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Effects of harmful algal blooms on stress levels and immune functioning in wetland-associated songbirds and reptiles

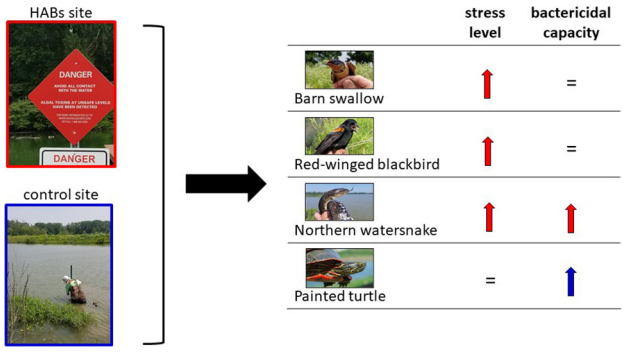
Jeanine M. Refsnider [a,*], Jessica A. Garcia [a], Brittany Holliker [a], Austin C. Hulbert [a], Ashley Nunez [a,b], Henry M. Streby [a]

[a] Department of Environmental Sciences, University of Toledo, Wolfe Hall Room 1235, 3050 West Towerview Blvd., Toledo, OH 43606-3390, USA
[b] Department of Biology, Ursinus College, 601 East Main St., Collegeville, PA 19426-1000, USA

HIGHLIGHTS

- Impacts of harmful algal blooms (HABs) on wildlife are poorly understood.
- We compared stress and immune function in wildlife from HAB and control wetlands.
- Stress levels were higher in HAB-exposed birds and snakes than control populations.
- Immune function was higher in HAB-exposed snakes but lower in HABs-exposed turtles.
- HABs cause physiological stress and may compromise immune functioning in wildlife.

GRAPHICAL ABSTRACT



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ABSTRACT

Harmful algal blooms (HABs), caused primarily by nutrient input from agricultural runoff, are a threat to freshwater systems worldwide, and are further predicted to increase in size, frequency, and intensity due to climate change. HABs occur annually in the Western Basin of Lake Erie (Ohio, USA), and these blooms become toxic when dominated by cyanobacteria that produce the liver toxin microcystin. Although we are making substantial inroads toward understanding how microcystin affects human health, less is known about effects of microcystin on wildlife exposed to HABs. Wetland-associated songbirds (barn swallows, *Hirundo rustica*, and red-winged blackbirds, *Agelaius phoeniceus*) and reptiles (Northern watersnakes, *Nerodia sipedon*, and painted turtles, *Chrysemys picta*) were sampled from wetlands exposed to chronically high microcystin levels due to a prolonged HAB event, and from unexposed, control wetlands. Physiological stress levels and several measures of immune functioning were compared between the HAB-exposed and control populations. Physiological stress levels, measured as heterophil:lymphocyte ratios, were higher in barn swallows, red-winged blackbirds, and Northern watersnakes exposed to a chronic HAB compared to unexposed, control individuals, but painted turtles did not differ in physiological stress levels between HAB-exposed and control individuals. Neither barn swallows nor red-winged blackbirds differed in immune functioning between populations, but HAB-exposed watersnakes had higher bactericidal capacity than control snakes, and HAB-exposed painted turtles had lower bactericidal capacity than control turtles. These results suggest that even when HABs do not cause direct mortality of exposed wildlife, they can potentially act as a physiological stressor across several taxa, and furthermore may compromise immune functioning in some species.

* Corresponding author.
E-mail address: jeanine.refsnider@utoledo.edu (J.M. Refsnider).

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1. Introduction

Algal blooms in aquatic systems are caused by nutrient input that fosters excessive growth of photosynthetic prokaryotes. Such blooms can have substantial effects on the ecosystem by blocking sunlight, depleting oxygen, and producing toxins. The frequency and severity of algal blooms are increasing worldwide due to excessive runoff from agriculture and sewage, with many lakes across the globe now experiencing large algal blooms annually (Tang et al., 2006; Wang et al., 2008). Such blooms can seriously impact both natural and human communities by killing fish, lowering water quality, and hurting local economies that depend on healthy aquatic systems.

When an algal bloom is dominated by a toxin-producing or otherwise harmful algal species, it can be described as a harmful algal bloom, or HAB. Cyanobacteria, which are the most common HAB-causing taxa in many shallow freshwater lakes, produce a variety of toxins, but one of the most common and harmful is microcystin, a cyclic peptide produced by algae in the genera *Microcystis* and *Planktothrix*. Microcystin has over 100 congeners (Zurawell et al., 2005), and of those microcystin-LR is known to cause liver, digestive, skin, and neurological damage in humans (Chen et al., 2009) and livestock (Chorus and Bartram, 1999). Microcystin can be fatal if ingested (Eriksson et al., 1990; Wang et al., 2021), but it can also be aerosolized, for example due to waves caused by boat wakes, and therefore it poses an inhalation risk as well (Backer et al., 2008). High concentrations of microcystin found in the tissues of shrimp, frog, and fish species suggest that humans or other animals that eat organisms exposed to HABs could face serious health risks (Papadimitriou et al., 2012). Cyanobacteria-dominated HABs are predicted to become more frequent and severe with climate warming, as higher temperatures and longer growing seasons will favor cyanobacteria over other types of phytoplankton (Paerl and Huisman, 2008; Wells et al., 2015; Gobler, 2020).

