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Mass mortality of eastern box turtles with upper respiratory disease following atypical cold weather

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ABSTRACT: Emerging infectious diseases cause population declines in many ectotherms, with outbreaks frequently punctuated by periods of mass mortality. It remains unclear, however, whether thermoregulation by ectotherms and variation in environmental temperature is associated with mortality risk and disease progression, especially in wild populations. Here, we examined environmental and body temperatures of free-ranging eastern box turtles *Terrapene carolina* during a mass die-off coincident with upper respiratory disease. We recorded deaths of 17 turtles that showed clinical signs of upper respiratory disease among 76 adult turtles encountered in Berea, Kentucky (USA), in 2014. Of the 17 mortalities, 11 occurred approximately 14 d after mean environmental temperature dropped 2.5 SD below the 3 mo mean. Partial genomic sequencing of the major capsid protein from 1 sick turtle identified a ranavirus isolate similar to frog virus 3. Turtles that lacked clinical signs of disease had significantly higher body temperatures (23°C) than sick turtles (21°C) during the mass mortality, but sick turtles that survived and recovered eventually warmed (measured by temperature loggers). Finally, there was a significant negative effect of daily environmental temperature deviation from the 3 mo mean on survival, suggesting that rapid decreases in environmental temperature were correlated with mortality. Our results point to a potential role for environmental temperature variation and body temperature in disease progression and mortality risk of eastern box turtles affected by upper respiratory disease. Given our findings, it is possible that colder or more variable environmental temperatures and an inability to effectively thermoregulate are associated with poorer disease outcomes in eastern box turtles.

KEY WORDS: Disease · Ranavirus · Mass die-offs · *Terrapene carolina* · Thermoregulation · Body temperature · Markov model

INTRODUCTION

Emerging pathogens are increasingly considered a major threat to ectotherm populations and assemblages worldwide (Duffus et al. 2015). Several pathogens, including ranaviruses, herpesviruses, and mycoplasma, have been linked to high morbidity in ectothermic vertebrate species and have been associated with die-offs in populations of amphibians and turtles (Green et al. 2002, Daszak et al. 2003, Feldman et al. 2006, Lesbarrères et al. 2012, Kane et al. 2016). In North America, mass die-offs linked to disease have occurred in populations of both wild and captive amphibians (Green et al. 2002, Daszak et al. 2003) and reptiles (Allender et al. 2011, Duffus et al. 2015). Because emerging infectious diseases pose a potential threat to global amphibian and reptile biodiversity, it has become increasingly important to better understand the distribution, progression, and impacts of diseases on native wildlife populations.

The prevalence of upper respiratory disease from pathogens such as ranavirus, herpesvirus, or myco-

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plasma can vary dramatically within populations of a single species, both among individuals and across time (Greer et al. 2009, Johnson et al. 2010, Hoverman et al. 2012, Souza et al. 2012, Crespi et al. 2015). The outcome and pathology of upper respiratory infections can also vary among populations (Pearman et al. 2004, Pearman & Garner 2005), with some populations experiencing high mortality (Green et al. 2002), and others exhibiting low mortality and few pathological signs when infected (Gray et al. 2009, Miller et al. 2009, Rothermel et al. 2013). One possible reason for the high degree of variation in morbidity from upper respiratory disease is that dynamics or the severity of progression may be greatly affected by environmental conditions, such as temperature (Hoverman et al. 2012, Echaubard et al. 2014, Brunner et al. 2015). Temperature likely plays a critical role in the timing and severity of upper respiratory epizootics and associated mortality in ectotherms (Rojas et al. 2005, Allender et al. 2013, Brunner et al. 2015). For example, ranavirus can be as much as 5 to 8 times more prevalent in larval amphibians during cold seasons than in summer (Gray et al. 2007). Additionally, in a study of 4 red-eared slider turtles *Trachemys scripta elegans* infected with ranavirus, mortality at lower temperatures was twice that at warmer temperatures (Allender et al. 2013). These results differ from those of other studies that suggest that warmer temperatures can exacerbate infections by altering the rate of viral replication and host-immune response (Brunner et al. 2015, Brand et al. 2016). Overall, temperature fluctuation (i.e. above or below average warm or cold events, respectively) at daily and seasonal time scales may also decrease resistance of ectothermic hosts to mortality-associated pathogens and increase the rate of host mortality (Raffel et al. 2006, 2013). However, previous studies of upper respiratory diseases in reptiles have predominantly focused on absolute temperature (Schumacher 1997, Allender et al. 2013, Jacobson et al. 2014), rather than seasonal variation in thermal regime (Sandmeier et al. 2013).

