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No Evidence of Long-Term Effects on Physiological Stress or Innate Immune Functioning in Northern Map Turtles a Decade After a Freshwater Oil Spill

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Abstract: Environmental perturbations such as chemical spills often have immediate and obvious effects on the health of ecosystems and individual organisms. However, the long-term effects of such perturbations are less evident and often less well-understood. In July 2010, a ruptured pipeline spilled diluted bitumen into the Kalamazoo River in Michigan, USA, ultimately oiling 56 km of the river channel. During spill cleanup operations, the most common vertebrate species rescued from the oiled river channel was the Northern Map Turtle (*Graptemys geographica*), of which over 2000 individuals were captured, cleaned of oil, and released. We returned to areas along the 2010 spill gradient to determine whether turtles from locations that had experienced different magnitudes of oiling in 2010 differed in baseline stress levels or immune functioning in 2020, which was 10 yr after the oil spill. In 2020, we collected blood samples from 100 individual Northern Map Turtles along the 2010 spill gradient and subsequently quantified the heterophil:lymphocyte ratio, bactericidal capacity, and natural antibody agglutination. We found no correlation between turtles' 2020 capture locations relative to the origin of the 2010 oil spill and either mean stress level (heterophil:lymphocyte ratio) or immune functioning (bactericidal capacity and natural antibody agglutination ability). Thus, we found no evidence that Northern Map Turtles sampled along a gradient of oil exposure resulting from the 2010 Kalamazoo River oil spill experienced long-term effects on physiological stress levels or immune functioning. Importantly, our sampling did not include any individuals that may have died during 2010–2020 from poor health after the oil spill and therefore is biased toward healthy turtles remaining in the population. Interestingly, we found that a subset of turtles known to have been oiled in 2010 actually had higher natural antibody titers than turtles sampled from a control site that presumably had never been exposed to oil. We cannot exclude the possibility of other long-term effects of the oil spill on the Map Turtle population, such as decreased reproductive output. We recommend that long-term monitoring of populations and ecosystems be included in spill mitigation plans to track the recovery of populations and ecosystems over time and to document any effects that may become evident only years after the spill event.

Key words: Bactericidal capacity; *Graptemys geographica*; Heterophil:lymphocyte ratio; Kalamazoo River; Natural antibody agglutination

Unpredictable, widespread environmental perturbations often result in the substantial mortality of organisms (e.g., red tides, Yamaguchi et al. 1981; heat waves, McKechnie and Wolf 2010; severe storms, Narwade et al. 2014). Organisms that survive such events may nevertheless experience delayed negative effects, which could result in overall decreased health or early mortality (e.g., Romero and Wikelski 2001, 2002). Often, an immediate effect of environmental perturbations is an increase in physiological stress levels in affected organisms (Wingfield et al. 1998; Angelier and Wingfield 2013). In vertebrates, an increase in plasma glucocorticoid (most commonly either corticosterone or cortisol) concentration due to activation of the hypothalamus–pituitary adrenal axis in response to a sudden environmental change suppresses nonessential processes such as reproduction or immune functioning and enhances processes associated with escape, survival, and recovery (e.g., Wingfield and Ramenofsky 1999; Sapolsky et al. 2000; Landys et al. 2006; Romero and Beattie 2021). For example, lizards in a low-quality habitat had elevated stress levels compared to individuals in a high-quality habitat, which may have stimulated food acquisition behavior or lipid storage in the inferior habitat (Josserand et al. 2017). Similarly, marine iguanas sampled during a severe El Niño event had higher concentrations of circulating corticosterone than conspecifics during a non-El Niño year (Romero and Wikelski 2001).

Although temporary increases in stress levels are critically important, for example by allowing an individual to escape or recover from a dangerous situation, prolonged periods of elevated stress levels can be harmful (e.g., Wingfield et al. 1998; McEwen and Wingfield 2003). In vertebrates, high concentrations of circulating glucocorticoids are often associated with depressed immune function, decreased body condition, or decreased reproductive output (e.g., Nelson and Demas 1996; Moore et al. 2000; Sapolsky et al. 2000). For example, chronically stressed snakes exhibited suppressed bactericidal capacity and reduced wound-healing ability compared to control animals (Neuman-Lee et al. 2015). Thus, when a physiological stress response is maintained by an individual over a prolonged timeframe, the individual's dynamic equilibrium becomes disrupted, resulting in stress-induced disease or allostatic overload (e.g., Romero and Beattie 2021). Therefore, organisms that initially survive a widespread environmental perturbation may nonetheless experience delayed negative effects from a long period of elevated stress levels. If such sublethal effects are experienced by numerous individuals, serious consequences could result at the population scale such as reduced recruitment (e.g., Jergenson et al. 2014).