Although we are making substantial inroads toward understanding how microcystin affects human health, to date, there has been comparatively less research into the effects of HABs on freshwater communities. In zooplankton and crayfish, high concentrations of microcystin were found in consumer species, suggesting that accumulation of algal toxins can occur in some members of aquatic communities (Vasconcelos et al., 2001; Kozlowsky-Suzuki et al., 2012). In adult fish, microcystin can cause severe liver damage (Malbrouck and Kestemont, 2006) and may also impair reproduction (Baganz et al., 1998; Liu et al., 2014). However, embryonic and larval fish appear to be far more susceptible to microcystin toxicity than later life stages, possibly due to increased environmental sensitivity of early life stages during development. In particular, larval fish exposed to microcystin exhibited liver damage, altered heart rate, immune suppression, growth inhibition, increased frequency of deformities, and decreased survival compared to control larvae (Lecoz et al., 2008; Adámek et al., 2011; Pavagadhi et al., 2013; Liu et al., 2014; Qi et al., 2016; Azevedo-Linhares et al., 2018). Similarly, in larval amphibians, microcystin was associated with liver and intestinal damage (Júnior et al., 2018; Su et al., 2020), while damage to reproductive organs was evident in adult frogs exposed to microcystin (Zhang et al., 2013). Taken in combination, these studies demonstrate that microcystin is particularly harmful during embryonic development in anamniotes such as fish and amphibians, where embryonic development occurs entirely within aquatic systems.

There remains a major knowledge gap involving the potential effects of microcystin-producing HABs on semi-aquatic vertebrates, particularly avian and non-avian reptiles. In vertebrates, chronically high stress levels can depress immune responses and make individuals more vulnerable to infection (McEwen and Wingfield, 2003; Millet et al., 2007). Environmental stressors, such as El Niño events or chemical contamination, can induce physiological stress responses in vertebrate animals, which can subsequently affect survival (e.g., Romero and Wikelski, 2001; Lattin et al., 2014). It is not currently known whether HABs can similarly induce a strong physiological stress response in vertebrates, but amphibian larvae exposed to HAB toxins exhibit organ damage (Su et al., 2020), which suggests that HABs likely act as an environmental stressor. Here, physiological stress levels and several indices of immune functioning were compared in two avian and two non-avian reptile species from a lake with chronically high microcystin levels, and from an unexposed, control site. The prediction being tested is that animals from the HAB-exposed site would exhibit higher stress levels, and lower immune functioning, than their counterparts from the unexposed, control site. This study provides the first baseline data on sublethal effects of microcystin-producing HABs in semi-aquatic wildlife.

Stress levels and immune functioning were compared in birds and reptiles at two sites: Grand Lake St. Marys (Mercer and Auglaize Counties, Ohio, USA) was the HAB-exposed site, and a series of inland wetlands within Ottawa National Wildlife Refuge (Ottawa County, Ohio, USA) was the control site (Fig. 1). Grand Lake St. Marys is a 59 km^2 manmade reservoir with a watershed that is 90% agricultural, which has resulted in extensive nutrient runoff from crops and poultry farms. The lake is so shallow (2 m average depth) that normal lake turnover does not occur in spring and fall. Combined, these factors have resulted in continuous, year-round blooms of microcystin-producing *Planktothrix* algae (Walls et al., 2018). The unexposed, control sampling site for this study was a series of wetlands within Ottawa National Wildlife Refuge, which borders the western basin of Lake Erie (Fig. 1). The wetlands sampled here were inland of and hydrologically disconnected from Lake Erie by constructed dikes and water intake control structures. The most common wetland-associated wildlife species at the two study sites were targeted for field-collection of blood samples: barn swallows (*Hirundo rustica*), red-winged blackbirds (*Agelaius phoeniceus*), Northern watersnakes (*Nerodia sipedon*), and painted turtles (*Chrysemys picta*). The four study species were sampled at both study sites in 2018 and 2019. Specifically, barn swallows and red-winged blackbirds were sampled during the June nesting period, painted turtles were sampled during the nesting period of June and early July, and Northern watersnakes were sampled from April – July, which is the time of their peak activity. All four species feed on aquatic prey items, thereby potentially exposing them to ingestion of microcystin through their food.

2. Material and methods

2.1. Microcystin concentrations

Microcystin concentrations are measured approximately biweekly at the HAB-exposed site, Grand Lake St. Marys, by the Ohio Environmental Protection Agency's Harmful Algal Blooms Monitoring Program; data are publicly available at http://wwwapp.epa.ohio.gov/gis/mapportal/HAB_Monitoring.html. Similarly, microcystin concentrations are measured approximately biweekly during June – October at fixed stations in Lake Erie by the National Oceanic and Atmospheric Administration's Great Lakes Environmental Research Laboratory; data are publicly available at https://www.glerl.noaa.gov/res/HABs_and_Hypoxia/WLEMicrocystin.html. Station WE16 is the closest monitoring station to the unexposed, control wetlands sampled here. Both monitoring programs quantify microcystin concentrations at the surface using a commercially available ELISA kit, which detects ADDA (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid) levels and measures intra-, extra-cellular, and total microcystin concentrations.