Ectotherms rely on behavioral thermoregulation to optimize physiological function and ecological performance (Huey 1991). Effective thermoregulation can affect nearly all physiological and ecological processes of ectotherms, including immune function (Huey & Stevenson 1979, Maniero & Carey 1997, Rohr et al. 2013). In response to infection, ectotherms will often seek out warmer microclimates to raise internal temperatures, inducing behavioral fever, which can reduce infection load and disease severity (Rowley & Alford 2013). Thus, disease outcome can be shaped by an individual's ability to effectively thermoregulate at temperatures that maximize immune function and/or inhibit pathogen growth (Kluger et al. 1996, Deen & Hutchison 2001, Allender et al. 2013, Nowakowski et al. 2016). However, once a disease has reached advanced stages, it can also reduce the thermal tolerances of ectotherms and may affect their ability to thermoregulate effectively (Sherman 2008, McGuire et al. 2014). To date, little information is available with which to understand how environmental temperatures or behavioral thermoregulation can shape morbidity or mortality of wild, free-ranging ectotherms from ranavirus or similar disease-causing pathogens (Brunner et al. 2015).

Here, we report a mass die-off of eastern box turtles *Terrapene carolina* exhibiting signs of upper respiratory disease coincident with rapid decreases in environmental temperatures. Because many of our study animals were outfitted with temperature recorders, we were able to examine how animal body temperatures and environmental temperatures were associated with timing of mortality and the disease outcome of animals that showed clinical signs of upper respiratory disease in the population. Our results suggest a link between environmental temperature, body temperature, and upper respiratory disease morbidity and mortality in a wild reptile population.

MATERIALS AND METHODS

Study area

We conducted our study in a 40 ha parcel in the Berea College Forest (BCF) in Madison County, Kentucky, USA. BCF is managed by Berea College and is used for experimental research studies, timber harvesting, recreation, and education. BCF is located on the western edge of the Northern Cumberland Plateau, and is dominated by mature hardwood forest primarily composed of oak–hickory species. Elevation at the site ranges from approximately 200 to 500 m.

Field techniques

From September 2013 to May 2014, we used intensive time–area constrained searches to detect and capture eastern box turtles at the study site (Walker 2012). We also located new box turtles opportunistically when we encountered them. Upon first capture, we recorded the sex, straight-line carapace length, plastron length, carapace width, shell depth (height) in cm, and uniquely marked the marginal scutes

using a triangular metal file (Cagle 1939). Additionally, we outfitted a subset of these turtles (12 adult males, 7 adult females) with a radio-transmitter to monitor movements (SOPER-2190; Wildlife Materials). We also outfitted these radio-tracked individuals with temperature loggers (Embedded data systems© thermochron iButton; DS1922T-F5) to monitor their carapace temperatures, which in small-bodied turtles are generally highly correlated with internal body temperatures (Bernstein & Black 2005, Chen & Lue 2008). Following the methods of Grayson & Dorcas (2004), we attached a radio-transmitter to the posterior carapace of each box turtle using marine grade epoxy (Loctite Five Minute Epoxy). To prevent water damage to temperature loggers, we covered each logger in plastic tool dip (Plasti-Dip International) prior to deployment. We used the temperature loggers to record turtle temperatures (±0.5°C) every 30 min. We released each box turtle at its point of capture after we attached all study equipment. We relocated telemetered adults every 7 to 10 d from June to September 2014, and recorded each location to the nearest 3 m using a handheld GPS (e.g. mean GPS accuracy). When each animal was relocated, we also assessed its general health, noting any clinical signs of disease, such as those typically associated with upper respiratory disease (i.e. nasal, ocular, or oral discharge, gasping for air, wheezing, swollen eyes, bobbing head; Allender et al. 2011, Goodman et al. 2013, Miller et al. 2015). External clinical signs of upper respiratory disease were not scored for severity. Lethargy was determined based on drooping of limbs or non-responsive posture. While tracking telemetered individuals, some additional sick or dead turtles were encountered incidentally. We used standard aseptic field techniques, handling each animal with new sterile latex gloves on each occurrence, and processed the individual to collect measurements. Two dead individuals were collected for testing, and further information is provided in the health assessment section below. Finally, we obtained daily mean ambient environmental temperatures (T_e) from a local weather station (Madison County; Berea, KY, KKYKENTU6). Using T_e, we generated daily deviations (DVM) from 3 mo mean T_e to assess temperature variation, and above or below average climate events throughout the entire 2014 study period.