Effects of environmental perturbations on the health of individuals are often examined during or immediately after the perturbation, which is the time period in which a direct stress response to the perturbation is greatest. For example, wild koalas that encountered habitat destruction and wildfires had higher physiological stress levels than control animals from an intact habitat (Narayan 2019). Similarly, songbirds and watersnakes exposed to algal toxins during a harmful algal bloom had higher stress levels than conspecifics from a control site (Refsnider et al. 2021). Importantly, however, environmental perturbations may also have long-

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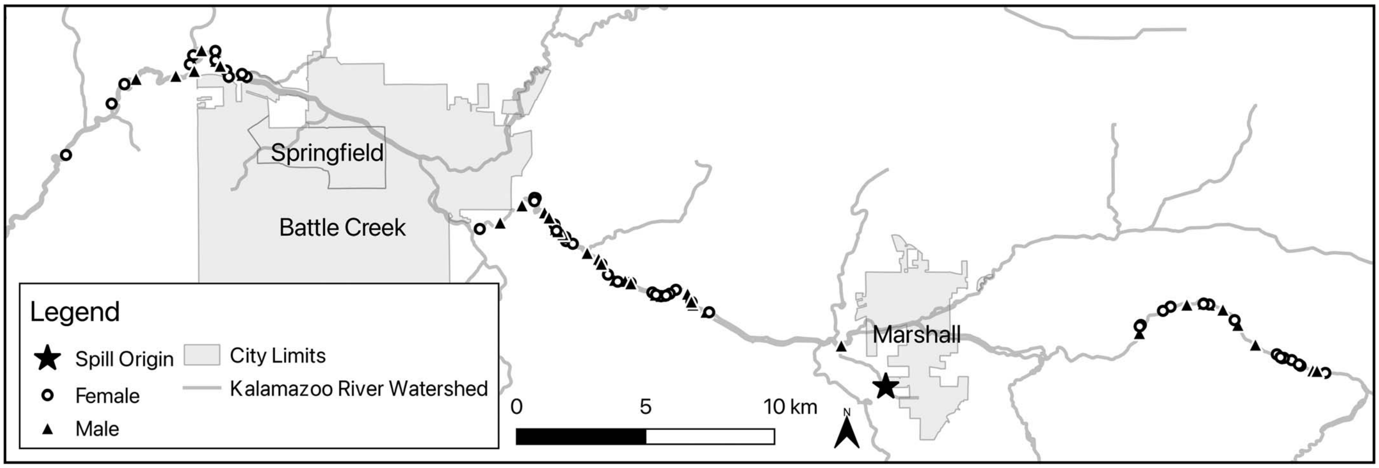

Fig. 1.—Capture locations of Northern Map Turtles (*Graptemys geographica*) sampled in July–September 2020 in the Kalamazoo River (Michigan, USA). The star indicates the location of the ruptured pipeline that caused the 2010 oil spill. Capture locations to the left (downstream) of the star were oiled during the 2010 oil spill; locations to the right (upstream) were not oiled and served as a control site.

term effects on individual health, particularly if stress levels are elevated for a prolonged period after the perturbation (Jessop et al. 2013). For example, an unpredictable event such as a chemical spill would likely result in an immediate and extreme stress response in affected individuals, but residual contamination in the environment could continue to act as a chronic stressor to inhabitants long after the initial contamination event (Romero and Wikelski 2002; Martin et al. 2010). The potential sublethal effects of environmental perturbations on the health of individual organisms long after the perturbation itself are generally poorly understood, particularly in comparison to our understanding of the immediate effects of a perturbation.

In July 2010, an oil pipeline near Talmadge Creek (Michigan, USA) ruptured and was reported to have spilled 3.2 million L (834,444 gal) of diluted bitumen (dilbit) crude oil (NTSB 2012). The crude oil entered Talmadge Creek and flowed downstream into the Kalamazoo River, where it continued to flow downstream with the current and ultimately affected nearly 56 km of river channel and banks (EPA 2016; Fig. 1). Initially the oil floated on the river surface, but subsequently, some oil mixed with the water column and sediment and eventually settled on the substrate (NTSB 2012). The U.S. Environmental Protection Agency later estimated that oil spill response operations recovered 4.5 million L (1,181,599 gal) of dilbit oil from the vicinity of the spill site (EPA 2016). Extensive cleanup and rehabilitation efforts by volunteers and private contractors began immediately after the oil spill and continued into 2011, with some additional work targeting specific areas in 2012 and 2013.