2.2. Capture methods

Adult barn swallows and red-winged blackbirds were captured using mist nets placed near active nests, and nestlings were sampled directly from nests when they were estimated to be within two days of fledging. Northern watersnakes were captured along wetland edges, mainly by hand but some in minnow traps baited with sardines. A few

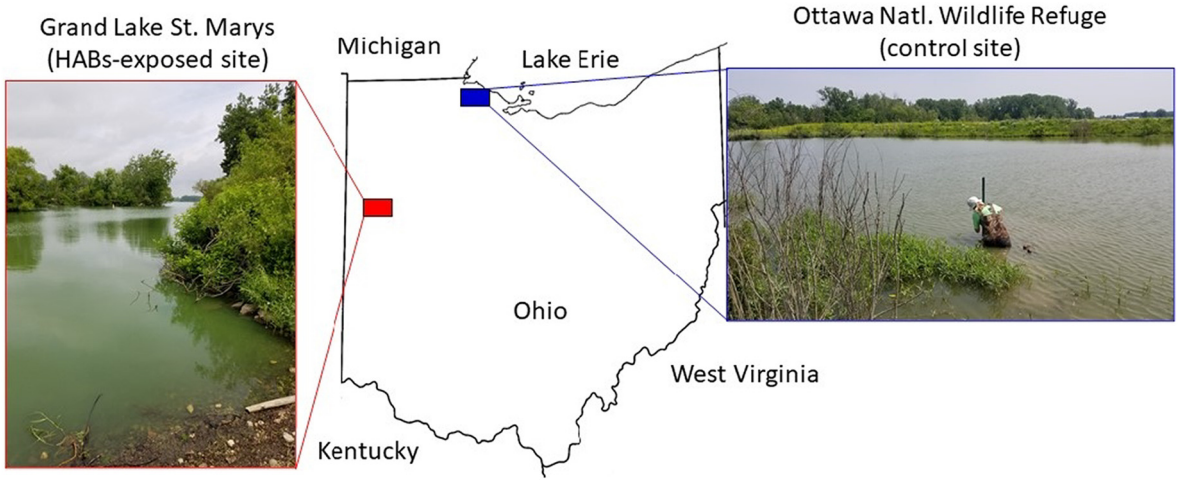

Fig. 1. Field sampling locations at harmful algal bloom-exposed site (Grand Lake St. Marys, Mercer and Auglaize Counties) and unexposed control site (Ottawa National Wildlife Refuge, Ottawa County) in Ohio, USA, in 2018–2019.

turtles were captured by hand, but the majority of turtles were captured in aquatic traps, including basking traps, hoopnet traps baited with sardines and corn, and fyke nets. Only adult watersnakes and turtles were sampled. Mist nets were checked every 15 min when open; traps were checked twice daily for snakes and turtles. Sex and standard morphological measurements were recorded for all individuals (wing chord, tarsus length, and mass for birds; snout-vent length or carapace length for snakes and turtles, respectively). Each animal was individually marked to ensure that no animal was sampled more than once. Birds were banded with a U.S. Fish and Wildlife Service-issued aluminum leg band, snakes were marked by clipping a unique combination of ventral scales (Blanchard and Finster, 1933), and turtles were marked by filing a unique combination of notches in the shell margin (Cagle, 1939).

A blood sample was collected from each individual immediately following capture. Blood samples never exceeded 5% of an individual's total mass, and were always <1% of body mass for birds. Blood samples were collected from birds by pricking the branchial artery with a needle and then collecting blood in a heparinized capillary tube. For snakes and turtles, blood was collected from the caudal vein using a heparinized, 28-ga syringe (as in Refsnider et al., 2015, Sanchez and Refsnider, 2017). Immediately following blood collection, for each individual, a blood smear was made on a glass slide, and the remainder of the blood sample was immediately centrifuged to separate the plasma from the packed blood cells. The plasma was subsequently drawn off using a pipette, aliquoted into separate tubes for subsequent immune assays, and flash-frozen in the field. All plasma samples were then stored at -80 °C at the University of Toledo. Birds and snakes were released at the exact site of capture immediately following collection of a blood sample. Turtles in 2018 were maintained in captivity for 48 h for an immune challenge assay (see below); in 2019 all turtles were released at the capture site immediately following collection of a blood sample.

2.3. Laboratory immune assays

Following field sampling, three laboratory assays were conducted to quantify physiological stress level and immune function in birds and snakes, and a fourth assay was conducted in turtles only. Physiological stress levels were measured by quantifying ratios of heterophils to lymphocytes (hereafter, H:L ratios) in the blood smears of all four species. Heterophils and lymphocytes are two types of white blood cells important in mounting an immune defense. An individual's H:L ratio becomes elevated when animals are exposed to a stressor; therefore, the higher the H:L ratio, the higher an individual's baseline level of physiological stress (Davis et al., 2008). Blood smear slides were air-dried in the field, and later stained with Stat-Quick Wright Giemsa and May-Grünwald Stain (ENG Scientific, Inc., Clinton, NJ). To quantify the circulating leukocyte profile of each individual, 100 blood cells were counted consecutively (excluding erythrocytes and thrombocytes) from each blood smear using a light microscope at 100× power and identified as a heterophil, lymphocyte, eosinophil, basophil, or monocyte (Kassab et al., 2009; Javanbakht et al., 2013). The H:L ratio for each individual was then calculated (as Sanchez and Refsnider, 2017, Garcia, 2018).