Linear mixed model thermal analysis

To examine changes in daily mean body temperature (T_b) over time associated with pathology, we grouped radio-tracked turtles into 1 of 3 groups: (1) animals that remained healthy, (2) those that showed external signs of upper respiratory disease (i.e. varying degrees of lethargy, discharge, or breathing difficulty) and recovered, or (3) those that showed signs (listed previously) and later died based on the last visual assessment that we made when we removed all study equipment from the animals on calendar day of year (DOY) 275 or when an animal was found dead. To compare T_b among the 3 groups, we used daily T_b from each individual within each group during the complete study period. To analyze data at the highest frequency while maintaining sufficient sample size, we partitioned T_b into 5 d periods (n = 8) of the year beginning with DOY 185 (first recorded mortality of non-telemetered turtle). Because our study focused on daytime behavior when turtles are presumed to be active, we removed temperature records occurring before sunrise and after sunset, as determined by the US Naval Observatory Astronomical Applications Department (http://aa.usno.navy.mil/data/index.php), when calculating T_b. We also excluded any temperature records from animals after the last day on which they were visually confirmed to be alive.

We used program R (Version 3.1.1) and the 'nlme' package (Pinheiro et al. 2016) to compare T_b among groups with linear mixed models (LMMs) for all 8 periods of the study season. We fit LMMs with an autoregressive (AR-1) correlation structure (Littell et al. 2000), which accounted for correlation between consecutive T_b records from each individual within each group. The AR-1 correlation structure was a function of DOY nested within the random effect of turtle ID (DOY|ID). We calculated mean T_b in each group from the model coefficients, and we calculated confidence intervals from the standard errors for the prediction means. We determined significance of model coefficients at $\alpha = 0.05$. We restricted our inference about differences among the 3 groups to DOY 180–224 because too few animals remained alive after DOY 224 in the group of animals that died to allow comparison. However, we predicted differences between recovered and healthy groups from DOY 225–245 because these animals were still recorded alive.

Markov model thermal analysis

To test the hypotheses that both the amount of time spent above and below ambient temperature and the transition probabilities between these 2

states differed by box turtle status (i.e. healthy, recovered, or dead), we used a 2-state continuous-time Markov model (Jackson 2011) to estimate the parameters of the transition probability matrix (TPM) of a discrete-time Markov chain (R package msm). We chose this approach instead of estimating parameters using a discrete-time Markov model because package msm can both produce parametric bootstrap confidence intervals and handle multiple time series, options which are not both available in other Markov model R packages. Discrete-time Markov chains specify that the transition probabilities between states are memoryless, that is, they only depend on the current state. Continuous-time Markov chains have the same memoryless property, but rather than determining state transitions with a TPM, a matrix of transition intensities—the instantaneous risk of moving between states—is used (Jackson 2011). Thus, if Q is the transition intensity matrix and t is an arbitrary amount of time, the discrete-time TPM is $P(t) = \exp(tQ)$ (Jackson 2011). We used a simple 2-state model where we defined the first state as a box turtle having T_b above ambient temperature (i.e. behavioral thermoregulation), and the second state as a box turtle having T_b below ambient temperature. A 2-state Markov model with no absorbing states has 2 parameters to estimate because, in the discrete time case, the probability of transitioning from state 1 to state 2, p_{12}, is $1 - p_{11}$, with p_{11} being the probability of remaining in state 1. The same holds for p_{21} and p_{22} and an analogous result holds for continuous-time models.

