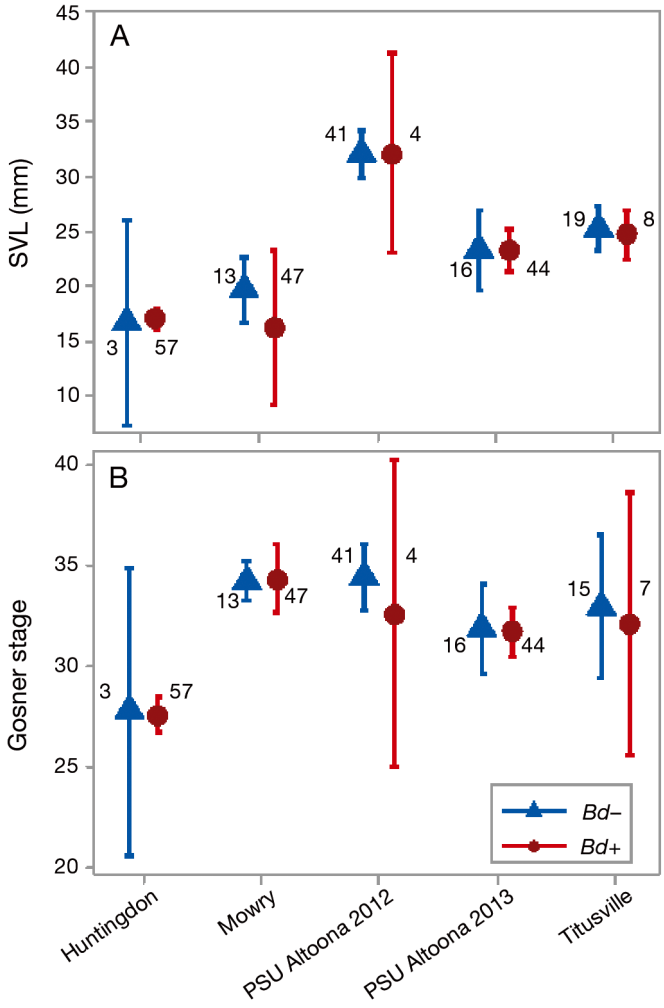

Fig. 1. Comparison of (A) average snout to vent length (SVL) and (B) developmental stage (Gosner stage) of northern green frog *Lithobates clamitans melanota* tadpoles from each population that were uninfected (*Bd*–, blue triangles) and infected (*Bd*+, red circles) with *Batrachochytrium dendrobatidis* (*Bd*). Error bars represent 95% confidence intervals, and numbers represent sample size

point where our sampling effort was unable to capture an infected individual, or that tadpoles must overwinter to become infected with *Bd*. If *Bd* zoosporangia persist in pond sediments or on mature adults throughout the year, then tadpoles could acquire infections while overwintering. We have no evidence to suggest that *Bd* is directly transmitted from post-wintering tadpoles to pre-wintering tadpoles, although it remains a possibility. Pre-wintering tadpoles would experience the 2 to 4 wk of exposure time that is necessary to become infected with *Bd* zoospores from conspecifics (Rachowicz & Vredenburg 2004) because post-wintering and pre-wintering green frog tadpoles co-occur in ponds from May through June. If pre- and post-wintering tadpoles inhabited different microhabitats, or co-occurred at low densities, we would expect transmission rates to be relatively low. It is possible that tadpoles become infected with *Bd* from adults, but since adult *Lithobates clamitans* remain in breeding ponds year-round, we would expect pre-wintering tadpoles to be infected with *Bd* from them also.