Innate immune competency was quantified in all four species using two assays. First, a bacteria-killing assay was conducted to measure the bactericidal capacity of complement proteins in the blood plasma to kill *E. coli* (Tieleman et al., 2005). In this assay, diluted plasma samples are applied to cultures of *E. coli* and given time for bactericidal activity in the plasma to kill the bacteria. The proportion of the *E. coli* inoculum killed compared to the number of *E. coli* colonies present in control samples represents the bactericidal capacity of an individual at the time of plasma collection. The methods of Millet et al. (2007) and Palacios et al. (2011) were followed, with a few species-specific modifications (Refsnider et al., 2015; Garcia, 2018). First, a pellet of lyophilized *E. coli* (Microbiologics, ATCC#8739, St. Cloud, MN, USA) was reconstituted in 40 mL of warm, phosphate-buffered saline (PBS), and a working *E. coli* solution was prepared by further diluting a fraction of reconstituted *E. coli* to 1:40, which produced approximately 100 colony-forming *E. coli* per 10 μL. The plasma samples were then diluted 1:10 in PBS. Sample reactions were prepared by adding 10 μL of working *E. coli* solution to 100 μL of the diluted plasma samples. Sample reactions were then incubated for 25 min at 40 °C (birds) or 28 °C (reptiles) to allow bacteria-killing to occur. Two replicate control reactions for each species were also prepared by adding 10 μL of working *E. coli* solution to 100 μL PBS, and then incubating the control plates for each species simultaneously with the *E. coli*-treated plasma samples for that species. Following incubation, two replicates of all sample and control reactions were plated using 50 μL aliquots on 4% tryptic soy agar. Plates were incubated at room temperature for 36 h, and then the number of *E. coli* colonies on each plate was visually counted. The number of colonies in each plate was divided by the mean number of colonies in the control plates for the corresponding species, and this value was subtracted from 1 to obtain the proportion of bacteria killed. For each plasma sample, the mean proportion of bacteria killed from the two replicate plates was used as the measure of each individual's bactericidal capacity.

As a second measure of innate immune competency, a natural antibody agglutination assay was conducted (Matson et al., 2005). Natural antibodies are produced constitutively and function by agglutinating and lysing foreign cells. This second measure of immune function assessed individuals' constitutive innate immunity in terms of ability

to adhere to foreign red blood cells (RBCs). The methods of Matson et al. (2005) were followed, with a few previously validated modifications for specific species (Palacios et al., 2011; Schwanz et al., 2011; Refsnider et al., 2015; Garcia, 2018). PBS was added to each well in a 96-well plate (12.5 μL for birds, 15 μL for both snakes and turtles). Then, 5 μL of either plasma (rows 2–11) or positive control (row 1; sheep anti-rabbit RBC serum for birds and turtles, rabbit anti-sheep RBC serum for snakes) was mixed with the PBS in column 1. Samples were serially diluted 1:2 for columns 2–8; row 12 served as a negative control and contained only PBS. Next, 10 μL of foreign red blood cells (2% rabbit RBCs for birds, 5% sheep RBCs for snakes, 5% rabbit RBCs for turtles) in Alsever's anti-coagulant (Hemostat Laboratories, Dixon, CA, USA) were added to all wells, which resulted in a final plasma dilution in column 1 of 13:100 for birds, and 1:8 for snakes and turtles. Plates of bird plasma samples were incubated at 37 °C for 90 min, snake samples were incubated at 28 °C for 90 min, and turtle samples were incubated at 28 °C for 120 min. Agglutination titers were then visually scored as $\log_2$ (1/D), where D is the final dilution of plasma where agglutination occurred (based on Fig. 1 in Matson et al., 2005). For example, for snakes and turtles, because the final plasma dilution in column 1 was 1:8, column 1 had a titer of 3, and column 8 had a titer of 10.

Lastly, for painted turtles captured in 2018 only, activity of the adaptive immune system was quantified using a phytohemagglutinin (PHA)-challenge assay, which measures localized skin swelling in response to an infection (here, PHA injected into the toe webbing of a hind foot; Martin et al., 2006). A 28-gauge syringe was used to inject 10 μL of 10 mg/mL PHA (L9017, SigmaAldrich Corp., St. Louis, MO) in 0.01 M PBS into the webbing between the fourth and fifth toe on a random hind foot of each individual. As a control, 10 μL of PBS was also injected into the webbing of the other hind foot. The thickness of the foot webbing was measured at the injection sites to the nearest 0.01 mm using digital calipers at four time points: immediately prior to injection (time 0), and then at 6 h, 24 h, and 48 h after injection. Each foot was measured twice at each time point, and the average of the two measurements was used as the final measurement of foot web thickness at each of the four time points (as in Schwanz et al., 2011, Sanchez and Refsnider, 2017). The change in foot web thickness at each of these four times indicates the magnitude of immune activity in response to the PHA challenge. For the PHA challenge only, turtles were maintained in captivity at the University of Toledo for 48 h following capture in individual plastic tubs under ambient lighting at room temperature; after the 48-h measurement, turtles were released at their exact site of capture (Garcia, 2018).

2.4. Statistical analyses

For each species at both the HAB-exposed and control site, the mean proportion of each of the five white blood cell types comprising the circulating leukocyte profile was determined. Then, for each species, chi-square (χ^2) goodness-of-fit tests were used to compare H:L ratios between individuals from the HAB-exposed and control sites. For each species, mean bactericidal capacity, measured as the proportion of *E. coli* colonies killed by each individual's plasma relative to control plates, were compared from HAB-exposed and control sites using two-way analyses of variance with year and sampling site as factors; age class (adult vs. juvenile) was also included as a factor for both bird species. Similarly, for each species, mean natural antibody agglutination titer, measured as the highest titer at which agglutination was observed, was compared from HAB-exposed and control sites using two-way analyses of variance with year and sampling site as factors; age class was included as a factor for both bird species.

To analyze skin-swelling response to PHA injection in painted turtles only, individuals' responses over time were first tested by fitting mixed-effects models of foot web thickness separately for control and PHA injections. Time since injection, study site (i.e., HAB-exposed or control site), and time x study site interaction were predictors, and individual identity was included as a random effect in each model. The peak swelling response in PHA-injected feet was determined as thickness at 6 h minus thickness pre-injection. A linear mixed model was then used to test whether the skin-swelling response to PHA was greater than the response to the control injection, and whether the response was influenced by study site. Treatment (control or PHA injection), study site, and study site x treatment interaction were predictors, and individual identity was included as a random effect. Finally, the peak swelling response at 6 h was compared between individuals from the HAB-exposed and control sites using *t*-tests. All statistical tests were conducted using R v.3.3.1 (R Core Team 2013), with $\alpha = 0.05$.