To determine whether state transition probabilities varied by box turtle status (i.e. healthy, recovered, or dead), we fit 2 models—one with no covariate effect on the 2 TPM parameters and one with a status effect on the TPM parameters—and compared them via Akaike's information criterion (AIC). Adding status covariates brings the number of parameters that require estimation to 6. We predicted that turtles that died would have the highest probability of remaining in state 2 before death and the lowest probability of remaining in state 1, the converse for healthy turtles, and intermediate values for recovered turtles. We produced 95% confidence intervals for the TPM parameters using parametric bootstrapping with B = 1000. The row-vector stationary distribution, $\boldsymbol{\pi}$, quantifying the proportion of time turtles spent in each state, was calculated by solving the equation $\boldsymbol{\pi}\mathbf{p} = \boldsymbol{\pi}$. Confidence intervals for the stationary distributions were calculated by modifying the bootstrapping routine in package msm.

Pathogen detection

Two of the 22 box turtles that showed external clinical signs of an upper respiratory disease (Miller et al. 2015) were sent to the National Wildlife Heath Center (NWHC; Madison, WI) for postmortem diagnostic examination (see Acknowledgements), including 1 radio-tracked animal and 1 that had not been radio-tracked. Turtles were necropsied and tissues were cultured for viruses, bacteria, and mycoplasmas. A ranaviral isolate from each turtle was subjected to partial genomic sequencing of the major capsid protein to identify the virus. Stomach and intestines of each turtle were examined for parasites, and most major organs were examined histologically. The other 20 turtles that showed external clinical signs in our study were not sent to NWHC for destructive testing because of rapid autolysis (i.e. decomposition) or non-retrieval because the turtle was still alive at time of last capture.

Survival analysis

To analyze survival data, we fit a Cox or proportional-hazard regression model in the 'survival' package (Therneau 2013). The model is expressed in terms of a single survival time value for each animal and corresponding mortality event, with censoring for individuals that did not die before the end of the study. The underlying hazard function assesses how the risk of death changes per unit time (DOY) and by categorical or continuous predictors. In addition, we only included individuals that were captured multiple times during the 2014 study season (n = 25), to account for known survival time, and if last capture was a mortality event or the end of the study period for each individual (i.e. last seen alive). If the last capture was the end of the study, and the individual was still alive, it was censored from the hazard analysis. The risk of dying was calculated relative to a continuous environmental effect parameter, viz. 'daily temperature deviation from 3 mo mean temperature' (DVM) taken at event time (mortality, last live capture, or end of study), and the categorical effect parameter 'sex.' Body temperatures were only available for animals with radio-transmitters, which did not include several of the additional marked animals in the study population that died. For this reason, we used DVM instead of body temperatures to regress against mortality events in our study. Finally, we tested for violations of the proportional hazards assumption using the function 'cox.zph,' and detected no violations.

RESULTS

We encountered and marked 76 adult box turtles (47 males [M], 29 females [F]) over the course of the study at BCF. A total of 22 animals (8 M, 14 F) showed varying degrees of clinical signs of an upper respiratory disease during the study (e.g. lethargy, discharge, breathing difficulty, and swollen eyes), including 7 of the 19 that we radio-tracked and 15 of 57 that we did not radio-track, but which were encountered and marked during the study. Of the 22 marked animals showing signs of upper respiratory disease, 17 died (5 M, 12 F) during a span of 76 d (DOY 184–259). Of the 17 deaths, 11 occurred within 15 d (DOY 224–238). Seven of the 19 turtles we radio-tracked showed clinical signs consistent with an upper respiratory disease. These signs first appeared in radio-telemetered turtles on approximately DOY 189, when several turtles were found close together in a small (5 m^2) ephemeral wetland (Fig. 1). Three of the 7 sick turtles were found dead on DOY 224 (2 turtles) and 245 (1 turtle), whereas the remaining 4 turtles recovered and survived, being seen alive at least 30 d after the last observed death at the field site, at which time equipment was removed from all animals and radio-tracking ceased. The most prevalent external clinical and behavioral signs of upper respiratory disease in all turtles that we observed occurred after DOY 224 and included swollen eyes, wheezing, extensive nasal discharge, and excessive lethargy. Individuals that appeared to have recovered ceased showing signs of upper respiratory disease at last capture. During this time period, we did not find any other species dead or similarly affected (e.g. amphibians).