During cleanup efforts after the 2010 oil spill, over 2000 Northern Map Turtles (*Graptemys geographica*), the most commonly captured turtle species in the Kalamazoo River, were removed from the river, cleaned of oil, individually marked, and released (Otten 2022). Over 25% of these turtles were recaptured in subsequent years, with many recaptures occurring in 2019 and 2020, or 9–10 yr after the 2010 oil spill (Otten et al. 2022). Extensive radiotelemetry and mark–recapture data from this population demonstrate that Map Turtles in the Kalamazoo River have mean home range lengths of 2.4 (males) and 3.9–6.6 river-km (females; Otten 2022). Moreover, both sexes have strong site fidelity and homing ability, such that 45% of turtles that were translocated and released outside their home ranges in the immediate aftermath of the 2010 oil spill were subsequently recaptured within their original home range, even when translocation distances were >50 river-km (Otten 2022). The strong site fidelity we have documented in Map Turtles in the Kalamazoo River suggests that individuals likely remain in the general vicinity of their capture sites, rather than traveling long distances over stretches of the river that were differentially affected by the 2010 oil spill.

In 2020, we sampled Northern Map Turtles from stretches of the Kalamazoo River that had been differentially affected by the 2010 oil spill to quantify physiological stress levels and innate immune functioning of individuals living in the river 10 yr after the oil spill. We hypothesized that individual health metrics would improve with greater distance from the origin of the 2010 oil spill. That is, we expected that turtles sampled from near the spill site, in areas most severely affected in 2010, would have higher physiological stress levels and lower innate immune functioning, than turtles sampled farther from the spill site, in areas that had been less affected by the 2010 oil spill. Thus, we predicted that long-term effects of oil contamination would manifest as higher stress levels and lower immune functioning in individuals currently inhabiting areas that had been most heavily affected by the environmental perturbation resulting from the 2010 oil spill. We also sampled turtles from a site upstream of the 2010 oil spill, which therefore had not been affected by the oil spill, to determine whether there were any differences between turtles living in previously contaminated sections of the river compared to an uncontaminated control site. As a secondary study objective, a subset of the turtles sampled in 2020 were known to have been oiled in 2010; therefore, we also compared stress level and immune functioning of this subset of turtles that were oiled in 2010 and sampled in 2020, to turtles sampled from the control site in 2020.

Materials and Methods

Study Species and Site

Northern Map Turtles inhabit rivers and large lakes and leave the water mainly to bask on woody debris or to nest, generally within 100 m of their aquatic habitat (Lindeman 2013; Nagle and Russel 2020). The sexes demonstrate pronounced dietary and size dimorphism, with females growing to much larger sizes than males and specializing on hard-shelled mollusks such as freshwater snails and mussels, whereas the smaller males feed mainly on soft-bodied aquatic invertebrates (Richards-Dimitrie et al. 2013; Gacheny et al. 2021). Their primarily aquatic lifestyle likely made Northern Map Turtles particularly vulnerable to oil exposure during the 2010 Kalamazoo River oil spill; indeed, in the months immediately after the pipeline rupture, spill response personnel rescued and cleaned over 2000 Northern Map Turtles, representing 60% of all rescued turtles, from oil-exposed stretches of the river (EPA 2016).

In July–September 2020, we sampled 71 Northern Map Turtles from approximately 40 river-km of the Kalamazoo River along a gradient that experienced differing magnitudes of oil exposure during the 2010 oil spill (Fig. 1). We also sampled 29 turtles from approximately 10–20 river-km upstream of the origin of the 2010 oil spill, which was not exposed to oil from the 2010 oil spill and that therefore served as an approximate control site. We captured Northern Map Turtles either by using dipnets from a kayak or by hand while snorkeling. Many turtles had previously been individually marked, either with a passive integrated transponder tag or with a unique set of notches in the marginal scutes, and because some of these previously marked turtles had been captured immediately after the 2010 oil spill, their capture histories included data on how heavily oiled those individuals had been upon their original capture in 2010 (Otten et al. 2022). Turtles that were captured for the first time during 2020 capture efforts were individually marked with a unique combination of shell notches (as in Cagle 1939). We recorded the capture location of each individual by using a hand-held GPS unit. We determined sex by using secondary sex characteristics and measured each individual (i.e., carapace and plastron length and width). We classified turtles as either subadults (5.9–7.0 cm for males, 5.5–16.0 cm for females) or adults (>7.0 cm for males, >16.0 cm for females) based on plastron length (Lindeman 2013); no turtles smaller than subadult size were included in the present study. We also collected a blood sample from the caudal vein using a heparinized, 28-ga syringe. Immediately after blood collection, for each individual, we made a blood smear on a glass slide, and then we centrifuged the remainder of the blood sample to separate the plasma from the packed blood cells. The plasma was drawn off using a pipette, aliquoted into separate tubes for subsequent immune assays, and flash-frozen. We stored plasma samples at –80°C at the University of Toledo. Turtles were released at their exact site of capture after collection of the blood sample.