3. Results

Reported below are the total microcystin concentrations measured by the microcystin monitoring programs at each study site during the sampling periods in each year of this study. At the HAB-exposed site, Grand Lake St. Marys, total microcystin concentrations were 50.4 μg/L in June 2018 and 40.9 μg/L in early July 2019 (Ohio Environmental Protection Agency Harmful Algal Blooms Monitoring; http://wwwapp.epa.ohio.gov/gis/mapportal/HAB_Monitoring.html). During all sampling periods throughout this study, Grand Lake St. Marys was under an "Avoid all contact with the water" advisory issued by the Ohio Environmental Protection Agency due to microcystin concentrations exceeding 20 μg/L, the exposure limit for recreational use. At the fixed monitoring station in Lake Erie closest to the unexposed, control wetlands (WE16), microcystin was below detectable levels during the sampling period in both years of this study. Microcystin was first detected at station WE16 on 16 July 2018 at 0.15 μg/L, and on 22 July 2019 at 0.29 μg/L (National Oceanic and Atmospheric Administration's Great Lakes Environmental Research Laboratory; https://www.glerl.noaa.gov/res/HABs_and_Hypoxia/WLEMicrocystin.html). Previous analysis of water samples collected from the same control wetlands used in this study were unable to detect microcystin in those water samples (test detection limit of 0.3 μg/L; Garcia, 2018).

At the HAB-exposed and control sites, respectively, blood samples were collected from a total of 24 and 16 barn swallows, 19 and 7 red-winged blackbirds, 14 and 24 Northern watersnakes, and 15 and 12 painted turtles. Circulating leukocyte profiles for all four species are shown in Table 1. In both barn swallows and red-winged blackbirds, H:L ratios were higher (indicating higher stress levels) in birds from the HAB-exposed site compared to the control site (barn swallows: means = 0.92 and 0.70; $n = 24$ and 16; $\chi^2 = 17.0$; $P < 0.0001$; red-winged blackbirds: means = 1.03 and 0.69; $n = 19$ and 7; $\chi^2 = 13.9$; $P = 0.0002$; Fig. 2a–b). Similarly, in Northern watersnakes, H:L ratios were higher in individuals from the HAB-exposed site compared to the control site (means = 0.07 and 0.05; $n = 14$ and 24; $\chi^2 = 4.8$; $P = 0.029$; Fig. 2c). However, there was no difference in H:L ratios between painted turtles from the HAB-exposed or control sites (means = 0.37 and 0.34; $n = 11$ and 12; $\chi^2 = 0.7$; $P = 0.41$; Fig. 2d).

Year did not affect bactericidal capacity in any of the study species, and age class did not affect bactericidal capacity in either bird species. Bactericidal capacity did not differ between individuals from the HAB-exposed or control sites in either barn swallows (means = 9.2% and 15.3%; $n = 15$ and 10; $F_{1,22} = 1.72$; $P = 0.20$; Fig. 3a) or red-winged blackbirds (means = 15.0% and 14.8%; $n = 7$ and 4; $F_{1,13} = 0.001$; $P = 0.97$; Fig. 3b). In contrast, Northern watersnakes from the HAB-exposed site had higher bactericidal capacity than did those from the control site (means = 46.0% and 14.1%; $n = 11$ and 17; $F_{1,25} = 5.89$; $P = 0.02$; Fig. 3c), whereas painted turtles from the HAB-exposed site had lower bactericidal capacity than did those from the control site (means = 5.2% and 36.2%; $n = 15$ and 11; $F_{1,24} = 26.21$; $P < 0.001$; Fig. 3d).

There was no effect of year on natural antibody agglutination titer in any of the four study species, and no effect of age class in either bird species. There was no difference in agglutination titer between individuals

Table 1
Circulating leukocyte profile (mean % eosinophils, basophils, lymphocytes, monocytes, and heterophils) and mean heterophil:lymphocyte (H:L) ratio, mean bactericidal capacity, and mean natural antibody agglutination titer in barn swallows, red-winged blackbirds, Northern watersnakes, and painted turtles sampled from control and harmful algal bloom-exposed sites in Ohio, USA, in 2018–2019. Additionally, mean peak skin swelling in response to injection with phytohemagglutinin was measured at each site in painted turtles only. Significant differences between sampling sites for a species are shown in bold.

	% eosinophils	% basophils	% lymphocytes	% monocytes	% heterophils	H:L ratio	bactericidal capacity (% colonies killed)	natural antibody agglutination titer	peak skin-swelling (mm)
Barn swallow									
Control site	0.5	0.1	59.0	0.5	40.5	**0.70**	15.3	4.2	–
Exposed site	0.3	0.4	52.8	0.4	46.8	**0.92**	9.2	4.7	–
Red-winged blackbird									
Control site	0.1	0.3	59.0	0.6	40.5	**0.69**	14.8	5.8	–
Exposed site	0.2	0.2	50.9	0.5	48.6	**1.03**	15.0	5.0	–
Northern watersnake									
Control site	16.8	0.2	78.0	1.5	3.6	**0.05**	**14.1**	4.9	–
Exposed site	17.6	0.1	75.0	2.4	4.9	**0.07**	**46.0**	5.9	–
Painted turtle									
Control site	7.8	6.4	61.3	3.8	20.6	0.34	**36.2**	5.2	0.107
Exposed site	4.8	5.3	63.0	3.8	23.0	0.37	**5.2**	5.8	0.035

from the HAB-exposed or control sites in barn swallows ($n = 19$ and 7; $F_{1,21} = 1.95$; $P = 0.18$), red-winged blackbirds ($n = 7$ and 5; $F_{1,8} = 1.55$; $P = 0.25$), or painted turtles ($n = 15$ and 12; $F_{1,25} = 0.26$; $P = 0.61$; Table 1). Northern watersnakes showed a non-significant trend wherein individuals from the HAB-exposed site tended to have a higher agglutination titer than individuals from the control site (means = 5.92 and 4.90; $n = 12$ and 10; $F_{1,19} = 3.60$; $P = 0.07$; Table 1).