The thermal tolerance range of *Bd* may explain both the infection of overwintering tadpoles, and the marked decrease in infection prevalence seen in tadpoles prior to overwintering. While early experiments suggested that optimal growth for *Bd* was between 17 and 25°C (Piotrowski et al. 2004), recent studies suggest that temperatures below 17°C are also suitable for *Bd* growth (Woodhams et al. 2008, Voyles et al. 2012, Stevenson et al. 2013). Furthermore, *Bd* is able to survive and produce zoospores at temperatures as low as 4°C (Piotrowski et al. 2004), and Stevenson et al. (2013) found that zoospore activity rates were highest at their lowest temperature treatment of 13°C. These findings suggest that *Bd* could infect, survive on, and accumulate in tadpoles while they were overwintering. In contrast to what occurs at cool temperatures, cultures of *Bd* can cease to grow at temperatures above 25°C and will die at temperatures above 30°C (Piotrowski et al. 2004). Therefore, tadpoles that use shallow littoral zones of ponds during the summer can expose themselves to temperatures near 30°C, potentially ridding them-

Table 2. Matrix of logistic regression odds ratios [95% CI] that report how much more likely a post-wintering tadpole from the row cohort will be infected with *Bd* than a post-wintering tadpole from the column cohort. ns: no significant difference

Cohort with higher infection rate	Cohort with lower infection rate			
	PSU Altoona 2013 cohort	Titusville	Mowry	PSU Altoona 2012 cohort
Huntingdon	6.08 [1.62–22.88]	38.46 [8.87–166.67]	67.11 [18.01–250.00]	144.92 [27.25–769.23]
PSU Altoona 2013 cohort	—	6.33 [2.31–17.33]	11.05 [4.56–26.73]	23.91 [7.02–81.37]
Titusville	—	—	ns	ns
Mowry	—	—	—	ns

selves of *Bd* zoospores. Amphibians have the ability to prevent the initial colonization of *Bd* through immune responses such as the secretion of antimicrobial peptides (AMPs) (reviewed by Rollins-Smith et al. 2011) that also combat against bacteria, viruses, and other fungi (Pask et al. 2013). AMPs have been detected in some species of tadpoles (Rollins-Smith et al. 2011), and if AMP production increases during the summer or fall in preparation for overwintering, it could provide another mechanism to explain temporal variation in *Bd* infections. This mechanism is purely speculative, and further research would be necessary to conclude whether this actually occurs.

There are 2 mechanisms by which our sampling methods could have failed to detect *Bd* in pre-wintering tadpoles. First, the surface areas of mouths of pre-wintering tadpoles are much smaller than those of post-wintering tadpoles. As a result, the mouths of pre-wintering tadpoles could have been too small to accumulate a detectable quantity of *Bd*. We conclude this was unlikely because the size of post-wintering tadpoles was not related to infection occurrence (tested with logistic regression analysis), and infection intensity was not correlated with size, nor developmental stage. Smith et al. (2007) found no relationship between tadpole size and *Bd* infections once developmental stage was accounted for. This led them to suggest that infection status was related to time of exposure, and they did not speculate that infection status was related to the surface area of mouth parts, per se.

It is also possible that mouth swabs were unable to collect enough infected cells to register an appreciable amount of *Bd* DNA, especially if *Bd* occurred in the intracellular, thallus stage in tadpoles. While we could have increased of probability of detecting *Bd* by using excised mouth tissue, we wanted to use the same methodology as a concurrent study that prohibited us from euthanizing tadpoles. Compared to using excised mouth tissue, Retallick et al. (2006) estimated that mouth swabs detected *Bd* in tadpoles between 11.7% and 90.0% of the time, with the highest end of detection rates occurring when tadpoles are exposed to *Bd* for 8 wk. We suspect that our detection rates were at the higher end of these estimates because (1) pre-wintering tadpoles were collected more than 8 wk into the breeding season, (2) the surgical swabs used in our study covered a larger surface area than the swabbing devices (toothpicks) used by Retallick et al. (2006), and (3) the pre-wintering tadpoles collected in our experiment were much larger (mean ± SD SVL = 24.6 ± 7.1 mm) than the tadpoles used by Retallick et al. (2006) (mean body length = 15.8 mm). Power analysis provided by Retallick et al. (2006) suggest that all but 1 pre-wintering population (PSU Altoona 2013 cohort) was sampled adequately enough to detect *Bd* with 95% certainty at a prevalence of 10 to 20% (assuming a population of 1000 individuals and detection rates between 54.2 and 90.0%). Considering that prevalence in post-wintering populations exceeded 20% in all but 1 cohort, we feel very confident in concluding that *Bd* prevalence decreased from spring-time highs, through the summer and fall months. We cannot be certain that tadpoles are free of *Bd* infections during the late-summer and fall months, but testing a total of 188 pre-wintering individuals from *Bd*-infected wetlands provided no evidence to the contrary. If tadpoles enter hibernation uninfected, then a possible source of infection could come from saprophytic colonies in the environment (Johnson & Speare 2005).