Quantifying Stress Levels and Immune Functioning

We measured each individual's physiological stress level by quantifying the ratio of heterophils to lymphocytes (hereafter, H:L ratio) in the individual's blood smear. Heterophils and lymphocytes are two types of white blood cells that contribute to the mounting of immune defenses. When an individual is exposed to a stressor, their H:L ratio becomes elevated; therefore, higher H:L ratios are indicative of higher baseline levels of physiological stress (Davis et al. 2008). Moreover, unlike glucocorticoid hormones that often are attenuated in chronically or repeatedly stressed animals, H:L ratios typically remain high in chronically stressed individuals, making H:L ratio a better metric than glucocorticoid concentration for detecting chronic stress in animal populations (e.g., Davis and Maney 2018; Gormally and Romero 2020). After the collection of blood samples, we air-dried blood smear slides, fixed them in 70% ethanol, and later stained them with Stat-Quick Wright Giemsa and May–Grünwald Stain (ENG Scientific, Inc.). Then, to quantify the circulating leukocyte profile of each individual, we counted 100 white blood cells in each blood smear (excluding thrombocytes) by using a light microscope at 100× power and identified each cell as a heterophil, lymphocyte, eosinophil, basophil, or monocyte (Kassab et al. 2009; Javanbakht et al. 2013). We calculated the H:L ratio for each individual as a measure of the individual's baseline physiological stress level (as in Refsnider et al. 2021).

We also quantified each individual's innate immune functioning by using two assays, namely, a bacterial killing assay and a natural antibody agglutination assay. First, we used the bacterial killing assay to measure the bactericidal capacity of an individual's blood plasma to kill *Escherichia coli* (Tieleman et al. 2005). For this assay, we applied diluted plasma samples to cultures of *E. coli* and then incubated the solution to allow time for bactericidal activity of the plasma to kill the *E. coli*. 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. We followed the methods of Millet et al. (2007) and Palacios et al. (2011), with modifications previously validated for Emydid turtles (Refsnider et al. 2021). First, we reconstituted a pellet of lyophilized *E. coli* (Microbiologics, ATCC#8739) in 40 mL of warm, phosphate-buffered saline (PBS), and then prepared a working *E. coli* solution by further diluting a fraction of reconstituted *E. coli* to 1:40, which produced approximately 100 colony-forming *E. coli* bacteria per 10 µL. We then diluted individual turtles' plasma samples to 1:10 in PBS. We prepared sample reactions by adding 10 µL of the working *E. coli* solution to 100 µL of the diluted plasma samples. We incubated sample reactions at 28°C to allow bacterial killing by complement proteins, natural antibodies, and antimicrobial peptides in the plasma. We also prepared two replicate control reactions per 20 individuals by adding 10 µL of the working *E. coli* solution to 100 µL PBS, which were incubated simultaneously with the *E. coli*-treated plasma samples. After the incubation, we prepared two replicate plates of all sample and control reactions using 50-µL aliquots on 4% tryptic soy agar. We incubated plates at room temperature for 36 h and then counted the number of *E. coli* colonies on each plate. The number of colonies in each plate was divided by the mean number of colonies in the control plates for the corresponding set of individual turtle samples, and this value was subtracted from 1 to obtain the proportion of bacteria killed. For each plasma sample, we used the mean proportion of bacteria killed from the two replicate

Table 1.—Circulating leukocyte profile, heterophil:lymphocyte (H:L) ratio, bactericidal capacity, and natural antibody agglutination titers in Northern Map Turtles (*Graptemys geographica*) sampled in July–September 2020 in the Kalamazoo River (Michigan, USA) along a gradient differing in oil exposure after an oil spill in 2010 (Oiled). An additional, unaffected 10-km stretch of the Kalamazoo River was also sampled, at 10–20 river-km upstream of the ruptured pipeline that caused the 2010 oil spill (Control). H:L ratio, bactericidal capacity, and natural antibody agglutination titer were not correlated with an individual's 2020 capture distance from the origin of the 2010 oil spill. Values are presented as mean ± standard error.