In painted turtles, the only species for which we conducted the PHA challenge, peak skin-swelling occurred 6 h after injection, and was significantly greater in feet injected with PHA compared to control-injected feet ($F_{1,17} = 6.92$, $P = 0.018$). There was no difference in the peak skin-swelling response at 6 h between turtles from the HAB-exposed or control sites (means = 0.035 and 0.107 mm; $n = 9$ and 11; $t = 1.44$, $P = 0.18$; Table 1).

4. Discussion

Environmental perturbations often cause direct mortality of organisms, but they can also have sublethal effects which may impact the health of individuals and the persistence of populations. Moreover, the effects of disruptions to the environment likely differ substantially across taxa. Harmful algal blooms in freshwater systems are predicted to become more frequent and severe due to climate warming (Paerl and Huisman, 2008; Wells et al., 2015; Gobler, 2020), and their impacts on biodiversity, human health, and local economies will likely increase concomitantly. To better understand the effects of HABs on wildlife species dependent on aquatic environments, physiological stress levels and immune functioning were compared in several species of birds and reptiles exposed to a chronic harmful algal bloom to animals from an

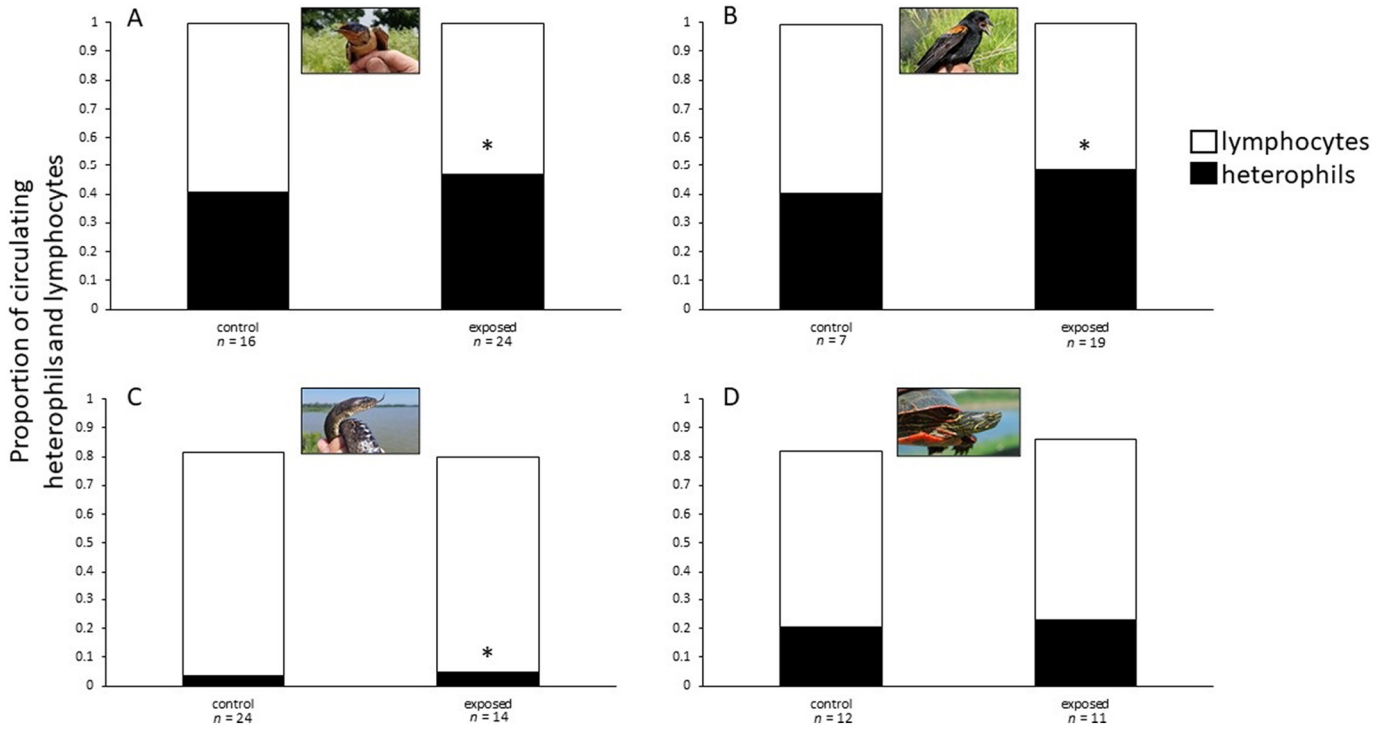

Fig. 2. Mean heterophil:lymphocyte (H:L) ratio in barn swallows (A), red-winged blackbirds (B), Northern watersnakes (C), and painted turtles (D) sampled from control and harmful algal bloom-exposed sites in Ohio, USA, in 2018–2019. Asterisks denote significant differences between groups. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