Our findings could have implications for the monitoring of disease in amphibian populations, as well as the design of human-created wetlands. Anuran larvae are an underutilized life stage in disease monitoring even though they pose several advantages over sampling post-metamorphic stages. Large sample sizes of anuran larvae can often be harvested with less effort than adults due to their high population densities, and they are effectively sampled during daylight hours. If researchers choose to sample larvae instead of adults, our results suggest that anuran larvae should be sampled in the Mid-Atlantic region of the USA prior to the warmest summer months (e.g. July and August) when *Bd* prevalence dramatically decreases in the population. If water temperatures are a cause of decreased *Bd* prevalence, then human-created wetlands could be designed to decrease *Bd* infection rates in larvae by creating shallow areas that are exposed to sunlight. If tadpoles have access to these microhabitats, and water temperatures exceed 25°C, tadpoles could inhibit the colonization and growth of *Bd* on themselves, and prevent chronically high prevalence rates in populations.

Acknowledgements. We thank L. Gromiller, C. Holsinger, C. Keller, and J. Skebo for their help with collecting and processing specimens. This research was supported through a Summer Student Research Grant and an Undergraduate Research Assistantship from the Office of Research and Sponsored Programs at Penn State Altoona College.

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Appendix

Table A1. Correlation results of *Batrachochytrium dendrobatidis* (*Bd*) intensity versus age (Gosner stage) and size (snout to vent length) of *Bd*-infected (*Bd*+) northern green frog *Lithobates clamitans melanota* tadpoles, as well as reference data comparing stage and size of *Bd*+ versus uninfected (*Bd*–) tadpoles used to construct Fig. 1

Site	Gosner stage							Snout to vent length (mm)						
Cohort	Correlation with *Bd* intensity		*Bd*+ ind.			*Bd*– ind.			Correlation with *Bd* intensity		*Bd*+ ind.			*Bd*– ind.		
	r	p	Median (range)	Mean (±SE)	n	Median (range)	Mean (±SE)	n	r	p	Median (range)	Mean (±SE)	n	Median (range)	Mean (±SE)	n
Titusville	–0.349	0.357	28.0 (26–41)	32.0 (±2.7)	7	37.0 (26–41)	32.9 (±1.7)	15	–0.017	0.965	25.5 (21–29)	24.6 (±1.0)	8	26.0 (18–31)	25.2 (±0.9)	19
PSU Altoona																
2012	–0.4	0.6	33.0 (27–37)	32.5 (±2.4)	4	36.0 (26–44)	34.3 (±0.8)	41	–0.8	0.2	31.5 (26–39)	32.0 (±2.9)	4	32.0 (17–45)	32.0 (±1.1)	41
2013	0.206	0.179	30.5 (26–38)	31.6 (±0.6)	44	29.5 (27–39)	31.8 (±1.0)	16	0.158	0.305	20.5 (15–38)	23.3 (±1.0)	44	20.0 (16–38)	23.2 (±1.7)	16
Mowry	0.137	0.657	35.0 (28–37)	34.2 (±0.7)	13	36.0 (25–40)	34.1 (±0.5)	47	0.451	0.122	20.0 (19–30)	16.1 (±3.2)	13	23.0 (17–33)	19.6 (±1.5)	47
Huntingdon	0.016	0.907	26.0 (26–31)	27.5 (±0.4)	57	26.0 (26–31)	27.7 (±1.7)	3	0.101	0.456	16.0 (12–33)	16.9 (±0.5)	57	15.0 (14–21)	16.7 (±2.2)	3

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Submitted: September 10, 2015; Accepted: July 13, 2016
Proofs received from author(s): September 2, 2016