Fig. 1. Several eastern box turtles *Terrapene carolina* were found at a drying, ephemeral wetland on day of year 189, just after the first animal in the population was found dead with signs of upper respiratory pathogen infection and just prior to the mass die-off

Ranavirus infection (frog virus 3 [FV3]-like) was confirmed as the cause of death in 1 of the radio-tracked animals, which was 1 of 2 animals sent to the NWHC for examination. Diagnosis was confirmed by histological examination and by partial genomic sequencing using tissue from the esophagus, liver, spleen, and kidney. No other disease-causing pathogens were found in this turtle in bacterial cultures and histological examinations. In addition, no other pathogens were investigated molecularly. The cause of death of the second box turtle could not be determined because of rapid autolysis and extensive scavenging of internal organs that occurred rapidly before the animal was found dead. While the NWHC diagnostic services case report stated that it was likely many of the other dead turtles at this location died of ranavirus infection, it is possible that other pathogens such as mycoplasma or herpesvirus (Kane et al. 2016) could have caused mortality in other individuals at our study site.

Body temperatures did not differ significantly among the turtles that were healthy, recovered, or died later, until DOY 210 to 214, when healthy box turtles were significantly warmer than those that recovered and those that died later (Fig. 2). From DOY 215–224, T_b differed significantly among all 3 groups. From DOY 224 to 245, T_b did not significantly differ between healthy and recovered turtles. Just 5 d before the 3 groups of turtles diverged significantly in T_b (~210 DOY), mean T_e dropped to nearly 2.5 SD below the 3 mo mean to 16°C (Fig. 3). Mean T_e also dropped rapidly to nearly 1.5 SD below the 3 mo mean around DOY 184 and DOY 195–200 (Fig. 3), 10–15 d before the 3 groups of turtles diverged in T_b.

Turtles that were healthy, recovered, or died later had different probabilities of T_b remaining within states above or below ambient temperature, and they had different probabilities of transitioning between states (Table 1). Turtles that died were significantly more likely to keep T_b below ambient temperature before death (79%), followed by turtles that recovered (66%) and healthy turtles (47%). When transitioning from above (state 1) to below ambient temperature (state 2), healthy and recovered turtles had a similar probability of keeping T_b above ambient

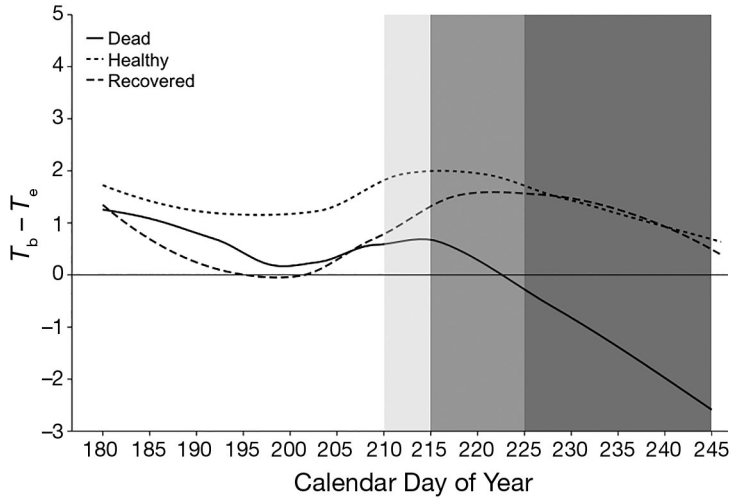

Fig. 2. Daily eastern box turtle *Terrapene carolina* body temperature (T_b) difference from environmental temperature (T_e) over day of year (DOY) 180–245. White space indicates no group is significantly different from any other. Light grey indicates that 'Dead' T_b and 'Recovered' T_b are significantly different from 'Healthy' T_b (DOY 210–215). Grey denotes when all groups are significantly different from each other (DOY 215–225). Dark grey is when 'Recovered' T_b and 'Healthy' T_b are not significantly different from each other (DOY 225–245)

(86–87%) and continuing to effectively thermoregulate T_b, whereas turtles that later died had a lower probability of remaining above ambient before death (76%). When transitioning from below (state 2) to above ambient (state 1), healthy turtles were most likely to effectively thermoregulate and return T_b to above ambient (53%), significantly different from turtles that recovered (34%) and those that later died (20%).