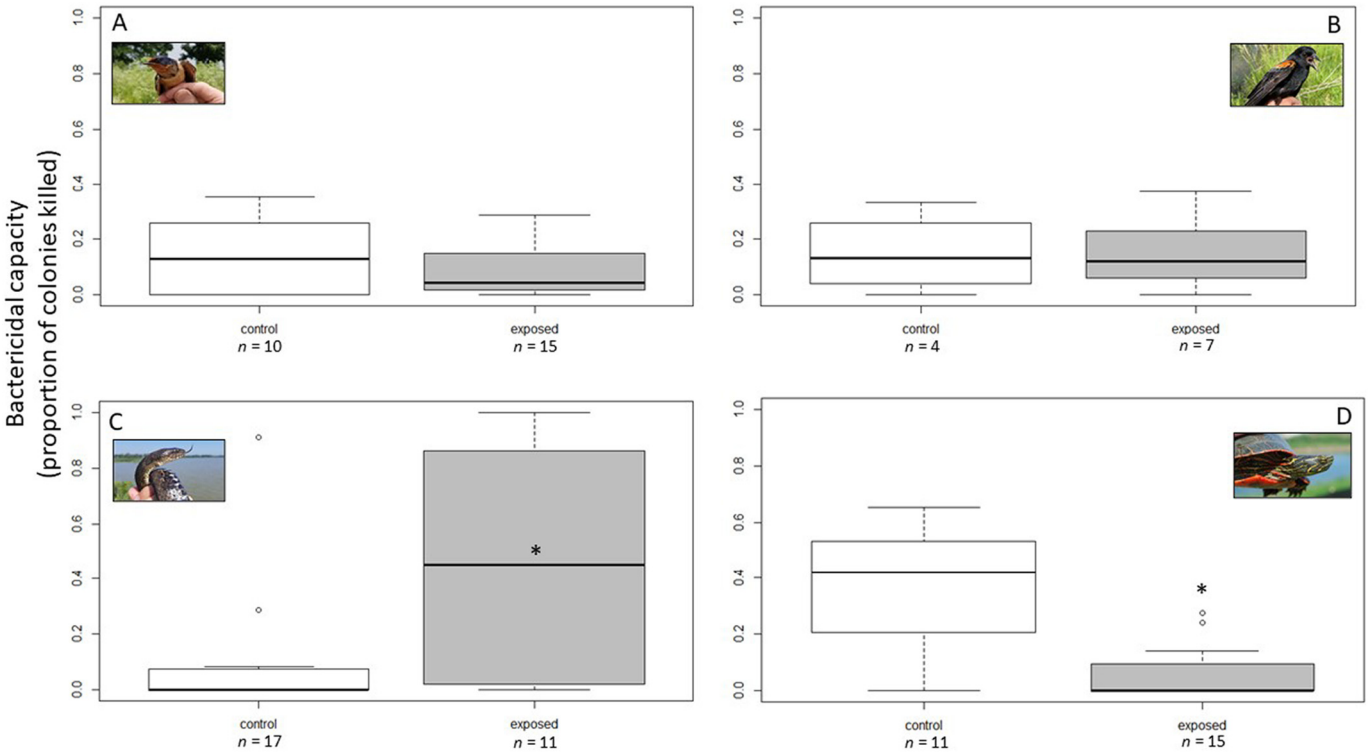

Fig. 3. Mean bactericidal capacity, measured as proportion of *E. coli* colonies killed in the presence of plasma complement relative to control samples, in barn swallows (A), red-winged blackbirds (B), Northern watersnakes (C), and painted turtles (D) sampled from control and harmful algal bloom-exposed sites in Ohio, USA, in 2018–2019. Asterisks denote significant differences between groups. Boxes indicate upper and lower quartiles with means indicated by dark horizontal lines; whiskers indicate range of data, and dots indicate outliers. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

unexposed, control site. Results from this study indicate that aquatic wildlife exposed to harmful algal blooms experience several sublethal effects, but these effects differ across taxa.

Overall, physiological stress levels were higher in barn swallows, red-winged blackbirds, and Northern watersnakes from the HAB-exposed site than from the control site. These results indicate that HABs represent an environmental stressor to some wildlife species. While temporary increases in physiological stress levels can be beneficial, for example, by allowing an individual to escape from hazardous conditions, a prolonged period of elevated stress levels can be harmful. The HAB-exposed study site represents an exaggerated state in that extremely high concentrations of the algal toxin microcystin are present year-round, whereas comparable HABs in larger lakes of natural origin usually occur seasonally. Nevertheless, even seasonal HABs in larger lakes such as Lake Erie span nearly half the annual active season of the reptile species sampled here, or half the time during which the bird species we studied are present on the breeding grounds sampled here. Therefore, if individuals' stress levels are elevated for the duration of a HAB, it is likely that such higher stress levels would trade off with other physiological parameters, such as immune functioning, body condition, and/or reproductive output. Importantly, in contrast with corticosterone concentrations that can rise immediately upon handling by a researcher (Cash et al., 1997; Romero and Reed, 2005), H:L ratios in reptiles may take hours to days to increase in response to a stressful event, allowing for a more accurate assessment of an individual's baseline level of physiological stress than can be measured by quantifying corticosterone levels (Davis et al., 2008).

In contrast to the results from birds and watersnakes, physiological stress levels in painted turtles did not differ between HAB-exposed or control sites. However, this result is perhaps not surprising, because painted turtles have previously been shown not to exhibit increases in physiological stress levels even when exposed to "stressful" conditions (such as a novel climate or high levels of human activity), indicating that physiological stress levels and immune functioning are decoupled in this species (Baxter-Gilbert et al., 2014; Refsnider et al., 2015; Polich, 2016; Sanchez and Refsnider, 2017).