Sampling location	Eosinophil %	Basophil %	Lymphocyte %	Monocyte %	Heterophil %	H:L ratio	Bactericidal capacity (% colonies killed)	Natural antibody agglutination titer
Oiled ($n = 71$)	0.10 ± 0.01	0.00 ± 0.00	0.67 ± 0.02	0.01 ± 0.00	0.21 ± 0.01	0.36 ± 0.03	0.21 ± 0.02	5.17 ± 0.13
Control ($n = 29$)	0.12 ± 0.02	0.00 ± 0.00	0.68 ± 0.02	0.00 ± 0.00	0.20 ± 0.02	0.32 ± 0.04	0.18 ± 0.03	4.61 ± 0.21

plates as the measure of each individual's bactericidal capacity. Repeatability between pairs of control plates within a single run of the assay ranged 3–19%.

As a second measure of innate immune competency, we conducted a natural antibody agglutination assay (Matson et al. 2005). Natural antibodies are produced constitutively, and their role in immune defense is to agglutinate to and lyse foreign cells. In this second measure of immune function, we assessed individuals' constitutive innate immunity in terms of ability to adhere to foreign red blood cells (RBCs). We followed the methods of Matson et al. (2005), with modifications previously validated for Emydid turtles (Refsnider et al. 2021). We added 15 µL of PBS to each well in a 96-well plate. Then, we mixed 5 µL of either plasma (rows 2–11) or positive control (Row 1; sheep anti-rabbit RBC serum) 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, we added 10 µL of foreign RBCs (5% rabbit RBCs) in Alsever's anticoagulant (Hemostat Laboratories) to all wells, which resulted in a final plasma dilution in Column 1 of 1:8. We then incubated plates at 28°C for 90 min. We visually scored agglutination titers 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, 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. We did not observe lysis in any of the samples, so lysis titers were not recorded.

Statistical Analysis

Analyses were conducted using R v3.6.3 (R Core Team 2020). For all turtles captured downstream of the oil spill origin, we used the Riverdist package (Tyers 2017) to measure the river distance as a continuous variable between each individual's 2020 capture location and the origin of the 2010 oil spill. We then used general linear regression in the Rcmdr package (Fox 2017) to determine whether individuals' H:L ratio, bactericidal capacity, or natural antibody agglutination ability were correlated with the distance from the oil spill at which they were sampled in 2020. Males and females were pooled because preliminary analyses showed no difference in mean H:L ratio, bactericidal capacity, or natural antibody agglutination ability between the sexes. We also compared mean H:L ratio, bactericidal capacity, or natural antibody agglutination ability between turtles sampled from stretches of the Kalamazoo River that had been oiled in 2010 to turtles sampled from the control site upstream of the 2010 oil spill using *t*-tests. Finally, we compared mean H:L ratio, bactericidal capacity, or natural antibody agglutination ability between the subset of adult turtles sampled in 2020 that were known to have been oiled immediately after the 2010 oil spill and turtles sampled from the control site in 2020 by using *t*-tests.

Results

During 23 July–15 September 2020, we sampled 100 Northern Map Turtles, as follows: 71 (35 males and 36 females) from stretches of the Kalamazoo River that had been oiled in 2010, and 29 (11 males and 18 females) from the control site upstream of the 2010 spill origin (Fig. 1). Of these 100 turtles, based on unique combinations of shell notches, 19 individuals (9 males and 10 females, all captured downstream of the oil spill origin) were known to have been captured, cleaned, and rehabilitated during cleanup operations immediately after the 2010 oil spill. At the time of their rescue from the oil spill, seven had been classified as "heavily oiled" (i.e., >50% of the individual's surface was oiled), eight as "moderately oiled" (i.e., oil on 10–50% of the body surface), two as "lightly oiled" (i.e., <10% of body surface was oiled); and two as "unoiled." Males and females did not differ in H:L ratio, bactericidal capacity, and natural antibody agglutination ability and were therefore pooled in subsequent analyses. Regardless of whether they were captured in oiled stretches of the river or from the control site, adult turtles had a slightly higher bactericidal capacity (mean = 22.8%) than subadults (mean = 14.7%, $t = 2.15$, $P = 0.036$); but the two age classes did not differ in H:L ratio or natural antibody agglutination ability.