Turtles that were healthy, recovered, or died later also had different proportions of time spent within each state (Table 2). The stationary distributions show that turtles that died spent significantly more of their time below ambient before death (54%), compared to healthy (19%) and recovered turtles (29%). Recovered turtles spent less time above ambient than healthy turtles, but the difference was not significant at $\alpha = 0.05$.

Survival analysis included 25 individuals, of which 12 died. The results showed a significant negative effect of DVM (estimate = −1.63 ± SE 0.53, p = 0.002, $R^2 = 0.51$) on survival (Fig. 4). As

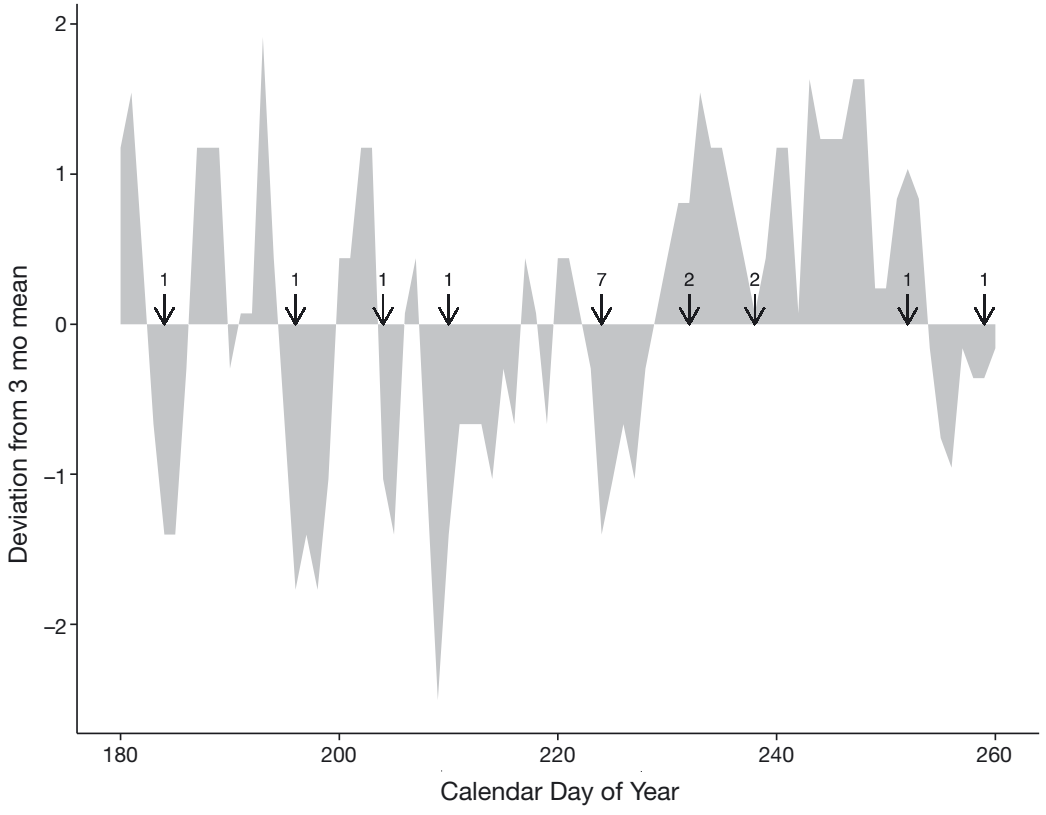

Fig. 3. Daily environmental temperature (T_e) deviation from 3 mo mean T_e (day of year, DOY 180–245) in 2014. Vertical arrows denote observed eastern box turtle *Terrapene carolina* mortalities during the study. We also provide the number of mortalities observed at each DOY arrow