The prediction that individuals exposed to HABs would have lower immune functioning than unexposed, control individuals was supported for bactericidal capacity in painted turtles, but not for any of the other three study species. In painted turtles, bactericidal capacity was lower in HAB-exposed individuals than in control individuals, but in Northern watersnakes, bactericidal capacity was higher in HAB-exposed individuals than in control individuals. Bactericidal capacity did not differ between HAB-exposed or control sites in either bird species. Thus, apparent effects of microcystin on immune functioning vary across taxa, but likely also vary depending on the precise immune axis being measured. For example, immune suppression resulting from microcystin exposure has been observed in larval zebrafish (Liu et al., 2014), and in bighead carp, individuals exposed to microcystin exhibited up-regulation of a chemokine involved in the recruitment of monocytes and neutrophils during inflammation, particularly in liver tissues (Li et al., 2013). If immune functioning is depressed in individuals exposed to algal toxins, such as fish or the turtles in the present study, then those individuals could be at increased risk of contracting infections from parasites or pathogens. Conversely, if immune functioning increases in species exposed to algal toxins, such as the watersnakes in this study, it is possible that the increased energy expenditure required to maintain enhanced immune activity could come at an energetic cost, for example by decreasing reproductive output or growth rates. Additional research is needed to quantify the potential costs of the enhanced bactericidal capacity observed here in HAB-exposed Northern watersnakes.

Neither natural antibody agglutination nor skin-swelling response to the PHA challenge differed between HAB-exposed or control painted turtles. The skin-swelling response to the PHA challenge is an indicator of immune activity by the adaptive immune system, and should not be interpreted as immune competence per se (Martin et al., 2006). Nevertheless, it is noteworthy that the skin-swelling response in painted

turtles did not differ between HAB-exposed and control individuals. We have previously found that female painted turtles have higher immune activity, as assessed by skin-swelling in response to PHA injection, during the reproductive season than do males (Sanchez and Refsnider, 2017). The reproductive season is energetically expensive for female painted turtles (due to egg production and overland travel to a nest site), but is also an annual, predictable event. Therefore, it is possible that immune activity in female turtles increases during the reproductive period in preparation for potential exposure to novel individuals or environments during nesting-related travel. In contrast, the unpredictable nature of HABs may preclude individuals from increasing their immune activity in preparation.

Microcystin has previously been shown to affect immune functioning in a variety of fish. For example, expression levels of several genes involved in immune activation and inflammatory responses were lower in fish exposed to microcystin compared to control individuals (Wei et al., 2009; Liu et al., 2014; Rymuszka and Adaszek, 2012). At the cellular level, microcystin affected immune cells in carp by impairing organization of their cytoskeleton (Rymuszka, 2013). In crucian carp, long-term dietary exposure to a low concentration of microcystin resulted in a stimulated immune response, but at high microcystin concentrations immune response became suppressed (Qiao et al., 2013). These results suggest that microcystin has complex effects on immune functioning across multiple levels of biological organization, and effects likely differ with dose, length of exposure, ontogenetic stage, and species.

The chronic HAB occurring in Grand Lake St. Marys, the HAB-exposed site, is likely fueled by extensive nutrient runoff from nearby crops and poultry farms. Indeed, 90% of Grand Lake St. Marys watershed is agricultural. Therefore, it cannot be ruled out that agricultural chemicals may contribute to the increased stress levels and altered immune functioning measured in wildlife from Grand Lake St. Marys, compared to the control sites which do not receive direct runoff from residential or agricultural land. However, the Ohio Environmental Protection Agency's "Avoid all contact with the water" advisory, which was in effect continuously over the duration of this study (including throughout the intervening winter), was due to microcystin concentrations exceeding (by at least 200%) the safe exposure level for recreational use of 20 μg/L. Furthermore, in a recent controlled exposure experiment, naïve bullfrog tadpoles from the control site in this study were housed in pond water containing 1 μg/L of microcystin, the concentration at which water is considered unsafe to drink. Within one week, liver damage and intestinal inflammation was evident in the microcystin-exposed tadpoles, but no sign of organ damage was observed in control tadpoles (Su et al., 2020). Therefore, it is likely that the extremely high level of the algal toxin microcystin is a predominant driver of altered stress levels and immune function observed at the HAB-exposed site in the current study. Laboratory exposure studies such as that conducted by Su et al. (2020) are now needed to establish a causal link between microcystin exposure and health effects on exposed wildlife.

4.1. Conclusions

Overall, results from this study indicate that harmful algal blooms can act as environmental stressors to aquatic amniotes, which can affect physiological stress levels and/or immune functioning across several common species of wetland-associated songbirds and reptiles. Importantly, these effects differ across taxa, and changes in immune function in response to exposure to a HAB do not appear to be consistently mediated by physiological stress levels. Thus, even if HABs do not cause direct mortality of exposed wildlife, they may have sublethal effects on the health of individual animals, which could potentially affect population persistence in aquatic systems that regularly undergo algal blooms dominated by microcystin-producing cyanobacteria. Future research should focus on how microcystin concentration and exposure duration affect wildlife taxa, and whether these impacts are exacerbated by other contaminants in aquatic systems.

CRediT authorship contribution statement

Jeanine M. Refsnider: Conceptualization, Formal analysis, Investigation, Writing - original draft. **Jessica A. Garcia:** Formal analysis, Investigation. **Brittany Holliker:** Investigation. **Austin C. Hulbert:** Investigation. **Ashley Nunez:** Investigation. **Henry M. Streby:** Investigation, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data statement

Data are deposited in Mendeley Data, V1, doi: 10.17632/pfpjwvrtcs.1.

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