Mean values for H:L ratio, bactericidal capacity, and natural antibody agglutination ability for turtles sampled in 2020 from previously oiled vs. control stretches of the Kalamazoo River are shown in Table 1. For turtles sampled in areas of the river that had been oiled in 2010, H:L ratio ($F_{1,66} = 1.14$, $P = 0.29$, $R^2 = 0.02$), bactericidal capacity ($F_{1,69} = 0.73$, $P = 0.40$, $R^2 = 0.01$), and natural antibody agglutination titer ($F_{1,58} = 0.07$, $P = 0.79$, $R^2 = 0.001$) were not correlated with an individual's 2020 capture distance from the origin of the 2010 oil spill (Fig. 2). Mean H:L ratio ($t = -1.00$, $P = 0.32$) and bactericidal capacity ($t = -1.04$, $P = 0.31$) did not differ between individuals sampled in 2020 that were known to have been oiled immediately after the 2010 oil spill, compared to turtles sampled in 2020 from the control site upstream of the 2010 oil spill origin. However, individuals sampled in 2020 that were known to have been oiled immediately after the 2010 oil spill had higher mean natural antibody agglutination titers than turtles sampled from the control site (means = 5.56 and 4.61 for turtles oiled in 2010 vs. turtles sampled from control sections; $t = -3.29$, $P = 0.002$; Fig. 3).

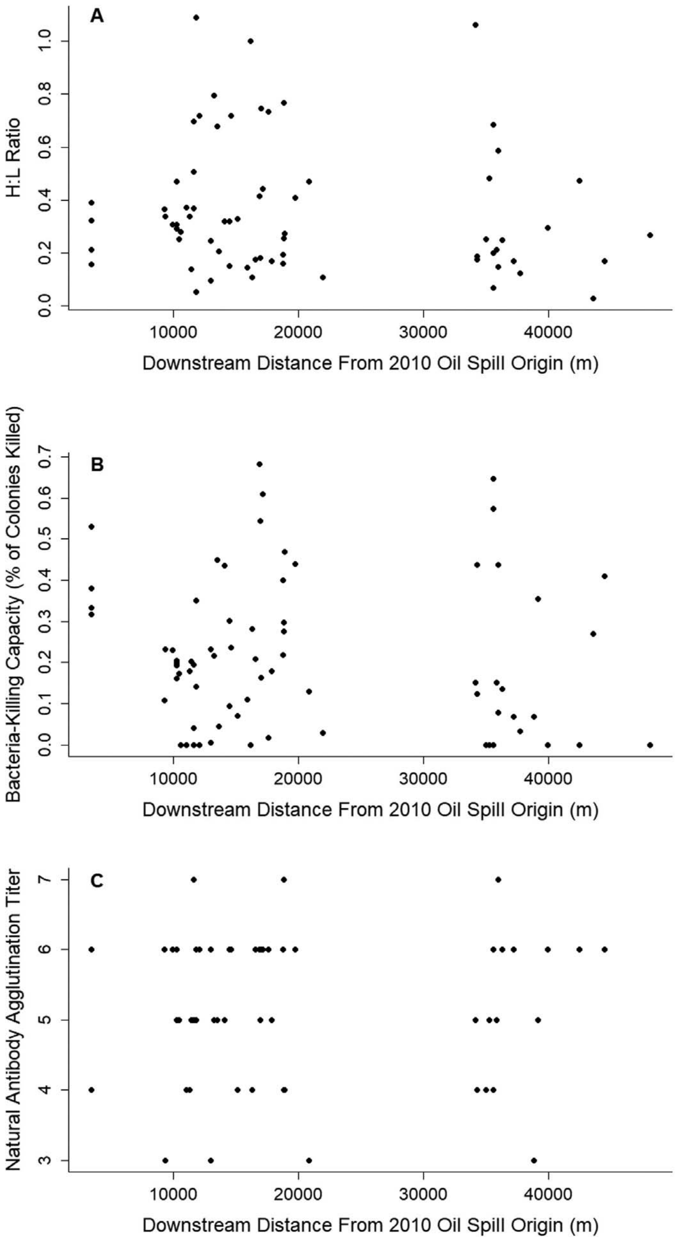

FIG. 2.—Physiological stress and immune function in Northern Map Turtles (*Graptemys geographica*) sampled in July–September 2020 from areas of the Kalamazoo River (Michigan, USA) that had been oiled in 2010. Mean stress levels (heterophil:lymphocyte [H:L] ratio) (A), bactericidal capacity (B), and natural antibody agglutination titer (C) were not correlated with an individual's 2020 capture distance from the origin of the 2010 oil spill.