Table 1. Markov model, 2-state. The state 'AT_e' denotes an eastern box turtle *Terrapene carolina* having body temperature (T_b) > environmental temperature (T_e), and the state 'BT_e' denotes a box turtle having $T_b < T_e$. Read each matrix as from state in each row to state in each column. Estimates are presented with 95% confidence intervals

Status	From state	To state	
		AT_e	BT_e
Dead	AT_e	0.76 (0.66, 0.85)	0.24 (0.15, 0.34)
	BT_e	0.20 (0.13, 0.31)	0.79 (0.69, 0.87)
Healthy	AT_e	0.87 (0.84, 0.90)	0.13 (0.09, 0.16)
	BT_e	0.53 (0.45, 0.63)	0.47 (0.36, 0.55)
Recovered	AT_e	0.86 (0.81, 0.89)	0.14 (0.11, 0.19)
	BT_e	0.34 (0.26, 0.43)	0.66 (0.57, 0.74)

Table 2. Stationary distributions for the time eastern box turtles *Terrapene carolina* spent in each state (see Table 1 for state definitions). Estimates are presented with 95% confidence intervals

Status	AT_e	BT_e
Dead	0.46 (0.34, 0.60)	0.54 (0.60, 0.66)
Healthy	0.81 (0.76, 0.85)	0.19 (0.15, 0.23)
Recovered	0.70 (0.61, 0.78)	0.30 (0.22, 0.38)

DVM increased, hazard rate (i.e. risk of mortality) decreased; conversely, as DVM decreased, hazard rate increased. The hazard ratio for risk of death was 0.19 (95% CI: 0.07–0.55), a result that corresponds to a 19% decrease in mortality (i.e. hazard) as DVM increases by 1 SD. Conversely, with each 1 SD decrease in DVM, relative risk of mortality increased by 81%. Finally, we did not detect a significant difference in survival between males and females (p = 0.34) or based on body size (p = 0.27).

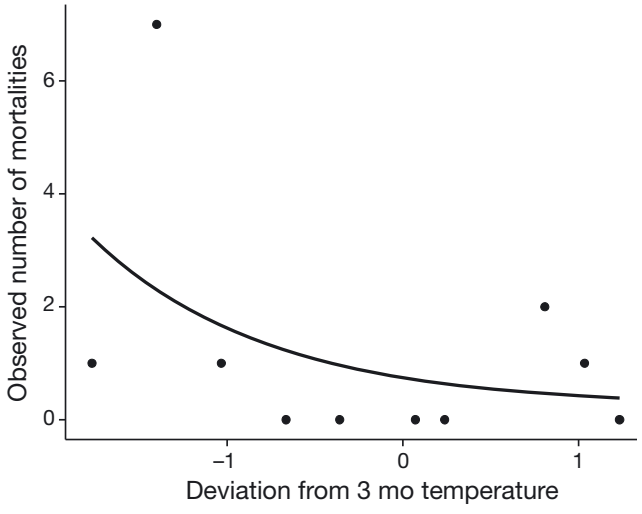

Fig. 4. Observed number of mortalities of eastern box turtles *Terrapene carolina* when daily environmental temperature (T_e) deviated from 3 mo mean T_e. Points represent raw data, and trend line is a loess smoothing fit

DISCUSSION

Epizootics have a range of impacts on ectotherms, from minor subclinical effects to complete local extirpation (Duffus et al. 2015). Mass die-offs associated with upper respiratory disease are distinguished by a rapid increase in infection prevalence soon followed by high mortality rates (Brunner et al. 2015). Reports of upper respiratory disease outbreaks in reptiles have involved both wild and captive turtles, mainly in North America (De Voe et al. 2004, Allender et al. 2006, Feldman et al. 2006, Johnson et al. 2008, Duffus et al. 2015, Kane et al. 2016), but have also included other turtle species worldwide (Marschang 2011). Most cases of die-offs in free-ranging turtles are noted in the summer months, but mortalities have been observed over an entire active season and across several years (Farnsworth & Seigel 2013, Brunner et al. 2015). Because upper respiratory infections are often rapidly lethal in turtles, the prevalence of infection within populations experiencing mass die-offs is often low (Johnson et al. 2010, Brunner et al. 2015). Additionally, turtles may die quickly before they are able to develop an antibody response (Johnson et al. 2010), reducing the prevalence of the pathogen within the population. For instance, only 1 surviving eastern box turtle out of 55 that were tested was seropositive 1 yr after a population experienced a ranavirus-associated mass die-off, suggesting that most infected turtles died or failed to develop an antibody response (Johnson et al. 2010). While ranavirus-related die-offs are often rapid and may leave few survivors, 1 study reported a major die-off that took place over several years (Farnsworth & Seigel 2013).

Previous studies have documented greater or longer survival in ectotherms that thermoregulate optimally and induce behavioral fever (Kluger et al. 1996, Deen & Hutchison 2001, Elliot et al. 2002). Hosts that are able to access thermal niche space that is detrimental to the pathogen (e.g. warm, dry microhabitats) may be able to clear infection or reduce their risk of becoming infected (Nowakowski et al. 2016). In our study, turtles may have avoided or delayed negative effects of upper respiratory disease by behaviorally thermoregulating at warmer T_b—23°C (Healthy) ver-

sus 21°C (Dead)—during a rapid drop in environmental temperature (1.4–2.5 SD below average, mean T_e: 16–19°C). Alternatively, advanced upper respiratory disease may have inhibited the ability of some turtles to effectively thermoregulate. In either case, mortality was associated with low body temperatures relative to healthy and recovered turtles. Maintenance of T_b within an optimal range may restrict pathogen growth or benefit host immunity, leading to a delay in mortality (Elliot et al. 2002, Nowakowski et al. 2016). Immune function of ectotherms can be suppressed at cold temperatures, whereas warmer, more optimal body temperatures are often associated with increased adaptive and innate immune responses (Bly & Clem 1992, Ribas et al. 2009).

Results of our survivorship analysis suggest that cold periods in our study (i.e. when T_e was ~1.6 SD below the 3 mo mean) were significantly correlated with the timing of mortality. While temperature may only play a minor role in survival, these results are congruent with other studies reporting that variation in T_e (i.e. above or below average temperatures) and the host's ability to thermoregulate can affect disease progression in turtles infected with ranavirus (Allender et al. 2013). Additionally, Raffel et al. (2013) found evidence that temperature variation decreases an ectotherm's resistance to a pathogen, thereby increasing the risk of mortality. In a controlled study, Allender et al. (2013) determined that red-eared sliders had higher viral copy number in turtles exposed to FV3 at 22°C compared with 28°C, suggesting that lower T_e increased disease progression. In addition, Rojas et al. (2005) found that salamanders exposed to ranavirus survived at 26°C, but all died at 18°C. Furthermore, a recent finding by Brand et al. (2016) suggested that a 2°C change alters disease outcomes in wood frog populations. Together, these studies suggest that minor differences in T_e can greatly affect disease outcome in ectotherms.

CONCLUSION

Here, we report a rapid mass die-off of eastern box turtles coincident with frequent drops in T_e below a 3 mo mean. We also report the first confirmed case of ranavirus infection (FV3-like) by the NWHC in a reptile from Kentucky. At our study site, 3 monitored animals that showed external signs of upper respiratory disease and died had lower T_b compared to 15 monitored animals that survived. Additionally, our survival analysis suggests that rapid drops in environmental temperature were correlated with mortality events; however, the main cause of mortality may be a combination of an upper respiratory disease and rapid variation in temperature. In summary, our observations suggest that turtles first contracted an upper respiratory pathogen infection (i.e. ranavirus, mycoplasma, or herpesvirus), and then sudden drops in temperature occurred, contributing to a mass mortality event. These results point to the importance of understanding host behavioral thermoregulation and fine-scale deviation in T_e in ranavirus-caused morbidity and mortality in ectotherms. Furthermore, these results also suggest a compelling link between body temperature in ectotherms, environmental temperatures, and disease outcome.

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