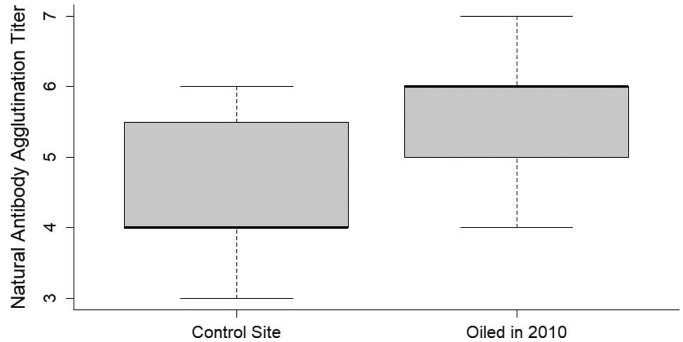

FIG. 3.—Box plot of agglutination titers in Northern Map Turtles (*Graptemys geographica*) sampled in July–September 2020 from the Kalamazoo River (Michigan, USA). Turtles that had been oiled immediately after the 2010 oil spill ($n = 17$) had higher mean agglutination titers than turtles sampled from the control site upstream of the origin of the 2010 oil spill ($n = 29$; $t = -3.29$, $P = 0.002$).

DISCUSSION

When organisms are exposed to unpredictable environmental perturbation, sublethal effects on individuals' health are often most evident in the period immediately after the event itself. For example, individuals exposed to wildfires or chemical spills may exhibit obvious injuries or abnormal behavior. However, long-term health effects may also result from environmental perturbations, and although they may be more cryptic than the immediate effects, they may be equally detrimental to a population's persistence. For example, koala populations that experienced land clearing and bushfires as environmental perturbations subsequently exhibited chronically elevated stress levels, potentially increasing their vulnerability to other threats such as disease, vehicle collisions, and dog attacks (Narayan 2019). We found no evidence that Northern Map Turtles sampled in 2020 differed in physiological stress levels or immune functioning based on their downstream location relative to the origin of the 2010 oil spill. However, mean natural antibody agglutination ability in 2020 was higher overall in turtles that were known to have been oiled immediately after the 2010 oil spill than that in turtles sampled from the control site.

One potential explanation for our results is that habitat recovery and animal rehabilitation efforts after the 2010 oil spill restored the Kalamazoo River sufficiently so that chronic health effects on the metrics we measured were not detectable 10 yr later. During our sampling in 2020, we captured 17 turtles that had been oiled and rehabilitated immediately after the 2010 oil spill, of which 15 had been classified as heavily or moderately oiled when originally captured (J. Otten, personal observation). The finding that these individuals did not have elevated stress levels or depressed immune functioning 10 yr after being oiled, cleaned, and released back into the river supports the effectiveness of habitat recovery and animal rehabilitation efforts after the oil spill.

Another potential reason that may explain, in particular, the similar physiological stress levels along the oil spill gradient and at the control site is that turtles often do not exhibit stress responses typical of other vertebrates, even when exposed to stressful environmental conditions. For example, Painted Turtles exposed to a novel climate did not show elevated corticosterone levels (Refsnider et al. 2015), and individuals of the same species exposed to toxic algal blooms did not show elevated H:L ratios compared to unexposed individuals (Refsnider et al. 2021). Similarly, nesting female Loggerhead Sea Turtles with injuries from recent shark attacks did not have higher corticosterone levels than uninjured conspecifics (Jessop et al. 2004). The reason for an apparent lack of stress response in turtles, despite otherwise stressful environmental conditions, could be that physiological stress levels may become attenuated during prolonged stressful environmental conditions to avoid impairing other important biological functions, such as immunity (Carbillet et al. 2019). Finally, our study included only turtles that were alive in 2020, and thus available to be sampled, and is necessarily biased toward healthy individuals (although it is worth noting that monthly survival rates in this population were >97% at 8–11 yr after the 2010 oil spill; Otten et al. 2022). It is possible that individuals that suffered negative health effects as a result of the oil spill died during the 10-yr period between the 2010 oil spill and our 2020 sampling period, similar to observations by Romero and Wikelski (2002). Therefore, we would have failed to sample sick individuals in this study because they would no longer have been available in the population to be sampled in 2020.

We do not believe that the similarities in H:L ratios, bactericidal capacity, and natural antibody agglutination ability in Northern Map Turtles sampled along the spill gradient are due to individuals traveling substantial distances throughout the Kalamazoo River, such that they were exposed to the entire gradient of oil-affected river and were not actually separated into different stretches of channel that experienced different magnitudes of oiling. Extensive radiotelemetry and mark–recapture data from this population demonstrate that both sexes have strong site fidelity and generally remain within a specific home range (mean = 2.4 river-km for males and 4.6 river-km for females; Otten 2022). Based on the capture histories of individually marked turtles, even when translocated over long distances immediately after the 2010 oil spill, a substantial proportion of Map Turtles subsequently returned to their original home ranges (Otten 2022; also see Jergenson et al. 2014). Therefore, turtles likely remain in the general vicinity of where we captured them in this study, rather than traveling among different stretches of the river that were differentially affected by the 2010 oil spill. Our spatial ecology results also demonstrated that individuals' home range sizes are similar between turtles living in areas nearest the oil spill origin compared to turtles living farther downstream in areas that were less heavily affected by the oil spill (Otten 2022). These similarities in Map Turtle spatial ecology along the main channel of the Kalamazoo River could indicate similarities in habitat quality 10 yr postspill, despite differences in the magnitude of oiling immediately after the 2010 oil spill (Otten 2022).

We did not observe any relationship between sampling location and H:L ratio, bactericidal capacity, or natural antibody agglutination ability in Northern Map Turtles relative to the origin of the 2010 Kalamazoo River oil spill. These results suggest that turtles currently living in sections of the Kalamazoo River that were the most heavily oiled in 2010 are in similar health to turtles sampled in areas either farther from the spill site or from an unoiled control site. It is important to note that we quantified only three metrics of individual health, and it is possible that other metrics may differ among turtles sampled at different locations along the spill gradient. For example, it would be interesting to determine whether the organs of turtles exposed to the 2010 oil spill contain detectable concentrations of polycyclic aromatic hydrocarbons or other compounds found in the dilbit oil that spilled into the Kalamazoo River. Previous research has found higher rates of deformities in hatchling turtles after an oil spill than in control turtles, which suggests that early life stages may be especially vulnerable to toxins present in spilled oil (Bell et al. 2006; Van Meter et al. 2006; Shaver et al. 2021). We did not assess reproductive output in turtles along the oil spill gradient, but understanding the potential long-term effect of oil spills on recruitment and population demography is a critical information gap.

We found that natural antibody agglutination titers were slightly higher in turtles that were known to have been oiled immediately after the 2010 oil spill than in turtles sampled from the control site. That this difference was evident despite the low sample size (17) of turtles known to have been oiled in 2010 suggests that oiling may have primed certain components of the turtles' immune system, such as the number or specificity of natural antibodies. We also found that, regardless of sampling location, adult turtles had a slightly higher bactericidal capacity than subadult turtles. Because this difference in immunity with age class was consistent across turtles sampled both from stretches of the river that had been oiled in 2010 and from the unoiled control stretch upstream from the 2010 oil spill, it likely reflects immune functioning relative to ontogeny and is not reflective of oil exposure history. Aging is often thought to result in decreasing immune functioning (Ujvari and Madsen 2005; Frasca et al. 2008), but Emydid turtles may be unusual in showing no evidence of declining immune responses during aging (Zimmerman et al. 2013).

Large-scale spills of oil and other chemicals are unpredictable and (fortunately) rare, making it difficult to directly study their effects on wild organisms in their natural habitat. It is even more difficult to attribute causality, or lack thereof, to any effects of a spill event on organisms or their environment long after a spill has occurred (e.g., Saba and Spotilla 2003; Luiselli et al. 2006). In our study, for example, were the lack of apparent health effects on Map Turtles due to successful habitat restoration efforts in the river channel itself or to the system's own resilience regardless of spill cleanup efforts; or were individual turtles simply unaffected by any lingering oil contamination? To maximize the effectiveness of restoration efforts and the efficiency of resources dedicated to such efforts, it is important to understand the factors associated with populations and ecosystems that have apparently recovered, as well as those that failed to recover after chemical spills and associated restoration efforts. Therefore, long-term monitoring of populations and ecosystems should be included in chemical spill mitigation plans to track the recovery of populations and ecosystems over time and to document any effects that may only become evident years after the spill event.

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University of Toledo's Institutional Animal Care and Use Committee (Protocols 108797 and 400109).

Supplemental Material

Data are deposited in Mendeley Data, V1, available at https://data.mendeley.com/datasets/6jnp59k59s.

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